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Proceedings of an FAO/IAEA Coordinated Research Project on Enabling Technologies for the Expansion of Screwworm SIT Programmes

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Cover caption. *Background*: SEM (scanning electron micrograph) of compound eye of *Drosophila melanogaster* showing multiple lenses and microtrichia © 2002 Robert Harding. *Inset: Top left:* Life cycle of OWS, *Chrysomya bezziana*, showing all life stages: eggs, three larval instars, puparia and adults (Photo: P. Crabb/M. Hall, Natural History Museum, London, UK). *Top right:* Male of *Wohlfahrtia magnifica* individually paint marked for behavioural studies, waiting for female flies at a mating station (an olive tree stump) on Crete island, Greece, the scene of a recent outbreak of wohlfahrtiosis (see Hall et al., pp. 72–79) (Photo: M. Hall, Natural History Museum, London, UK). *Bottom:* Female NWS, *Cochliomyia hominivorax*, reared in London, UK, from one of 91 larvae removed from the scalp of a woman on her return from a visit to Trinidad, the first recorded case of imported NWS myiasis in the UK (Mallon, P., Evans, M., Hall, M. and Bailey, R. (1999) "Something moving in my head" *The Lancet*, 354, 1260) (Photo: H. Taylor/M. Hall, Natural History Museum, London, UK).

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Enabling technologies to improve area-wide integrated pest management programmes for the control of screwworms

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Abstract. The economic devastation caused in the past by the New World screwworm fly *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae) to the livestock industry in the U.S.A., Mexico and the rest of Central America was staggering. The eradication of this major livestock pest from North and Central America using the sterile insect technique (SIT) as part of an area-wide integrated pest management (AW-IPM) programme was a phenomenal technical and managerial accomplishment with enormous economic implications. The area is maintained screwworm-free by the weekly release of 40 million sterile flies in the Darien Gap in Panama, which prevents migration from screwworm-infested areas in Columbia. However, the species is still a major pest in many areas of the Caribbean and South America and there is considerable interest in extending the eradication programme to these countries. Understanding New World screwworm fly populations in the Caribbean and South America, which represent a continuous threat to the screwworm-free areas of Central America and the U.S.A., is a prerequisite to any future eradication campaigns. The Old World screwworm fly *Chrysomya bezziana* Villeneuve (Diptera: Calliphoridae) has a very wide distribution ranging from Southern Africa to Papua New Guinea and, although its economic importance is assumed to be less than that of its New World counterpart, it is a serious pest in extensive livestock production and a constant threat to pest-free areas such as Australia. In the 1980s repeated introductions and an expansion of Old World screwworm populations were reported in the Middle East; in the 1990s it invaded Iraq and since late 2007 it has been reported in Yemen, where a severe outbreak of myiasis occurred in 2008. Small-scale field trials have shown the potential of integrating the SIT in the control of this pest and various international organizations are considering using the release of sterile insects as part of an AW-IPM approach on a much wider scale. *Wohlfahrtia magnifica* (Schiner) (Diptera: Sarcophagidae) is a screwworm of temperate regions, which, although of limited agricultural importance, has invaded several new locations in the past few years. This special issue reports on the results of a 6-year project funded by the Joint Food and Agriculture Organization of the United Nations/International Atomic Energy Agency (FAO/IAEA) Programme of Nuclear Techniques in Food and Agriculture entitled 'Enabling Technologies for the Expansion of the SIT for Old and New World Screwworm'. A major goal of the project was to better understand population genetic variation in screwworms as an aid to the identification of isolated populations. The project also addressed issues related to genetic sexing, cuticular hydrocarbons, population dynamics, genetic transformation and chromosome analysis.

Key words. genetic sexing, population genetics, screwworm, sterile insect technique.

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Introduction

Different species of screwworm are responsible for traumatic myiasis in many parts of the tropical, subtropical and temperate world. In the New World, successful area-wide integrated pest management (AW-IPM) programmes including the use of the sterile insect technique (SIT) have led to the eradication of the New World screwworm *Cochliomyia hominivorax* (Coquerel) from the southern U.S.A., Mexico, Central America, Panama and some islands in the Caribbean. The species occupying a similar ecological niche in the Old World are *Chrysomya bezziana* Villeneuve and, in more temperate regions, *Wohlfahrtia magnifica* (Schiner). There is evidence that the latter two species are expanding their distributions into new areas. The success of AW-IPM programmes with an SIT component in control of *C. hominivorax*, coupled with the significance of the damage wrought by screwworm pests, has encouraged other countries and regions to consider using a similar approach. Expansion of the approach to new regions and species will benefit from improved information on potential target populations and from the development of improved strains for release.

In support of future AW-IPM programmes against these screwworm pests, the Joint Food and Agriculture Organization of the United Nations/International Atomic Energy Agency (FAO/IAEA) Programme of Nuclear Techniques in Food and Agriculture funded a 6-year co-ordinated research project (CRP) entitled 'Enabling Technologies for the Expansion of the SIT for Old and New World Screwworm', with the participation of 11 scientists from nine countries. The results of this CRP are reported in this special issue. A major goal of the CRP was to better understand population genetic variation in screwworms as an aid to the identification of isolated populations as targets for AW-IPM programmes. The CRP also addressed issues related to genetic sexing, cuticular hydrocarbons and mating compatibility, population dynamics, genetic transformation and chromosome analysis. This special issue is devoted to the results generated during the CRP, together with several invited contributions.

The species

The New World screwworm fly is a major parasitic pest of warm-blooded hosts which causes primary myiasis in wildlife, livestock, pets and humans. Humans living in conditions of poor hygiene are particularly at risk, and the infestation can become rapidly debilitating when it occurs in the nasal and frontal sinuses, eyes, ears or mouth. The pest is, however, mainly present in livestock, as was demonstrated in 1935 when 230 000 cases were detected in livestock in Texas, compared with only 55 in humans (FAO, 1992). Gravid female screwworm flies are attracted to open wounds caused either by animal husbandry practices (dehorning, castration etc.) or occurring naturally (e.g. tick bites). A female oviposits on average 200 eggs in single or multiple egg masses in the vicinity of the wound. First instar larvae hatch after 11–24 h and start feeding immediately on living tissue. During the next 4–8 days, the larvae continue feeding and develop into mature third-stage larvae, which drop to the ground and burrow into the soil for pupation. The pupal period is largely temperature-dependent; in Texas it fluctuated between 7 days in summer to over 50 days in winter. The lifecycle is short and can be completed in 21 days under optimal conditions.

Larvae from a single oviposition can kill smaller animals and multiple infections can kill mature cattle. Therefore, larval screwworm infestations have serious economic implications for the livestock industry. Local conditions such as the distribution and density of livestock and wildlife populations, husbandry practices, commercial movement of animals, human population density, socioeconomic conditions, public health services, climatic factors and geography dictate the severity of the infestations and the economic magnitude of the problem. The eradication of *C. hominivorax* from the U.S.A., Mexico and the remainder of Central America is undoubtedly the most successful example of the application of the SIT as part of an AW-IPM programme. In 2000, the programme's direct annual producer benefits were estimated at US$ 896 m, US$ 328 m and US$ 87 m for the U.S.A., Mexico and Central America, respectively (Vargas-Terán *et al.*, 2005). Adding the positive effect on the general economy increases the annual benefits to more than US$ 4 bn for the entire screwworm-free zone (Wyss, 2002). To prevent reinvasion from South America, a biological buffer zone has been established through the weekly release of 40 million sterile flies in the Darien Gap, Panama.

However, this screwworm-free region remains at risk because of endemic populations in the Caribbean; *C. hominivorax* remains one of the major livestock and wildlife pests in Cuba, the Dominican Republic and Haiti, the economies of which are still dominated by agriculture. The Caribbean territories affected by *C. hominivorax* have a total surface area of 203 460 km^2 and the total annual costs in treatment and prevention are estimated to exceed US$ 75 m (1999 figures) (Vargas-Terán & Novy, 2001). Furthermore, the species is highly mobile and individual flies have been recorded travelling 290 km (Spradbery, 1994). In addition, there is some evidence that flies may be carried by wind to infest new areas, as is suspected to have been the case in the re-infestation of the Virgin Islands in 1977 (Rawlins, 1985). However, the most common way for the fly to be introduced is through the movement of larvae-infested animals or humans, as was demonstrated by its accidental introduction into the Libyan Arab Jamahiriya in 1988 (Lindquist *et al.*, 1992). The presence of *C. hominivorax* in the Caribbean poses a constant threat of re-infestation to countries in the region where the pest is non-endemic or has been eradicated and this is becoming especially significant with increased international trade and globalization. The costs of eradication from the Caribbean territories using an AW-IPM strategy were estimated in 2001 to be US$ 140 m (Vargas-Téran & Novy, 2001). *Cochliomyia hominivorax* is also present in all countries in South America, with the exception of Chile, and it has been estimated that the potential annual producer benefits following eradication would be US$ 2.8 bn (Wyss, 2002). Before embarking on such programmes, it is important to generate the required baseline information on the distribution, genetics and mating compatibility of populations in this region.

The Old World screwworm fly *Ch. bezziana* causes traumatic myiasis in many warm-blooded animals throughout the tropical regions of the Old World, with the exception of Australia (Spradbery, 1994). Since early reports of repeated introductions

in the Middle East (Kloft *et al.*, 1981), it has expanded its range there. It invaded Iraq in the 1990s and since late 2007 has been reported from Yemen, where a severe outbreak of myiasis occurred in 2008. This is probably the result of livestock transport (official trade and – in some areas – smuggling of livestock) and human interventions such as tree planting and irrigation, which lead to the creation of habitats suitable for the species. It is widely distributed in the Indian subcontinent and Southeast Asia and is present in tropical and subtropical sub-Saharan Africa. Good data and statistics are available on livestock production in the Asia-Pacific region and in the Middle East, but there is a severe paucity of data on the incidence of *Ch. bezziana* and its relevance to animal production. Although the myiasis caused by this species is also classified as a 'List B' disease (transmissible diseases that are considered to be of socioeconomic and/or public health importance within countries and that are significant in the international trade of animals and animal products) by the Office International des Epizooties (OIE), cases are rarely reported by the affected countries.

An economic feasibility study in Malaysia showed that economic losses of US$ 5 m can be attributed annually to *Ch. bezziana*, but that current livestock numbers, animal husbandry practices and fly rearing costs did not justify the implementation of an AW-IPM programme against this species at the time (Grindle, 2001). However, there are other areas where these restrictions are absent. In fact, the Australian authorities established an experimental production facility in Kluang, Malaysia, where procedures for mass rearing were developed and tested. In addition, pilot release projects have already demonstrated the technical feasibility of using the SIT against *Ch. bezziana* (Spradbery *et al.*, 1989; Mahon, 2002). In November 2001 the Australian authorities reviewed their *Ch. bezziana* Emergency Preparedness Strategy (EPS) (Office of the Chief Veterinary Officer [OCVO], 2002). The EPS foresees the construction of a multi-insect rearing factory, which could be used to provide sterile insects following the detection of Old World screwworm in Australia. After the 1996 outbreak in Iraq and the subsequent trans-boundary threat to the entire region (Kloft *et al.*, 1981), a regional project was set up by the IAEA to help prevent the spread of *Ch. bezziana* to neighbouring countries, and to assess the severity of the infestation in the Middle East and the feasibility of an AW-IPM control programme in conjunction with the Arab Organization for Agricultural Development (AOAD). Recently, AOAD, with the support of the FAO and IAEA, has begun to develop plans for the possible construction of a pilot rearing facility in the region.

A third screwworm genus, *Wohlfahrtia*, has members present in the temperate regions of the world; the flesh fly *W. magnifica* is an important cause of myiasis of livestock throughout the Mediterranean Basin, Europe and across to China. It occurs across Turkey, Iraq and Iran and penetrates into Asia. In Iran and Iraq its distribution partly overlaps that of *Ch. bezziana*, with which it can be confused. Unlike the other screwworms, females of *W. magnifica* are larviparous, depositing first instars into wounds or body orifices of their warm-blooded hosts. This species is also extending its range, with new outbreaks recently recorded in Crete and Morocco. It is the least well studied of the three screwworms and the least important economically.

The co-ordinated research project

The CRP focused on two main topics as relevant to an expansion of AW-IPM programmes for these pests: (a) an assessment of population genetic variation in field populations of screwworms, and (b) the development of a genetic sexing strain for *C. hominivorax*.

Population genetic analysis can identify similarities and differences at the DNA level in insect populations from different geographic regions. This information can be used to assess the level of genetic exchange between populations and hence their degree of isolation from one another. This can provide AW-IPM programmes with important decision-making tools related to the identification of target populations in terms of defining the size of their geographic distributions, as well as the sustainability of establishing free areas in relation to their degree of isolation. Population genetic information can also be used to help identify the origin of outbreaks in new areas, although frequently the level of discrimination can only be used to exclude populations that were not the source of the introduction. Identification of outbreak origins can lead to improved quarantine and animal health regulations. We now have very powerful molecular tools to generate and analyse population genetic information, but frequently our major difficulty lies in obtaining sufficient samples from the field. This can be a problem for species such as screwworms, where attractants for adult flies are not always effective. All the data reported in this issue were derived from larvae sampled from infested animals and considerable resources and organization were required to obtain the samples.

A major cost associated with SIT implementation is that of fly production and release, and half of this is represented by the rearing and releasing of sterile females which do not contribute to control (Robinson *et al.*, 1999). Therefore, the development of a genetic sexing strain that enables the elimination of females at an early stage in their development has obvious economic benefits, which have already been estimated for *C. hominivorax* (LaChance, 1979). The release of only males in an SIT programme has additional advantages such as: (a) the increased effectiveness of sterile male flies in the field in the absence of sterile females, and (b) the prevention of loss of sterile sperm in assortative matings between sterile males and females. The advantages of using genetic sexing strain in SIT programmes have been clearly documented for the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) (Robinson *et al.*, 1999; Rendón *et al.*, 2000, 2004). There are two approaches to developing a genetic sexing strain.

The first of these is the classical Mendelian genetic approach, which consists of: (a) the isolation of selectable markers such as a pupal colour mutation or a temperature-sensitive mutation, and (b) the linking of the selectable marker to the male sex by inducing a male-linked translocation. In these strains, females are associated with a particular pupal colour or with temperature sensitivity and can be removed from production by pupal sorting or raising the temperature. An important requirement of this approach is information on the position in the chromosome of the selectable marker and the translocation breakpoint. This information can be obtained by analysing polytene chromosomes; the development of a polytene map for *C. hominivorax* was included in the CRP.

The second method concerns the molecular approach, which requires the development of germ-line transformation and the

use of gene constructs which lead to inducible female lethality when introduced into the germ-line. If successful, the molecular approach will require the release of sterile transgenic males. Both approaches have their advantages and disadvantages and were initially included in the CRP, but changes in the mandate of a key laboratory in the project forced a shift in focus away from the Mendelian approach.

The issue of assortative mating, mentioned above, is also related to research carried out in the CRP on cuticular hydrocarbons of *C. hominivorax*. It is well established that prolonged mass rearing of screwworm flies results in the loss or masking of the female contact sex pheromone. In nature male screwworms will only proceed with mating when the female's specificity is confirmed through tarsal contact and detection of the pheromone. In strains of both Old and New World screwworm, asymmetric mating isolation has therefore been associated with longer colonization (Hammack, 1987), and its importance for programmes including the SIT has recently been modelled (Vreysen *et al.*, 2006). Selective mating between different screwworm strains, which has been related to differences in these compounds (Mangan, 1988), has been observed in laboratory cages, where wild male screwworm flies mated almost exclusively with wild females, but sterile male screwworm flies did not discriminate and mated equally with sterile and wild females (R. Garcia, unpublished data, quoted in Vreysen *et al.* [2006]). Geographic variation in cuticular hydrocarbons and the effects of mass rearing could compromise the effectiveness of sterile flies in the field.

Results

Sampling

Population genetic analyses at different levels of scale and using different DNA sequences were carried out for *C. hominivorax*, *W. magnifica* and *Ch. bezziana.* For *C. hominivorax*, samples were analysed over the entire distribution in the Caribbean and South America, with the exception of the Amazon Basin, the putative origin of the species. Logistical difficulties have prevented the obtaining of samples from this important region. Also not included were samples from the strains (Costa Rica 92, Panama 95) released in Panama and Jamaica. The distribution of *Ch. bezziana* is extremely large and, as a consequence, sampling has been very patchy and there are several large gaps, including all of India and the Horn of Africa. During the course of the larval sampling much new information was collected on the host preference, prevalence, and epidemiology of the three screwworm species (Hall *et al.*, 2009; Coronado & Kowalski, 2009; Farkas *et al.*, 2009). During sampling for *Wohlfahrtia* in Northern Spain, a new species was collected and described (Hall *et al.*, 2009).

Population genetics of Cochliomyia hominivorax

Extensive population genetic analyses are reported on populations of *C. hominivorax* from eight countries in South America and four Caribbean islands using both mitochondrial and nuclear markers, restriction fragment length polymorphisms (RFLPs) and sequence data (McDonagh *et al.*, 2009; Torres & Azeredo-Espin, 2009; Lyra *et al.*, 2009). A major finding was that the population from Cuba was remarkably dissimilar to any of the other analysed populations, including those from the other Caribbean islands. However, there was also evidence that the populations on the Caribbean islands themselves constitute independent entities connected by restricted gene flow. It is not clear why the population from Cuba is so different from all the others. From the analysis of the populations from South America, both east and west of the Andes, there is no strong evidence to suggest drastic population structuring, although there was a high level of variability among populations from this region. It is unlikely, but not to be excluded, that further sampling will change these conclusions. These results suggest that there are no naturally isolated populations of New World screwworm in South America and this will need to be taken into account when future AW-IPM programmes are planned.

The detailed information now available for many *C. hominivorax* populations from South America and the Caribbean should enable the origin of any future outbreaks in eradicated areas to be determined with improved precision. In relation to potential new outbreaks, life-table analysis provides important information on population growth rate and the likelihood that an infestation will become established. Based on an extensive literature survey, together with an analysis of unpublished data, new estimates have been developed for important population parameters, including survivorship, recruitment and the rate of density-independent population growth (Matlock & Skoda, 2009).

Population genetics of Chrysomya bezziana

Population genetic analysis of *Ch. bezziana* shows some structuring that possibly reflects the wide geographical range of the species over various land masses from sub-Saharan Africa to Papua New Guinea. Previous analysis has shown that there is a distinct geographical race in sub-Saharan Africa (Hall *et al.*, 2001), but the data presented here do not support a single Asian lineage (Ready *et al.*, 2009). There was very limited genetic diversity in the populations from the Persian Gulf region to Southeast Asia. By contrast, the lack of gene flow between populations from different Indonesian islands suggests that their elimination would not be followed by reinvasion. The absence of mtDNA haplotypes from Indonesian and African populations in the Middle East populations so far analysed may suggest that the large-scale transport of sheep is not leading to the spread of this species (Hall *et al.*, 2009). However, the current expansion in the distribution of this species may suggest otherwise. Although populations have been sampled from many regions, no flies from the Horn of Africa have yet been obtained to ascertain whether they belong to the African or Asian lineages.

Population genetics of Wohlfahrtia magnifica

Two mtDNA lineages for *W. magnifica* have been demonstrated, one in the extreme west of Europe and the second

elsewhere in Europe and eastwards towards the Middle East (Hall *et al.*, 2009). In general there is much more diversity in the western than the eastern lineage. Two distinct populations were identified within Morocco and local veterinarians assumed that the northern population was a new introduction as they had not registered cases previously. However, this is unlikely given the level of genetic diversity shown by this population. It is possible that this population represents a re-emergence of an extant population (Hall *et al.*, 2009). Individuals from the new outbreak on the island of Crete share haplotypes with populations in mainland Greece, Hungary and Eastern Europe.

Genetic sexing

In relation to the development of genetic sexing strain for *C. hominivorax*, progress has been made in two areas. Firstly, a complete polytene chromosome map has been developed (Batista *et al.*, 2009). This species has a diploid chromosome number of 12, with five pairs of autosomes and a pair of sex chromosomes (XY). The sex chromosomes do not polytenize, so there are five polytene elements representing the five autosomes. Each arm of the chromosome has a characteristic banding pattern that can be used to identify it. As well as being essential for the analysis of genetic sexing strains, these chromosomes can also be used to reveal population differences (Coluzzi *et al.*, 2002), and to demonstrate the localization of DNA sequences through *in situ* hybridization (Zacharopoulou *et al.*, 1992). Using *in situ* hybridization it should be possible to identify the genomic location of the microsatellites used in the population genetic analysis as well as the insertion sites in transgenic strains. Secondly, genetic transformation techniques have been developed that will enable the introduction of specific gene constructs that can lead to female lethality (Handler *et al.*, 2009). Several transgenic strains carrying fluorescent protein markers were developed and it was possible to cryopreserve several of these strains for future studies (Leopold, 2007); one of these is a potential candidate as a marker strain for release.

Insecticide resistance

Control of *C. hominivorax* in South America is focused on wound treatment and/or animal dipping using organophosphorous (OP) insecticides such as coumaphos. However, the introduction of other insecticides, such as pyrethroids, which have been associated with the development of OP resistance in *Lucilia cuprina* Wiedemann (Diptera: Calliphoridae), is causing some concern. The development of resistance to OP chemicals would present major problems in the control of this pest and molecular methods to assess the presence of resistant alleles in natural populations would be very useful. Mutations at certain positions in specific esterases confer OP resistance in many species (Oakeshott *et al.*, 2005) and these have now been identified in *C. hominivorax* (Carvalho *et al.*, 2009).

Cuticular hydrocarbons

Cochliomyia hominivorax sex pheromones have proven to be difficult to identify, although recently progress has been made with the identification of two biologically active long chain secondary alcohols (Carlson *et al.*, 2007). These were synthesized compounds, but it has now been shown that they are present in the extracts of recently colonized females (Akasaka *et al.*, 2009). Changes in these compounds as a result of longterm mass rearing can reduce the effectiveness of a sterile insect release (Mangan, 1988).

The future

The sterile fly release barrier for *C. hominivorax* in Panama will continue to be maintained in order to protect the pest-free areas in Central America, some Caribbean islands, Mexico and the U.S.A., but the populations in the Caribbean and South America will continue to pose a threat of re-infestation. This was the background to the current programme in Jamaica. The difficulties experienced in eliminating the population in Jamaica, despite the release of many more sterile flies than are usually required, serves as a warning that what appears to be a relatively straightforward operation can run into serious problems (Vreysen *et al.*, 2007). It also emphasizes the fact that the use of the SIT involves more than simply releasing sterile insects. Population genetic data for Cuba show that this population is highly differentiated from all others; whether this extends to other components of fly biology important for SIT application is uncertain.

Although population genetic data can be very useful in identifying genetic relationships between populations, it says very little about whether the populations are compatible in terms of mating. For SIT effectiveness, the mating phenotype is much more critical than the DNA genotype. However, because of the documented genetic differences between all the island populations, any AW-IPM programme will need to be preceded by careful evaluations of all aspects of mating compatibility, population dynamics in time and space, and the collection of good baseline data. These more technical components will have to be embedded in an efficient programme organization in order to ensure success (Vreysen *et al.*, 2007). Laboratory cage mating studies using wild Jamaican and Cuban flies and the release strain did indeed provide evidence of asymmetric mating, with wild males refusing to mate with released females (R. Garcia, unpublished data, quoted in Vreysen *et al.* [2006]). This may be related to qualitative and quantitative differences in the profile of the cuticular sex pheromones between released flies and wild flies, and analyses of the sex pheromone of field populations from Trinidad and Tobago, Dominican Republic, Peru, Ecuador and Columbia and of a release strain need to be carried out.

Strategies to prevent the introduction of screwworms into new areas can benefit from an understanding of the geographic origin of the screwworms in detected cases of myiasis so that quarantine and animal health regulations can be improved. Although the population genetic data for all three screwworm species will help with this, backtracking from outbreaks to origins using population genetic data is not straightforward as, very

frequently, there are no population-specific markers available and probability estimates are all that can be provided. The data can, however, often be used to exclude particular populations as the source. For *C. hominivorax*, the database is quite extensive but probably not yet sufficient to provide unequivocal answers about the geographic origin of an outbreak. For *Ch. bezziana* and *W. magnifica* the database is more limited, but the work on population genetics of the three species will continue in a new CRP which will integrate screwworm population genetic data with other biological data into a geographic information system (GIS). This will be a challenging project but could provide unique insights that correlate population genetic variation with variation in environmental and other parameters.

Whether a screwworm population will establish after its introduction depends on many factors, including the number of individuals introduced, the local climate and the availability of hosts, whereas its spread will depend on the reproductive potential of the species and its ability to disperse from the original site of introduction. The CRP provided an updated analysis of many of these population biology parameters for *C. hominivorax* that will help to predict the probability of establishment and the spread of introductions.

The improved dispersal and survival data presented in this volume will also help to improve the efficiency of the continuous sterile fly release barrier in Panama by optimizing its size and position, as well as fly release rates and flight lines. A major contribution to cost saving for barrier maintenance would be access to a strain producing only male insects and, as indicated above, some progress has been made towards this end. Considering the permanence of the barrier and the costs of future expansion of the eradication programme into South America, further investment in this area could have a very large benefit/cost return.

The future of AW-IPM programmes for *Ch. bezziana* is uncertain, but the technology to run them has been developed and successfully tested (Spradbery *et al.*, 1989) and is available to deal with new introductions. Economics, stakeholder commitment, pest status and the absence of an effective organization are all critical issues that have to be addressed before area-wide use of the SIT for this species can be contemplated. However, the recent expansion of its range into areas of the Middle East, and the extensive losses it is causing, have revived interest in the development of an AW-IPM programme and there are plans for the construction of a pilot rearing facility in the region.

Conclusions

The population genetic data reported in these papers provides no evidence of significant levels of population structuring in New World screwworms in South America, which has relevance for any future AW-IPM programme in this region in two ways. It suggests, firstly, that it will be difficult to identify naturally isolated populations and, secondly, that there may be no mating incompatibility between different populations. However, studies on mating compatibility between different wild populations and strains to be used for a sterile release have not been carried out. Furthermore, much remains to be investigated in variation in cuticular hydrocarbons between field populations and mass reared strains. The development of a genetic sexing strain for *C. hominivorax* is still some way off, but given the longterm status of the sterile fly release barrier in Panama, or of any future eradication campaigns, investment in this area would seem very worthwhile. The availability of a genetic transformation system for this species needs to be exploited to achieve this goal. Expansion of the distribution of *C. bezziana* into new areas of the Middle East is of great concern and may well lead to the development of an AW-IPM programme in some areas of the region.

Conflicts of interest

The authors declare no conflicts of interests.

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Characterization and utilization of microsatellite loci in the New World screwworm fly, *Cochliomyia hominivorax*

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Abstract. New World screwworm populations in North and Central America have been the targets of virtually continuous eradication attempts by sterile insect technique (SIT) since the 1950s. Nevertheless, in some areas, such as Jamaica, SIT control programmes have failed. Reasons for the failure of SIT-based control programmes in some locations are unknown, but it has been hypothesized that failure may be related to mating incompatibility between sterile and wild fly populations or to the existence of sexually incompatible cryptic species. This paper outlines the development of a suite of four new microsatellite loci which can be used to study intra-specific relationships between populations of *Cochliomyia hominivorax* from the Caribbean and South America, which represent those populations involved in, or earmarked for, forthcoming SIT control. Cross-amplification with the secondary screwworm, *Cochliomyia macellaria*, was also successful with three of the new loci. We present results which suggest that populations from Trinidad and Jamaica form distinct groupings of flies and that *C. hominivorax* from Trinidad appears particularly distinct.

Key words. *Cochliomyia hominivorax*, Calliphoridae, microsatellite, myiasis, population genetics, screwworm.

Introduction

The New World screwworm fly (NWSF), *Cochliomyia hominivorax* (Diptera: Calliphoridae), is one of the most destructive insect parasites of livestock in the western hemisphere. Historically, *C. hominivorax* has a broad tropical and subtropical geographic range stretching from the southern U.S.A. down as far as Argentina, and it is annually responsible for millions of dollars of economic losses to the livestock industry, attributable to both animal deaths and the cost of insecticidal treatment and prevention (Klassen & Curtis, 2005; Vargas-Terán *et al.*, 2005). Screwworm eggs are laid onto open wounds or in body orifices and larvae develop by feeding on the protein-rich tissue of the host (e.g. cattle, sheep, pigs and even humans); such infestations cause a disease condition known as myiasis. Since the 1950s, New World screwworm populations in North and Central America have been the target of virtually continuous eradication attempts by sterile insect technique (SIT). Nevertheless, in some areas, such as Jamaica, SIT control programmes have failed (A. Robinson, International Atomic Energy Agency [IAEA], personal communication, 2007). Reasons for the failure of SIT-based control programmes in some locations are unknown, but it is hypothesized that failure may be related to mating incompatibility between sterile and wild flies (as a result of the effects of inbreeding in sterile flies and/or particular local adaptations associated with the reproductive process in target populations) or to the existence of sexually incompatible cryptic species (Richardson *et al.*, 1982a, 1982b). Accordingly, this paper outlines the development of a suite of microsatellite loci which can be used to study intra-specific phylogenetic relationships and associated biogeographic patterns in *C. hominivorax* populations from the Caribbean and South America, which represent those populations involved in, or earmarked for, forthcoming SIT control.

Materials and methods

Microsatellite library development

Microsatellite loci for *C. hominivorax* were isolated using a modification of the enrichment protocols of Armour *et al.*

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(1994) and Gibbs *et al.* (1997). Briefly, genomic DNA was extracted from colony-bred *C. hominivorax* pupae using the PUREGENE™ DNA isolation kit (Gentra Systems, Inc., Minneapolis, PA, U.S.A.) according to the manufacturer's instructions; DNA was extracted from pupal tissue to avoid potential dietary contamination of insect DNA, which can sometimes be problematic when extracting DNA from either the larvae or adults of carrion-feeding or parasitic insects. The fly colony was maintained at the laboratory of Professor A.M.L. Azeredo-Espin, Universidade Estadual de Campinas, Campinas, SP, Brazil. Approximately 2.5 μg of DNA was digested with the *Mbo*I (ABgene) restriction enzyme. In order to limit the loss of DNA, and because the majority of the fragment sizes produced by the digest were approximately of the size required (100–700 bp), *Mbo*I fragments were not size-selected. After cleaning up the digest by running the DNA through a MinElute polymerase chain reaction (PCR) purification spin column (Qiagen Inc., Valencia, CA, U.S.A.), *Sau* linkers were ligated to the ends of the fragments. The DNA was enriched for microsatellite repeat motifs by overnight hybridization to polynucleotides consisting of the following repeats: $(GA)_n$, $(CT)_n$, $(GTT)_n$, $(CAA)_n$, $(CAT)_n$, $(AC.GT)_n$ and $(GAAA.TTTC)_n$ (Amersham Biosciences Ltd, Little Chalfont, U.K.), which had previously been bound to Hybond nylon membranes (Amersham Biosciences Ltd). These enriched DNA fragments were stripped from the filters, purified by ethanol precipitation and subject to 30 rounds of hotstart PCR using primers complementary to the *Sau* linkers. The linkers were removed by a second digestion with *Mbo*I and purified by ethanol precipitation. The DNA was then ligated into a bluescript vector (Stratagene Corp., La Jolla, CA, U.S.A.) and the plasmids were then transformed into EZ QIAGEN competent cells (Qiagen Inc., Valencia, CA, USA) through heat shock. Transformed cells were grown on selective media and those containing an insert were identified by the disruption of β-galactosidase activity (producing white colonies when grown on Xgal). Successfully transformed clones were grown in 96-well microtitre plates and 800 clones were duplicated onto Hybond N membranes and screened by hybridization to α^{32}P-radiolabelled AT and CG sequences (Amersham Biosciences Ltd), which identified 38 positives. Inserts were sequenced in 34 clones, using the M13 forward primer, by Lark Technologies Inc. (Saffron Walden, U.K.). Primers for PCR were designed with PRIMER 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) for 16 loci, of which four were polymorphic and generated PCR products that were clear and easy to score.

Microsatellite screening

The four pairs of primers developed in this project were combined with additional primer sets, previously designed by Torres *et al.* (2004), to amplify a total of 12 microsatellite loci in 31 NWSF specimens. These specimens were generally collected as larvae, each corresponding to an individual infection from a different host. They originated from nine sample sites spread across four countries and island states in South America and the Caribbean (Table 1). The small sample sizes reflect problems in sample collection and preservation, as well as a desire to include flies from several populations in order to increase the likelihood of detecting polymorphism in the microsatellite loci. A single sample of the secondary screwworm, *Cochliomyia macellaria*, from Campinas, São Paulo State, Brazil was also included in the analysis to allow the cross-species utility of the primers to be tested. The PCR was carried out in a Thermo Hybaid Express thermocycler, in 15 μL volumes containing approximately 5 ng of template DNA, 500 nM of each primer, 0.75 units Biotaq DNA polymerase (Bioline), 1.5 μL of the manufacturer's buffer (32 mM $[NH_4]_2SO_4$, 125 mM Tris-HCL, pH 8.8 and 0.02% Tween 20), 200 μM of each dNTP and 15 mM $MgCl_2$. The PCR programme used was 5 min denaturing at 94 °C, followed by 35 cycles of 30 s at 94 °C, 1 min at the annealing temperature (Table 2) and 1 min at 72 °C, ending with a 10-min final elongation stage at 72 °C. The PCR products were resolved on polyacrylamide gels, visualized by silver nitrate staining (Bassam *et al.*, 1994) and alleles were scored manually.

Phylogenetic and population genetics methods

Population genetics statistics were calculated using the program GENEPOP (http://genepop.curtin.edu.au/). Given the number of simultaneous tests for departures from the Hardy–Weinberg equilibrium (HWE) undertaken, Bonferroni corrections were employed (Rice, 1989).

Table 1. Details of screwworm fly specimens analysed.

Sample name	Date collected	Country of origin	Sample site
Cochliomyia hominivorax			
CA 01, 02, 03, 04, 05	January, 2003	Uruguay	Canelones, Suarez
CO 01, 02, 03, 04, 05	January, 2003	Uruguay	Colonia, Colonia
CL 01, 02, 03, 04, 05	January, 2003	Uruguay	Cerro Largo, Bañados de Medina
MG-Br	November, 2000	Brazil	Minas Gerias State
SP-Br	November, 2000	Brazil	São Paulo State
Jam 01, 02, 03, 04	May, 2005	Jamaica	Kingstown
Trin 01, 02, 03, 05	Jul–Aug, 2005	Trinidad	Northwest Trinidad
Trin 04, 10	August, 2005	Trinidad	North Trinidad
Trin 06, 07, 08, 09	August, 2005	Trinidad	South Trinidad
Cochliomyia macellaria	November, 2000	Brazil	São Paulo State

Table 2. Genetic characterization of polymorphic microsatellites in the New World screwworm fly, *Cochliomyia hominivorax*, isolated in this study and previously by Torres *et al*. (2004).

Locus	Primer sequence (5′–3′)	Repeat motif	Annealing temperature, °C	Fragment size range	Clone size	*n*	Alleles, *n*	H_O	H_E
ChEX01*	TTTTATATTCTTGCTTCACA CAAATGAAATTGTTCAAAGT	$(TA)_3$ $(TAT)_3$	50	161–173	153	32	4	0.4063	0.7108
ChEX02*	GCTTCAGATTCTTGTTCAAA ATCCGAAGATGGTCAAAAT	$(AT)_4$	50	186–224	184	31	9	0.7419	0.8387
ChEX03*	GATCGGTTTATGTATTACCAAGA GCGATACTCAGTCGAGAAAA	$(AG)_3$ $(TAAA)_3$	57	218–260	217	31	6	0.4516	0.7800
ChEX04*	GATCCTCGAGTTGACCAATA CCCCATATACCTGGAAGTAA	$(TGTA)_3$	57	136–159	143	29	3	0.7586	0.5305
Ch01†	GCATTATACAGACTCAAACAC GTTTGTCTGGTTATAAGCTT	$(CA)_8$	58	117–143	123	30	7	0.4000	0.7514
Ch05†	CAATCAACACAAACTCACG CACCGTTAGTATCGCATG	$(AC)_{25}$	58	117–137	156	31	8	0.8065	0.7774
Ch09†	ACTATACCGGCCTCACAC TTGTTGTTGTATGAGTGGTG	$(CA)_7CG(CA)_4$	56	104–134	101	25	3	0.2800	0.5331
Ch10†	TGTGGCAACATTTCTTTTG TTGGCAAAAAATGTCTCTG	$(CA)_9(CAAA)_2$ $(CA)_3CAAA$	56	244–270	165	31	4	0.3548	0.6742
Ch11†	TTGCTGCAACATACTGGG TAATTTGTTGGCGGCTTC	$(CA)_{26}$	58	138–168	178	18	6	0.5000	0.7460
Ch12†	ATGTCCATCACTTCAAGCAC TTTGCAGAGGAGTGAGCG	$(AC)_9TCACCC(AC)_5$ $TCACCC(AC)_5$	58	72–88	101	24	8	0.3750	0.4131
Ch14†	TGCTGTTGGGTGTTGTAG TTATGATGACCTTCTCTACG	$(CA)_{11}$	58	178–208	186	31	9	0.7096	0.8519
Ch15†	ATCACAAGAGTAAACATTTC TGTGTGTGTAAGACATGC	$(CA)_2AA(CA)_7$	58	164–218	178	14	12	0.4286	0.8995

*Microsatellite loci isolated in this study.
†Microsatellite loci isolated by Torres *et al*. (2004).

The small sample sizes and problems of sample preservation in this project limited the use of the data for examining population relationships using allele frequencies; sample sizes were in some instances too small to allow accurate estimates of genetic distances. Instead, an alternative approach of using multilocus genotypes as a measure of similarity between individual flies was employed. This method had been employed previously in the study of human evolutionary trees and allowed the construction of trees of individuals that reflected their geographic origin with a high degree of accuracy (Bowcock *et al.*, 1994).

Accordingly, the genetic distance between individual flies, based on a pairwise measure of allele sharing, was calculated with MICROSAT Version 1.5 (Minch *et al.*, 1996), and the neighbour-joining method was used to reconstruct a tree (Fig. 1) from the resulting distance matrix using PHYLIP 3.6 software (Felsenstein, 1995). The stability of tree nodes was assessed by bootstrapping using 1000 resampled datasets. Trees were rooted by comparison with the secondary screwworm, *C. macellaria*, which in previous phylogenetic analyses of the Calliphoridae has been shown to be a suitable sister taxon to *C. hominivorax* (e.g. Stevens, 2003). A second analysis (results not shown) was

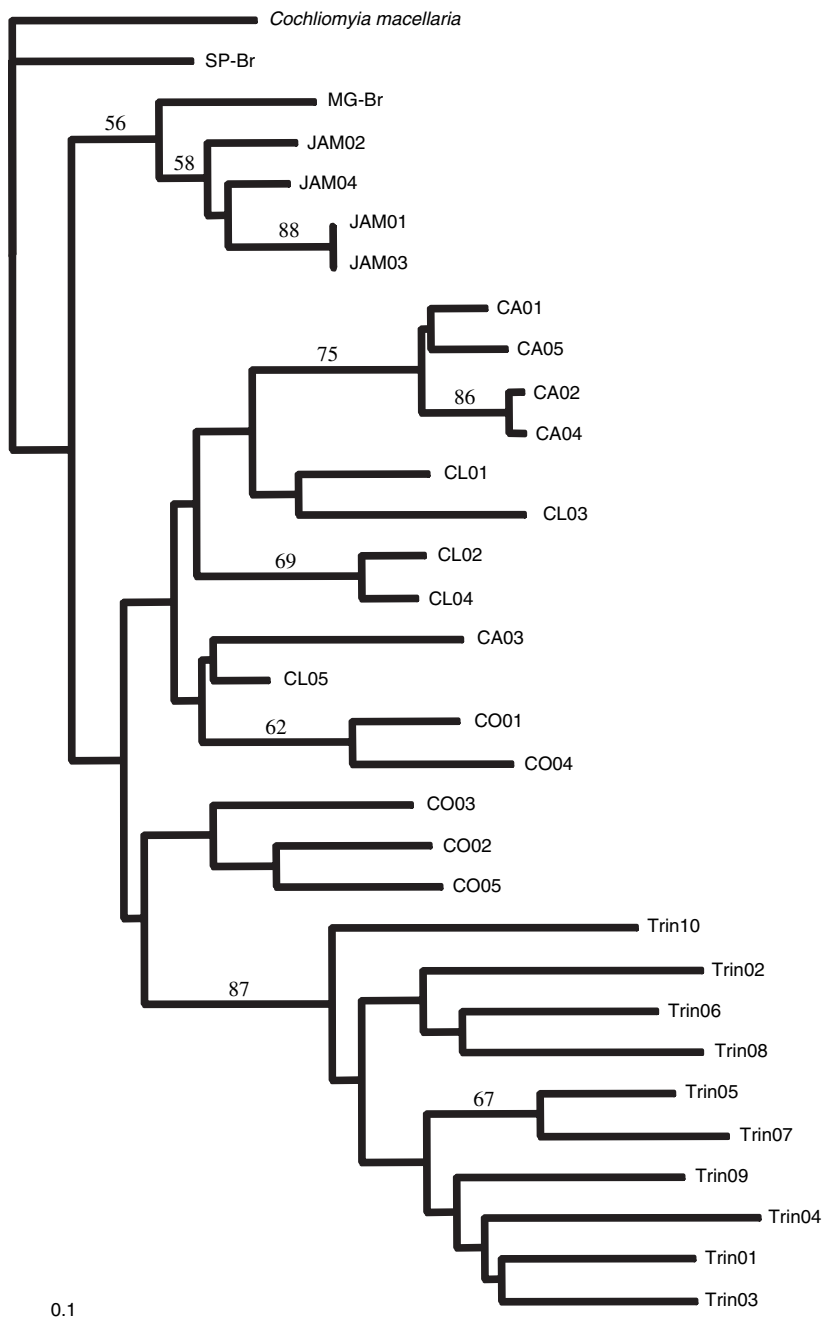

Fig. 1. Neighbour-joining phylogram based on pairwise allele sharing distances, rooted on the secondary screwworm fly, *Cochliomyia macellaria*. Only bootstrap values > 50% are shown.

performed with PAUP 4.0 (Swofford, 2003) by scoring the presence or absence of each allele at each locus in order to construct an overall profile of similarity between specimens. Programme defaults for parsimony-based analysis were used, with 1000 bootstrap replicates. As before, the cladogram was rooted on *C. macellaria.*

Results

Population genetics analysis

In the four microsatellite loci isolated in this study, the number of alleles per locus ranged from three to nine, the observed heterozygosities (H_O) varied from 0.2800 to 0.8065 and the expected heterozygosities (H_E) varied from 0.5305 to 0.8995. Using GENEPOP, all loci demonstrated significant departures from HWE expectations, except Ch11 ($P < 0.05$) and ChEX04 after Bonferroni corrections for simultaneous tests (Rice, 1989). Linkage disequilibrium was detected in 22 pairs of loci ($P < 0.05$, which, after Bonferroni corrections, remained significant in the following pairs of loci: Ch01/Ch10; Ch01/Ch14; Ch01/ChEX01; Ch05/ChEX01; Ch14/ChEX01; Ch05/ChEX02; ChEX01/ChEX02; Ch14/ChEX03; ChEX01/ChEX03 and ChEX02/ChEX03). These results were not unexpected as the samples analysed were unlikely to represent a single inter-breeding population; in the case of heterozygote deficits in tests for HWE they may also reflect the presence of null alleles. Specimens were then divided by country of origin as it was thought that flies from the same region were more likely to represent inter-breeding populations, but because of the small sample sizes from Brazil and Jamaica, tests were carried out only on the largest collections of flies (i.e. those from Trinidad and Uruguay). Subsequently, the number of significant deviations from HWE in the combined sample from Trinidad fell to four loci; however, in the case of Uruguay, 11 loci still showed significant deviation from HWE ($P < 0.05$, no Bonferroni corrections applied). Amplification by PCR with the *C. macellaria* sample was successful in five loci (CH01, CH12, ChEX01, ChEX03 and ChEX04).

Phylogenetic analysis

The phylogram derived using genetic distances between individual flies based on a pairwise measure of allele sharing (Minch *et al.*, 1996) provided support for distinct groupings of flies from Trinidad and Jamaica, with the clade of Trinidad flies receiving relatively strong bootstrap support of 87%. Overall, bootstrap support for a single monophyletic group of flies from Uruguay was not apparent, but relatively high support for smaller groupings within the Uruguayan flies suggested that *C. hominivorax* population structure on the mainland of South America may be more complex than a single inter-breeding unit.

The second analysis, performed using PAUP 4 (Swofford, 2003) by scoring the presence or absence of each allele at each locus, produced a similar tree (cladogram not shown). The resulting parsimony-based tree was very similar to that in Fig. 1. Again, there was strong support (92%) for a distinct Trinidad group; Jamaican flies also grouped together, but support for this grouping (which included a Brazilian specimen) was less strong. As before, flies from Uruguay did not form a single monophyletic clade, but showed a similarly complex pattern to that observed in the phylogram (Fig. 1).

Discussion

Overall, the phylogenetic analyses and tests for departures from HWE suggest that a group of flies, distinct from those on the mainland, exist in Trinidad (where they may represent a single inter-breeding population). Flies from Jamaica may also be distinct, but support for this is less clear-cut. Previous molecular studies on flies from this region support the suggestion that *C. hominivorax* from Jamaica may be genetically distinct (Taylor *et al.*, 1996).

The population structure of *C. hominivorax* on mainland South America was more difficult to describe and no distinct subunits could be clearly defined. A previous study looking at variation in a number of mitochondrial gene markers (Lyra *et al.*, 2005) also found no evidence of subpopulation differentiation in *C. hominivorax* in Uruguay and concluded that Uruguayan *C. hominivorax* form a single panmictic population. However, in the current study there was at least some weak support for groups of flies that generally reflected sampling locations. Significant departures from HWE expectations (specifically deficits of heterozygotes) when data from different sample sites were combined also suggested that *C. hominivorax* from Uruguay are not panmictic and that population structuring may exist between screwworm flies in at least some areas of mainland South America. We propose that the higher level of genetic resolution provided by the use of microsatellite markers in the current study may account for the differences in interpretation of data between this study and that of Lyra *et al.* (2005). It is interesting to speculate that the failure of *C. hominivorax* from Uruguay to conform to expectations of HWE may also reflect complex patterns of extinction and re-colonization at the extreme of the species' range.

The use of the microsatellite loci isolated in this study, together with those from Torres *et al.* (2004), clearly demonstrate the utility of such markers to elucidate patterns of population structure in *C. hominivorax*, a species of considerable veterinary significance. However, larger and more extensive sample collections are clearly required for the reliable assessment of fine-scale genetic structure that is needed as a basis for future control strategies. Concerted effort on sample collection would certainly allow a more thorough exploration of the hypotheses concerning population differentiation in NWSFs of South America and the Caribbean Islands, in particular, the existence of cryptic species. Such work would be all the more conclusive if it were to be carried out in tandem with appropriate ecological and behavioural studies.

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Conflicts of interest

All authors declare no conflicts of interests.

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Phylogenetic analysis of New World screwworm fly, *Cochliomyia hominivorax*, suggests genetic isolation of some Caribbean island populations following colonization from South America

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Abstract. Larval infestations of the New World screwworm (NWS) fly, *Cochliomyia hominivorax*, cause considerable economic losses through the direct mortality and reduced production of livestock. Since the 1950s, NWS populations in North and Central America have been the target of virtually continuous eradication attempts by sterile insect technique (SIT). Nevertheless, in some areas, such as Jamaica, SIT-based control programmes have failed. Reasons for the failure of SIT-based programmes in some locations are unknown, but it is hypothesized that failure may be related to the mating incompatibility between sterile and wild flies or to the existence of sexually incompatible cryptic species. Accordingly, the current research investigates intraspecific phylogenetic relationships and associated biogeographic patterns between NWS populations from the Caribbean and South America, which represent those populations involved in, or earmarked for, forthcoming SIT programmes. Uniquely, this study also includes analyses of two North American samples, collected in Texas in 1933 and 1953 prior to initiation of the SIT-based eradication programme. The study utilizes three nucleotide datasets: elongation factor-1α (nuclear); cytochrome oxidase subunit 1 (mitochondrial), and 12S rRNA (mitochondrial). Phylogenetic analysis of these data, representing populations from across the Caribbean, South America and Texas, indicates sub-structuring of fly populations on several of the larger Caribbean islands, suggesting a period of isolation and/or founder effects following colonization from South America; significantly, our findings do not support a North American origin for Cuban flies. The importance of these findings in the light of proposed SIT programmes in the region is discussed.

Key words. *Cochliomyia hominivorax*, 12S rRNA, Calliphoridae, cytochrome oxidase subunit 1, elongation factor-1α, evolution, New World screwworm fly, sterile insect technique.

Introduction

Since its first use in Florida in 1958, the sterile insect technique (SIT) has successfully been used to eradicate the New World screwworm (NWS) fly, *Cochliomyia hominivorax* (Coquerel), from all of mainland North America and most of Central America (Klassen & Curtis, 2005). Today, *C. hominivorax* has been eradicated as far south as Panama, where an ongoing weekly release of 50 million sterile males close to the border with Colombia has created a sterile fly barrier in Darien Province to prevent the reintroduction of NWS fly from South America (Klassen & Curtis, 2005; Vargas-Terán *et al.*, 2005). *Cochliomyia hominivorax*

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has also been eradicated from the Virgin Islands and Puerto Rico in the Caribbean, and the Jamaican government initiated a SIT programme in 1999. No such programmes have been attempted on Cuba or Hispaniola, leaving, as a consequence, reservoirs for the potential reintroduction of *C. hominivorax* into areas where the fly has been eliminated (Klassen & Curtis, 2005).

However, despite the overwhelming success of the NWS fly SIT programme in the U.S.A. and Central America, since its outset significant differences in the efficacy of the technique have been apparent, with variations in induced egg sterility ranging from 100% to 15%. For example, whereas the first field trials conducted in Florida resulted in almost 100% egg sterility, further field trials using the same southern U.S.A. strain of irradiated flies produced very different results: the release of sterile male flies on an island off the coast of Venezuela resulted in observed egg sterility of just 15% (Klassen & Curtis, 2005). Most recently, a SIT programme in Jamaica in 1999–2005, using flies originating from Mexico, also failed (A. Robinson, International Atomic Energy Agency, personal communication, 2007) and the programme is currently suspended. Reasons for the failure of SIT-based control programmes in some locations are unknown, but it is hypothesized that failure may be related to the mating incompatibility between sterile and wild flies, or to the existence of sexually incompatible cryptic species, as proposed by Richardson *et al.* (1982a, 1982b). (N.B. The existence of cryptic species within *C. hominivorax* remains controversial. See, e.g.: LaChance *et al.* [1982]; Taylor & Peterson [1994].) To investigate these hypotheses, an assessment was undertaken, in collaboration with the International Atomic Energy Agency (IAEA), Vienna, of genetic variation within and between populations of *C. hominivorax* using DNA sequence data from nuclear and mitochondrial (mtDNA) genes to type fly specimens and populations from across South America, the Caribbean and from two, now extinct, southern U.S.A. populations.

Although both nuclear and mitochondrial DNA sequence data have been used to explore population genetic variability in *C. hominivorax*, molecular studies have focused heavily on analysis of mitochondrial markers (e.g. Taylor *et al.* 1996; Azeredo-Espin & Lessinger, 2006). In insects and arthropods in general, the majority of mtDNA-based evolutionary and population studies have frequently used only single protein coding genes from a limited range of those potentially available (Shao & Barker, 2007), with many mtDNA genes being largely disregarded and others receiving only sporadic attention (Caterino *et al.*, 2000). Nevertheless, phylogenetic studies of insects based on mtDNA have been far more frequent than those using nuclear DNA (Lessinger *et al.*, 2000), with cytochrome oxidase subunit I (COI) being among the most commonly used (Zhang & Hewitt, 1997). In addition to practical and operational considerations (which certainly account for part of its continued popularity), mtDNA also appears particularly useful for inferring relationships between closely related and recently diverged species, a result of the apparently more rapid lineage arrangement, compared with nuclear DNA (Wells *et al.*, 2007).

However, in previous investigations of the mitochondrial genome of *C. hominivorax*, studies have found COI to be among the most conserved gene sequences among several dipteran species (Lessinger *et al.*, 2000). Accordingly, in addition to COI, this study also utilized sequence data from a second mitochondrial gene, 12S, and a nuclear gene, elongation factor-1α (EF-1α). All three genes have been widely used as phylogenetic markers in insects (Caterino *et al.*, 2000); EF-1α is almost the only single-copy nuclear gene to have been used as a molecular marker in such studies (Caterino *et al.*, 2000).

As outlined above, genetic differences between populations and the possible existence of reduced mating success between divergent populations and/or the existence of sexually incompatible cryptic species (as proposed by Richardson *et al.*, 1982a, 1982b) may be critical factors in determining the ultimate success of screwworm eradication programmes.

Materials and methods

Samples and DNA extraction

Details of all specimens analysed in this study are given in Table 1. Briefly, specimens of *C. hominivorax* were obtained from various locations across South America and the Caribbean, including Brazil, Colombia, Cuba, the Dominican Republic, Ecuador, Jamaica, Peru, Venezuela, Trinidad and Uruguay (Fig. 1). Two potentially key islands, Cuba and the Dominican Republic, were sampled more intensively (Fig. 2). Two Calliphoridae outgroup taxa from the closely related subfamily Luciliinae (Stevens, 2003) were used.

All specimens were stored in 100% ethanol at 4 °C, except the Uruguayan samples, which arrived as DNA extracts direct from the University of Campinas, Brazil, and were re-hydrated with distilled water for a minimum of 1 h prior to polymerase chain reaction (PCR) amplification. Where adult flies specimens were available, thoracic flight muscle tissues were extracted, avoiding contamination from ingested protein, parasites or eggs (Stevens, 2003), and the remainder of the adult fly kept as a voucher specimen. The entire specimen of second- and third-stage larvae was used. DNA extraction was carried out using a salt extraction method (Aljanabi & Martinez, 1997). Successfully extracted DNA samples were stored at − 20 °C until analysis.

'*Historical*' *North American samples and DNA extraction*

Two specimens belonging to historical Texan populations of *C. hominivorax*, on loan from the collection held at the Natural History Museum, London, were also sampled. DNA extractions from these specimens, which had been dried and pinned in 1933 and 1953, were carried out using a DNeasy® Blood & Tissue Kit (Qiagen Ltd., Crawley, U.K.). DNA extraction was performed by removing thoracic flight muscle from the ventral side of the thorax, leaving as much of the taxonomically important morphology intact as possible.

To ensure that DNA obtained was that of the historic specimen and not a result of contamination from contemporary *C. hominivorax* material, all molecular work for the historical samples was carried out in a separate 'clean' room, using a

Table 1. *Cochliomyia hominivorax* specimen details.

Sample site location		Code	Capture date	Life stage	Accession number		
					COI	12S	EF-1α
Cuba, east	Las Tunas	A	Oct 2005	L3	FM867778	FM866410	FM867795
					FM867779	FM866409	FM867796
	Santiago de Cuba	B	Oct 2005	L3	FM867776	FM867706	FM867797
					FM867777	FM866411	FM867798
Cuba, west	Pinar del Río	C	Nov 2005	L3	FM867769	FM867714	FM867805
					FM867768	FM867713	FM867806
Cuba, centre	Ciego de Ávila	D	Oct 2005	L3	FM867780	FM866408	FM867794
					FM867781	FM866407	FM867793
Cuba, Isla de la Juventud	Ciro Redondo	E	Nov 2005	L3	FM867774	FM867708	FM867799
					FM867775	FM867707	FM867800
	Micro	F	Nov 2005	L2, L3	FM867771	FM867712	FM867803
					FM867770	FM867711	FM867804
	Melvis	G	Nov 2005	L2, L3	FM867773	FM867709	FM867802
					FM867772	FM867710	FM867801
Dominican Republic, northwest	Monte Cristi	H	Oct 2005	A	FM867761	FM867721	FM867811
					FM867760	FM867722	FM867812
	Duarte	I	Oct 2005	L3	FM867762	FM867720	FM867814
					FM867763	FM867719	FM867813
Dominican Republic, south	Neiba	J	Oct 2005	L2, L3	FM867759	FM867724	FM867818
					FM867758	FM867723	FM867817
	Polo	K	Oct 2005	L3	FM867756	FM867726	FM867815
					FM867757	FM867725	FM867816
Dominican Republic, centre	Santo Domingo	L	Oct 2005	L2, L3	FM867764	FM867718	FM867809
					FM867765	FM867717	FM867810
	Nigua	M	Sep 2005	A	FM867767	FM867716	FM867807
					FM867766	FM867715	FM867808
Brazil	Minas Gerais	–	Nov 2000	A	FM867785	FM866402	FM867788
	São Paulo				FM867784	FM866403	FM867789
Jamaica		–	Jan 2005	A	FM867752	FM867731	FM867823
					FM867753	FM867730	FM867822
Trinidad		–	Aug 2005	L3	FM867746	FM867736	FM867829
					FM867747	FM867737	FM867828
Venezuela/Colombia border		–	May 2005	L3	FM867743	FM867740	FM867832
					FM867742	FM867741	FM867833
Uruguay		–	–	DNA extract	FM867744	FM867739	FM867830
					FM867745	FM867738	FM867831
Colombia	Chigorodo	–	Mar/Apr 2007	L2, L3	FM867783	FM866404	FM867790
	Choco				FM867782	FM866405	FM867791
	Turbo				FM867787	FM866406	FM867792
Ecuador	Napo	–	Jun 2007	L2, L3	FM867786	FM867729	FM867821
	Esmeraldas				FM867755	FM867727	FM867819
	Guayas				FM867754	FM867728	FM867820
Peru	Chiclayo	–	May 2007	L2, L3	FM867749	FM867734	FM867826
	Tambopata				FM867748	FM867735	FM867827
U.S.A.	Texas	–	1953	A	FM867751	FM867732	FM867825
			1933		FM867750	FM867733	FM867824

L2, first-stage larva; L3, second-stage larva; A, adult.

separate set of pipettes, etc. Extracted DNA was then stored at − 20 °C until analysis.

DNA sequences

Each DNA extraction was subjected to PCR amplification of an approximately 680-bp region of the mitochondrial COI, an approximately 780-bp region of EF-1α and a 600–700-bp region of the mitochondrial 12S gene.

Using published primers (Otranto *et al.*, 2005), COI sequence data were obtained from approximately half of the Cuban and Dominican Republic samples. Accordingly, a new set of COI primers were designed (two forward, two reverse; Table 2) in an attempt to amplify the remaining templates. Initial attempts to utilize more generic arthropod EF-1α primers (provided by

Fig. 1. Sampling locations within South and Central America.

J. K. Moulton, personal communication, 2004) proved unsuccessful and a new set of primers specific for *C. hominivorax* were designed. All 12S sequences were amplified using published primers (Lessinger & Azeredo-Espin, 2000), although successful PCR product sequencing was consistently only obtained for the reverse primer (SR-J-14233). At least two sequences were amplified for each gene from each of the locations (Table 1).

Standard PCR amplification involved 1 μL template DNA, 2.5 μL reaction buffer (160 mM $[NH_4]_2SO_4$, 670 mM Tris-HCI [pH 8.8 at 25 °C], 0.1% Tween-20), 1.25 μL $MgCl_2$ (50 mM), 2.5 μL dNTP mix (giving a final concentration of 0.2 mM), 0.5 μL forward and reverse external primers (10 mM), 1 μL of BIOTAQ™ Red DNA polymerase, and sterile H_2O to a final volume of 25 μL. All amplifications were carried out in Hybaid thermal cycler machines (Thermo Hybaid, Ashford, U.K.). COI reactions using the primers of Otranto *et al.* (2005) included an initial denaturation step of 94 °C for 5 min, followed by 35 cycles of 95 °C for 40 s, 48 °C for 30 s and 72 °C for 1 min, and a final elongation step of 72 °C for 10 min (Yu *et al.*, 2005). Reactions using the new screwworm-specific COI and EF-1α primers used modifications of the same generic PCR protocol (Hoelzel & Green, 1992). The new COI primer reactions employed a protocol whereby an initial denaturation step of 95 °C for 5 min was followed by 35 cycles of 55 °C for 1 min, 72 °C for 1 min, 94 °C for 30 s and 55 °C for 1 min, and a final elongation step of 72 °C for 10 min, modified from generic PCR practice. The EF-1α reactions used an initial denaturation step of 95 °C for 5 min, followed by 35 cycles of 58 °C for 1 min, 72 °C for 1 min, 94 °C for 30 s and 55 °C for 1 min, with a final elongation step of 72 °C for 10 min. All 12S reactions were carried out using an initial denaturation step of 94 °C for 2 min, followed by 30 cycles of 94 °C for 30 s, 58 °C for 1 min and 60 °C for 2 min, with a final elongation step of 60 °C for 10 min, (Lessinger & Azeredo-Espin, 2000). All PCR reactions using 'historical' North American material were carried out in a separate, clean room to avoid possible contamination.

The resulting PCR products were separated by gel electrophoresis, with appropriate bands cut out and purified using a MinElute® PCR purification kit (Qiagen UK), according to the manufacturer's protocol. Purified PCR products were sequenced on an ABI 3730 automated sequencer (Applied Biosystems Inc., Carlsbad, CA, U.S.A.), using a commercial sequencing facility (Lark Technologies, Inc., Saffron, Walden, UK).

Sequence alignment and phylogenetic analysis

Sequence fragments were checked for quality and edited manually before being assembled into a single consensus

Fig. 2. Sampling locations within Cuba (1, Ciro Redondo; 2, Micro; 3, Melvis; 4, Pinar del Río; 5, Ciego de Ávila; 6, Las Tunas; 7, Santiago de Cuba) and Dominican Republic (8, Monte Cristi; 9, Neiba; 10, Polo; 11, Duarte; 12, Santo Domingo; 13, Nigua).

Table 2. Primer details.

	Forward primer	Sequence 5′–3′	Reverse primer	Sequence 5′–3′
EF-1α				
External amplification	B1	CCCATYTCCGGHTGGCACGG	C1	GTCTCATGTCACGDACRGCG
Internal amplification	F. Int	GGTGGYATCGGHACAGTACC	Int. R1	CAGCRAMGTACCCACGACGC
COI				
External amplification*	COI F1	ATAGACGTTGAYACWCGAGC	COI R1	ATCCATTGCACTAATCTGCC
Internal amplification†	COI Int.F1	GTATTTGCTATTATRGCCGG	COI Int.R4	AATCCTAARAAATGTTGGGG

*Position on *Cochliomyia hominivorax* mtDNA genome (AF260826) 3492–4171 bp.
†Position on *Cochliomyia hominivorax* mtDNA genome (AF260826) 3759–3895 bp.

sequence, using AutoAssembler 2.0 (Applied Biosystems, Inc.), from which the consensus sequence was exported. Sequences were then aligned using ClustalX (Thompson *et al.*, 1997), with final adjustments performed by eye. Phylogenetic analyses were carried out using PAUP, (Swofford, 1998), with both maximum parsimony (MP) and maximum likelihood (ML) criteria being used to infer trees for individual gene datasets and a combined dataset.

Parsimony tree inference was employed using an equal weighting scheme, gaps treated as missing data, tree bisection and reconnection (TBR) swapping, and random sequence addition, with 1000 replicates per search. The parsimony-based partition homogeneity test (incongruence length difference [ILD] test) (Farris *et al.*, 1995) was used to evaluate the phylogenetic congruence between the genes and to verify whether the three datasets were combinable.

For likelihood analyses, parameters produced from a series of nested hierarchical likelihood-ratio tests using MODELTEST Version 3.06, (Posada & Crandall, 1998), with heuristic searching (for 100 000 rearrangements), TBR branch swapping and random sequence addition with 10 repeats, were used to implement the most appropriate model of evolution for each of the three genes and the combined dataset separately. Node support for all tree topologies was assessed using bootstrap analysis, using 100 replicates for ML analyses (restricted for computational time limits) and 1000 replicates for all MP analyses, with additional Bremer decay indices (Bremer, 1988) calculated on all parsimony trees.

When differences between taxa are very small, a reduction in the accuracy of algorithmic-based distance methods can result in incorrect tree topology. By comparison, methods based on optimality criteria, such as parsimony and ML, provide the advantage of choosing one tree over another based on a mathematical ranking system, whereas algorithmic methods cluster taxa according to the conventions of the particular algorithm being used, although even then the best tree may not be found (Swofford & Sullivan, 2003). Parsimony has also been shown to be valuable under circumstances where rates of evolution are low (Swofford & Sullivan, 2003), such as between gene sequences from populations of the same species. Accordingly, data were analysed by both parsimony and ML methods, with detailed analysis of inter-gene phylogenetic congruence and levels of relative support for clades performed using parsimony-based methods.

Results

Phylogenetic analysis: EF-1α

The EF-1α sequence dataset consisted of 743 aligned nucleotide sites, of which 50 were found to be parsimony informative. Parsimony analysis of the EF-1α data found a large number of most-parsimonious trees (MPTs) (L = 101). A strict consensus tree resulted in a loss of all resolution within the Caribbean and South American NWS taxa, but grouped together both U.S.A. samples, albeit with no node support.

The best-fit ML model for the EF-1α dataset was found to be a Hasegawa–Kishino–Yano (HKY) (Hasegawa *et al.*, 1985), including invariable sites (+I), and rate variation among sites (+G) (base frequencies = [A 0.2339; C 0.3010; G 0.2259]; number of substitution types = 2; T ratio = 1.6875; rate = gamma [rate variation]; shape = 0.8377; proportion invariant sites = 0.6982).

Two ML trees were found for the EF-1α dataset (not shown) and node support was evaluated using bootstrap values. Strong support for monophyly of the NWS samples was found, although intraspecific bootstrap support was very low.

Phylogenetic analysis: 12S

The 12S sequence data contained 1239 characters, of which just 53 were parsimony informative sites. A strict consensus tree of MPTs (L = 409) resulted in a lack of meaningful resolution within the NWS taxa.

The best-fit ML model for the 12S dataset was found to be Felsenstein 81 (F81) (Felsenstein, 1981), including rate variation among sites (+G) (base frequencies = [A 0.4230; C 0.1164; G 0.0689]; number of substitution types = 1; rate = gamma; shape = 0.6480; proportion invariant sites = 0).

The ML analysis found a single tree which displayed a lack of resolution for the South American and Caribbean samples, but grouped the two U.S.A. samples together, with a strong bootstrap value of 99%.

Phylogenetic analysis: COI

The COI sequence data, although the smallest dataset, contained 78 parsimony informative sites. Moreover, by contrast with the EF-1α and 12S datasets, a strict consensus of all MPTs (L = 274) did conserve some resolution within the screwworm

clade (Fig. 3), with a distinct 10-taxon 'Cuban clade' (with the exception of one Brazilian sample) preserved, although with relatively low bootstrap support.

The best-fit ML model for the COI dataset was found to be a transitional model (TIM), including invariable sites (+I), and rate variation among sites (+G) (base frequencies = [A 0.3083; C 0.1625; G 0.1511]; number of substitution types = 6 [generalized time-reversible]; r matrix = [A–C 1.0000; A–G 3.3693; A–T 2.1386; C–G 2.1386; C–T 7.5244]; rate = gamma; shape = 0.7496; proportion invariant sites = 0.4459).

Two ML trees were found, again with the noticeable Cuban clade in both topologies. Mapping bootstrap values on to one of the ML trees revealed strong support for the Cuban clade (Fig. 3).

Combined dataset

The parsimony-based ILD test, performed on a partitioned combined three-gene (COI, 12S and EF-1α) dataset, revealed a significant difference ($P < 0.01$, $n = 1$) in topology between the genealogies. This suggests the data partitions have significantly different signals under a 95% significance level, and as such are not compatible. However, in an attempt to identify clades which are congruent between datasets, a combined dataset analysis was performed.

Maximum parsimony analysis of the combined data resulted in MPTs with a length of 924, with a strict consensus preserving an even larger 12-taxon Cuban clade, including the Minas Gerais taxon from Brazil (not shown); this Cuban grouping received high Bremer support in the combined dataset. Throughout the combined tree, regions of apparent incongruence between the genealogies (as implied by the ILD test) are identified by negative Bremer support values, indicating where the data partition did not support a particular node. From this it is possible to see that the Cuban clade is not one of the areas of conflict between genealogies, although some of the topology *within* this clade does receive negative Bremer values. The large number of MPTs found in all datasets (combined and single-gene) reflect the largely uniform nature and lack of resolving power of these sequence data for *C. hominivorax*.

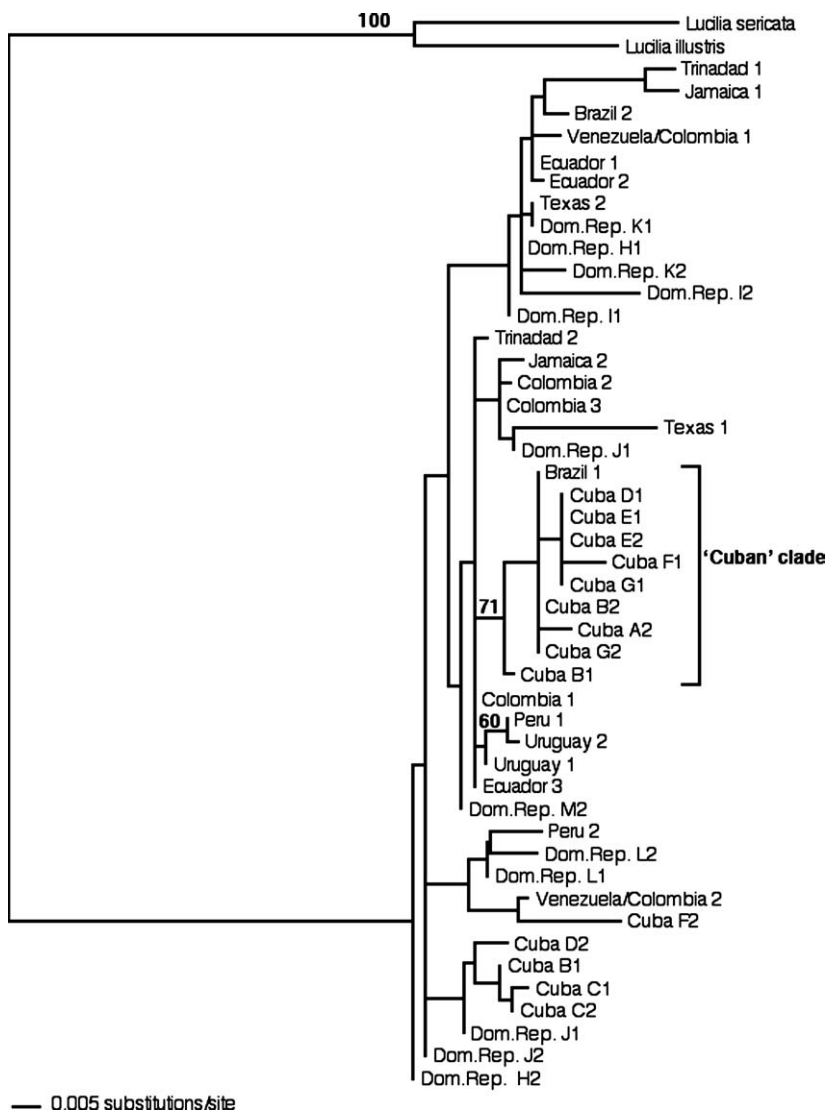

Fig. 3. Rooted phylogram constructed by maximum likelihood analysis of COI sequence data for *Cochliomyia hominivorax*. Bootstrap values are shown at each node with > 50% bootstrap support. Two *Lucilia* species were used as the outgroup.

The best-fit ML model was found to be a generalized time-reversible (GTR) (Tavaré, 1986) including invariable sites (+I), and rate variation among sites (+G) (base frequencies = [A 0.3418; C 0.1735; G 0.1361]; number of substitution types = 6; r matrix = [A–C 1.9887; A–G 2.7059; A–T 2.7872; C–G 1.0890; C–T 4.6986]; rate = gamma; shape = 0.6723; proportion invariant sites = 0.5283).

Conversely, node support on the single ML tree, found during the likelihood analysis, (Fig. 4), resulted in a loss of support for the original 10-taxon Cuba grouping. However, grouping of the samples from Texas away from the remaining South American and Caribbean samples is very strongly supported (Fig. 4), suggesting a clear North-South genetic divide.

Discussion

Regional genetic variability of NWS fly populations

Both COI and multi-gene parsimony-based genealogies group populations from Cuba together. Indeed, the Cuban clade was the only major grouping within the South American and Caribbean samples to be supported. The majority of the Dominican Republic specimens were generally also found to group together in the ML trees (not shown), albeit without node support, signifying that specimens from geographically diverse populations appear to show a greater affinity for one another than they do for flies from other locations. Significantly, however, Dominican Republic populations appeared to be more closely related to flies from other areas of South America than they did to populations from Cuba.

One notable anomaly within the Cuban clade is the presence of a Brazilian sample (Minas Gerais). Whereas the remaining non-Cuban or non-Dominican Republic taxa appear indiscriminately placed within the tree, the Minas Gerais sample appears strongly associated with Cuban screwworm populations. This may be the result of one of a number of factors, including: historical lineage sorting; transportation of fly-infested livestock, or, in view of the low number of informative characters identified, stochastic convergent evolution. In addition, the possibility of a laboratory error cannot be ruled out.

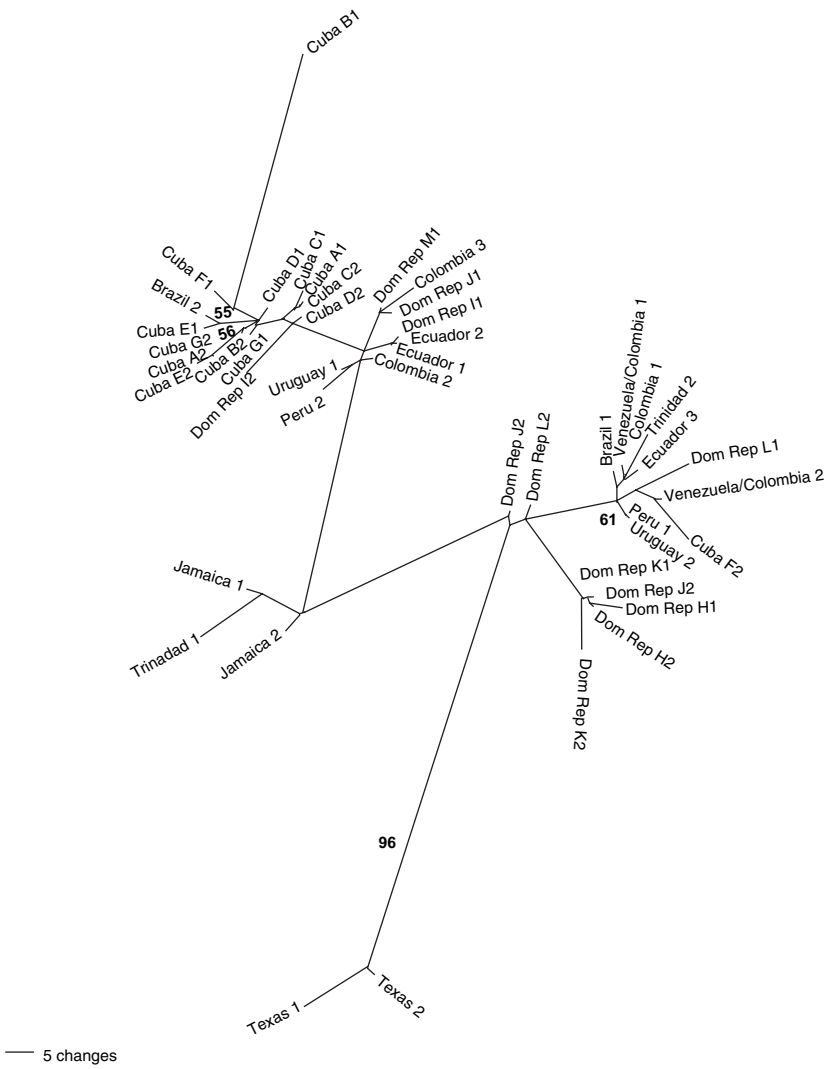

Fig. 4. Unrooted phylogram constructed by maximum likelihood analysis of the combined three-gene (COI, EF-1α, rRNA 12S) sequence dataset for *Cochliomyia hominivorax*. Bootstrap values are shown at nodes with > 50% bootstrap support.

Possibly the most striking relationship found in this study was that of the strongly supported separation of the two Texan samples from the South American and Caribbean samples, suggesting a possible north/south divide. Previously, a nucleotide divergence of $> 2\%$ was observed between Jamaican and Mexican samples (Roehrdanz, 1989) and it has been suggested that such a result supports a north/south differentiation, with Jamaican populations originally colonized from South America (Azeredo-Espin & Lessinger, 2006).

A previous mtDNA-based study by Taylor *et al.* (1996), which incorporated the findings of Roehrdanz (1989), compared *C. hominivorax* samples from around the Caribbean, Central and South America, and showed haplotypes to subdivide into three lineages. The lineages identified by Taylor *et al.* (1996) were 'North and Central America' (which contained Costa Rican and Cuban samples, the only significant clade in their cladogram, with bootstrap support of 90%), 'South America', and 'Jamaica' (with the Jamaican and one of the Trinidad samples sharing a comparatively different haplotype of their own), although total geographic conformity was not supported and only some support for partial phylogeographic structuring was present. Although samples from the Caribbean in Taylor *et al.*'s (1996) study were limited, the authors noted that the single Cuban sample appeared to be more closely related to Central American populations, whereas the two Dominican Republic samples grouped with those from South America, suggesting the possibility of multiple origins of *C. hominivorax* throughout the Caribbean.

Significantly, although the results of our study provide support for a separate Cuban clade, they do not link this Cuban group with a North American lineage, as previously hypothesized by Taylor *et al.* (1996). Furthermore, although the distinctiveness of the majority of Cuban samples may be indicative of a sustained period of isolation and/or founder effects, their placement relatively close to other Caribbean and South American samples and well apart from the Texan specimens (Fig. 4) suggests that, on the contrary, Cuban *C. hominivorax* may have originated from a southern lineage, which colonized the Caribbean islands from South America.

Mitochondrial vs. nuclear genealogy

Population studies using mtDNA have commonly used sequence data from a single gene, with COI one of the most commonly used (Liu *et al.*, 2006). It is now widely recognized, however, that the use of single-gene phylogenies (especially mtDNA gene phylogenies) to represent overall species evolution can frequently be misleading (e.g. Stevens & Wall, 1996; Stevens *et al.*, 2002; Wells *et al.*, 2007) and multi-gene phylogenies are now recognized as essential. Where intraspecific relationships based on single-gene sequences are sometimes poorly resolved (Shao & Barker, 2007), multiple concatenated nucleotide sequences may contain sufficient genetic differentiation to resolve such relationships. Similarly, combined molecular and morphological studies have identified the possible existence of distinct geographical 'races' in the Old World screwworm fly, *Chrysomya bezziana*, (Hall *et al.*, 2001).

In the current study, the use of a multi-gene analysis was found to both increase the size of the Cuban clade, previously reflected in only one of the three single-gene phylogenies (COI), and to increase support for the grouping of Cuban samples. This suggests that although the phylogenetic signal within the COI dataset provides most of the discriminatory power to identify a Cuban clade, the two other genes also offer some, albeit weak, support for such a grouping. As noted, however, a congruence test (ILD) performed on the partitioned three-gene dataset (COI, 12S and EF-1α) indicated significant incongruence between the overall genealogies. This potentially lends further support to the affinity between the Cuban samples because, even with significant conflict between genealogies, a Cuban grouping is still preserved.

When comparing the three genealogies, it seems the mitochondrial COI sequence data reveal a greater degree of geographic differentiation within *C. hominivorax* than either the mitochondrial 12S or the nuclear EF-1α data. However, although the phylogenetic signal in the COI data may be explained as the result of the uniparental inheritance and non-recombining nature of the mitochondrial gene, the lack of similar signal in the 12S data suggests that some regions of the mtDNA are experiencing faster rates of evolution than others. Further mtDNA-based phylogenetic analyses of *C. hominivorax* will provide an interesting insight into whether such intraspecific relationships are universal throughout the *C. hominivorax* mitochondrial genome. One suggested explanation for such a discrepancy between phylogeographic patterns between the nuclear and mitochondrial molecular markers refers to the increased movement of livestock between and within North, Central and South America over the past 400–500 years (Taylor *et al.*, 1996). For example, since the transportation of livestock began to increase significantly from the 16th century onwards, populations of *C. hominivorax* that were formerly isolated or that experienced only limited gene flow have had considerably more opportunity to mix (Taylor *et al.*, 1996; Azeredo-Espin & Lessinger, 2006).

Of the Caribbean islands, only the Virgin Islands and Puerto Rico have successfully eradicated *C. hominivorax* to date, although the Jamaican government initiated a SIT programme in 1999. However, no such programmes have yet been undertaken on Cuba or Hispaniola, with the result that these islands harbour reservoirs of *C. hominivorax*; this could potentially lead to the reintroduction of the fly to regions across the Caribbean and Central America that are currently screwworm-free (Klassen & Curtis, 2005).

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Conflicts of interest

All authors declare no conflicts of interests.

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Population genetics of New World screwworm from the Caribbean: insights from microsatellite data

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Abstract. Diseases affecting livestock can have a significant impact on animal productivity and on trade of live animals, meat and other animal products, which, consequently, affects the overall process of economic development. The New World screwworm, *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae), is an important parasitic insect pest in Neotropical regions. This species has been successfully eradicated from North and most of Central America by the sterile insect technique, but continues to affect the development of the livestock sector in most Caribbean economies. Here, we provide some insight into the patterns of genetic variation and structure and gene flow of *C. hominivorax* populations from the Caribbean. Analysis of populations from 10 geographical sites in four islands revealed a moderate genetic variability within the populations. Surprisingly, a high population differentiation was found even in intra-island comparisons between populations. This observation can reflect either highly structured populations resulting from a lack of gene flow or a source–sink dynamic. Our study also suggests that New World screwworm populations can recover very rapidly from population contractions. This is valuable information that should be required prior to any investment in large-scale efforts aiming at controlling this pest.

Key words. Microsatellites, myiasis, population genetics, screwworm, source–sink dynamics, sterile insect technique.

Introduction

The increasing demand for animal food products has triggered a rapid development of the livestock sector in many Latin American economies (United Nations Food & Agriculture Organization [FAO], 2006). The Caribbean region, by contrast, exhibits relatively low production and productivity indices when compared with its potential. One of the major constraints to the development of a competitive animal production system in the Caribbean is animal health (Bundy & Grey, 1982).

Parasitic diseases are an undeniable threat to the livestock sector in the tropical region, but more so in the Caribbean, where the majority of animals are kept in backyard smallholder systems that are difficult to reach with most veterinary services. Among the most important parasitic pests in the West Indies is the New World screwworm (NWS), *Cochliomyia hominivorax* (Coquerel). New World screwworm myiasis occurs when the larval stage of the fly infests tissues of warm-blooded vertebrates. This causes significant reductions in the quality of leather and in milk and meat production. This insect pest also represents a serious public health problem in the Caribbean region, where screwworm infestations in humans are frequently reported (Vargas-Terán *et al.*, 2005).

Historically, *C. hominivorax* was widely distributed from the southern U.S.A. to central Argentina. However, the species has been successfully eradicated from North and most of Central America by the sterile insect technique (SIT) (Wyss, 2000), but it maintains a high rate of incidence in Caribbean countries such as Cuba, the Dominican Republic, Jamaica, Haiti, and Trinidad and Tobago (Vargas-Terán *et al.*, 2005). To promote sustainable

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development of the animal production sector in the West Indian economies and to prevent (re-)invasions into screwworm-free areas, an international effort is underway to establish suppression projects and evaluate strategies for NWS eradication. This involves, *inter alia*, collecting data on the damage and costs associated with control and on the distribution and density of the fly in this region. In this context, knowledge of the genetic variability and structure of NWS populations is important to supporting and improving the application of techniques to eradicate target pest populations. Important issues such as the existence of reproductive isolation and the extent and potential routes of gene flow can be addressed by using modern genetics techniques coupled with adequate sampling of natural populations.

In the past few years, genetic markers have been used to characterize the genetic variability and structure of NWS field populations. Krafsur & Whitten (1993) examined isozyme loci in 11 Mexican populations and reported that screwworm populations in Mexico belonged to a single panmitic population. Taylor *et al.* (1996) also used isozyme loci to study two Brazilian populations and compared the results with previous data from Costa Rica (Taylor & Peterson, 1994) and partial data from Mexico (Krafsur & Whitten, 1993), also reporting that NWS represented a single panmitic population. By contrast, subsequent analyses of Brazilian populations, using different types of molecular markers in both the mitochondrial and nuclear genomes, suggested a reduced gene flow between interconnecting structured populations (Infante-Vargas & Azeredo-Espin, 1995; Infante-Malachias *et al.*, 1999; Azeredo-Espin & Lessinger, 2006).

One possible explanation for the discrepancies among the different data is that different levels of sub-structuring were present in different locations. However, it is clear that the available information is insufficient to infer patterns of genetic variation and structure among NWS populations across their current geographical range.

Among the several classes of molecular markers, microsatellite loci stand out as co-dominant markers, with a high number of alleles per locus, high polymorphism and a high expected heterozygosity value. Because of these features, microsatellites have been extremely useful for investigating issues regarding population structure, gene flow and mating systems, even in populations which have low levels of allozyme and mitochondrial gene variation. Microsatellites, or simple sequence repeats (SSRs), are short sequences made up of a single motif with no more than six base pairs that are tandemly repeated (Goldstein & Schlötterer, 1999). They are found in large numbers and are relatively evenly spaced throughout the genome of eukaryotic organisms analysed to date.

The recent isolation and characterization of polymorphic microsatellite markers for *C. hominivorax* (Torres *et al.*, 2004; Torres & Azeredo-Espin, 2005) enables investigation into the genetic variability and population structure of this pest across its current geographical distribution. Here, the following questions are addressed: (a) What is the extent of genetic diversity in NWS populations in the Caribbean islands? (b) How is the variability between different geographical populations structured? (c) What are the possible implications of these results in the design and implementation of control strategies for this important livestock pest?

Materials and methods

Fly samples

New World screwworm (*C. hominivorax*) samples were obtained from 10 distinct geographic locations (Fig. 1, Table 1). Screwworm larvae were collected from infested wounds in sheep, cattle, dogs and pigs. One sample from Maracaibo, Venezuela was also included in the analysis to allow comparisons of the variability levels between islands and continental samples. Larvae were fixed in 100% ethanol and genomic DNA was extracted from individual larvae using the phenol/chloroform procedure (Infante-Vargas & Azeredo-Espin, 1995). Analysis of related individuals was avoided by choosing wounds in different animals and/or farms and by classifying larvae from the same wound by instar.

Microsatellite analysis

Twelve previously characterized microsatellite markers (Torres *et al.*, 2004; Torres & Azeredo-Espin, 2005) were used in this study. Polymerase chain reaction (PCR) amplifications were performed in a PT-100 thermal cycler (MJ Research, Inc., Waltham, MA, U.S.A.), with an initial denaturing step of 3 min at 96 °C, 35 cycles of 45 s at 94 °C, a primer-specific annealing temperature (Torres *et al.*, 2004; Torres & Azeredo-Espin, 2005), and 1 min at 72 °C, followed by a final 30-min extension at 72 °C. All PCR processes were performed in final reaction volumes of 13 μL containing 5–10 ng DNA template, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 mg bovine serum albumin/mL, 20 μM of each dNTP, 0.6 nM of each primer, and 1 unit of *Taq* DNA polymerase (Invitrogen, Inc., Carlsbad, CA, U.S.A.). The PCR products were analysed on denaturing 6% polyacrylamide gels in a sequencing electrophoresis apparatus and the alleles were visualized using a standard silver staining protocol. PCR products of one cloned allele for each locus (using 1 : 1000 dilution of plasmid DNAs) and the 10-bp DNA ladder (Invitrogen, Inc.) were used as size references. Products of each newly identified allele were also included in the subsequent electrophoresis to avoid misidentification of alleles.

Data analyses

The number and frequency of alleles, the allele size range and the observed (H_O) and unbiased expected (H_E) heterozygosities under Hardy–Weinberg equilibrium were determined per locus for each location. Allelic richness, which corrects the observed number of alleles for differences in sample sizes, was computed using MICROSATELLITE ANALYSER (MSA) Version 4.05 (Dieringer & Schlötterer, 2003). MICROCHECKER Version 2.2.0 (Van Oosterhout *et al.*, 2003) was used to test for technical artefacts, such as null alleles, stuttering and large allele dropout.

Each locus and population was tested for deviations from Hardy–Weinberg equilibrium expectations using exact tests as implemented in GENEPOP (Raymond & Rousset, 1995). Genotypic linkage disequilibrium among all pairs of loci within each site was investigated using Fisher's exact test as implemented

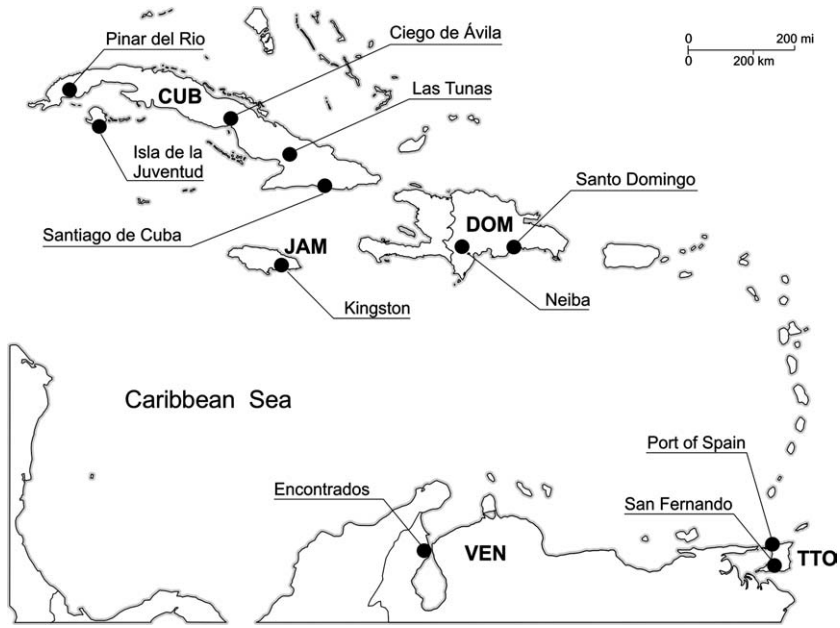

Fig. 1. Collection sites for New World screwworm samples. CUB, Cuba; DOM, Dominican Republic; JAM, Jamaica; TTO, Trinidad and Tobago; VEN, Venezuela.

in GENEPOP. An unbiased estimate of the exact probability was obtained using the Markov chain algorithm of Guo & Thompson (1992). To test for differences in genetic variability, non-parametric Wilcoxon's signed rank tests were performed on differences in the heterozygosity and allelic richness of geographical population pairs.

Recent reductions in population size were tested using the software BOTTLENECK (Cornuet & Luikart, 1996; Piry *et al.*, 1999), under the infinite alleles model (IAM), the stepwise mutation model (SMM) and the two-phase mutation model (TPM) under 30% multi-step changes.

To evaluate the level of differentiation between NWS populations, unbiased estimates of F_{ST}, θ, were calculated using the FSTAT computer program (Goudet, 1995). The significance of pairwise F_{ST} estimates was tested by permuting genotypes among populations (Goudet *et al.*, 1996). Population structure among islands was investigated either by analysing each sampled location independently or by pooling insular samples (the two sub-locations in Trinidad and Tobago and the five sub-locations in Cuba). Sequential Bonferroni corrections (Rice, 1989) were applied for multiple tests performed simultaneously.

Population sub-structure was also inferred using a Bayesian clustering method implemented in *structure* Version 2.2 (Pritchard *et al.*, 2000). Posterior probabilities were calculated assuming *K* from 1 to 10 using a burn-in length of 100 000 and 500 000 Markov chain Monte Carlo replications. Three replicates for each *K* were compared to verify the consistency of the estimated likelihood of the data.

Results

Microsatellite variation

The number of alleles, and the expected and observed heterozygosity per locus and per population are given in Table 2. Analysis of 226 NWS genotypes revealed a moderate to high degree of polymorphism across the seven sampling locations.

Table 1. Samples of *Cochliomyia hominivorax* analysed in this study.

Collection site (abbreviation)	Collection date	*n*	Latitude	Longitude
Encontrados, Venezuela (Encon VEN)	June 2006	30	09°02′ N	72°13′ W
Port of Spain, Trinidad and Tobago (PSpain TTO)	July–August 2005	17	10°40′ N	61°28′ W
San Fernando, Trinidad and Tobago (SFerna TTO)	July–August 2005	12	10°16′ N	61°27′ W
Neiba, Dominican Republic (Nb DOM)	October 2005	10	18°29′ N	71°25′ W
Santo Domingo, Dominican Republic (SDo DOM)	October 2005	11	18°28′ N	69°63′ W
Pinar del Río, Cuba (PinRio CUB)	November 2005	18	22°25′ N	83°41′ W
Juventud Island, Cuba (JuvIsl CUB)	November 2005	28	21°53′ N	82°54′ W
Ciego de Ávila, Cuba (CAvila CUB)	October 2005	40	21°50′ N	78°46′ W
Las Tunas, Cuba (LTunas CUB)	October 2005	12	20°58′ N	76°57′ W
Santiago de Cuba, Cuba (StCuba CUB)	October 2005	26	20°01′ N	75°49′ W
Kingston, Jamaica (Kingst JAM)	February 2005	18	17°59′ N	76°47′ W

Table 2. Genetic diversity in *Cochliomyia hominivorax* populations from the Caribbean.

	Encon VEN	PSpain TTO	SFerna TTO	Nb + SDo DOM	PinRio CUB	JuvIsl CUB	CAvila CUB	LTunas CUB	StCuba CUB	Kingst JAM
CH01										
N_a (*A*)	4 (3.39)	5 (4.81)	3 (3.00)	2 (2.00)	1 (1.00)	2 (1.94)	2 (1.99)	2 (2.00)	2 (2.00)	2 (1.90)
H_O	0.4333	0.3529	0.4167	0.2632	0.0000	0.1765	0.2000	0.3333	0.3462	0.0000
H_E	0.5949	0.5294	0.3659	0.4623	0.0000	0.1633	0.2582	0.2899	0.2919	0.1079
CH05										
N_a (*A*)	7 (5.16)	4 (3.98)	4 (4.00)	2 (1.87)	3 (2.66)	5 (4.26)	7 (4.82)	2 (2.00)	3 (2.78)	4 (3.86)
H_O	0.6333	0.6471	1.0000	0.1053	0.2778	0.8235	0.4750	0.0833	0.2308	0.5000
H_E	0.5847	0.6078	0.6812	0.1024	0.2524	0.6422	0.4288	0.0833	0.2466	0.4937
CH09										
N_a (*A*)	3 (2.75)	2 (2.00)	2 (2.00)	3 (3.00)	3 (2.61)	4 (3.33)	3 (2.97)	4 (4.00)	4 (3.56)	3 (2.99)
H_O	0.5333	0.1765	0.5000	0.5000	0.3889	0.3939	0.5000	0.8182	0.4286	0.6667
H_E	0.4367	0.3369	0.3913	0.5667	0.4937	0.6452	0.5835	0.6970	0.5819	0.5952
CH10										
N_a (*A*)	5 (4.49)	3 (2.99)	5 (5.00)	5 (4.90)	2 (2.00)	2 (1.89)	2 (2.00)	3 (3.00)	3 (2.87)	3 (2.62)
H_O	0.4000	0.1765	0.2000	0.0909	0.3636	0.0000	0.4872	0.5000	0.1905	0.2222
H_E	0.7020*	0.5045*	0.7211*	0.6277*	0.5195	0.1560	0.4945	0.5217	0.5424*	0.2095
CH11										
N_a (*A*)	5 (4.37)	3 (3.00)	5 (4.98)	2 (1.99)	4 (4.00)	3 (2.97)	4 (3.62)	4 (4.00)	4 (3.11)	6 (5.13)
H_O	0.3333	0.5294	0.8333	0.2105	0.7692	0.1429	0.1351	0.2000	0.2778	0.4667
H_E	0.7317*	0.6756	0.8007	0.2731	0.7138*	0.5981*	0.6897*	0.7105	0.4905	0.6713
CH12										
N_a (*A*)	8 (6.20)	4 (3.63)	4 (4.00)	5 (4.94)	6 (5.76)	4 (3.89)	5 (4.96)	3 (3.00)	5 (4.67)	8 (6.66)
H_O	0.6207	0.3529	0.5000	0.4211	0.5556	0.7576	0.7250	0.8333	0.7200	0.4444
H_E	0.7374	0.5971	0.6703	0.7582	0.7825	0.6084	0.7839	0.6812	0.7371	0.7635*
CH14										
N_a (*A*)	7 (5.61)	4 (3.98)	3 (3.00)	5 (4.85)	4 (3.86)	7 (5.97)	4 (3.41)	2 (2.00)	4 (3.81)	4 (3.84)
H_O	0.3333	0.5294	0.1667	0.3158	0.6667	0.7000	0.2162	0.0000	0.5000	0.0000
H_E	0.6757*	0.6578	0.6630	0.7354*	0.6270	0.7458	0.5883*	0.1594	0.6418	0.5348*
CH15										
N_a (*A*)	6 (5.13)	5 (4.67)	4 (3.89)	3 (2.46)	3 (2.76)	4 (3.33)	7 (5.43)	4 (4.00)	4 (3.34)	4 (3.56)
H_O	0.3214	0.3846	0.6667	0.3158	0.2222	0.4706	0.7250	0.1111	0.5385	0.5000
H_E	0.7721*	0.7754*	0.5362	0.3172	0.5651	0.5615	0.7576	0.6078	0.6418	0.6895
CH20										
N_a (*A*)	4 (3.39)	5 (4.81)	3 (3.00)	2 (2.00)	1 (1.00)	2 (1.94)	2 (1.99)	2 (2.00)	2 (2.00)	2 (1.90)
H_O	0.4333	0.3529	0.4167	0.2632	0.0000	0.1765	0.2000	0.3333	0.3462	0.0000
H_E	0.5949	0.5294	0.3659	0.4623	0.0000	0.1633	0.2582	0.2899	0.2919	0.1079
CH21										
N_a (*A*)	7 (5.16)	4 (3.98)	4 (4.00)	2 (1.87)	3 (2.66)	5 (4.26)	7 (4.82)	2 (2.00)	3 (2.78)	4 (3.86)
H_O	0.6333	0.6471	1.0000	0.1053	0.2778	0.8235	0.4750	0.0833	0.2308	0.5000
H_E	0.5847	0.6078*	0.6812	0.1024	0.2524*	0.6422*	0.4288	0.0833	0.2466*	0.4937
CH24										
N_a (*A*)	3 (2.75)	2 (2.00)	2 (2.00)	3 (3.00)	3 (2.61)	4 (3.33)	3 (2.97)	4 (4.00)	4 (3.56)	3 (2.99)
H_O	0.5333	0.1765	0.5000	0.5000	0.3889	0.3939	0.5000	0.8182	0.4286	0.6667
H_E	0.4367*	0.3369	0.3913	0.5667	0.4937	0.6452	0.5835*	0.6970	0.5819	0.5952
CH26										
N_a (*A*)	5 (4.49)	3 (2.99)	5 (5.00)	5 (4.90)	2 (2.00)	2 (1.89)	2 (2.00)	3 (3.00)	3 (2.87)	3 (2.62)
H_O	0.4000	0.1765	0.2000	0.0909	0.3636	0.0000	0.4872	0.5000	0.1905	0.2222
H_E	0.7020	0.5045	0.7211	0.6277	0.5195	0.1560	0.4945	0.5217	0.5424	0.2095
Todos										
N_a (*A*)	6.67 (5.50)	4.58 (4.44)	4.17 (4.14)	4.25 (3.97)	4.08 (3.79)	4.75 (4.21)	5.00 (4.27)	3.83 (3.83)	4.92 (4.23)	5.33 (4.73)
H_O avg	0.5174	0.5276	0.5444	0.4147	0.4277	0.4448	0.4952	0.4173	0.4562	0.4781
H_E avg	0.7057*	0.6402*	0.6107*	0.5612*	0.5384*	0.5597*	0.6019*	0.5386*	0.5591*	0.6056*

*Denotes a significant ($\alpha = 0.05$) deviation from Hardy–Weinberg equilibrium.

A, allelic richness; H_E, expected heterozygosity; H_O, observed heterozygosity; N_a, number of alleles.

Encon, Encontrados; PSpain, Port of Spain; SFerna, San Fernando; Nb, Neiba; SDo, Santo Domingo; PinRio, Pinar del Río; JuvIsl, Juventud Island; CAvila, Ciego de Ávila; LTunas, Las Tunas; StCuba, Santiago de Cuba; Kingst, Kingston.

CUB, Cuba; DOM, Dominican Republic; JAM, Jamaica; TTO, Trinidad and Tobago; VEN, Venezuela.

In total, 122 alleles were detected in the analysed populations. The highest allelic richness, 5.50, was observed in the Encontrados (Venezuela) sample (Table 2). This population also had the highest number (nine) of unique alleles (those found in only one population). Unique alleles were detected in all but the population from Pinar del Río (Cuba), which also had the lowest allelic richness (3.83). Moreover, two loci were monomorphic in this population. Fifteen alleles were unique in the pooled Cuban sample, thus indicating some degree of differentiation between these populations and the other populations.

Heterozygosities within the analysed populations varied greatly (Table 2). The highest diversity in terms of expected heterozygosity was found in the Venezuelan population ($H_E = 0.7057$), whereas the lowest levels of diversity were observed in the Pinar del Río and Las Tunas populations from Cuba ($H_E = 0.5384$ and $H_E = 0.5386$, respectively).

Of the 120 population/locus combinations, 53 showed significant departures from Hardy–Weinberg expectations and 22 remained significant after applying the sequential Bonferroni correction. Deviations from the Hardy–Weinberg equilibrium were observed in all populations except Las Tunas (Cuba). In all cases, departures from expectations were the result of an excess of homozygotes. Among the possible factors that might account for these deviations is the Wahlund effect, as most samples were collected from wounded dogs in veterinary clinics. Hence, it is difficult to infer the origin of the animals and the passively transported larvae. Moreover, the samples from Dominican Republic and Jamaica, which originated from different sublocations, were pooled because of the small number of individuals at each sub-location. Another factor that may also have caused the observed deviations is the presence of null alleles. These result from mutations such as substitutions, insertions or deletions in one or both priming sites, preventing the binding of the DNA strand and primers (Callen *et al.*, 1993) and non-amplification of the allele. At the population level this can lead to a misinterpretation of the number of heterozygotes and, consequently, of Hardy–Weinberg deviations. However, null alleles had a negligible effect on our conclusions, as the outputs of our analyses were not significantly altered by excluding affected loci and adjusting genotype frequencies (data not shown). Linkage disequilibrium was found in only two of 646 comparisons among the loci and populations analysed, but no common pair of loci showed non-random associations in more than one population.

In order to test the influence of sterile insect releases on NWS populations, we should, ideally, compare samples collected before and after such attempts. As such collections were not available, we used the average allelic richness and expected hererozygosity across islands as an approximation of variability for populations not under control. We then tested the difference between genetic variability in Kingston (Jamaica) and in Isla de la Juventud (Cuba) and the averaged genetic variability among islands, and found no reduction in genetic variability (Wilcoxon signed rank test, $P > 0.05$; data not shown). In fact, surprisingly, variability in Jamaica was significantly higher than the island average (Wilcoxon signed rank test, $P > 0.05$).

The pairwise difference between the genetic variability in the continental sample (Encontrados, Venezuela) and that in the island populations was small, but significant in all comparisons. After the correction for multiple tests, however, the differences in allelic richness did not remain significant for populations from Port of Spain (Trinidad and Tobago), Ciego de Ávila (Cuba) or Kingston (Jamaica). In terms of expected heterozygosity, we found no evidence of a reduction in genetic variability after the correction. As we used only one continental population, these results could be misleading if the variability levels of the analysed population were not representative of mainland populations overall. To test for such a bias, we performed the same comparisons with seven previously analysed Uruguayan samples (Torres *et al.*, 2007). In the former study, we had genotyped nine microsatellite markers, and the three additional loci were included for this analysis. There were no significant differences between the Venezuelan and Uruguayan samples, but five island populations showed reduced expected heterozygosity compared with the Uruguayan average. Confirming our previous result, populations from Kingston (Jamaica) and Isla de la Juventud (Cuba) did not show reductions in expected heterozygosity after the correction for multiple tests (Table 3). When the difference in allelic richness was tested, all comparisons were significant (Table 3).

Population differentiation

The overall F_{ST} value for all samples was highly significant ($F_{ST} = 0.1570$, $P < 0.0001$). The pairwise F_{ST} estimates (Table 4) indicated a high level of population differentiation, and were significant for all but one comparison, Port of Spain × San Fernando in Trinidad and Tobago (0.0474). Inter-landmass pairwise F_{ST} estimates ranged from 0.0987 between Port of Spain (Trinidad and Tobago) and Encontrados (Venezuela), to 0.2598 between Isla de la Juventud (Cuba) and Kingston (Jamaica). These estimates were apparently higher than intra-island comparisons, which ranged from 0.0474 between Port of Spain and San Fernando (Trinidad and Tobago), to 0.1508 between Las Tunas and Pinar del Río (Cuba).

A model-based clustering method in *structure* (Pritchard *et al.*, 2000) was used to estimate the number of populations based on posterior probabilities, and to assign individuals to those populations. The number of clusters was inferred by calculating the probability P(X|K) of the data, given a certain prior value of K (number of clusters) over a number of Monte Carlo Markov chain (MCMC) iterations. The clusters are characterized by different allele frequencies and individuals are probabilistically assigned to one or more clusters, according to their allele distribution. Seven groups with distinctive multi-locus allele frequencies were identified. Two of the identified clusters consisted of more than one geographical population. One of them contained the two populations from Trinidad and Tobago and the other included three Cuban populations, Ciego de Ávila, Las Tunas and Santiago de Cuba. The remaining populations were grouped according to geographical origin (Fig. 2).

Bottlenecks

Bottleneck analyses were performed by comparing observed gene diversity with expected diversity on the basis of the observed

Table 3. Pairwise comparisons of genetic variability between island and continental populations. Genetic variability from each island population was compared with the variability in the Venezuelan sample (VEN) and with the averaged genetic variation among Uruguayan samples (URU avg). *P*-values for Wilcoxon signed rank tests are shown.

	VEN		URU avg	
Study area	*A*	H_E	*A*	H_E
Encon VEN	–	–	**0.004639**	0.211900
PSpain TTO	0.021240	0.010500	**0.001221**	**0.004639**
SFerna TTO	**0.006100**	0.038570	**0.000244**	0.021240
Nb + SDo DOM	**0.003418**	0.021240	**0.000244**	**0.008057**
PinRio CUB	**0.000244**	0.006104	**0.000244**	**0.000240**
IslJuv CUB	**0.003418**	0.038570	**0.001221**	0.021240
CAvila CUB	0.017090	0.017090	**0.001709**	**0.006104**
LTunas CUB	**0.008055**	0.010500	**0.000732**	0.013430
StCuba CUB	**0.001221**	0.006104	**0.000244**	**0.002441**
Kingst JAM	0.038575	0.046140	**0.000732**	0.026120

Significant comparisons after correction for multiple tests are shown in bold.
A, allelic richness; H_E, expected heterozygosity.
Encon, Encontrados; PSpain, Port of Spain; SFerna, San Fernando; Nb, Neiba; SDo, Santo Domingo; PinRio, Pinar del Río; JuvIsl, Juventud Island; CAvila, Ciego de Ávila; LTunas, Las Tunas; StCuba, Santiago de Cuba; Kingst, Kingston.
CUB, Cuba; DOM, Dominican Republic; JAM, Jamaica; TTO, Trinidad and Tobago; VEN, Venezuela.

number of alleles (Cornuet & Luikart, 1996), under the IAM, SMM and TPM under 30% multi-step changes. If a given population has recently undergone a bottleneck, the H_E calculated from allele frequencies is higher than the H_E at equilibrium (H_{EQ}), estimated from the observed number of alleles in a sample (Cornuet & Luikart, 1996). This observation is expected because the number of alleles is affected faster than the expected heterozygosity if there is a reduction in the effective population size. Most populations showed significant evidence for population bottlenecks, under IAM, but only Port of Spain (Trinidad and Tobago) and Ciego de Ávila (Cuba) had a significant result under both IAM and TPM. No evidence for bottleneck was detected in Santiago de Cuba (Cuba) and Kingston (Jamaica). After the correction for multiple tests, however, only Port of Spain (Trinidad and Tobago) had statistical support for a bottleneck (IAM, $P = 0.00061$).

Discussion

The NWS fly is characterized by high levels of genetic variability across its geographical distribution. There is some controversy, however, as to how this variation is distributed; it is not clear if it can be explained by differences among individuals within geographical populations or between samples from the same geographical region or between different regions.

Here, using microsatellites, we examined 10 populations from Caribbean islands where we found moderate to high levels of genetic variability. Deviation from Hardy–Weinberg equilibrium was noted in all but one population. The excess of homozygotes was also observed in former microsatellite analyses of screwworm populations (Torres *et al*., 2007). In Uruguayan populations, this observation was attributed to the occurrence of demographic

Table 4. F_{ST} estimates for all population pairwise comparisons.

Study area	PSpain TTO	SFerna TTO	Nb + SDo DOM	PinRio CUB	IslJuv CUB	CAvila CUB	LTunas CUB	StCuba CUB	Kingst JAM
Encont VEN	0.0987*	0.1394*	0.1487*	0.1416*	0.1625*	0.1363*	0.1269*	0.1120*	0.1656*
PSpain TTO	–	**0.0474**§	0.1455†	0.1994‡	0.1943†	0.1301*	0.1694†	0.1390†	0.1817*
SFerna TTO		–	0.2024†	0.2466‡	0.2461‡	0.1967*	0.2265†	0.2136†	0.2016*
Nb + SDo DOM			–	0.2360†	0.2498†	0.1882*	0.2067†	0.1537†	0.2129†
PinRio CUB				–	0.1675§	**0.1194***	**0.1508‡**	**0.0995‡**	0.1564†
IslJuv CUB					–	0.1194*	0.1507§	0.1281‡	0.2598†
CAvila CUB						–	**0.0475***	**0.0718***	0.1693*
LTunas CUB							–	**0.1036†**	0.2424*
StCuba CUB								–	0.1764†
Kingst JAM									–

*$P < 0.001$; †$P < 0.01$; ‡$P < 0.05$); §Not significant. Intra-island comparisons are shown in bold.
Encon, Encontrados; PSpain, Port of Spain; SFerna, San Fernando; Nb, Neiba; SDo, Santo Domingo; PinRio, Pinar del Río; JuvIsl, Juventud Island; CAvila, Ciego de Ávila; LTunas, Las Tunas; StCuba, Santiago de Cuba; Kingst, Kingston.
CUB, Cuba; DOM, Dominican Republic; JAM, Jamaica; TTO, Trinidad and Tobago; VEN, Venezuela.

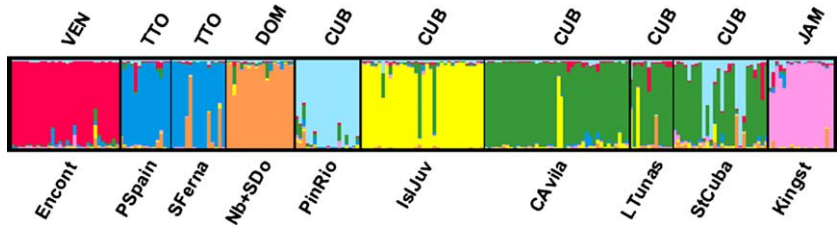

Fig. 2. Population structure of New World screwworm as inferred by the model-based clustering method. Individual multi-locus genotypes are plotted as vertical lines, in which the coloured segments represent the membership coefficients for each of the seven estimated clusters. Pre-defined populations are labelled with sampling location below the figures and the island/country above.

changes caused by seasonal fluctuations and/or persistent insecticide treatment (Torres *et al.*, 2007).

Oceanic island populations are expected to have lower genetic variability than their mainland counterparts for non-endemic species. Island populations lose genetic variation at the moment of establishment because of their small number of founders and, after foundation, genetic variation is lost as they typically have lower population sizes than mainland populations. Natural selection also influences the loss of genetic variation; selection for a favourable allele will increase the rate of loss. A literature survey of publications comparing genetic variation in mainland and island populations (Frankham, 1998) showed that an overwhelming majority (165 of 202) of mainland populations had higher heterozygosities than island populations of the same species. The average reduction was 29%. Similarly, recent observations of other insect pest populations showed that genetic variability in mainland and island populations can have a two-fold difference (e.g. *Anopheles funestus* Giles [Michel *et al.*, 2005] and *Ceratitis capitata* [Wiedemann] [Bonizzoni *et al.*, 2000]).

Based on these previous results, a similar situation in NWS populations was expected. Interestingly, the levels of genetic variability in some of the Caribbean populations were not drastically reduced compared with continental samples (Table 1). This is particularly intriguing because it is generally believed that *C. hominivorax* was introduced into the Caribbean by domestic livestock and trade and that, since their introduction, Caribbean populations have remained isolated from continental influence. However, the presence of such levels of variability requires some additional explanation, which may include: (a) colonization of the Caribbean islands results from several events mediated by successive introductions from South America and/or North America; (b) founder populations were large and did not go through a severe bottleneck that would cause great loss in allele variation; (c) introduction was followed by a rapid increase in population size, or (d) the species was present on the islands before animal domestication.

Differences in genetic variability were further analysed by focusing on islands where attempts to control NWS have been made. An area-wide eradication programme in Jamaica was launched in 1998, using the release of sterile insects in combination with suppression of the NWS population through the treatment of wounds, epidemiological surveillance and quarantine measures (Dyck *et al.*, 2005). Although the programme encountered a number of problems, there was a reduction in screwworm cases as a consequence of the reduction of fly density. However, we did not find any evidence for a recent bottleneck. Surprisingly, the Jamaican population presented similar and even higher levels of variability than populations on other islands. Likewise, the levels of variability of the Jamaican population did not significantly differ from the island average. Naturally, this may reflect a higher level of genetic variability in this population before the releases, but the test for recent bottlenecks also failed to detect any evidence of population contraction. The same was observed for the population from Isla de la Juventud (Cuba), where a suppression trial has been ongoing since 2000 (Méndez *et al.*, 2005). These two observations possibly reflect a striking capacity for recovery, not only of population sizes (as shown by the increase in cases of infestation), but also of the genetic variability of populations. These results, however, must be interpreted with caution because a strong field component has not been employed to assess fly density. Instead, the data rely on the number of screwworm cases reported to monitor the effect of sterile releases, which may not reflect the actual population size. This highlights the need for a fine-scale assessment of population density and dynamics to further evaluate the effect of SIT on wild populations.

In the Caribbean populations, a high level of population differentiation was observed, as might be expected for island populations. Surprisingly, however, we found high levels of differentiation among different geographical populations in Cuba, with pairwise F_{ST} estimates as high as 0.1508 for differentiation between the populations of Las Tunas and Pinar del Río. This finding was reinforced by the fact that the populations of the main Cuban island were grouped into two different clusters by *structure*. This observation is in stark contrast with the low levels of sub-structuring observed among Uruguayan populations separated by similar distances (Lyra *et al.*, 2005; Torres *et al.*, 2007).

The low differentiation in Uruguay could be attributed to the high dispersal capacity of NWS, as migration is assumed to prevent genetic differentiation at neutral markers, but the lack of isolation by distance suggested a more complex structure, involving the extinction and recolonization of entire populations (Torres *et al.*, 2007). The same dynamics may be shaping the intra-island genetic structure observed in Cuba. Recurrent insecticide treatment and isolated control efforts can cause mass population mortality and local extinction of screwworm populations. A re-colonization by a large founder population might cause a demographic turnover if this population were to spread rapidly, in which case the influence of such dynamics on global genetic differentiation would depend on the number and variability of source populations (Hanski & Gaggiotti, 2004).

Small islands are, in principle, ideal locations in which to implement eradication programmes. Island populations are generally much easier to extinguish than mainland populations (Frankham, 1998) because the target population is usually clearly

defined and it is isolated from other populations that could act as a source of individuals for re-colonization. One recent case, however, opened a new discussion concerning the extent to which genetic variability and population structuring influence the success of pest eradication attempts.

The eradication programme in Jamaica was launched in 1998, but, by the end of 2004, little progress had been achieved as a result of several logistical problems (Dyck *et al.*, 2005). Among the factors potentially responsible for the lack of progress was the absence of baseline data on NWS population ecology and dynamics. Knowledge of the genetics of target populations has since become an important component in the planning of new pest management strategies. The use of molecular genetics to define population structure is a well-established, practical alternative to direct methods, such as mark and recapture.

The current study has provided baseline information about the genetic variability and structuring of NWS populations from Caribbean islands. Different scenarios can be interpreted from these results. If the high differentiation (even within the same island) reflects the existence of populations that are highly structured because of a lack of gene flow between them, populations can be delimited into well-defined eradication units, which can be independently managed because they carry a low risk of recolonization.

However, the pattern of genetic variation among populations may also be evidence of source–sink dynamics (Hanski & Gaggioti, 2004). Efforts to eradicate a fraction of a population, or a sink population within an unidentified source–sink dynamic, would inevitably result in rapid recolonization and a waste of resources (Robertson & Gemmell, 2004). In such cases, the most effective control strategy for *C. hominivorax* populations involves an area-wide approach that includes the co-ordinated use of several pest control methods through an integrated pest management strategy. Although large-scale approaches are logistically difficult, such a strategy would ensure that temporary refuges and source populations are also targeted.

Given the limited nature of the information on NWS ecology and population dynamics available to date, it is not possible to distinguish between these two population models; thus fine-scale sampling and analysis associated with field research will be necessary to further investigate the processes that shape the patterns observed in NWS populations. Hence, additional studies should complement our findings and provide support for those who make decisions on the planning and implementation of new area-wide control programmes for this important livestock pest.

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Conflicts of interest

All authors declare no conflicts of interests.

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Complex patterns of genetic variability in populations of the New World screwworm fly revealed by mitochondrial DNA markers

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Abstract. *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae), the New World screwworm fly, is an important agent of traumatic myiasis, which is endemic in the Neotropical region and which has great economic impact on the development of the livestock industry. International efforts have been aimed at designing programmes to control and eradicate this species from endemic areas. Thorough knowledge of the population genetics of an insect pest is a fundamental component to ensuring the success of a pest management strategy because it enables the determination of an appropriate geographic scale for carrying out effective treatments. This study undertook an analysis of mtDNA polymerase chain reaction-restricted fragment length polymorphism (PCR-RFLP) in 34 populations of *C. hominivorax* from 10 countries, encompassing almost all the current distribution of the species. Results showed high levels of mitochondrial DNA variability ($\pi = 2.9\%$) and a complex pattern of population genetic structure for this species. Significant population structure ($\Phi ST = 0.5234$) and low variability were found in Caribbean populations, suggesting that, in general, islands constitute independent evolutionary entities connected by restricted gene flow. By contrast, high variability and low, but significant, differentiation was found among mainland populations ($\Phi ST = 0.0483$), which could not be attributed to geographic distance. Several processes may be acting to maintain the observed patterns, with different implications for establishing control programmes.

Key words. *Cochliomyia hominivorax*, current geographic distribution, mtDNA, PCR-RFLP, population structure.

Introduction

Cochliomyia hominivorax Coquerel, the New World screwworm (NWS) fly, is a myiasis-causing fly endemic in the Neotropical region (Guimarães *et al.*, 1983). The larvae of this insect pest invade and feed on the living tissues of warm-blooded vertebrates, enlarging wounds and attracting female flies to oviposit. Infestation without treatment may lead to the animal's death. *Cochliomyia hominivorax* has a very wide host range, from wildlife to man, but is most notorious when affecting livestock; the species causes injuries to leather and is responsible for declines in milk and beef production, and, consequently, substantial profit losses to livestock breeders (Hall & Wall, 1995; Vargas-Terán *et al.*, 2005).

Historically, the geographic distribution of this insect extended from the southern U.S.A. to Uruguay and part of Argentina (Hall & Wall, 1995). During the second half of the last century, *C. hominivorax* was successfully eradicated from North and most of Central America through the use of the sterile insect technique (SIT) and a sterile fly barrier system is now in place in Panama to prevent reinvasion. The insect is still present in several islands in the Caribbean and South America

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(Klassen & Curtis, 2005). The fly was first found outside the Americas in 1988, in Libya, from which, after a major SIT programme, it was eradicated in 1991 (Lindquist *et al.*, 1992; Wyss, 2000).

Because of the economic importance of *C. hominivorax* to the livestock industry, international efforts have been aimed at designing programmes to control and eventually eradicate this species from other endemic areas (International Atomic Energy Agency/UN Food & Agriculture Organization [IAEA/FAO], 2000). A fundamental component of a successful pest management strategy is a good knowledge of the genetic diversity and structure of field populations. Insights into these aspects of the species' biology can help greatly in, for example, predicting geographic spread patterns, understanding the species' colonization and establishment potential, and determining appropriate geographic scales for effective treatments (Krafsur, 2005).

In the past few years the population genetics of this species have been studied with different techniques, giving rise to various speculations and conflicting reports about population structure and genetic diversity and their effects on control strategies (see Azeredo-Espin & Lessinger, 2006). Richardson *et al.* (1982) analysed karyotypes in natural populations of *C. hominivorax* in North America and proposed that the species was a complex of reproductively isolated populations, but subsequent investigations did not find evidence of any sub-structuring (LaChance *et al.*, 1982; McInnis, 1983). Studies of genetic variation based on allozyme techniques also resulted in divergent conclusions. Krafsur & Whitten (1993) and Taylor *et al.* (1996) analysed populations from North and Central America and concluded that they constitute a single panmitic population. However, analysis of five Brazilian populations revealed a high geographic differentiation across southeastern Brazil (Infante-Malachias, 1999). Analyses of mitochondrial DNA markers revealed high levels of variability in *C. hominivorax* populations, but with different levels of population differentiation. Roehrdanz and co-workers (Roehrdanz & Johnson, 1988; Roehrdanz, 1989; Taylor *et al.*, 1991) analysed populations from North and Central America and found a small divergence among mainland populations, but a high divergence between these and the Jamaican population. Infante-Vargas & Azeredo-Espin (1995) analysed populations from southern Brazil and suggested that these populations were divergent. More recently, NWS populations from Uruguay were analysed by mtDNA polymerase chain reaction-restricted fragment length polymorphism (PCR-RFLP) and microsatellite markers (Lyra *et al.*, 2005; Torres *et al.*, 2007) and results suggested that the populations were very similar in the extreme south of the distribution, where mtDNA markers did not reveal variation and microsatellite markers revealed a small, but significant, variation.

There is no conclusive explanation for the discrepancies between different studies regarding the genetics of *C. hominivorax*, but one generic interpretation is that they result from the analyses of distinct populations and/or the use of different genetic markers.

Thus, it is clear that a more extensive study on genetic diversity and differentiation in this species throughout its geographic distribution, using a single genetic marker, would greatly help in highlighting the evolutionary scenario of *C. hominivorax* populations. The investigation reported here comprised an extensive survey of mtDNA PCR-RFLP in 34 *C. hominivorax* populations, which attempted to characterize genetic variability and the patterns of its distribution across the species' current geographic range. We have included a discussion on the possible influences of several processes that might be acting to maintain the patterns observed and on the implications of our results for establishing control programmes.

Materials and methods

Fly samples and DNA extractions

A total of 883 individuals from 34 *C. hominivorax* populations in 10 countries across almost all the species' current distribution range (Table 1, Fig. 1) were analysed. Samples were obtained as third instars from wounded cattle, sheep, dogs or pigs, between January 2003 and June 2007, except the sample from Campo Grande-MS (Brazil), which comprised 12 adults collected in a trap. Collected larvae were fixed in 100% ethanol or allowed to pupate in sawdust and were taken to the laboratory for species identification (Guimarães *et al.*, 1983). The adults that emerged and the fixed larvae were used for DNA extractions. Samples from each wound were identified and DNA extractions from two or three individuals per wound were conducted. Total DNA from each individual was extracted with the phenol : chloroform method (Infante-Vargas & Azeredo-Espin, 1995), with volumes adapted for microcentrifuge tubes. The extractions were stored at − 20 °C.

The Uruguayan populations analysed in this study were the sum of samples collected in 2003 and analysed by Lyra *et al.* (2005), together with new samples collected in 2004. Temporal analysis indicated that samples from the different years were not significantly different (data not shown) and so they were pooled. Samples from the Dominican Republic, as well as from Ecuador, originated from a few sub-locations and were pooled in the analysis because of the small number of individuals sampled in each sub-location; the geographic references considered were from the locality with the highest number of individuals sampled.

PCR amplification and restriction procedures

PCR amplifications and restriction procedures were performed as described in Lyra *et al.* (2005) with some slight modifications. Briefly, two specific mtDNA regions were amplified using the University of British Columbia-insect mtDNA oligonucleotide set (Simon *et al.*, 1994). One region included complete sequences of cytochrome oxidase c subunits 1 and 2 (COI/COII: ~ 2360 bp), which was amplified using the primers C1-J-2195 and L2-N-3014. The other region included the complete control region and partial rRNA 12S sequences (A+T-rich/12S: ~ 2100 bp), which was amplified using the primers TM-N-193 and SR-J-14233. Standard PCR conditions as described in Lessinger & Azeredo-Espin (2000) and Litjens *et al.* (2001) were used.

To improve the amplification of some samples, the regions were amplified using a nested PCR, with two sets of primers in two distinct reactions for each region. Three new primers were

Table 1. Geographic locations of *Cochliomyia hominivorax* populations sampled, population codes (ID codes) and number of individuals analysed.

Country	Location	ID code	Latitude	Longitude	Samples, *n*, T/A
CB	Pinar del Rio	CPR	22°25′ N	83°41′ W	14/6
	Ciego de Ávila	CCA	21°50′ N	78°46′ W	18/13
	Santiago de Cuba	CSC	20°01′ N	75°49′ W	17/13
	Ciro Redondo	CCR	21°53′ N	82°54′ W	14/11
DR	Santo Domingos	DRP	18°28′ N	59°63′ W	25/14
JM	Kingston	JAM	17°59′ N	76°47′ W	14/8
T & T	Puerto España	TET	10°40′ N	61°28′ W	22/12
VE	Encontrados	VEN	09°02′ N	72°13′ W	20/10
	Barquisimeto	VBA	10°03′ N	69°19′ W	23/11
	Juangriego	VJU	11°04′ N	63°57′ W	10/1
CO	Turbo	COT	08°05′ N	76°43′ W	35/15
EC	Sto Dom. de los Colorados	ECO	00°15′ S	79°10′ W	29/13
BR	Santa Maria das Barreiras	BSM	08°52′ S	49°42′ W	26/20
	Cocalinhos	BCO	14°22′ S	51°00′ W	11/6
	Costa Rica City	BCR	18°32′S	53°07′ W	25/5
	Goiânia	BGO	16°43′ S	49°15′ W	28/10
	Goianira	BGN	16°32′ S	49°22′ W	16/8
	Caiapônia	BCA	16°57′ S	51°48′ W	68/31
	Campo Grande	BCG	20°27′ S	54°36′ W	12/12
	S.S. do Paraíso	BSS	20°55′ S	46°59′ W	18/6
	Campinas	BCP	22°48′ S	47°03′ W	17/9
	Estiva	BES	22°27′ S	46°01′ W	42/18
	Carambeí	BCI	24°55′ S	50°05′ W	26/15
	Fagundes Varela	BFV	28°52′ S	51°41′ W	9/5
	Sto Antonio das Missões	BSA	29°04′ S	56°19′ W	22/11
	Pinheiro Machado	BPM	31°04′ S	53°23′ W	24/9
PY	Ybytymí	PYB	25°46′ S	56°41′ W	28/14
UY*	Bañados de Medina	UBM	32°23′ S	54°21′ W	46/20
	Paso Muñoz	UPM	31°27′ S	56°23′ W	36/10
	San Antonio	UST	31°24′ S	57°58′ W	31/16
	Dayman	UDA	31°33′ S	57°57′ W	24/11
	Cerro Colorado	UCC	33°52′ S	55°33′ W	44/27
	Colonia	UCO	34°28′ S	57°51′ W	43/15
	Juaquín Suarez	UJS	34°44′ S	56°02′ W	46/21
Total					883/426

*Sum of data from Lyra *et al.* (2005) and this work.
T/A, number of individuals sampled/number of individuals considered for genetic comparisons between populations.
CB, Cuba; DR, Dominican Republic; JM, Jamaica; T & T, Trinidad and Tobago; VE, Venezuela; CO, Colombia; EC, Ecuador; BR, Brazil; PY, Paraguay; UY, Uruguay.

used: two flanking the COI/COII region at the genes *ATP8* (A8-N-3931: 5′AAT TGG TGC TAT TTG AGG 3′) and *tRNA-Trp* (TW-J-1287: 5′ACT AAT AGC CTT CAA AGC 3′), and one downstream from the *12S* rRNA subunit, at the *tRNA-Val* gene (TV-J-14122: 5′ TTC AAT GTA AAT GAA ATG C 3′). We used the TV-J-14022 with the N2-N-327 primer (Oliveira *et al.*, 2006), to amplify the A+T-rich/12S region. An initial reaction was performed with the external primers for each region and the products of these amplifications were used as templates for the second reaction, as described above.

For the construction of the three new mtDNA oligonucleotides, the sequence data for homologous regions from the *tRNA-Trp*, *tRNA-Val* and *ATP8* genes of *C. hominivorax* and other Calliphoridae available in GenBank were aligned separately using ClustalX (Thompson *et al.*, 1997). The primers were selected based on conserved aligned regions, and the structural stabilities and thermodynamic properties of each primer were analysed using Gene Runner software (Hastings Software Inc., Hastings on Hudson, NY, U.S.A.). The nomenclature and relative position for these new primers were assigned according to Simon *et al.* (1994). The three primers were tested for different species of Calliphoridae and Muscidae (data not shown).

The PCR products of COI/COII were digested with the diagnostic restriction endonucleases *Ase* I and *Msp* I and A+T-rich/12S fragments were digested with the restriction endonuclease *Dra* I, as described in Lyra *et al.* (2005). The fragments were separated by electrophoresis in 2.0% agarose gels, stained with ethidium bromide (EtBr) and photographed on Edas Kodak 290 (Eastman Kodak Company, Rochester, NY, U.S.A.); previously identified restriction fragment patterns were run on the gels as control. The size of the fragments was estimated by comparison with the molecular size standard DNA Ladder Plus 1 Kb (12 Kb

Fig. 1. Map of South America and the Caribbean region, showing collecting localities for sampled populations of *Cochliomyia hominivorax* used in this study. Identification codes correspond to localities given in Table 1.

to 100 bp; Invitrogen Corp., Carlsbad, CA, U.S.A.) using regression analysis, carried out manually. Digestions with enzymes that produced different restriction patterns were repeated to confirm that the observed patterns did not result from partial digests. Fragments of < 200 bp were not included in the analysis, resulting in a few inconsistencies that were either related to the sizes of the originally amplified PCR products or to the total size recovered from the sum of the digested fragments (Table 2).

Diversity indices and population genetic structure

Data analyses were conducted as described in Lyra *et al.* (2005). For haplotype frequency estimations and genetic comparisons, each haplotype found in a wound was considered only once. This approach was taken to avoid a bias in the analysis that might arise by sampling the same mitochondria because sibling larvae have gregarious behaviour (Lyra *et al.*, 2005); for this reason, the number of *C. hominivorax* individuals considered in the analysis of genetic variation was reduced to 426 (Table 1). The population from Juangriego, Venezuela was not included in population differentiation analyses because of the small number of samples obtained (just one wound sampled).

The genetic diversity within populations was interpreted using the estimates of haplotype diversity (*Hs* = probability that two randomly chosen haplotypes will be different; Nei, 1987) and nucleotide diversity (π = the proportion of nucleotide sites that are different when any two haplotype sequences are randomly compared; Nei & Tajima, 1981), computed using the Restriction Enzyme Analyses Package (REAP; McElroy *et al.*, 1992).

Population structure was assessed by calculating nucleotide divergence for all pairs of populations (Nei & Tajima, 1981; REAP, McElroy *et al.*, 1992), Slatkin's linearized F_{ST} matrix (Slatkin, 1995) and analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992), computed using ARLEQUIN Version 3.01 (Excoffier *et al.*, 2005). The relationship among populations was explored by constructing an UPGMA (unweighted pair group method with arithmetic mean) tree using MEGA Version 4.0 (Tamura *et al.*, 2007) and with a multidimensional scaling analysis diagram (MSD; STATISTICA Version 6.1; StatSoft, Inc., Tulsa, OK, U.S.A.), with both the matrix of nucleotide divergence and Slatkin's linearized F_{ST}s. The partition of haplotype diversity between groups formed in this tree and MSD was investigated by AMOVA. The degree of isolation of populations, or between groups of populations, was interpreted using the Φ_{ST} parameter (Michalakis & Excoffier, 1996) and the significance of the variance components was tested by the non-parametric permutation method (10 000 permutations, Excoffier *et al.*, 1992).

A Mantel test (Smouse *et al.*, 1986) was carried out to test for significant isolation by distance, when population subdivision was found. The test was conducted using matrices of geographic distance and nucleotide divergences between populations as input data and performed using ARLEQUIN (Excoffier *et al.*, 2005).

Results

Haplotype frequencies and within-population diversity

The diagnostic restriction patterns for each mtDNA region and the composed haplotypes recorded are shown in Table 2. Amplifications of the A+T-rich/12S sequences showed size polymorphism, but little variation in restriction sites. Six different sizes of fragments were obtained and the most common fragment (~ 2100 bp) accounted for 99% of examined individuals. Other sizes appeared just once in a specific locality (~ 0.2% each) and the nature of the size variation is still under investigation. Digestion of the A+T-rich/12S sequences with the enzyme *Dra* I produced seven diagnostic patterns (Table 2). Patterns A and B were digestion products of the 2100-bp fragment (restriction site polymorphism), and patterns C, D, E, F and G were identified when the different size fragments were digested (fragment size polymorphism). Pattern A appeared in approximately 98.8% of the samples analysed. The COI/COII sequences presented higher restriction site polymorphism than A+T-rich/12S, but did not present size polymorphism. For this region, the *Msp* I enzyme yielded seven diagnostic patterns, whereas the *Ase* I enzyme presented two different patterns (Table 2).

No evidence of heteroplasmy was noted in the specimens analysed. Although *C. hominivorax* presents polymorphic restriction patterns for both mtDNA regions studied, the patterns

Table 2. (A) Diagnostic restriction patterns obtained by polymerase chain reaction-restricted fragment length polymorphism of *Cochliomyia hominivorax.* Capital letters indicate polymorphic restriction patterns for each region. (B) Composite haplotypes based on the restriction pattern obtained for each enzyme and their frequency in the total sample.

(A) Restriction patterns							(B) Haplotypes		
Region	Enzyme	Pattern	Fragments (bp)				Haplotype	*Dra*I *Ase*I *Msp*I	Frequency, %
A+T/12S	*Dra* I	A	800	320	200	200	H1	A A C	51.0
		B	1000	320	200		H2	A A D	0.2
		C*	1800	320	200	200	H3	A B A	18.6
		D*	1700	320	200	200	H4	A B B	13.6
		E*	1650	320	200	200	H5	B A C	0.2
		F*	1900	320	200	200	H6	A B E	0.2
		G*	1300	320	200	200	H7	C A C	0.2
							H8	A A A	14.0
COI/COII	*Ase* I	A	1100	460	350	220	H9	A B C	0.2
		B	1100	680	350		H10	D B A	0.2
	Msp I	A	1500	480			H11	A A F	0.2
		B	1400	480			H12	E A C	0.2
		C	1500	300			H13	A B G	0.2
		D	870	680	300		H14	A B H	0.2
		E	1400	300			H15	D A C	0.2
		F	1100	550	300		H16	F B B	0.2
		G	850	750	480		H17	B A A	0.2
		H	1000	500	480		H18	G A A	0.2

*Fragments obtained with the restriction of size polymorphic amplifications; fragments $<$ 200 bp were not considered.

obtained here do not overlap diagnostic restriction patterns obtained for the secondary screwworm, *Cochliomyia macellaria* (Fabricius), by Litjens *et al.* (2001).

Eighteen different haplotypes were identified based on the restriction patterns obtained for each enzyme in the two mtDNA regions analysed (Table 2), providing a measure of diversity and discrimination between populations. Table 2 shows the frequency of each haplotype in the total sample and Table 3 shows the distribution and frequency of the haplotypes found in the different populations sampled. Fourteen of the 18 haplotypes recorded were locally distributed and accounted for a very low proportion (~ 2.8% in total) of the individuals examined. The other four haplotypes were common between 17 or more populations. The most common haplotype (H1) was found in all populations, except the Cuban populations, and accounted for 51% of the total sample. Haplotypes H3, H4 and H8 represented, respectively, 18.6%, 13.6% and 14% of the individuals examined (Table 2). H3 was widely distributed among populations, H4 was present only in populations from South America (mainland haplotype) and H8 was the most frequent in Cuban populations (83–100%), but was found in South American populations with low frequencies (~ 5.1%) (Table 3).

Estimates of gene diversity within populations (*Hs* and π) are shown in Table 3. Populations from the Caribbean, except Trinidad and Tobago, presented low levels of diversity, with high frequencies of just one haplotype. Higher levels of diversity were found in populations from both Venezuela (VBA) and central–southern Brazil (e.g. BSS and BFV).

Population structure

The population structure was revealed by AMOVA (Table 4) and represented in UPGMA tree and MDS diagrams (Fig. 2). Nucleotide divergences ranged from D = 0 to D = 0.0363 (average D = 0.00549 $\pm$ 0.000001), with higher divergences found between Caribbean island populations or between these and mainland populations. Nucleotide divergences and linearized F_{ST}s presented very similar results.

Analyses indicated a moderate and non-homogeneous level of genetic differentiation of *C. hominivorax* across its current geographic distribution. The Caribbean represented the area of most variation between populations, whereas South America showed low population differentiation (Table 4, Fig. 2). Cuban populations were highly divergent (Fig. 2) from all other Caribbean and mainland populations. Populations from Jamaica and the Dominican Republic were more similar to one another and grouped with mainland populations (Fig. 2A), but represented marginal populations in the MSD (Fig. 2B), revealing that they are a little different from the mainland population. The population from Trinidad and Tobago, despite its status as a Caribbean island population, was very similar to some of the mainland populations and appeared together with these populations in the UPGMA tree and MSD. There was no clear pattern for population clusters on the mainland, but the MSD revealed some populations in a periferical distribution, or with little differentiation (Ecuador [ECO], Goiânia [BGO], Costa Rica City [BCR] and Pinheiro Machado [BPM]).

Table 3. Genetic diversity and haplotype distribution within populations of *Cochliomyia hominivorax*. Population ID codes correspond to the localities given in Table 1.

Population		*Hs*	π	Hap, *n*	Haplotype frequency
	CPR	0.2637 ± 0.14746	0.009022	2	H3 (17%), H8 (83%)
CB	CCA	0.0000 ± 0.00000	0.000000	1	H8 (100%)
	CSC	0.2831 ± 0.10948	0.009911	3	H3 (7.5%), H8 (85%), H17 (7.5%)
	CCR	0.1732 ± 0.10812	0.003468	2	H8 (90%), H18 (10%)
DR	DRP	0.1376 ± 0.08371	0.003536	2	H1 (93%), H8 (7%)
JM	JAM	0.2333 ± 0.12562	0.006189	2	H1 (88%), H8 (12%)
TeT	TET	0.5072 ± 0.04451	0.030616	2	H1 (59%), H3 (41%)
	VEN	0.3368 ± 0.10981	0.020527	2	H1 (80%), H3 (20%)
VE	VBA	0.7579 ± 0.06338	0.049380	5	H1 (40%), H3 (30%), H4 (10%), H14 (10%), H15 (10%)
	VJG	nd	nd	1	H10 (nd)
CO	COT	0.4046 ± 0.07765	0.024193	2	H1 (73%), H3 (27%)
EC	ECO	0.5415 ± 0.07503	0.017697	3	H1 (61%), H3 (8%), H8 (31%)
	BSM	0.6000 ± 0.07302	0.030486	4	H1 (60%), H3 (15%), H4 (10%), H8 (15%)
	BCO	0.7111 ± 0.08600	0.029129	3	H1 (33%), H3 (33%), H8 (33%)
	BCR	0.5455 ± 0.14361	0.021156	2	H1 (80%), H11 (20%)
	BCG	0.5072 ± 0.09287	0.025430	3	H1 (20%), H3 (40%), H4 (40%)
	BGO	0.6737 ± 0.04925	0.035128	3	H1 (37.5%), H3 (37.5%), H4 (25%)
	BGN	0.7000 ± 0.05063	0.042586	5	H1 (68%), H3 (16%), H4 (10%), H8 (3%), H13 (3%)
BR	BCA	0.5119 ± 0.06748	0.032358	3	H1 (67%), H3 (25%), H8 (8%)
	BSS	0.5455 ± 0.06155	0.050644	2	H1 (50%), H4 (50%)
	BCP	0.6667 ± 0.07483	0.028079	3	H1 (55%), H3 (22.5%), H8 (22.5%)
	BES	0.5524 ± 0.04620	0.043660	3	H1 (56%), H3 (5%), H4 (39%)
	BCI	0.5241 ± 0.09216	0.034715	4	H1 (66%), H3 (7%), H4 (20%), H8 (7%)
	BFV	0.8000 ± 0.08884	0.040097	4	H1 (20%), H3 (40%), H4 (20%), H8 (20%)
	BSA	0.7273 ± 0.05051	0.035243	4	H1 (36.5%), H3 (36.5%), H4 (9%), H8 (18%)
	BPM	0.7059 ± 0.06997	0.048979	4	H1 (33%), H3 (11%), H4 (45%), H16 (11%)
PY	PYB	0.6243 ± 0.07950	0.036381	4	H1 (57%), H3 (22%), H4 (14%), H8 (7%)
	UBM	0.7026 ± 0.03178	0.043842	4	H1 (40%), H2 (5%), H3 (30%), H4 (25%)
	UPM	0.5684 ± 0.08629	0.036196	3	H1 (60%), H3 (30%), H4 (10%)
	UST	0.6210 ± 0.07046	0.037881	4	H1 (56%), H3 (25%), H4 (13%), H7 (6%)
UY	UDA	0.5887 ± 0.11396	0.025067	5	H1 (64%), H3 (9%), H5 (9%), H8 (9%), H9 (9%)
	UCC	0.6569 ± 0.03814	0.042428	4	H1 (48%), H3 (18%), H4 (30%), H6 (4%)
	UCO	0.6161 ± 0.06221	0.045371	4	H1 (53%), H3 (7%), H4 (33%), H12 (7%)
	UJS	0.6504 ± 0.03128	0.037409	3	H1 (38%), H3 (43%), H4 (19%)
Average		0.5263 ± 0.00114	0.029482 ± 0.000006	3	

Hs, haplotype diversity; Hap, *n*, number of haplotypes found in each locality; π, nucleotide diversity; nd, not determined.
CB, Cuba; DR, Dominican Republic; JM, Jamaica; T & T, Trinidad and Tobago; VE, Venezuela; CO, Colombia; EC, Ecuador; BR, Brazil; PY, Paraguay; UY, Uruguay.

Based on the significance of AMOVA, isolation by distance was tested in Caribbean and mainland populations. Caribbean results indicated that 22.2% of genetic differentiation between populations could be explained by distance (Mantel $r = 0.47$, $P = 0.0319$). No association was detected for mainland populations (Mantel $r = 0.058$, $P = 0.27$), indicating that the degree

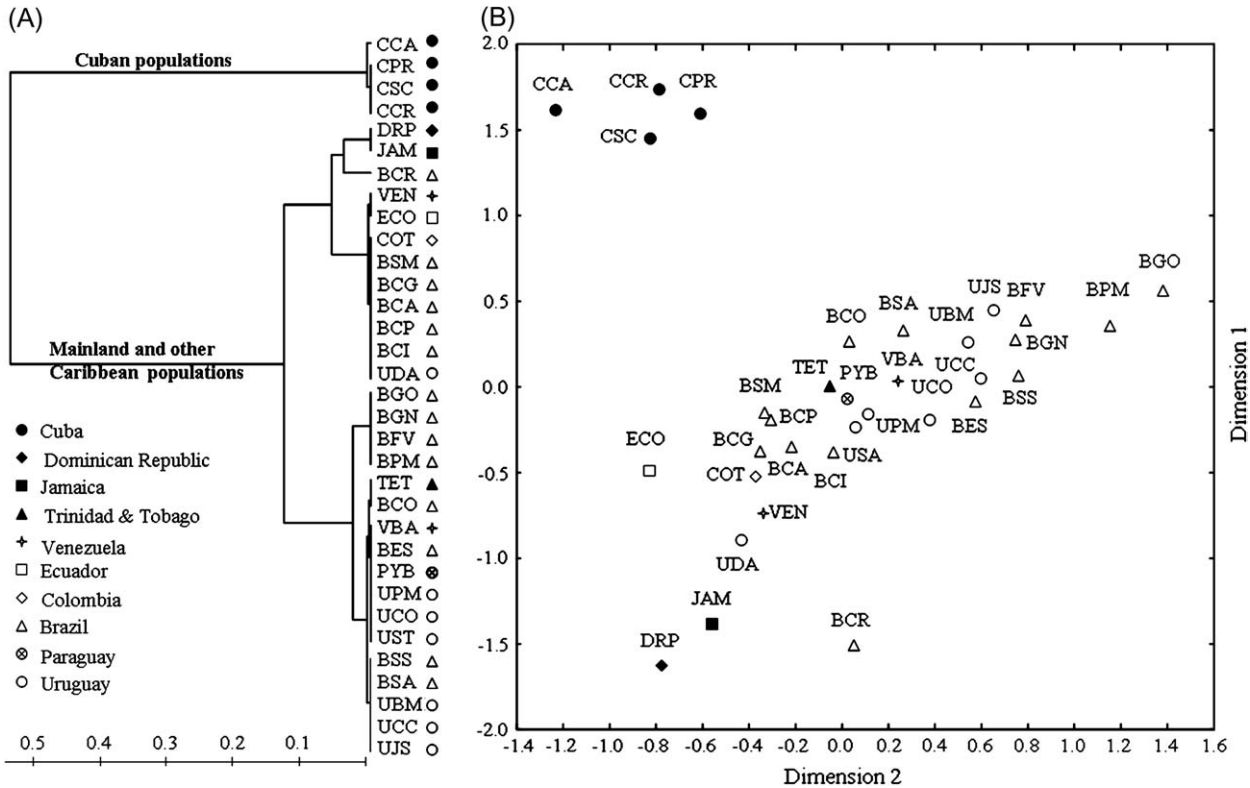

Fig.2. (A) UPGMA (unweighted pair group method with arithmetic mean) tree and (B) multidimensional scaling analysis diagram (MSD) showing the clustering of *Cochliomyia hominivorax* populations, obtained with Slatkin's linearized F_{ST} matrix. Caribbean populations are indicated by black symbols; mainland populations are indicated by open symbols (see legend in [A]). Population identification codes correspond to the localities given in Table 1.

of population differentiation was not related to the extent of geographic distance between sampled locations.

As low but significant differentiation was found between South American populations, and differentiation was not correlated to geographic distance, an exploratory analysis was conducted to identify the major area of variation in mainland populations. Populations were apportioned into different groups, according to geographic region and an AMOVA was conducted for each group. The groupings were: Northern (populations from Venezuela, Colombia, Ecuador and northern Brazil); Southern (populations from Uruguay, Paraguay and southern and southeastern Brazil), and Central (populations from midwestern Brazil). Results from AMOVA are shown in Table 4; only the Central group presented significant variation between populations and the genetic variation was not correlated with geographic distance (Mantel test, $r = -0.089$, $P = 0.5804$).

Discussion

Genetic diversity

This study on the NWS fly is one of the few studies of the genetic diversity and population structure of an insect pest across almost all of its geographic distribution. These results showed high levels of mitochondrial DNA variability and a complex pattern of genetic variation distribution for this species.

Analysis by PCR-RFLP of mitochondrial regions, particularly COI/COII, was valuable and sufficient to provide information at the population level for *C. hominivorax*, as previously suggested for the species (Litjens *et al.*, 2001; Lyra *et al.*, 2005), especially given the geographic scale studied here. Although the A+T/12S region did not present high restriction site polymorphism, it presented remarkable size variation. Intraspecific size variation in the control region has been documented in different groups of insects (e.g. Rondan Dueñas *et al.*, 2002; Snäll *et al.*, 2002; Oliveira *et al.*, 2007) and the analysis of this variation has provided important data on the population structure of insect species (Mardulyn *et al.*, 2003; Townsend & Rand, 2004). For this reason, the mtDNA control region of *C. hominivorax* populations is being analysed by DNA sequencing to establish whether it might be useful to characterize variation at the intraspecific level.

The genetic variability reported herein for *C. hominivorax* suggests that it is a highly polymorphic species and results obtained using mtDNA PCR-RFLP markers are consistent with conclusions reached using independently derived data from populations from North America, Central America, Brazil and Uruguay (Roehrdanz, 1989; Infante-Vargas & Azeredo-Espin, 1995; Taylor *et al.*, 1996; Lyra *et al.*, 2005). The level of genetic diversity with respect to mtDNA observed for this species falls

Table 4. AMOVA results obtained for the distribution of genetic variation among populations or groups of *Cochliomyia hominivorax*. See text for details about mainland population groups.

	ΦST	*P*-value
All populations	**0.1304**	< 0.00001
Caribbean	**0.5234**	< 0.00001
South America (mainland)	**0.0483**	0.0058
Northern	0.0044	0.4663*
Central	**0.1584**	0.0049
Southern	0.0097	0.3055*
Caribbean vs. mainland	**0.1180**	< 0.00001

*Non-significant values.
Significant values for population differentiation are in bold.

well within the range expected for intraspecific variation (e.g. Martin & Simon, 1990; Chapco *et al.*, 1992) and is comparable with diversities found in other insect pests (Estoup *et al.*, 1996; Baliraine *et al.*, 2004; Grapputo *et al.*, 2005).

Higher levels of genetic variability found within South American populations of *C. hominivorax*, by contrast with the lower levels found in Caribbean island populations, strongly support the hypothesis that South America represents the centre of origin of the NWS fly (Infante-Vargas & Azeredo-Espin, 1995; Infante-Malachias *et al.*, 1999). However, the presence of highly diverse populations in both central–southern Brazil and Venezuela hampers a more precise conclusion about the precise geographic origin of the species.

The low diversity indices found in Caribbean island populations, except that from Trinidad and Tobago, may be explained by a general model of the founder event, followed by genetic drift (Hedrick, 2005). However, the current results contrast with the higher genetic diversity obtained for the Caribbean populations with microsatellite markers (Torres & Azeredo-Espin, 2009), revealing that some alternative explanations for this more complex scenario of population structure in the Caribbean require elucidation.

The lack of congruence between nuclear and mtDNA genetic diversity may arise for other reasons, for example: (a) a rapid increase in population size after a recent founder event with a small number of females would recover nuclear diversity, but not mitochondrial diversity; (b) populations from islands may result from an ancient colonization, which also allows species to recover nuclear, but not mitochondrial, diversity, and (c) island populations may have arisen from multiple introductions of the same mtDNA haplotype. The complexity of the patterns observed may be a result of different histories of *C. hominivorax* introduction for the different islands studied (see Discussion) and, therefore, on the basis of our data we were unable to select any particular hypothesis as a more probable explanation for the scenario of diversity in the Caribbean.

Population differentiation

Comparisons among NWS populations revealed moderate subdivision on the geographic scale studied and indicated that the distribution of variation is non-uniform. High levels of differentiation were found among Caribbean populations and between them and mainland populations, by contrast with the low variation found among mainland populations.

Results suggested that, in general, island populations constitute independent evolutionary entities, connected by a greatly restricted gene flow. Nuclear data also support the conclusions from this study regarding the population structure for *C. hominivorax* in the Caribbean area (Torres & Azeredo-Espin, 2009).

Analogous comparisons for this pattern of population structure between island and mainland populations have been found in other insect species (Estoup *et al.*, 1996; De La Rúa *et al.*, 2001; Baliraine *et al.*, 2004; Shao *et al.*, 2004) and geographic isolation has been interpreted as the main cause of this genetic differentiation in island populations. Indeed, inter-island and mainland geographic isolation probably plays a major role in causing the extremely high levels of genetic differentiation among NWS populations in the Caribbean islands. However, the Trinidad and Tobago population is a more diverse population and, in this case, distance from the mainland may be insufficient to prevent gene flow.

Cuban populations were the most differentiated populations sampled in this study. The four populations from Cuba were very similar to one another with respect to mtDNA markers, but presented some differentiation in relation to nuclear markers (Torres & Azeredo-Espin, 2009). This difference between results may reflect a possible unequal contribution of the sexes in mediating gene flow, probably mediated by longer female dispersion (Hightower *et al.*, 1965; Mayer & Atzeni, 1993), or it may represent differences in the mode of inheritance and polymorphism of the markers, as previously suggested for Uruguayan NWS populations (Torres *et al.*, 2007).

As Cuban populations presented unexpectedly high frequencies of haplotype H8 (which is rare in other populations), low diversity indices and high genetic divergences, we are conducting fine-scale analyses with DNA sequences to obtain more conclusive data about the relationship between the Cuban and other populations. Results strongly suggest that the haplotype H8 from Cuba is different from those present in South American and other Caribbean populations (data not shown) and that mtDNA diversity is very small. More extensive studies are being conducted with these samples to resolve whether the assemblage is endemic to Cuba or represents a recent introduction.

Based on results obtained with mtDNA markers for diversity and population structure in Caribbean populations of *C. hominivorax*, we propose that islands have different histories of colonization. It is possible that the NWS in Jamaica, Dominican Republic and Trinidad and Tobago is of South American origin (via single or multiple introductions), whereas Cuban populations possibly originated from somewhere in Central or North America that was not sampled for this study. The Jamaican population was previously studied by Roehrdanz & Johnson (1988), who also suggested a South American origin. An extensive and periodical investigation in the Caribbean area would help us to draw more precise conclusions about the profile of introduction for different populations.

This study found a general pattern of low populational subdivision in South America, except in mainland populations located in midwestern Brazil, which showed substantial genetic variation. Few studies have been previously conducted in South American populations and these obtained different results for population structure. In Brazil, populations mainly from the state of São Paulo revealed moderate to high populational subdivision for NWS (Infante-Vargas & Azeredo-Espin, 1995 [RFLP of mtDNA]; Infante-Malachias *et al.*, 1999 [random amplification polymorphic DNA {RAPD-PCR}; Infante-Malachias, 1999 [allozymes]). New World screwworm populations from Uruguay have also been analysed by mtDNA PCR-RFLP and microsatellite markers (Lyra *et al.*, 2005; Torres *et al.*, 2007) and results suggest that the populations are very similar in the extreme south of the species' distribution.

The current study, in addition to these previous studies, strongly indicates that genetic variation in *C. hominivorax* populations is not distributed uniformly across the continent. Population structures for insect pests are being reported in different continents and are often associated with isolation by distance (Krafsur, 2002; Baliraine *et al.*, 2004). In this study, genetic differentiation between mainland populations could not be attributed to geographic distance, which implies a more complex pattern of cause for population differentiation.

Different patterns of genetic differentiation on the continent have been discussed for other insect pests. Grapputo *et al.* (2005) suggested that the genetic diversity partitioning in North American populations of the Colorado potato beetle, *Leptinotarsa decemlineata* L., is mainly the result of founder events during range expansion, agricultural management and insecticide application. Scataglini *et al.* (2006), working with boll weevil, *Anthonomus grandis* Bohmen, populations from South America, proposed that different populations were the result of recent invaders and ancient populations isolated by events of historical fragmentation. Similarly, we need alternative explanations to understand the population subdivisions in the *C. hominivorax* population of South America.

One possible cause for genetic diversity partitioning in the NWS is the potential influence of human activities. Management policies to control this pest and strong selection imposed by the use of insecticides (Carvalho *et al.*, 2006) may partially explain differences in local populations. As *C. hominivorax* is mainly livestock-associated, the movement of animals across landscapes may be an important but often overlooked process influencing the population dynamics of this insect pest. Other factors, such as seasonal regimes, events of extinction and re-colonization and local selection regimes may have contributed to this complex pattern of variability distribution in some regions. Unfortunately, on the basis of the data from this study it was not possible to formulate a more precise explanation of local differentiation and our results highlight the need to evaluate factors that may influence population structure by, for example, fine-scale analysis and the use of supplemental ecological data.

Over the past few years various studies have attempted to assess the population structure of *C. hominivorax* throughout its geographic distribution, but with discordant results. Based on the results of this study, we conclude that populations of *C. hominivorax* are diverse and structured with respect to mtDNA. The species presents a complex pattern of genetic distribution throughout the current range, with few isolated populations and some panmictic populations. The distinct patterns of genetic variation observed can be explained only by complex demography and their maintenance needs to be investigated in order to consider the influences of several processes, separately and in combination, in the different regions and geographic scales studied.

Implications from the results for control programmes

The current study provides different perspectives through which we might interpret patterns of population subdivision in *C. hominivorax* populations across its current distribution, from isolated evolutionary units to non-differentiated populations. Our results indicate that some implications for the development of control programmes need to be discussed. The programme for the eradication of *C. hominivorax* in North and Central America, which has implemented the integrated use of SIT over the last 45 years, has been very successful (Vargas-Terán *et al.*, 2005) and, at present, the pest is restricted to several Caribbean islands and South America.

Some new efforts have been made in designing NWS eradication programmes in Cuba, Hispaniola and Jamaica (FAO, 1999, 2003; Dyck *et al.*, 2005). However, in Cuba, only the first part of the eradication campaign programme was concluded and the release of sterile insects was not initiated because of diplomatic problems and the absence of donors (FAO, 1999; García-Rodriguez, 2003). In Jamaica, the programme began releasing sterile flies in 1999, but by mid-2004 little progress had been made (Dyck *et al.*, 2005). Although most of the problems encountered in the Jamaican programme were attributed to several logistical factors, the importance of collecting baseline data on NWS population ecology and dynamics, before initiating sterile insect release, was recognized (Dyck *et al.*, 2005). Roehrdanz (1989) found extensive genetic diversity ($> 2\%$) between Jamaican and mainland populations of *C. hominivorax*, and it is still not known if this may have contributed to problems in the efficiency of the eradication programme in Jamaica (FAO, 2003; Dyck *et al.*, 2005).

Data presented here suggest that Caribbean islands may be ideal locations for the implementation of eradication programmes because their NWS populations can be delimited into units that are relatively isolated from one another. Before initiating an SIT programme, however, it is important to establish the extent of genetic diversity and divergence that may disrupt the control programme because we found different levels of genetic variation in populations analysed.

New World screwworm populations from South America demonstrate low differentiation. The first implication of these results is that, as gene flow appears to be high enough to maintain extensive genetic homogeneity among almost all mainland populations, control programmes on a small geographic scale are unlikely to be effective. However, this study found local differentiation for some Brazilian populations and, as yet, little is known about Amazonian populations and whether the forest or the Andes may be considered as geographic barriers, suggesting that fine-scale analysis is needed to supplement our results in this area.

Therefore, prior to the development and implementation of any eradication strategies in mainland South America, comprehensive efforts to establish the most effective methods of control should be undertaken. These should involve a diverse range of areas and should include the undertaking of extensive, fine-scale genetic studies, as well as government and livestock producer involvement and personnel training (see Dyck *et al.*, 2005).

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Conflicts of interest

All authors declare no conflicts of interests.

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Phylogeography and recent emergence of the Old World screwworm fly, *Chrysomya bezziana*, based on mitochondrial and nuclear gene sequences

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Abstract. A previous study had identified an African and an Asian race of the Old World screwworm fly, *Chrysomya bezziana* Villeneuve (Diptera: Calliphoridae), based on the 3′ terminal 279 basepairs (bp) of the mitochondrial *cytochrome b* gene. The current study improved the phylogeographic resolution of *cytochrome b* for this species by characterizing more of the gene (the 3′ terminal 715 bp) and by sampling more geographical populations, including Oman, Iran, Hong Kong and the Indonesian islands of Sulawesi and East Sumba. Strong support was found for recognizing an African race, but not for a monophyletic Asian race. The cladistic and genealogical relationships among the Asian populations were complex. There was sufficient genetic homogeneity throughout separate regions (mainland Asia and each Indonesian island) to suggest that there are no reproductive barriers within each region that might necessitate the production of more than one strain for control by the sterile insect technique (SIT). Primers were designed for the amplification by polymerase chain reaction of two nuclear loci, the highly conserved *elongation factor-1*α gene and the less conserved *white* gene, and the preliminary results indicated that these genes showed the same pattern of small-scale regional variation as *cytochrome b*. The *cytochrome b* haplotypes are useful markers for identifying the geographical origins of any emerging infestations of the species: the absence of Indonesian and African haplotypes in the Middle East demonstrates that the large-scale transport of livestock is not spreading Old World screwworm.

Key words. *Chrysomya bezziana*, *elongation factor-1*α gene, emergence, mitochondrial *cytochrome b* gene, Old World screwworm fly, phylogeography, *white* gene.

Introduction

Hall *et al.* (2001) reviewed genetic studies of the Old World screwworm fly, *Chrysomya bezziana* and concluded that it occurs as two geographical races (African and Asian), based on a phylogenetic analysis of the 3′ terminal 279 basepairs (bp) of the mitochondrial gene *cytochrome b*. They concluded that the 1.8–2.5% pairwise divergence between the African and Asian mtDNA lineages could not have accumulated during the last 10 000 years (the Holocene epoch), during which humans domesticated the hoofed animal hosts now frequently exploited by *Ch. bezziana*, because mtDNA shows a pairwise divergence of *c.* 2.3% per million years in insects (Brower, 1994).

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The current study aimed to improve the phylogeographic resolution of *cytochrome b* for this species by characterizing more of the gene (715 bp) and by sampling more geographical populations. In addition, some of the new populations were characterized at two nuclear loci, the highly conserved *elongation factor-1*α (*EF-1*α) gene (Danforth & Ji, 1998; Esseghir *et al.*, 2000) and the less conserved *white* gene (Bennett & Frommer, 1997; Gomulski *et al.*, 2001), to test for any speciation or genetic divergence masked by cytoplasmic sweeps affecting the mtDNA. Collections were focused on the Arabian/Persian Gulf region in the Middle East and on Indonesia because these are regions where the species can be economically important (Al-Izzi *et al.*, 1999; Wardhana *et al.*, 2003) and where knowledge of population genetics could help plan interventions using the sterile insect technique (SIT) (Spradbery, 1994).

Materials and methods

Populations and preparation

Samples came from three African countries, five countries bordering the Gulf (Middle East) and five Southeast Asian countries (Table 1). Second or third instars were removed with forceps from wounds of several host species (cattle, horses, goats, sheep, pigs, dogs) and stored in 80% analytical grade ethanol, at ambient field temperatures or at 4 °C (London). Larvae collected after 2002 (specimen codes MHa200 onwards) were killed by immersion in boiling tap water for 15 s to enhance preservation (Adams & Hall, 2003) before storage in ethanol. Adult flies were caught with nets, starved for 24 h, killed by freezing and stored dry on pins at ambient temperatures.

DNA extraction from single specimens

Away from the molecular biology laboratory, flame-sterilized micro-needles and forceps were used to dissect and scrape out muscle tissue from individual specimens, from three median segments of each larva and from the entire thorax of each adult. DNA was extracted from the tissues using the DNAzol® kit (Invitrogen Corp., Carlsbad, CA, U.S.A.) according to Chomczynski *et al.* (1997), dissolved in 1x Tris-EDTA solution and stored short-term at 4 °C and longterm at − 20 °C.

Primers and PCR amplification of target genes

Primers were synthesized by MWG Biotech (UK) Ltd (Milton Keynes, U.K.). Fragment sizes include primers. Negative and positive controls were used. The 3′ terminal of the mitochondrial gene *cytochrome b* was amplified within either a 761-bp fragment (CB) using the primers CB1-SE (5′–3′ TATGTACTACCATGAGGACAAATATC) (Esseghir *et al.*, 2000) and PDR-WR04 (5′–3′ ATTTCACGCTCATTAACT) (Hall *et al.*, 2001), or, if the genomic DNA was degraded, a 338-bp fragment (CB3) using the primers CB3-PDR (5′–3′ CAYATTCAACCWGAATGATA) and PDR-WR04. Polymerase chain reaction (PCR) conditions were those reported by Hall *et al.* (2001), except that the *Taq* polymerase was part of the total reagent master-mix and two different thermal cyclers were used with equal success: the PE 9700 0.2 mL (Perkin Elmer, Inc., Waltham, MA, U.S.A.) and the Techne Genius 2 (Barloworld Scientific Ltd, Stone, Staffs, U.K.).

A 401-bp intronless fragment of the *EF-1*α gene was amplified using the primers EF-F05 (5′–3′ CCTGGACATCGTGATTTCAT) and EF-F06 (5′–3′ TTACCTTCAGCGTTACCTTC), with PCR conditions according to Testa *et al.* (2002) except that a single annealing temperature of 48 °C was used for 35 cycles.

A 617-bp fragment from exon 3 of the *white* gene was amplified using the primers WEC-F21 (5′–3′ GTTTGTGGCGTAGCCTATCC) and WEC-R12 (5′–3′ AATGTCACTCTACCYTCGGC), based on an alignment of the nucleotide sequences of Diptera (Bennett & Frommer, 1997; Gomulski *et al.*, 2001) and favouring the calliphorid *Lucilia cuprina* Wiedemann (Garcia *et al.*, 1996). Again, the PCR conditions were those of Testa *et al.* (2002) except that a single annealing temperature of 42 °C was used for 35 cycles.

DNA sequencing and editing

DNA fragments amplified by PCR were fractionated on 1.2–1.5% horizontal, submerged agarose gels before purification with glassmilk (GeneClean II Spin Kit; QBiogene, Cambridge, U.K.) and cycle-sequenced in both directions using an ABI PRISM® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Version 1.1) and semi-automated sequencing systems ABI 377A and ABI 3730x1 (Applied Biosystems, Inc., Foster City, CA, U.S.A.) with 1 pmol of primers. DNA sequences were manually edited and aligned in Sequencher™ 3.1–4.6 (Gene Codes Corp., Ann Arbor, MI, U.S.A.).

Phylogenetic analyses of aligned DNA sequences

PAUP* Version 4.0b 10 (Swofford, 2002) was used for phylogenetic analyses, with 1000 replicates for all bootstrap analyses. Population level genealogical networks were constructed in TCS (Clement *et al.*, 2000) using 95% parsimony.

Results

Phylogeographic analysis of the mitochondrial cytochrome b gene

Haplotype diversity. The 3′ terminal 279 bp were obtained for all specimens, either by amplifying only the short CB3 fragment (African populations: see Hall *et al.* [2001]) or by amplifying the larger CB fragment (most of the other populations). Only nine unique sequences (haplotypes CB3_bezz1001–1009) were identified (Table 1; GenBank accessions: FJ373321–FJ373329), with a single haplotype occurring in all populations except Iraq01 (CB3_bezz1005 with CB3_bezz1007) and the Indonesian island of Sulawesi (CB3_bezz1006 with CB3_bezz1009).

Table 1. *Chrysomya bezziana* characterized at mitochondrial *cytochrome b* locus (CB3 and CB haplotypes), nuclear *EF-1*α locus (diploid genotype) and nuclear *white* locus (diploid genotype).

Specimens: larva (L), adult (A)	Populations, wild or colony (C)	CB haplotypes 2007	CB3 haplotypes 2007 (2001)	*EF-1*α genotype	*White* genotype
Five (L)*	Zimbabwe 02		**CB3_bezz1001 (1)**		
One (L)*	Cameroon 07		CB3_bezz1001 (1)		
Two (L)*	Cameroon 07		**CB3_bezz1002 (2)**		
One (L)*	Chad 04		**CB3_bezz1003 (3)**		
One (L)*	Chad 04		**CB3_bezz1004 (4)**		
Two (L)*	Iraq 08		**CB3_bezz1005 (5)**		
Two (L)*	Iraq 09		CB3_bezz1005 (5)		
Two (L)*	Kuwait 11		CB3_bezz1005 (5)		
Two (L)*	Myanmar 13		CB3_bezz1005 (5)		
Two (L)*	Bahrain 18		CB3_bezz1005 (5)		
Two (L)*	Iran 19		CB3_bezz1005 (5)		
Three (L)	UAE 22		CB3_bezz1005 (5)		
Three (L)	UAE 23		CB3_bezz1005 (5)		
Two (L)*	UAE 24		CB3_bezz1005 (5)		
Three (A)*	Malaysia_A [C]		CB3_bezz1005 (5)		
Three (A)*	Malaysia_B [C]		CB3_bezz1005 (5)		
Two (L)*	PNG New Britain 14		**CB3_bezz1006 (6)**		
Two (L)*	PNG 16 [C]		CB3_bezz1006 (6)		
Five (L)*	PNG 17 [C]		CB3_bezz1006 (6)		
MHa09 (A)	Malaysia [C]	**CB_bezz01**	**CB3_bezz1005**		01 01
MHa10 (A)	Malaysia [C]	CB_bezz01	CB3_bezz1005		
MHa11 (A)	Malaysia [C]	CB_bezz01	CB3_bezz1005	01 01	01 01
MHa12 (A)	Malaysia [C]	CB_bezz01	CB3_bezz1005	01 01	01 01
MHa20 (L)	Malaysia [C]	CB_bezz01	CB3_bezz1005		
MHa321 (L)	Iran, Boushehr Province, Daier	CB_bezz01	CB3_bezz1005		
MHa322 (L)	Iran, Boushehr Province, Daier	CB_bezz01	CB3_bezz1005		
MHa436 (L)	Hong Kong	CB_bezz01	CB3_bezz1005		
MHa437 (L)	Hong Kong	CB_bezz01	CB3_bezz1005		
MHa439 (L)	Hong Kong	CB_bezz01	CB3_bezz1005		
MHa458 (A)	Malaysia	CB_bezz01	CB3_bezz1005		
MHa66 (L)	South Iran 01	**CB_bezz02**	CB3_bezz1005		
MHa69 (L)	South Iran 01	CB_bezz02	CB3_bezz1005		
MHa240b (L)	South Iran 02	CB_bezz02	CB3_bezz1005		
MHa241b (L)	South Iran 02	CB_bezz02	CB3_bezz1005		
MHa242b (L)	South Iran 02	CB_bezz02	CB3_bezz1005	01 01	
MHa245b (L)	South Iran 02	CB_bezz02	CB3_bezz1005		
MHa260b (L)	Iran03	CB_bezz02	CB3_bezz1005		05 07
MHa261ab (L)	Iran03	CB_bezz02	CB3_bezz1005		05 07
MHa262ab (L)	Iran03	CB_bezz02	CB3_bezz1005	01 02	05 07
MHa263ab (L)	Iran03	CB_bezz02	CB3_bezz1005	01 01	05 07
MHa265 (L)	Iraq01 [C]		CB3_bezz1005	01 05 or 03 04	01 01
MHa266 (L)	Iraq01 [C]	CB_bezz02	CB3_bezz1005	01 05 or 03 04	01 05
MHa267 (L)	Iraq01 [C]	CB_bezz02	CB3_bezz1005		01 05
MHa268 (L)	Iraq01 [C]	CB_bezz02	CB3_bezz1005	04 05	01 01
MHa332 (L)	Iran, Boushehr Province, Daier	CB_bezz02	CB3_bezz1005		
MHa333 (L)	Iran, Boushehr Province, Daier	CB_bezz02	CB3_bezz1005		
MHa336 (L)	Iran, Boushehr Province, Deilam	CB_bezz02	CB3_bezz1005		
MHa337 (L)	Iran, Boushehr Province, Deilam	CB_bezz02	CB3_bezz1005		
MHa338 (L)	Iran, Boushehr Province, Deilam	CB_bezz02	CB3_bezz1005		
MHa339 (L)	Iran, Boushehr Province, Deilam	CB_bezz02	CB3_bezz1005		
MHa340 (L)	Iran, Hormozgan Province, Dashtestan	CB_bezz02	CB3_bezz1005		

Continued

Table 1. Continued.

Specimens: larva (L), adult (A)	Populations, wild or colony (C)	CB haplotypes 2007	CB3 haplotypes 2007 (2001)	*EF-1*α genotype	*White* genotype
MHa341 (L)	Iran, Hormozgan Province, Dashtestan	CB_bezz02	CB3_bezz1005		
MHa342 (L)	Iran, Hormozgan Province, Dashtestan	CB_bezz02	CB3_bezz1005		
MHa343 (L)	Iran, Hormozgan Province, Dashtestan	CB_bezz02	CB3_bezz1005		
MHa344 (L)	Iran, Hormozgan Province, Dashtestan	CB_bezz02	CB3_bezz1005		
MHa345 (L)	Iran, Hormozgan Province, Dashtestan	CB_bezz02	CB3_bezz1005		
MHa346 (L)	Iran, Hormozgan Province, Dashtestan	CB_bezz02	CB3_bezz1005		
MHa347 (L)	Iran, Hormozgan Province, Dashtestan	CB_bezz02	CB3_bezz1005		
MHa359 (L)	Iran, Hormozgan Province, Minab	CB_bezz02	CB3_bezz1005		
MHa364 (L)	Iran, Hormozgan Province, Minab	CB_bezz02	CB3_bezz1005		
MHa365 (L)	Iran, Hormozgan Province, Minab	CB_bezz02	CB3_bezz1005		
MHa374 (L)	Iran, Boushehr Province, Genaveh	CB_bezz02	CB3_bezz1005		
MHa447 (L)	Iraq	CB_bezz02	CB3_bezz1005		
MHa450 (A)	Malaysia	CB_bezz02	CB3_bezz1005		
MHa456 (A)	Malaysia	CB_bezz02	CB3_bezz1005		
MHa457 (A)	Malaysia	CB_bezz02	CB3_bezz1005		
MHa459 (A)	Malaysia	CB_bezz02	CB3_bezz1005		
MHa269 (L)	Iraq01 [C]	**CB_bezz03**	**CB3_bezz1007**	01 03	05 05
MHa352 (L)	Oman	**CB_bezz04**	**CB3_bezz1008**		
MHa353 (L)	Oman	CB_bezz04	CB3_bezz1008		
MHa354 (L)	Oman	CB_bezz04	CB3_bezz1008		
MHa355 (L)	Oman	CB_bezz04	CB3_bezz1008		
MHa163 (L)	Sulawesi	**CB_bezz05**	**CB3_bezz1006**	01 01	01 02
MHa165 (L)	Sulawesi	**CB_bezz06**	**CB3_bezz1009**		
MHa166 (L)	Sulawesi	CB_bezz06	CB3_bezz1009		
MHa155 (L)	Sulawesi	CB_bezz06	CB3_bezz1009		01 08
MHa156 (L)	Sulawesi	CB_bezz06	CB3_bezz1009	01 01	01 01
MHa164 (L)	Sulawesi	CB_bezz06	CB3_bezz1009	01 01	05 05
MHa157 (L)	Sulawesi	CB_bezz06	CB3_bezz1009		04 05
MHa158 (L)	Sulawesi	CB_bezz06	CB3_bezz1009	01 03	04 05
MHa169 (L)	East Sumba	**CB_bezz07**	**CB3_bezz1009**	01 01	06 06
MHa170 (L)	East Sumba	CB_bezz07	CB3_bezz1009	01 01	06 06
MHa171 (L)	East Sumba	CB_bezz07	CB3_bezz1009		
MHa172 (L)	East Sumba	CB_bezz07	CB3_bezz1009	01 01	05 06
MHa173 (L)	East Sumba	CB_bezz07	CB3_bezz1009		
MHa174 (L)	East Sumba	CB_bezz07	CB3_bezz1009		
MHa153 (L)	East Sumba	CB_bezz07	CB3_bezz1009		
MHa154 (L)	East Sumba	CB_bezz07	CB3_bezz1009		
MHa151 (L)	East Sumba	CB_bezz07	CB3_bezz1009		06 06
MHa152 (L)	East Sumba	CB_bezz07	CB3_bezz1009	01 03	06 06
MHa159 (L)	East Sumba	CB_bezz07	CB3_bezz1009	01 03	06 06
MHa160 (L)	East Sumba	CB_bezz07	CB3_bezz1009	01 01	06 06
MHa151 (L)	East Sumba	**CB_bezz08**	**CB3_bezz1009**		06 06
MHa152 (L)	East Sumba	CB_bezz08	CB3_bezz1009	01 03	06 06
MHa159 (L)	East Sumba	CB_bezz08	CB3_bezz1009	01 03	06 06
MHa160 (L)	East Sumba	CB_bezz08	CB3_bezz1009	01 01	06 06

*Specimen details and *cytochrome b* characterization given by Hall *et al.* (2001).UAE, United Arab Emirates; PNG, Papua New Guinea.

The 3′ terminal 715 bp obtained from the CB fragment showed greater diversity than the CB3 fragment, with a total of eight haplotypes (CB_bezz01-08) found in the Asian samples (Table 1; GenBank accessions: FJ373330–FJ373337), compared with five for CB3, and two haplotypes co-occurring in most Gulf countries and Malaysia (CB_bezz01 with CB_bezz02) and in the Indonesian islands of Sulawesi (CB_bezz05 with CB_bezz06) and East Sumba (CB_bezz07 with CB_bezz08).

In all cases, the 3′ terminal 279 bp were identical in both the CB3 and CB fragments from the same specimen. Heteroplasmy was recorded in four larvae from East Sumba, each of which had both of the haplotypes CB_bezz07 and CB_bezz08 (Table 1).

Phylogenetic analysis. Rooted on the CB3 sequences of the outgroups *Chrysomya albiceps* (Wiedemann) and *Chrysomya megacephala* (F) (Hall *et al.*, 2001; GenBank accessions: FJ373319, FJ373320), a bootstrap parsimony analysis with a branch-and-bound search (Fig. 1) gave strong support (96%) for the monophyly of all the haplotypes of *Ch. bezziana* and strong support (85%) for the monophyly of all four haplotypes of this species restricted to sub-Saharan Africa, but there was only weak support (48%) for the monophyly of all the Asian haplotypes of *Ch. bezziana*.

A parsimony analysis of the longer CB sequences was more informative about relationships among the Asian haplotypes of *Ch. bezziana*. A bootstrap analysis with a branch-and-bound search gave strong support (76%) for the monophyly of three of the four haplotypes of *Ch. bezziana* restricted to Indonesia (CB_bezz06, CB_bezz07 and CB_bezz08, but not CB_bezz05).

Geographic distribution of haplotypes. The geographical distribution of each CB3 haplotype (Table 1) was restricted to one region of single countries, except for the widespread occurrence of CB3_bezz1005 from the Gulf to Hong Kong and the Malaysian peninsula and of CB3_bezz1009 shared by the large islands of Sulawesi and East Sumba. CB haplotyping indicated geographical localization within these Indonesian islands, with CB_bezz06 occurring only in Sulawesi and CB_bezz07 and CB_bezz08 restricted to East Sumba.

Cladistic analysis (using branch-and-bound searches) is not always appropriate for investigating intra-specific relationships

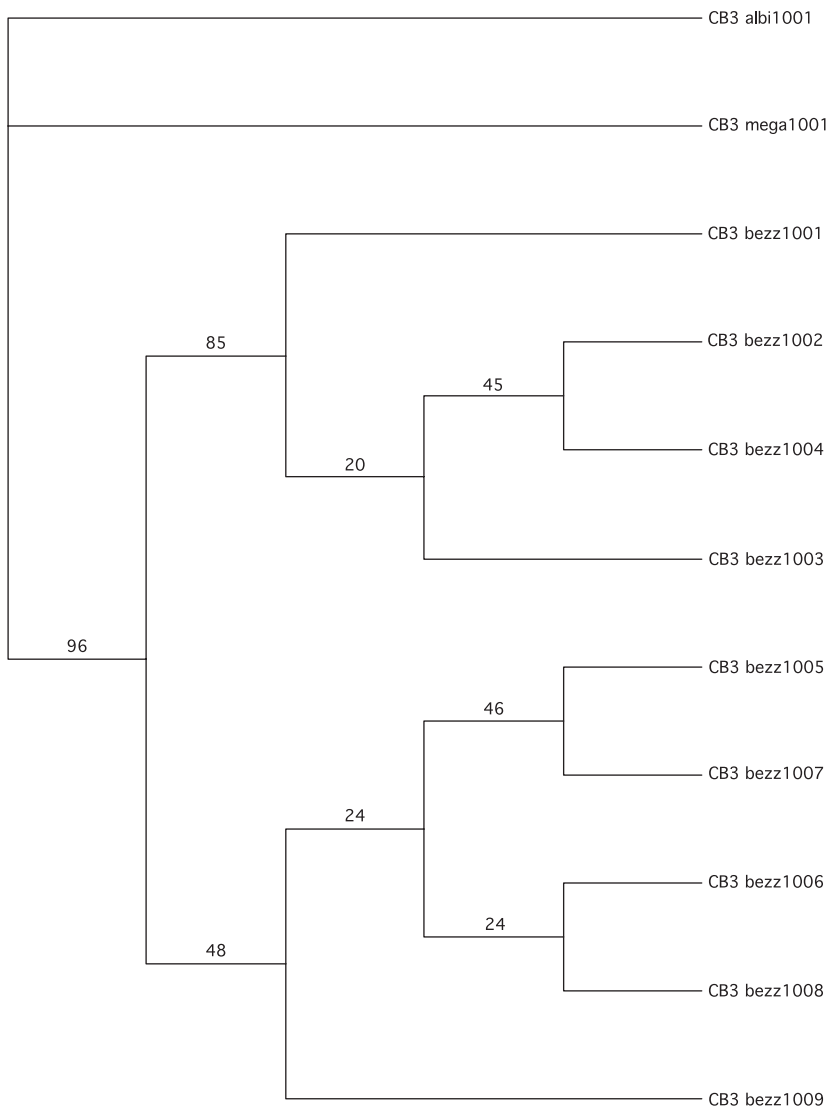

Fig. 1. A 50% majority-rule phylogenetic tree (plus other compatible groups) of the CB3 haplotypes of mitochondrial *cytochrome b* of *Chrysomya bezziana* (bezz) and the outgroups *Chrysomya albiceps* (albi) and *Chrysomya megacephala* (mega). The tree was produced by a bootstrap parsimony analysis (1000 replicates) with a branch-and-bound search in PAUP*. Bootstrap support for each branch is shown as a percentage.

because of incomplete lineage sorting and so population-level genealogical networks were constructed in TCS (Templeton *et al.*, 1992; Clement *et al.*, 2000). The TCS analysis of CB3 haplotypes (Fig. 2) indicated that the haplotype found in Oman (CB3_bezz1008) could be ancestral and the well-differentiated sub-Saharan cluster of haplotypes were closest to haplotype CB3_bezz1009 from Indonesia. The TCS analysis of CB haplotypes from Asia (Fig. 3) indicated that one of the haplotypes widespread on the mainland (CB_bezz01) could be ancestral, three of the four haplotypes restricted to Indonesia formed a cluster (CB_bezz06, CB_bezz07 and CB_bezz08) and the fourth (CB_bezz05) had a divergent origin.

Phylogeographic analysis of the nuclear EF-1α gene

Three of the 361 (0.83%) nucleotide sites analysed were polymorphic and all substitutions were synonymous at third-base sites. Five alleles were identified (bezz_EF01–bezz_EF05) in the 19 specimens characterized (Table 1; GenBank accessions: FJ373338–FJ373342), and the genotypes could not be unambiguously scored for only two specimens. Allele bezz_EF01 predominated (minimum frequency 0.71) and was found in all the countries sampled (Iraq, Iran, Malaysia, Indonesia). The less frequent alleles were geographically restricted, and were found only in Iran (bezz_EF02), only in Iraq and Indonesia (bezz_EF03), or only in Iraq (bezz_EF04 and bezz_EF05). The population from Iraq was most diverse, and showed four alleles in four specimens, compared with that from East Sumba, which showed two alleles in six specimens.

Phylogeographic analysis of the nuclear white gene

Nine of the 577 (1.56%) nucleotide sites analysed were polymorphic and all substitutions were synonymous. The 192 amino acids were aligned with GenBank sequences of *Anopheles gambiae* Giles (Besansky *et al.*, 1995), *Drosophila melanogaster* Meigen (Davison *et al.*, 1985), *Drosophila simulans* Sturtevant (Kirby & Stephan, 1995), *Bactrocera tryoni* (Froggatt) (Bennett & Frommer, 1997), *Ceratitis capitata* Wiedemann (Gomulski *et al.*, 2001) and *Lucilia cuprina* Wiedemann (Garcia *et al.*, 1996). The sequence of *Ch. bezziana* differed least (by just three amino acids) from that of *L. cuprina*, and these two calliphorid sequences formed a well-supported clade (100%) in a bootstrap parsimony analysis with a branch-and-bound search.

Seven alleles were identified (bezz_white01, bezz_white02, bezz_white04–bezz_white08) in the 25 specimens characterized (Table 1; GenBank accessions: FJ373344–FJ373349), and the genotypes were unambiguously scored for all specimens. A bootstrap parsimony analysis with a branch-and-bound search found two well-supported (> 70%) clades: alleles bezz_white05, bezz_white06 and bezz_white07; and alleles bezz_white01, bezz_white04 and bezz_white08. Alleles from both clades were found in the Gulf and in Sulawesi, indicating an absence of phylogeographic structure.

Allele bezz_white01 was found throughout the sampled range of *Ch. bezziana* (in the Gulf, Malaysia and Indonesia), but it did not predominate anywhere (frequency 0.32) and was not found in Iran (Table 1). Allele bezz_white05 was widespread and locally abundant, occurring in Iraq (frequency 0.50), Iran (frequency 0.50) and Sulawesi (frequency 0.40). Two alleles were restricted to single regions: bezz_white07 in Iran (frequency 0.5), and bezz_white06 in the island of East Sumba (frequency 0.93).

Discussion

Building on the study by Hall *et al.* (2001), additional geographical populations were sampled from Oman, Iraq, Iran, Hong Kong, Malaysia and the Indonesian islands of Sulawesi and East Sumba. All were characterized by sequencing the 3′ terminal 715 bp of the *cytochrome b* gene (CB fragment), and some

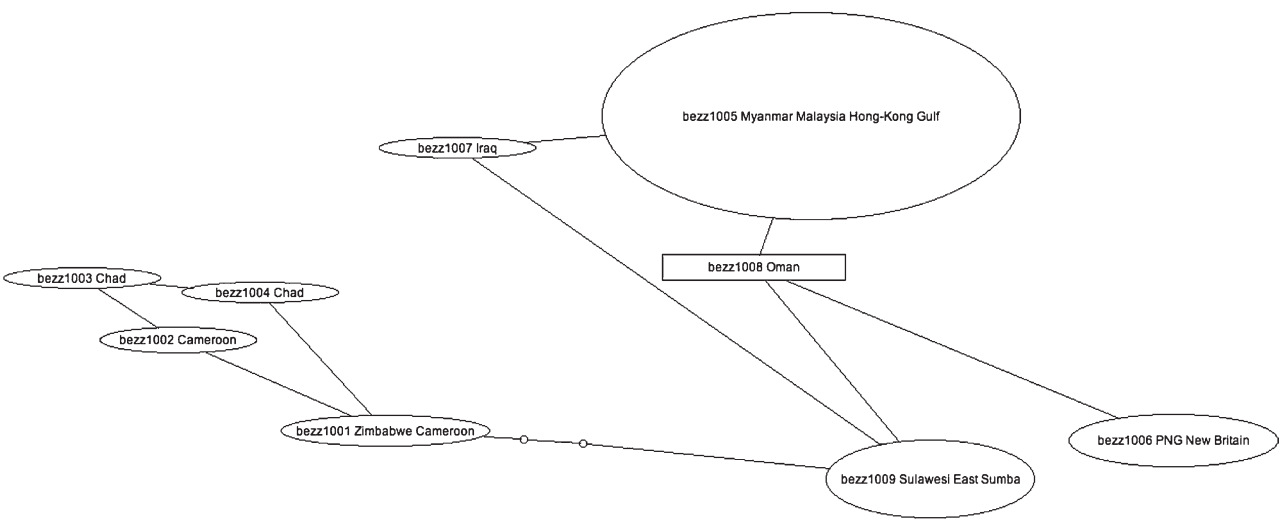

Fig. 2. Genealogical network (TCS) of the CB3 haplotypes of *Chrysomya bezziana* of mitochondrial *cytochrome b*. The areas of the ovals and rectangle (indicating that haplotype CB3_bezz1008 is ancestral) are proportional to the number of specimens characterized. Each line represents one pairwise nucleotide difference; missing haplotypes are indicated by small circles.

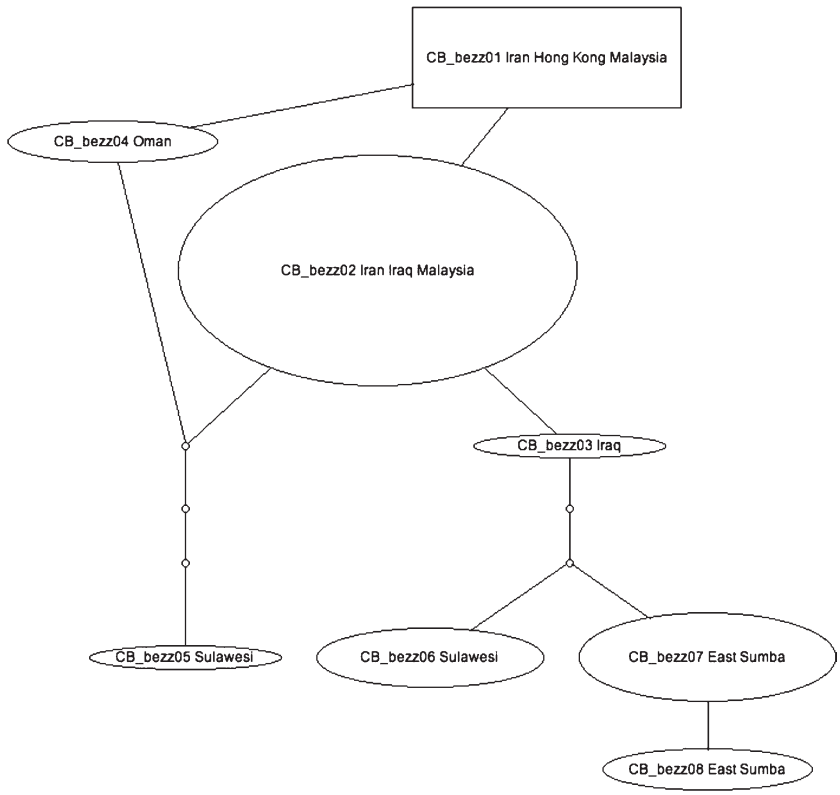

Fig. 3. Genealogical network (TCS) of the CB haplotypes of *Chrysomya bezziana* mitochondrial *cytochrome b*. The areas of the ovals and rectangle (indicating that haplotype CB_bezz01 is ancestral) are proportional to the number of specimens characterized. Each line represents one pairwise nucleotide difference; missing haplotypes are indicated by small circles.

individuals were characterized by sequencing fragments of two nuclear loci for which primers were designed. Unfortunately, it was not possible to obtain samples sufficiently large for population genetics studies, and it proved impossible to source northeast African populations, perhaps because of the economic risks of acknowledging the presence of a pest that is on List B of notifiable diseases published in the Animal Health Code of the Office International des Epizooties (OIE).

Parsimony analysis of the 3′ terminal 279 bp of the *cytochrome b* gene (the original CB3 fragment) again indicated that *Ch. bezziana* is a single phylogenetic species within which there is a distinctive geographical race in sub-Saharan Africa, as reported by Hall *et al.* (2001), but the addition of haplotypes from the new locations did not support the recognition of a single Asian lineage. Within Asia, a parsimony analysis of the longer CB fragment gave strong support only for a clade containing three of the four haplotypes restricted to Indonesia. All these findings were consistent with the genealogical networks constructed in TCS, which is probably a more appropriate method for analysing the relationships among intra-specific mtDNA haplotypes (Templeton *et al.*, 1992).

Inclusion of the new Asian data in the analysis of the *cytochrome b* gene indicated more geographical population structure in the Old World screwworm (Table 1) than reported by Hall *et al.* (2001), with new haplotypes found to be restricted to the Gulf (CB_bezz04 in Oman) and the two Indonesian islands of Sulawesi (CB_bezz06) and East Sumba (CB_bezz07, with the very similar CB_bezz08). However, there remained a remarkable lack of diversity in Iraq, Iran and mainland Southeast Asia (Hong Kong, Malaysian peninsula), with only two very similar haplotypes predominating (CB_bezz01 and CB_bezz02). The absence of these two haplotypes in Indonesia and the occurrence there of island-specific predominant haplotypes provides strong evidence that the Old World screwworm is not dispersing easily within or beyond this large archipelago. From this, it is concluded that the species might well be controlled on Indonesian islands by using SIT, without a high risk of reinvasion. Based on a pairwise divergence rate of 2.3% for insect mtDNA sequences (Brower, 1994; Esseghir *et al.*, 2000), the Indonesian clade of haplotypes (CB_bezz06, CB_bezz07, CB_bezz08) must have been diverging from the haplotypes now found on mainland Asia for at least 182 000 years. The process of distinguishing allopatric divergence from dispersal noise for insect groups that have invaded the Indo-Australian tropics (Holloway, 1998) is not straightforward, and such an analysis for *Ch. bezziana* should await a more comprehensive sampling of Indonesian islands and others nearby.

The lack of mtDNA diversity on mainland Asia might be a result of natural and/or human-assisted dispersion, but this genetic homogeneity suggests reproductive compatibility throughout a region within which SIT might be applied using a single production unit for rearing sterile males of a single strain. However, it should be cautioned that mtDNA homogeneity does not always indicate chromosomal homogeneity, because cytoplasmic sweeps can sometimes homogenize only the former (e.g. for *D. simulans* [Turelli & Hoffmann, 1991]). Only a small number of flies were characterized at two nuclear loci, but the current preliminary results are consistent with a shared geographical pattern of small-scale regional genetic variation of

both mtDNA and nuclear DNA, rather than with local cytoplasmic sweeps associated with greater nuclear diversity. Thus, the highly conserved *EF-1*α gene showed most diversity in the Gulf – the putative origin of *Ch. bezziana* according to the genealogical analyses of mtDNA (Figs 2 and 3) – and the more polymorphic *white* gene had some alleles restricted to Iran and Indonesia.

The current phylogeographic investigations should be followed up with experimental testing of the reproductive compatibility of Old World screwworm from different lineages in preparation for SIT (Spradbery, 1994). At least in the short-term, the new mtDNA markers reported here will be useful for identifying the geographical origins of any emerging infestations of this species. The absence of Indonesian and African mtDNA haplotypes in the Gulf demonstrates that the large-scale transport of livestock is not spreading the species, even though livestock shipped through endemic regions can be infested *en route* (Rajapaksa & Spradbery, 1989; Spradbery, 2001) and the spread of *Ch. bezziana* by ship within the Gulf has been reported (Kloft *et al.*, 1981; Rajapaksa & Spradbery, 1989).

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Conflicts of interest

All authors declare no conflicts of interests.

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Genetic diversity of populations of Old World screwworm fly, *Chrysomya bezziana*, causing traumatic myiasis of livestock in the Gulf region and implications for control by sterile insect technique

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Abstract. Fly larvae were collected from 181 cases of traumatic myiasis in livestock in 10 regions of four countries in the Middle East Gulf region: Iran, Iraq, Saudi Arabia and Oman. The predominant fly species responsible for cases was the Old World screwworm (OWS) fly, *Chrysomya bezziana* Villeneuve (Diptera: Calliphoridae). In cases from Iran and Oman, which included non-OWS fly species, OWS fly was found solely responsible for 67.6% of cases and jointly with other fly species for a further 12.7% of cases. The major hosts were sheep and goats, together comprising 84.6% of the total, which reflects their predominance among the livestock of these Gulf countries. The major site of wounding on sheep and goats was the tail (40.3%), followed by female genitalia (14.0%). The 3′ terminal 715 nucleotides of the mitochondrial *cytochrome b* gene were sequenced for 178 larvae of OWS. Five haplotypes were identified: three had been recorded previously in the region (two were common throughout and one was unique to Oman), and two were newly identified, one from southern Iraq and the other from Saudi Arabia, both in regions sampled for the first time. The haplotypes varied from one another only at one or two nucleotide sites, equivalent to an intraspecific difference of 0.14–0.28% across the entire 715-bp fragment. There was a single statistically significant association between host species and haplotype in Saudi Arabia, a first such record for OWS fly. The small degree of genetic diversity between geographical populations of OWS fly within the Gulf region suggests that a single Gulf colony could be used to implement the sterile insect technique within an integrated control programme.

Key words. *Chrysomya bezziana*, mitochondrial *cytochrome b* gene, Old World screwworm fly, traumatic myiasis, Iran, Iraq, Oman, Saudi Arabia.

Introduction

The Old World screwworm (OWS) fly, *Chrysomya bezziana* Villeneuve (Diptera: Calliphoridae), is a major agent of traumatic myiasis throughout the tropical regions of the Old World and the island of New Guinea (Spradbery, 1994). The species is most successful under hot and wet conditions and, therefore, the mostly hot and dry regions surrounding the Middle East Gulf are not those in which it would be expected to flourish (Sutherst *et al.*, 1989). Nevertheless, human interventions in the form of tree planting and irrigation, which supplements natural watercourses and raises the humidity of an area, can make even naturally unfavourable locations seasonally suitable for habitation by OWS fly (Siddig *et al.*, 2005). A similar situation was found in Libya,

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where the New World screwworm (NWS) fly, *Cochliomyia hominivorax* (Diptera: Calliphoridae), was introduced in 1988: although the climate there was not naturally suited to NWS, human changes to the environment, especially irrigation, made it possible for the species to become established (UN Food & Agriculture Organization [FAO], 1992; Spradbery, 1994).

Cases of traumatic myiasis caused by OWS were first recorded in Iraq in 1996 (Abdul Rassoul *et al.*, 1996; Al-Izzi *et al.*, 1999; Al-Taweel *et al.*, 2000) and have led to calls for an area-wide programme to control the species throughout the Gulf region (Al-Izzi, 2002). In view of the success of the sterile insect technique (SIT) in eradicating NWS not only from huge areas of its natural range, but also from its Libyan foothold in Africa (FAO, 1992), SIT is being considered as a potential tool for use in the Gulf region. One of the important elements of a successful SIT campaign is sexual compatibility between the released strain of flies and the local population. In addition, other genetically determined factors may be important, such as insecticide resistance and pathogenicity. Therefore, an assessment of the genetic composition of this species in the Gulf region was considered an important early step in the planning of any management programme. To this end, samples of OWS from 10 regions (six new) in four countries in the region were collected for genetic analysis, extending the work already undertaken (Hall *et al.*, 2001; Ready *et al.*, 2009). Regions included locations in Iran, Iraq, Oman and, sampled by us for the first time, Saudi Arabia.

Cases in Iraq in 1996 prompted a renewed interest in OWS in the Gulf region, but human myiasis caused by OWS was reported from Iran as early as 1978 (Jdalayer *et al.*, 1978). Its importance as a livestock pest in Iran was not described until an outbreak of OWS myiasis occurred in Khoozestan Province, mainly among lambs and adult sheep (Navidpour *et al.*, 1996; Haddadzadeh *et al.*, 1997). In Saudi Arabia, older records of OWS infestation also refer to human cases (Ansari & Oertley, 1982; Kersten *et al.*, 1986), but there have been more recent reports of its importance to the livestock industry (Alahmed, 2002; El-Azazy & El-Metenawy, 2004). The presence of OWS in Oman was confirmed by Spradbery *et al.* (1992), who reviewed past records for the Gulf region and concluded that all Gulf countries were endemic for OWS fly.

The present study was undertaken to determine the genetic diversity of OWS fly in four countries that encircle the Gulf, using the comparative sequence analysis of a fragment of the mitochondrial *cytochrome b* gene (Hall *et al.*, 2001; Ready *et al.*, 2009). In addition, data were collected on other species that cause traumatic myiasis in the region and on important epidemiological factors, such as host range and wound locations.

Materials and methods

Populations sampled

Local veterinary services in four Gulf countries made 10 regional surveys for cases of traumatic myiasis, covering (* indicates first time of sampling; Fig. 1): Boushehr and Hormozgan Provinces, Iran (September 2003 to January 2004); Diyala and Basrah* Provinces, Iraq (September 2004 to March 2006); Al-Kharj*, Al-Muzahimiyah* and Al-Ehsaa*, Saudi Arabia (November 2004 to June 2006), and the Al-Batina, Al-Sharqiah* and Interior* Districts, Oman (January to May 2005).

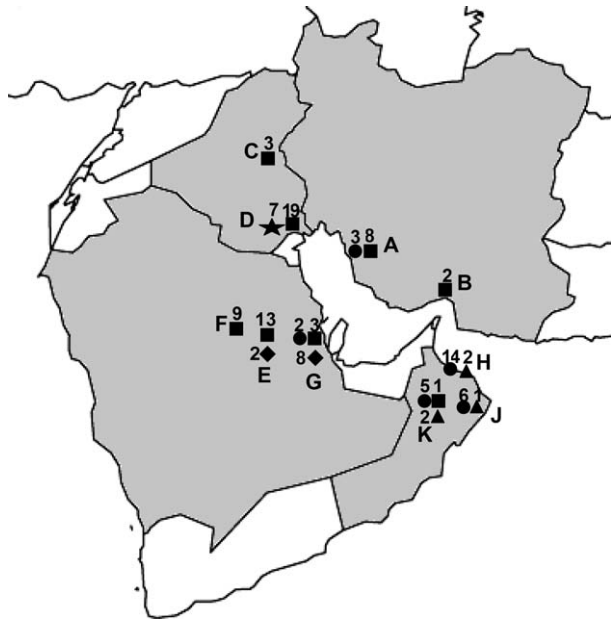

Fig. 1. Map of regions sampled showing locations where different *cytochrome b* haplotypes (CB_bezzNN) were identified. A, Boushehr Province, Iran; B, Hormozgan Province, Iran; C, Diyala Province, Iraq; D, Basrah Province, Iraq; E, Al-Kharj, Saudi Arabia; F, Al-Muzahimiyah, Saudi Arabia; G, Al-Ehsaa, Saudi Arabia; H, Al-Batina District, Oman; J, Al-Sharqiah District, Oman; K, Interior District, Oman. Numbers above or adjacent to haplotypes indicate numbers of adult equivalents identified at each location. ●, CB_bezz01; ■, CB_bezz02; ▲, CB_bezz04; ★, CB_bezz17; ♦, CB_bezz19.

Larvae from wounds in livestock were sampled either by visiting farms or by collecting from animals that were brought by the owners to veterinary clinics. When an animal with traumatic myiasis was identified, the location of the wound was recorded and larvae were removed using forceps. Samples were collected according to a standard protocol, with up to 10 larvae removed from each wound. Where possible larvae were killed by immersion in hot water (> 80 °C) for 15–30 s (Adams & Hall, 2003) and then stored in 80% analytical grade ethanol at ambient temperatures and at 4 °C in London, with all larvae from one wound in one sample tube. During an initial identification sift in Iraq and Saudi Arabia all non-screwworm cases were rejected, so that only screwworm cases were sent to London for molecular analysis. However, larvae of all species collected in Iran and Oman were sent to London for analysis. Larvae were identified in London according to standard identification guides (Zumpt, 1965; Spradbery, 1991; Hall, 2004).

DNA extraction from single specimens

Two specimens randomly selected from each sample tube, representing individual infested wounds, were dissected. In a

standard entomology laboratory, separated from the molecular biology laboratory, flame-sterilized micro-needles and forceps were used to remove muscle tissue blocks from three median segments of individual larvae. The tissue blocks were put into individual Eppendorf tubes, which were placed on crushed ice. Remaining tissues were also placed into separate individual Eppendorf tubes, coded as for the muscle samples, and retained for future morphological studies. DNA was extracted from the larval tissues using the DNAzol® kit (Invitrogen Corp., Carlsbad, CA, U.S.A.) (Chomczynski *et al.*, 1997), dissolved in 1× Tris-EDTA solution and stored short-term at 4 °C and longterm at − 20 °C. Some larvae were poorly preserved and either DNA could not be extracted from them or it was too degraded to produce fragments of sufficient length for the primers used.

PCR amplification and cycle sequencing of cytochrome b *gene*

The 3′ terminal 715 nucleotides of the mitochondrial *cytochrome b* gene were amplified in one fragment (CB) using the primers CB1-SE (5′–3′ TATGTACTACCATGAGGACAAATATC) and PDR-WR04 (5′–3′ ATTTCACGCTCATTAACT) (Ready *et al.*, 2009). The protocols were those followed by Ready *et al.* (2009), using the software Sequencher™ 3.1–4.6 (Gene Codes Corp., Ann Arbor, MI, U.S.A.) to manually edit and align the sequences prior to export into PAUP* software (Swofford, 2002) for phylogeographic analysis of the unique haplotypes (CB_bezzNN).

Adult equivalents and multiple infestations

In calculating the haplotype frequency it was necessary to deal in units of 'adult equivalents' (AEs) because larvae in a single wound may be siblings from a single oviposition. Therefore, when two specimens of the same haplotype were recorded in the same wound, the frequency score for the haplotype in that wound was recorded as 1 AE, unless the larvae were clearly of such differing sizes that they derived from two female ovipositions, in which case 2 AE was recorded. If the two specimens in a wound were of differing haplotypes, each haplotype was given a score of 1 AE for that wound. This has the potential to underestimate the number of multiple infestations by the same haplotype, which might be exaggerated for the more common haplotypes.

The minimum frequency of multiple infestations was determined based only on those infestations for which at least two specimens had been sequenced.

Results

Species causing traumatic myiasis and stage of OWS recorded

In all but three cases reported, only a single larval infested wound was recorded on each host animal. The contribution of non-OWS species in Iraq and Saudi Arabia is unknown because of the initial identification sift, which was of sufficiently high quality that only a single case of non-OWS larvae was submitted (OWS with a single larva of a *Lucilia* species from Saudi Arabia). Of the cases of traumatic myiasis submitted from Iran and Oman, 80.4% (82/102) were the result of infestation by OWS larvae and 19.6% (20/102) by only larvae of other species. Of the OWS cases, 15.9% (13/82) were mixed infestations with other species (Table 1). In Iran mixed infestations including OWS were found with *Chrysomya megacephala* (F) (9×), *Lucilia sericata* (Meigen) (1×) and *Calliphora* species (2×). *Chrysomya megacephala* was also responsible for some cases (3×) where it was the only species recorded or where it was recorded only with *L. sericata* (3×). There was also a single case of *Chrysomya albiceps* (Wiedmann). All the cases involving *Ch. megacephala* were found in Boushehr Province, whereas all of those involving *Ch. albiceps* and *Calliphora* were found in Hormozgan Province. Cases with *Lucilia* were found in both provinces. In Oman there was a single mixed infestation of OWS with *Ch. megacephala* and three cases where *Ch. megacephala*, *Ch. albiceps* or *Lucilia* species were found alone (Table 1). Most non-screwworm myiasis in Oman (10 cases)

Table 1. Numbers of wounds infested by each fly species recorded as causing traumatic myiasis. (N.B. Non-Old World screwworm cases from Iraq and Saudi Arabia were not included.)

	Country									
	Iran		Iraq		Saudi Arabia		Oman		Total	
Fly species	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Chrysomya bezziana	30	61.2	45	100	33	97.1	39	73.6	147	81.2
Chrysomya bezziana plus other species	12	24.5	0	0	1	2.9	1	1.9	14	7.7
Chrysomya megacephala	3	6.1	0	0	0	0	1	1.9	4	2.2
Chrysomya megacephala plus other species	3	6.1	0	0	0	0	0	0	3	1.7
Chrysomya albiceps	1	2.0	0	0	0	0	1	1.9	2	1.1
Lucilia species	0	0	0	0	0	0	1	1.9	1	0.6
Sarcophagidae	0	0	0	0	0	0	10	18.9	10	5.5
Total	49	100	45	100	34	100	53	100	181	100

was caused by one or more unidentified sarcophagids (not *Wohlfahrtia magnifica* [Schiner]), never associated with OWS (Table 1). The non-OWS cases from Oman were recorded either in the Interior (11/13) or in Al-Sharqiah (2/13), none were recorded in Al-Batina.

In almost all wounds where OWS was recorded, the oldest specimens of OWS were third instar larvae. Where the larval stage was recorded, second instar larvae were the oldest stage in only 1.7% of cases (2/116).

Hosts of OWS

Sheep and goats were the most common hosts (84.6%) infested by OWS, with similar proportions of each species affected in Iran and Saudi Arabia, but more sheep in Iraq and more goats in Oman (Table 2). Analysis of wound sites was confined to these hosts.

Old World screwworm wound sites on sheep and goats

Overall, the most common sites of wounds were on the tail, followed by the female genitalia (Table 3). When wound sites in all countries were compared, there was a statistically significant effect of country (9×4 contingency table, $\chi^2 = 51.0$, d.f. $= 24$, $P = 0.001$). However, it was clear that the distribution of wound sites in Oman differed from those in other countries, mainly as a result of a smaller proportion of tail wounds and, when data for Oman were excluded, comparison of the wound sites in Iran, Iraq and Saudi Arabia showed no significant differences (9×3 contingency table, $\chi^2 = 21.5$, d.f. $= 16$, $P = 0.16$).

Old World screwworm haplotype identity

Five CB haplotypes of *cytochrome b* were identified in this study (Fig. 1, Table 4). Haplotypes CB_bezz01, CB_bezz02 and CB_bezz04 had been recorded previously (Ready *et al.*, 2009), but two were new records, CB_bezz17 and CB_bezz19 (Table 4; GenBank accessions FJ379596 and FJ379597). The haplotypes differed from one another at just one or two nucleotide sites, equivalent to a difference of just 0.14% or 0.28% over the 715 nucleotide positions examined. Phylogenetic relationships were investigated among these five haplotypes, together with a sixth Gulf haplotype not isolated here but previously recorded from Iraq (Ready *et al.*, 2009) (Fig. 2).

The original shorter CB3 sequence of 279 positions (Hall *et al.*, 2001) was contained within the 715-bp CB fragment. Analysis of this short fragment showed that the specimens from the current study belonged to one of four CB3 haplotypes: CB3_bezz1005 (within CB_bezz01 and CB_bezz02) and CB3_bezz1008 (within CB_bezz04), both recorded by Ready *et al.* (2009), and two new CB3 haplotypes, CB3_bezz1012 (within CB_bezz17) and CB3_bezz1013 (within CB_bezz19). The CB3 haplotypes were identified based on three variable nucleotide positions, numbers 73, 201 and 210 of the 279-bp segment, equivalent to positions 509, 637 and 646 of the 715-bp CB fragment (Table 4).

Haplotype frequency by geographic OWS population

In all countries except Oman, haplotype CB_bezz02 was most common, representing 67–77% of AEs recorded (Table 5). In Oman, CB-bezz02 represented only 3.2% of the population and, instead, the most common haplotype was CB_bezz01, at 81%. In the present study CB_bezz01 was found in all countries except Iraq. Three countries had unique haplotypes: CB_bezz04, CB_bezz17 and CB_bezz19 were found only in Oman, Iraq and Saudi Arabia, respectively.

The haplotype frequency distributions in each country were compared by 5×2 contingency tables. All comparisons demonstrated statistically significant differences between countries (χ^2 test, d.f. $= 4$, $P < 0.001$) except for that between Saudi Arabia and Iran ($\chi^2 = 7.0$, $P = 0.1362$).

There were too few samples from Iran to suggest any within-country geographic variation in haplotype frequency between provinces, but CB_bezz02 was recorded only from Hormozgan. Similarly, in Iraq there were too few samples to compare provinces, but CB_bezz17 was found only in the southern province of Basrah. Within each district of Oman, CB_bezz01 predominated over CB_bezz04: the single case infested with specimens of CB_bezz02 was recorded in the Interior District. Only in

Table 2. Numbers of host species infested by larvae of *Chrysomya bezziana*.

	Country									
	Iran		Iraq		Saudi Arabia		Oman		Total	
Host	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Sheep	18	42.9	24	53.4	16	55.2	5	12.5	63	40.4
Goat	20	47.6	4	8.9	10	34.5	35	87.5	69	44.2
Cattle	4	9.5	11	24.4	2	6.9	0	0	17	10.9
Buffalo	0	0	1	2.2	0	0	0	0	1	0.6
Horse	0	0	1	2.2	0	0	0	0	1	0.6
Camel	0	0	0	0	1	3.4	0	0	1	0.6
Dog	0	0	4	8.9	0	0	0	0	4	2.6
Total	42	100	45	100	29	100	40	100	156	100

Table 3. Locations of wounds infested by *Chrysomya bezziana* in sheep and goats, by country.

	Country									
	Iran		Iraq		Saudi Arabia		Oman		Total	
Wound site	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%
Head	3	7.9	2	7.4	2	8.0	9	23.1	16	12.4
Body	1	2.6	1	3.7	1	4.0	5	12.8	8	6.2
Udder	0	0	0	0	0	0	3	7.7	3	2.3
Umbilical	7	18.4	0	0	0	0	3	7.7	10	7.8
Tail	16	42.1	14	51.9	13	52.0	9	23.1	52	40.3
Anus	1	2.6	1	3.7	2	8.0	0	0	4	3.1
Female genitalia	7	18.4	3	11.1	1	4.0	7	17.9	18	14.0
Male genitalia	0	0	0	0	0	0	2	5.1	2	1.6
Legs	3	7.9	6	22.2	6	24.0	1	2.6	16	12.4
Total	38	100	27	100	25	100	39	100	129	100

Saudi Arabia was there evidence of within-country geographic variation of haplotype frequency. The AE ratios in El-Kharg, El-Mezahmia and El-Ehsaa for haplotypes CB_bezz01: CB_bezz02: CB_bezz19 were, respectively, 0:13:2, 0:9:0 and 2:3:8. Whereas these ratios in El-Kharg and El-Mezahmia did not differ statistically ($\chi^2 = 1.3$, d.f. $= 2$, $P = 0.52$), the ratios in both those locations differed from that in El-Ehsaa ($\chi^2 > 11.8$, d.f. $= 2$, $P < 0.0028$) as a result of the predominance of newly recorded haplotype CB_bezz19 in El-Ehsaa and the predominance of CB_bezz02 elsewhere.

Haplotype frequency in different hosts

It was not possible to make any meaningful general evaluation of haplotype frequency in different hosts because there were either too few infestations with the less common haplotypes or because the differences between host frequencies in different countries did not allow pooling for comparison (Table 6). The one interesting host association that does stand out is that of haplotype CB_bezz19 in Saudi Arabia, which was not found in sheep, unlike CB_bezz02, which was most common in sheep. However, CB_bezz19 was found in only two of the three locations in Saudi Arabia. When the data from only Al-Kharj and El-Ehsaa were compared, the ratios for infestations on sheep : goats : cattle were 9:4:1 for CB_bezz02 and 0:6:2 for CB_bezz19, demonstrating a statistically significant difference between these two haplotypes in their host associations ($\chi^2 = 8.7476$, d.f. $= 2$, $P = 0.0126$). Because numbers were small and some expected values < 5, an exact test was also conducted on a 2×2 contingency table, which combined infestations on goats and cattle. This test also produced a statistically significant result with a two-tailed $P = 0.00405$.

Frequency of unambiguous multiple OWS infestations

Multiple infestations, with ovipositions by more than one female, were unambiguously recorded in Iraq (18.8%, 3/16), Saudi Arabia (21.4%, 6/28) and Oman (8.7%, 2/23). There were no multiple infestations recorded in Iran (0/13) and therefore the overall minimum frequency of multiple infestations in the Gulf region was 13.8% (11/80).

Discussion

In all countries involved in this study, the principal agent of traumatic myiasis was OWS fly. The unsifted samples from Iran and Oman indicated the potential diversity of other species in multiple species infestations (Table 1). Spradbery *et al.* (1992) found that 85.4% of livestock myiasis cases in Oman were caused by OWS, but they also found that 10.4% of cases were infested with *Wohlfahrtia nuba* (Wiedemann). Spradbery *et al.*

Table 4. Nucleotide characterization of the five *cytochrome b* haplotypes of *Chrysomya bezziana* found in the Gulf region. Character states are shown for the 18 nucleotide positions that show variation among the known haplotypes of *Ch. bezziana*. Those four sites that are variable in the haplotypes found in the Gulf region are shaded.

Haplotype	28	40	73	74	76	196	250	264	271	284	367	380	493	509	631	637	646	650
CB_bezz01	C	C	A	G	A	A	T	A	T	G	C	G	C	T	T	A	G	G
CB_bezz02	C	C	A	G	A	A	T	A	T	A	C	G	C	T	T	A	G	G
CB_bezz04	C	C	A	G	A	A	T	A	T	G	C	G	C	T	T	A	A	G
CB_bezz17	C	C	A	G	A	A	T	A	T	G	C	G	C	T	T	G	G	G
CB_bezz19	C	C	A	G	A	A	T	A	T	G	C	G	C	C	T	A	G	G

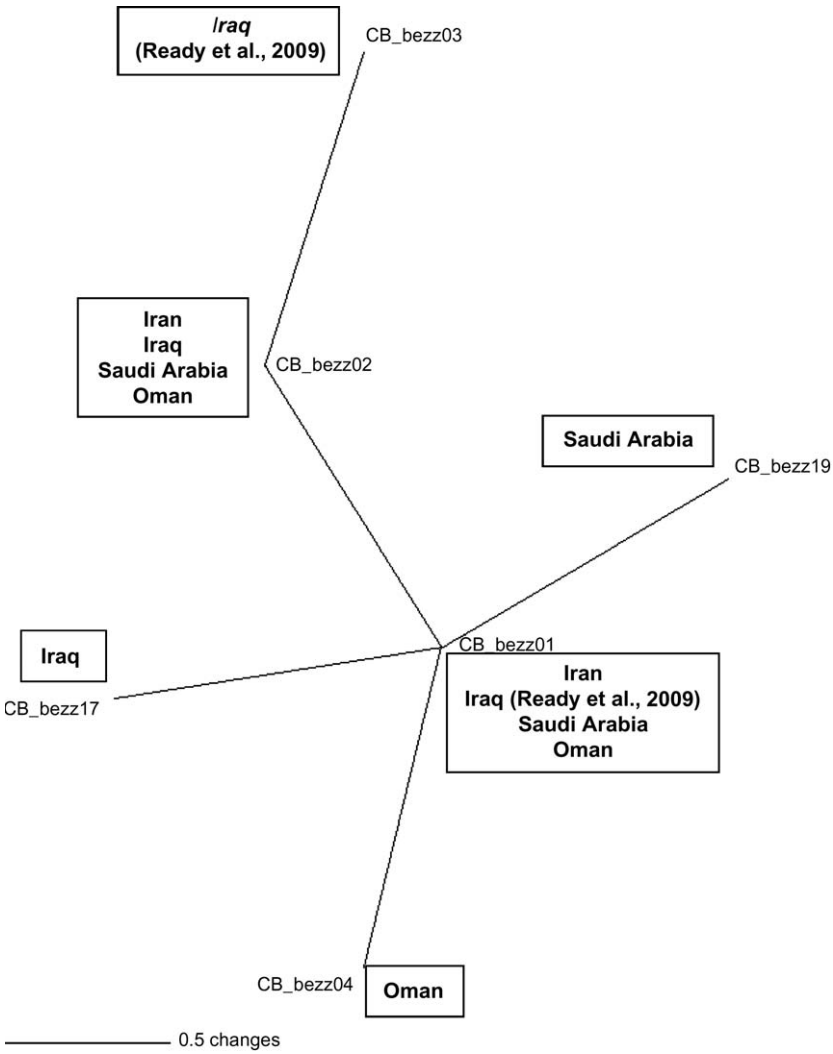

Fig. 2. Unrooted neighbour-joining tree generated by using untransformed genetic distances between six *cytochrome b* haplotypes of *Chrysomya bezziana* (CB_bezz01–CB_bezz04, CB_bezz 17 and CB_bezz19). The numbers of nucleotide differences between each haplotype are indicated by the branch lengths (see scale). Countries in which each haplotype were identified are indicated. Data for CB_bezz03 and CB_bezz01 in Iraq are sourced from Ready *et al.* (2009).

(1992) reared larvae to adulthood to permit identification; if this had been possible in the current study it is likely that some of the unidentified sarcophagid larvae collected in Oman (18.9% of cases, Table 1) would have been identified as *W. nuba*. El-Azazy & El-Metenawy (2004) concluded that *W. nuba* was an important myiasis-causing agent in Saudi Arabia, responsible for almost one-third of cases in all areas studied. Whereas the current study recorded a variety of species involved in traumatic myiasis in Iran, Navidpour *et al.* (1996) recorded that 99.6% of cases resulted from OWS fly alone. This probably reflects the outbreak nature of the infestation studied by these authors.

The predominant host animals recorded in the current study were sheep and goats, which reflects the livestock census data for the regions under study. This can be shown by reference to two countries as examples. In a 1999 census, the ratio of sheep to goats in Saudi Arabia was 1.00 : 0.35, but a 2004–2005 census in Oman gave this ratio as 1.00 : 4.31 (source: www.fao.org). The ratio of sheep to goats as hosts in the current study was 1.00 : 0.63 in Saudi Arabia, but 1.00 : 7.00 in Oman.

In all countries except Oman, the main site of OWS infestation in sheep and goats was the tail (Table 3). The tail was less important as a site of wounding in Oman, probably because goats represented a greater proportion of host animals and they do not have a fatty tail, unlike local breeds of sheep in the Gulf region. The importance of determining the site of infestation is related to the potential application of prophylactics for control of OWS. Selective application of prophylactics to the hind quarters of sheep and goats would help to protect not only the tail region, but also the genitalia, anus, udder and umbilicus (a high proportion [69%] of OWS infestations in our study were located on these sites). Including perineal infestations, these sites constituted 73% of cases in sheep and goats in the study by Spradbery *et al.* (1992) and $>$ 98% of cases in the study by Navidpour *et al.* (1996).

The 3′ terminal 715 bp obtained from the CB fragment showed five different haplotypes, three previously recorded in the Gulf region (Ready *et al.*, 2009) and two new records, CB_bezz17 and CB_bezz19, found in Iraq and Saudi Arabia,

Table 5. Frequency of haplotypes of *Chrysomya bezziana* in each country sampled, given in numbers and percentage of adult equivalents (AEs) within each country.

			Cytochrome b haplotype									
			CB_bezz01		CB_bezz02		CB_bezz04		CB_bezz17		CB_bezz19	
Country	Larvae, *n*	AE, *n*	AE, *n*	%	AE, *n*	%	AE, *n*	%	AE, *n*	%	AE, *n*	%
Iran	25*	13	3	23.1	10	76.9	0	0	0	0	0	0
Iraq	42	29	0	0	22	75.9	0	0	7	24.1	0	0
Saudi Arabia	59	37	2	5.4	25	67.6	0	0	0	0	10	27.1
Oman	52	31	25	80.6	1	3.2	5	16.1	0	0	0	0
Total	178	110	30	–	58	–	5	–	7	–	10	–

*Two of the 25 specimens recorded here were analysed by Ready *et al.* (2009).

respectively. Because the shorter 279-bp fragment of CB3 is included within the region of the CB fragment where variation that gave rise to the new CB haplotypes occurred, two new CB3 haplotypes were also identified, CB3_bezz1012 and CB3_bezz1013. As before (Ready *et al.*, 2009), CB_bezz04 was only recorded from Oman. The dominant mt-DNA haplotypes were CB_bezz01 and CB_bezz02, reported previously from Iraq and Iran as well as from Southeast Asia, where both haplotypes occurred in Malaysia and only CB_bezz01 occurred in Hong Kong (Ready *et al.*, 2009). A rare haplotype, CB_bezz03, recorded previously in a single specimen from Iraq (Ready *et al.*, 2009), was not recorded in the current study. It is interesting to note that previous more extensive studies of the genetic identity of populations of OWS within the Gulf region failed to identify any specimens of African origin, with African haplotypes as described by Hall *et al.* (2001) and Ready *et al.* (2009), despite the trade in livestock between the Horn of Africa and the Gulf countries (Knips, 2004). This suggests that this trade with Africa is not resulting in a movement of OWS into the Gulf, unless the haplotypes in the Horn of Africa differ from those of sub-Saharan Africa, which is yet to be resolved.

The observation of a statistically significant difference in host records between the two most common haplotypes of OWS in Saudi Arabia is of great interest and worthy of further investigation with a larger sample size. The analysis compared OWS populations from the same locations, exposed to the same hosts, but CB_bezz02 was found mainly in sheep and CB_bezz19 mainly in goats. It is perhaps surprising that this discrimination should occur between two small ruminants rather than, for example, between small and large ruminants. We are not aware of any other reports of host-associated genetic differentiation of myiasis-causing flies, but there are comparable reports from studies of herbivores, such as lineages of Thysanoptera associated with leek or tobacco host plants (Brunner *et al.*, 2004).

The current study confirms previous work (Hall *et al.*, 2001; Ready *et al.*, 2009) which demonstrated a very limited genetic diversity of OWS from the Gulf region to the furthest edges of mainland Southeast Asia – diversity within Asia only became noticeable in more isolated island populations in Indonesia (Ready *et al.*, 2009). Therefore, it suggests that SIT within the Gulf region should not be compromised by genetic incompatibility if a single Gulf fly strain was used as a component of an integrated programme for the control of OWS fly.

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Table 6. Numbers of hosts infested by the five different *cytochrome b* haplotypes (CB_bezzNN) of *Chrysomya bezziana* in each of the four countries studied.

	Iran		Iraq		Saudi Arabia			Oman		
Host	bezz01	bezz02	bezz02	bezz17	bezz01	bezz02	bezz19	bezz01	bezz02	bezz04
Sheep	2	5	15	3	0	16	0	5	0	2
Goat	1	5	2	0	1	5	6	20	1	3
Cattle	0	0	3	3	1	1	2	0	0	0
Buffalo	0	0	0	1	0	0	0	0	0	0
Horse	0	0	1	0	0	0	0	0	0	0
Camel	0	0	0	0	0	1	0	0	0	0
Dog	0	0	1	0	0	0	0	0	0	0
Total	3	10	22	7	2	23	8	25	1	5

(N.B. The identity of four hosts in Saudi Arabia was not recorded, 2 × bezz02 and 2 × bezz19, c.f. Table 5).

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Conflicts of interest

All authors declare no conflicts of interests.

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Morphological and mitochondrial DNA characters for identification and phylogenetic analysis of the myiasis-causing flesh fly *Wohlfahrtia magnifica* and its relatives, with a description of *Wohlfahrtia monegrosensis* sp. n. Wyatt & Hall

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Abstract. *Wohlfahrtia magnifica* (Schiner) (Diptera: Sarcophagidae) is a major cause of traumatic myiasis in livestock in Central and Eastern Europe and in countries bordering the Mediterranean. The present study explored the utility of external body characters, genitalia characters and mitochondrial DNA characters for identification of this and related species in the subfamily Paramacronychiinae. Sequence analyses of the 3′ terminal 273 bp of the mitochondrial *cytochrome b* gene revealed two lineages of *W. magnifica*, one from Spain and France and the other from the rest of Eurasia, differing by only two base pairs. Phylogenetic analysis of *cytochrome b* showed that *W. magnifica* and *Wohlfahrtia vigil* Walker were sister species; this conclusion was not contradicted by a phylogenetic analysis of the morphological characters. Based on *cytochrome b*, the genetic distance between specimens of *W. vigil* from Europe and North America was sufficiently large to justify the recognition of more than one species. A new species, *Wohlfahrtia monegrosensis*, from northern Spain, was described, based on morphology and *cytochrome b*. A unique combination of external body characters of males or females were diagnostic for *W. magnifica*, the *W. vigil* group and *Wohlfahrtia bella*, but only the genitalia characters were diagnostic for all nine species studied.

Key words. *Wohlfahrtia magnifica*, *Wohlfahrtia monegrosensis*, *cytochrome b*, mitochondrial DNA, morphology, Sarcophagidae.

Introduction

Myiasis is the infestation of live vertebrates, including humans, with the larvae of true flies, Diptera (Zumpt, 1965). Traumatic or wound myiasis is the infestation of cutaneous tissues at sites of wounding or in body orifices (Hall & Farkas, 2000). Subcutaneous tissues can also be affected in a serious infestation. Three fly species are economically important as obligate larval parasites causing traumatic myiasis: the Old World screwworm fly (OWS) *Chrysomya bezziana* Villeneuve (Diptera: Calliphoridae), the New World screwworm fly (NWS) *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae) and Wohlfahrt's wound myiasis fly *Wohlfahrtia magnifica* (Schiner) (Diptera: Sarcophagidae). Taxonomic knowledge of groups containing any insect pest, including those that cause traumatic myiasis in humans and animals, is vital for their correct identification. The latter is essential in any control campaign, especially when a pest is new to a region. This was exemplified following the introduction of NWS to Libya, when correct and timely identification of the pest

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species led to a rapid and ultimately successful eradication campaign (Lindquist *et al.*, 1992).

Wohlfahrtia is a genus of 26 species, including the new species described here, *Wohlfahrtia monegrosensis*, placed in the subfamily Paramacronychiinae of the family Sarcophagidae (Diptera) (Pape, 1996; Lehrer, 2003). Most species in this subfamily, including *Wohlfahrtia*, are densely covered with greyish microtomentum with a more-or-less distinct abdominal pattern of a median stripe and dark lateral spots, and character states considered autapomorphic for the subfamily are all associated with the male terminalia: sternite 5 is small, with shallow posterior emargination; tergite 6 is fused to syntergosternite 7–8; the surstyli are fused to the epandrium and the acrophallus is long and curved (Pape, 1996). Within this subfamily, *Wohlfahrtia* can be diagnosed by the following characters: the male is without proclinate orbital bristles; the parafacial plate is bare or has a few scattered setulae; the costal spine is at most as long as crossvein r–m, and the abdominal microtomentum is densely silvery-grey with distinct black spots that sometimes coalesce posteriorly (Pape, 1996). Most species in this genus are confined to arid parts of the Palaearctic and Afrotropical regions, especially northern Africa, the Middle East and central Asia, but one species occurs in North America.

The larval feeding habits in the genus *Wohlfahrtia* are diverse (Table 1). The most economically important species is *W. magnifica*, the larvae of which are obligate parasites that cause a serious traumatic myiasis, mainly in livestock, but also in humans and, rarely, in wildlife (Farkas *et al.*, 1997; Hall, 1997; Hall & Farkas, 2000). *Wohlfahrtia magnifica* is widely distributed throughout the southern and central parts of the Palaearctic region, from Morocco in the west to China in the east. An expansion of its range onto the Mediterranean island of Crete occurred in 1999 (Sotiraki *et al.*, 2003, 2005).

The only species of *Wohlfahrtia* to occur in the New World is *Wohlfahrtia vigil* (Walker), found in the U.S.A. and Canada, as well as the Palaearctic region (Hall & Farkas, 2000). The definition of this species has been problematic because of variable external morphology, and a number of different names have been used, mainly *W. vigil* and *Wohlfahrtia opaca* (Coquillett) in the Nearctic region and *Wohlfahrtia meigenii* (Schiner) in the Palaearctic region (Pape, 1996). There also appear to be regional differences in the larval feeding habitats of this species (see Discussion). Nevertheless, all forms have been synonymized under the name *W. vigil* by Pape (1996) because to date no reliable morphological characters have been found to distinguish Nearctic and Palaearctic specimens.

Table 1. Larval feeding habits of species in the genus *Wohlfahrtia*.

Larval feeding habit	Species
Obligate parasite	*magnifica*, *vigil*?
Facultative parasite	*bella*, *indigens*, *nuba*, *trina*, *vigil*?
Necrophagous	*balassogloi**, *brunnipalpi*, *fedtschenkoi**, *intermedia**, *pavlovskyi**, *stackelbergi**, *villeneuvi*
Insectivorous	*erythrocera*
Unknown	*atra*, *africana*, *aschersoni*, *cheni*, *grunini*, *ilanramoni*, *monegrosensis*, *musiva*, *pachytyli*, *seguyi*, *smarti*, *triquetra*

*Source: Rohdendorf & Verves (1978).

The causal agent in cases of traumatic myiasis is usually identified to species using mature larvae taken from infested wounds. It is common for the appearance and biology of the adult fly to be almost unknown among farmers and veterinarians dealing with a myiasis problem. Whereas the correct identification of third instars of *W. magnifica* is relatively straightforward (Spradbery, 1991), identification of the adults of *Wohlfahrtia* can be more complex. However, identifications of adults are required when they are captured during trapping surveys of myiasis-causing species.

The genitalia of adult males often exhibit the most reliable characters for recognizing new species and identifying individual sarcophagids (Pape, 1987). Identification of females is far less satisfactory and, for some taxa, identification can only be arrived at if the female is taken *in copulo* with a male. Members of the genus *Wohlfahrtia* have some useful external characters, which have been used in conjunction with genitalia characters to generate workable identification keys to the species. However, it is often impractical to dissect genitalia of all *Wohlfahrtia* specimens collected and thus, in practice, only a few specimens will be dissected and, as a result, males and females collected with them, showing the same external character states, will be given the same identification. As indicated by Salem (1938), the small number of external characters and their variability makes identification of *Wohlfahrtia* difficult. Characters of both male and female genitalia provided better results, but were not ideal.

The current report tests the diagnostic value of commonly used external morphological characters by subjecting data from nine *Wohlfahrtia* species to a phenetic analysis to determine which taxa could be unambiguously identified solely on these characters. A phylogenetic analysis of these taxa using external and genitalia morphology characters, and mitochondrial DNA analysis for some taxa, to look for subgeneric structure in the group was attempted. The main objective of all these approaches was to be able to distinguish unambiguously the males and females of the economically important obligate parasite *W. magnifica* from any other species in the genus. The relationship between North American and European specimens of *W. vigil* was examined and, in addition, the description of a new species, *W. monegrosensis*, from northern Spain, was undertaken. Both species are sympatric with *W. magnifica* and have a similar morphology; hence it is important for epidemiological studies to be able to identify *W. magnifica* unambiguously.

Materials and methods

Morphological analysis

Nine of the 26 species in the genus *Wohlfahrtia* were included in the present analysis (Table 2). Taxa were selected primarily according to their sympatry with *W. magnifica*, on the basis that they might, therefore, need to be identified in epidemiological studies. The secondary criterion required that specimens be

Table 2. Geographical origin of the material examined for morphological analysis.

Wohlfahrtia species	Geographical region	Number of specimens	
		Male	Female
W. aschersoni	Egypt	1	0
	Oman	1	1
	Palaearctic region	9	10
W. bella	Canary Islands	1	2
	Tenerife	0	1
	Morocco	1	0
	Pakistan	2	1
	Palaearctic region	3	0
	Palestine	0	1
	Spain	0	1
	Tadzhikistan	1	1
W. brunipalpis	Gambia	0	1
	Saudi Arabia	3	4
	Sudan	0	1
	Unknown	1	0
W. erythrocera	Algeria	1	0
	Eritrea	2	2
	India	0	1
	Pakistan	3	0
	Saudi Arabia	2	0
	Somalia	2	2
	Unknown	1	0
W. indigens	Canary Islands	1	1
	Egypt	1	0
	Ethiopia	0	1
	Oman	0	1
	Palaearctic region	1	2
	Saudi Arabia	3	0
	Somalia	2	1
	Sudan	1	0
	U.A.E.	0	1
	Unknown	1	0
W. magnifica	Hungary	12	10
	Italy	1	0
	Palaearctic region	1	0
W. monegrosensis sp. *novo*	Spain	12	12
W. nuba	India	3	2
	Saudi Arabia	1	0
	Sudan	7	7
	U.A.E.	0	2
	Unknown	2	0
W. vigil	Canada	2	4
	Hungary	0	1
	Nearctic region	0	1
	Palaearctic region	2	0
	Romania	3	1
	U.S.A.	4	4

available in sufficient numbers in the collections of the Natural History Museum (NHM), London.

A total of 23 adult characters, commonly used in identification keys, were selected for the analysis (Table 3). These pertained to the: head (three); thorax (one); wings (one); abdomen (three); male genitalia (nine), and female genitalia (six). An initial character set was arrived at by listing all the characters used in Verves' (1986) key to species. This list was then refined by direct observation, uninformative characters were excluded, new characters were added and others were redefined if the original description in Verves was felt to be too subjective. Body characters were scored for pinned adult specimens (seven to 24 individuals per species) and genitalia characters were scored for available genitalia preparations (one to six individuals per species).

PAUP* software (Swofford, 1993) was used to search for phylogenetic relationships. For each sex, a dataset containing every individual examined, scored for eight external morphological characters, was subjected to distance measure analyses, using the neighbourhood joining (NJ) methodology. Maximum parsimony (MP) (with heuristic searches) was also used to identify any phylogenetic signal within the genus based on a full combined dataset containing all 23 external body and genitalia characters of both sexes. Missing data, where characters were missing from the available specimens, were denoted by '?'. All multistate characters were treated as unordered, with the exception of the length ratio of antennal segments a3: a2 (Table 3), where the gap weighting coding method (Thiele, 1993) was used and the character treated as an ordered multistate.

Molecular analysis

Mitochondrial DNA (*cytochrome b*) was chosen for sequencing because previous studies of medically important Diptera have indicated that the fragment is suitable for studying geographical variation (Esseghir *et al*, 1997; Ready *et al.*, 1997; Hall *et al.*, 2001): it is maternally inherited, rarely or never recombines and has a relatively rapid rate of nucleotide substitution.

Many of the pinned adult specimens in the NHM collections were old or available in low numbers and, therefore, not suited to destructive molecular analysis. Sequences were obtained instead from recently collected specimens of five species, both larvae and adults (Table 4).

DNA extraction

Muscle tissues were dissected from the middle abdominal segments of larvae or from the thoracic flight muscles of adults. The DNA extraction technique was that of Ish-Horowicz with minor variations (Ready *et al.*, 1997). The DNA pellet was dried under vacuum and dissolved in 30 μL 1× TE. In some cases with old specimens, further purification was carried out with glassmilk (Geneclean II®; MP Biomedicals, Solon, OH, U.S.A.) and DNA was dissolved in distilled water.

PCR conditions

Three pairs of primers were used for the characterization of *cytochrome b* sequences. Primer pair CB3-PDR with N1N-PDR was used to amplify and sequence a 550-bp fragment of

Table 3. Adult morphological characters used with anatomical nomenclature following Verves (1986).

Body region	Character	Characters states
Head	Length ratio of antennal segments a3: a2	Gap weighting coding was applied to the data following Thiele (1993)
	Parafacial plate vestiture	(0) bare; (1) with a few small hairs close to the eye margin
	Colour of first and second antennal segments	(0) reddish-yellow; (1) orange–red to red–brown; (2) black with distal third reddish
Thorax	Presutural acrostichal setae	(0) absent; (1) weak and hair-like; (2) well developed
Wing	Basicosta and epaulet colour	(0) yellow to brownish-yellow; (1) brownish-black to black
Abdomen	Shape of median spot on tergite 3	(0) roundish, not extended forward; (1) triangular, extending forward
	Shape of median spot on tergite 4	(0) not extending forward as far as the fore margin; (1) extending forward to the fore margin
	Shape of black spots on tergite 5	(0) absent or very small and restricted to around the base of the setae; (1) restricted to the hind margin; (2) extending to the fore margin
Female genitalia	Tergite 7 with isolated sclerotized regions at the base of some of the hairs	(0) absent; (1) present
	Extensive sclerotization forming a plate on tergite 7	(0) as a single plate; (1) as two plates with a membranous region in between; (2) no plate
	Pigment patch on hypoproct	(0) absent; (1) present
	Shape of sclerotized plate on sternite 6	(0) an extended oval with no corners; (1) tapering to a point at both ends
	Marginal setae on sternite 7	(0) absent; (1) present
	Shape of sclerotized plate on sternite 8	(0) in one piece; (1) as two pieces
Male genitalia	Shape of phallosome apex	(0) blunt or squared off; (1) bifid; (2) tapering
	Phallosome in profile	(0) straight or almost; (1) curved
	Shape of ventral lobes on the phallosome	(0) narrow at base, broad at tip; (1) broad at base and narrow at tip; (2) narrowing at base and tip
	Angle at which the ventral lobes meet the phallosome	(0) acute; (1) right angle; (2) obtuse
	Point of attachment of ventral lobes on phallosome	(0) near tip; (1) mid-point; (2) near base
	Distribution of discal setae on sternite 5	(0) fairly even, slight increase laterally; (1) more than four times as many laterally compared with central region
	Shape of posterior margin on sclerotized plate of tergite 5	(0) more-or-less straight; (1) curved
	Setae on tergite 5	(0) marginal setae obviously stronger and longer than the discal setae; (1) not much difference
	Point of attachment of the major setae on the anterior paramere	(0) around mid-point; (1) near base; (2) near tip

cytochrome b-NADH (Ready *et al.*, 1997; Hall *et al.*, 2001). In addition, primer pairs PDR-WF01 with PDR-WR02, and PDR-WF03 with PDR-WR04, were used to amplify and sequence a 196-bp fragment and an overlapping 132-bp fragment, respectively, within the last 300 bp of *cytochrome b* (Hall *et al.*, 2001). These primers had been developed at the NHM specifically to analyse sequences from calliphorid species, but worked well with specimens of the family Sarcophagidae. Polymerase chain reaction (PCR) products were checked by submerged horizontal electrophoresis using 1.5% agarose gels before purification with glassmilk (Geneclean II®; BIO 101 Inc.).

Sequencing and analysis

Direct sequencing of each DNA strand was performed with each of the PCR primers using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Inc., Carlsbad, CA, U.S.A.) and automated sequence analysers (models 373 and 377; Applied Biosystems, Inc.). The alignment of the DNA was carried out with Sequence Navigator (Applied Biosystems, Inc.), Sequencher 3.1.1™ (Gene Codes Corp., Ann Arbor, MI, U.S.A.) and ClustalV (Higgins & Sharp, 1989) software. Phylogenetic analysis was run using PAUP* (Swofford, 2002).

Results

Description of Wohlfahrtia monegrosensis *Wyatt & Hall sp.n.*

On the basis of its morphology, this species most closely resembles *Wohlfahrtia smarti* from Somalia and can only be reliably identified by differences in the male genitalia, detailed below.

Table 4. Haplotypes of *Wohlfahrtia* identified by analysis of an alignment of 149-bp of mitochondrial DNA (*cytochrome b* gene) from 27 specimens.

Haplotype	Individual code
CB3_magn1001 *W. magnifica*, Hungary, Italy, Israel, Iraq	Wm1, Wm3, Wm4, Wm5, Mne1, Mne2, Msa1, Msa2, Mnk1, Mdo2, Mul2
CB3_magn1002 *W. magnifica*, Spain, France	Wm6, Wm7, Wm8, Wmf2, Wmf3, Wmf4
CB3_vigi1001 *W. vigil*, Romania, Hungary	Wv1, Wv2, Vhu1
CB3_vigi1002 *W. vigil*, New Mexico, U.S.A.	Wv3
CB3_asch1001 *W. aschersoni*, Sharjah, U.A.E.	Wa1, Wa2
CB3_bell1001 *W. bella*, Canaries	Wb1, Wb2
CB3_mone1001 *W. monegrosensis*, Spain	Nsp1, Nsp2

Male

Head. The frons at the narrowest point is slightly narrower than the width of an eye, at approximately 0.3× head-width; it is silvery-grey pollinose, with the parafrontalia slightly more densely silver pollinose than the darker area between. Parafacials are approximately 1.5× the width of the antennal flagellum, with a few short setulae on the lower half towards the eye margin. Eyes are bare. Antennae are mostly blackish, but the pedicel is a paler brownish-yellow towards the tip. The flagellum is approximately twice as long as the pedicel. The arista is very short-haired; hair length is approximately equal to arista width; the arista is mostly dark in colour, but a distinct, narrow, pale yellowish-white band is usually present near the middle. The vibrissae are situated slightly above the mouth margin. The hairs of the gena and occiput are entirely black. The palpi are brownish-yellow and somewhat darkened at the tip.

Thorax. The mesonotum is pale grey pollinose and shows three narrow, dark vittae when viewed from behind, one situated centrally between the rows of acrostichal setae, and the other two between the dorsocentral and intra-alar rows of setae. An additional inconspicuous and narrower pair of dark vittae is present anterior to the suture, situated between the central and lateral vittae. There are 2 + 3 pairs of dorsocentrals; the first postsutural pair is weak. Acrostichals are restricted to a strong prescutellar pair and some weak presutural setae can also be differentiated. The scutellum has three pairs of marginal setae; the apical pair is crossed, and a pair of discal setae is also present. Both anterior and posterior spiracles are dark blackish-brown. The halteres are brownish-yellow. Calypters are white.

Wings. The wing membrane is hyaline with no distinct dark or coloured markings. The tegula is pale brown and the basicosta pale creamy yellow. There is a row of 5–9 setae at the base of R4 + 5 extending approximately two-thirds of the way from the base of the vein to the crossvein r–m.

Legs. Legs are entirely blackish in colour. The femorae are covered with rather dense, fine hair-like setae ventrally, lacking strong bristles; some of these hairs are wavy at the tip and the longest are slightly longer than the greatest width of the femur. Slightly stronger rows of anteroventral and posteroventral setae are present on the hind femur in addition to this hairing. The hind tibia has a rather weakly developed fringe of hairs on the posteroventral surface, the strongest of which is as long as the tibia is wide. A few weak, hair-like setae can also be differentiated on the anteroventral surface.

Abdomen. The abdomen is densely pale grey and dusted on the dorsal surface, with three sharply defined, shiny, dark brown spots on the posterior margins of syntergite 1 + 2 and tergites 3–5 (Fig. 1). These spots extend forward for less than half the length of the tergite except on syntergite 1 + 2, where the spot extends for most of the tergite length, and tergite 3, where it narrows and extends anteriorly, reaching the anterior margin of the segment. The dark spots on these basal tergites do not have any strong setae arising from them. The central spot on tergite 4 is also weakly extended anteriorly on some specimens; this and the central spot of tergite 5 are associated with two strong setae, whereas the large lateral spots on these tergites are associated with one seta only. The ventral surface of the abdomen has fine, wavy-tipped hairs on the sternites, which are slightly longer than the width of the hind femur. This surface is also less densely pollinose and has a dull reddish background colour, which contrasts clearly with the dorsal surface when viewed laterally (Fig. 2). The protandrial segment is orange–red, the background colour is partially obscured by grey pollinosity, and there are two transverse rows of strong setae, the anterior of which is somewhat stronger. The epandrium is a shiny orange–red. Figures 3 and 4 show the cerci of *W. monegrosensis*.

Genitalia. The genitalia of *W. monegrosensis* and *W. smarti* Salem are extremely similar, but have the following differences.

Fig. 1. Dorsal abdominal surface of male *Wohlfahrtia monegrosensis*. Scale bar = 1.0 mm.

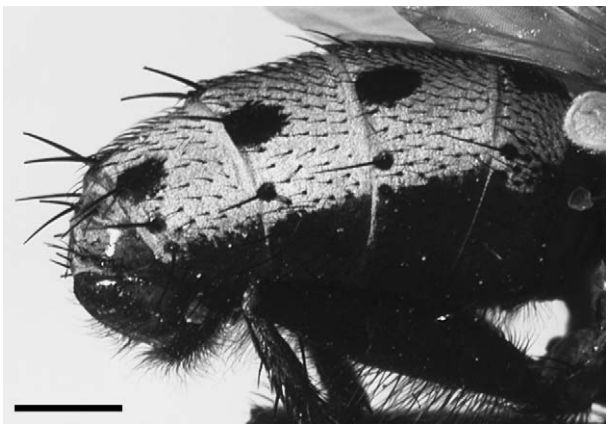

Fig. 2. Lateral view of abdomen of male *Wohlfahrtia monegrosensis*, illuminated from behind. Scale bar = 1.0 mm.

In the *W. monegrosensis* aedeagus (Fig. 5), the distiphallus is almost parallel-sided but is rounded and slightly broadened at the tip; the two lobes of the vesica are prominent and somewhat cup-shaped, and project roughly at right angles from the main body of the distiphallus; the lobes are finely toothed around the edges (visible at high magnifications only), and have a downward-pointing thorn-like structure immediately above them; the parameres are strongly curved with hook-like tips, and gradually narrow towards the tip; the gonopods are broad and almost parallel-sided, and somewhat rounded at the tip with a small, tooth-like structure at the centre of the distal edge. *Wohlfahrtia smarti* (Fig. 6) has a curved distiphallus with a membranous tip; the lobes of the vesica are narrower; the thorn-like structure above the vesica is not present; the parameres are curved, almost uniformly narrow and hooked at the tip; the gonopods are somewhat narrower than in *W. monegrosensis* and are slightly curved backwards, not parallel-sided, more strongly convex on the inner surface and have a slightly hooked tip.

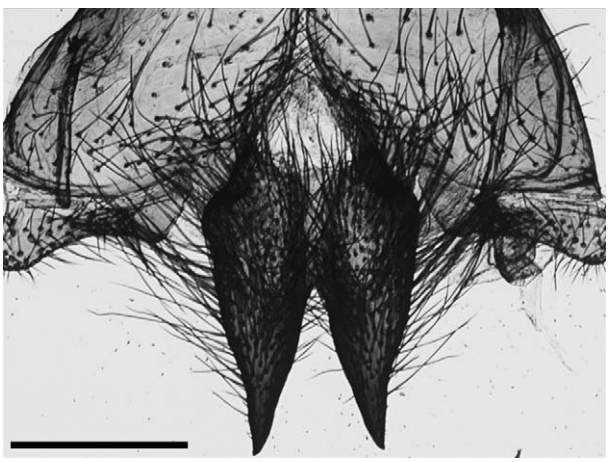

Fig. 3. View from behind of cerci of *Wohlfahrtia monegrosensis*. Scale bar = 0.5 mm.

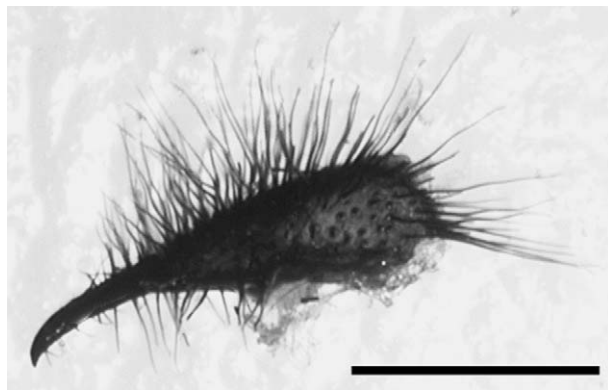

Fig. 4. Lateral view of cercus of *Wohlfahrtia monegrosensis*. Scale bar = 0.5 mm.

Female

The female shares most of the male's characters, with the following differences. The frons is somewhat wider, making it as wide or slightly wider than the width of an eye (when viewed from the front), approximately 0.33–0.35× head-width at its narrowest point. Two pairs of strong proclinate setae are seen on the upper half of the parafrontalia. The legs are without fringes of fine hairs on the femorae and tibiae; the femorae have rows of strong setae on the ventral surfaces. The long, fine hairs on the ventral surface of the abdomen in the male are absent in the female; only scattered, short adpressed setae are present. The terminalia are mostly reddish-brown. Tergite 6 is entire, not divided dorsally, and has a row of strong marginal setae. The two sclerites of tergite 7 are both triangular, rather narrow, and taper to a point dorsally; there are three marginal setae. Tergite 8 has a group of 3–4 fine setae on either side. Sternite 6 has a row of fairly strong marginal setae, which are somewhat weaker in the middle. Sternite 7 has much finer marginals (Figs 7 and 8).

The body length of *Wohlfahrtia monegrosensis* is 6.5–10.5 mm.

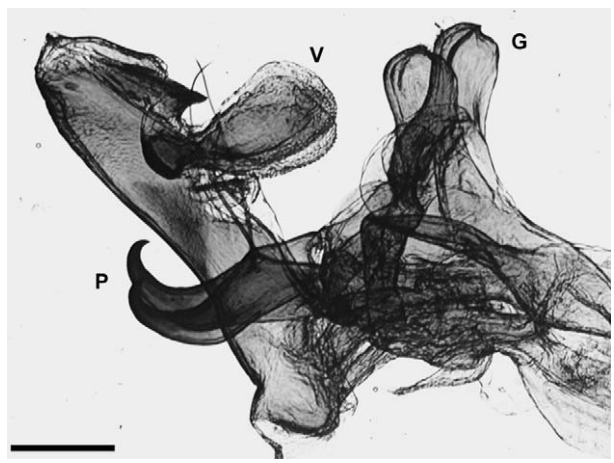

Fig. 5. Aedeagus of *Wohlfahrtia monegrosensis* with vesica (V), gonopods (G) and parameres (P) indicated. Scale bar = 0.2 mm.

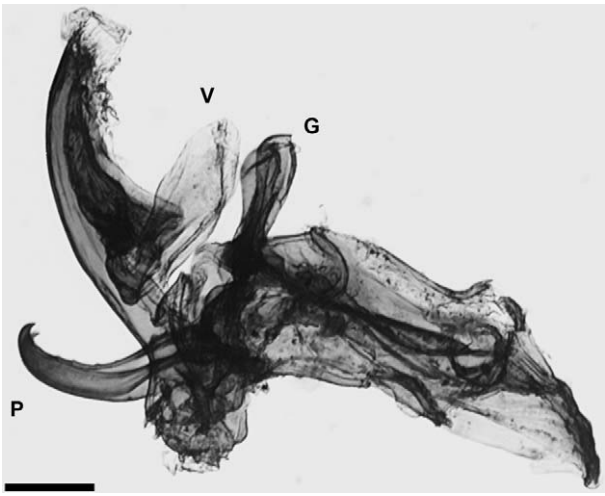

Fig. 6. Aedeagus of syntype of *Wohlfahrtia smarti* with vesica (V), gonopods (G) and parameres (P) indicated. Scale bar = 0.2 mm.

Type material

Holotype ♂, SPAIN: Aragon, Los Monegros, Los Polvorosas, ex Lucitrap, 18–22 August 1995 (J. Blasco-Zumeta). Paratypes: same data as holotype, 15♂♂ 7♀♀; same locality, 5–12 July 1995, 17♂♂ 3♀♀ (J. Blasco-Zumeta), same locality, 21–26 September 1995, 2♂ (M. J. R. Hall, J. Lucientes & N. P. Wyatt); Montes de la Retuerta de Pina, ex Lucilure trap, 22–25 June 1995 (M. J. R. Hall, J. Lucientes & N. P. Wyatt), 1♂; 4 km. N. of Pina del Ebro, ex Lucitrap, 21–26 September 1995, 1♀ (M. J. R. Hall, J. Lucientes & N. P. Wyatt), exact locality not given, Los Monegros, same date and collectors as previous, 1♂ 3♀♀. Type series all deposited in the Natural History Museum, London.

Fig. 7. Dorsal abdominal surface of female *Wohlfahrtia monegrosensis*. Scale bar = 1.0 mm.

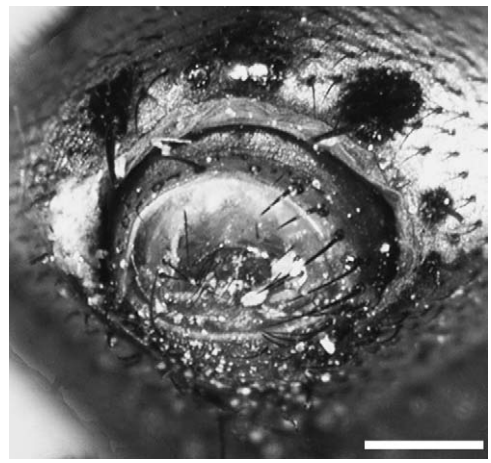

Fig. 8. Rear view of terminal abdominal segments of female *Wohlfahrtia monegrosensis*. Scale bar = 0.5 mm.

Analysis of adult morphology

Species identification. The morphological characters used to describe *Wohlfahrtia* species are usually found on the male genitalia. Nine characters were scored for the male genitalia dissected from between one and six specimens of each species, from which it was confirmed that each species has a unique combination of these characters and little intraspecific variation, none of which confuses identification. Six characters were scored for the female genitalia dissected from between one and six specimens of each species, from which it was also confirmed that each species has a unique combination of these characters and little or no intraspecific variation.

A distance measure NJ search was used to cluster all males and all females based on their score for eight external body morphology characters. Searches of males (Fig. 9) and females (Fig. 10) each returned a single tree of minimum length. Examination of both trees showed that each returned single clusters containing every individual of the same three taxa, *Wohlfahrtia bella*, *W. magnifica* and *W. vigil*, suggesting that at least these three taxa can be reliably identified using external body morphology alone. Inability to retrieve the other six taxa does not indicate that they are not good taxa, but merely that genitalia characters are additionally required for their unambiguous identification. Principal components analysis (PCA) of body character data confirmed the reliable separation of individuals of *W. bella*, *W. magnifica* and *W. vigil* for both sexes and, in addition, of *W. monegrosensis* for females (Figs 11 and 12). The pairs of characters contributing most strongly to each component were the same for both sexes (i.e. the length ratio of the antennal segments and parafacial plate vestiture to component 1; the colour of antennal segments and presutural acrostichal setae to component 2) (Table 3). The relative spread of points for *W. vigil* was much greater along a diagonal

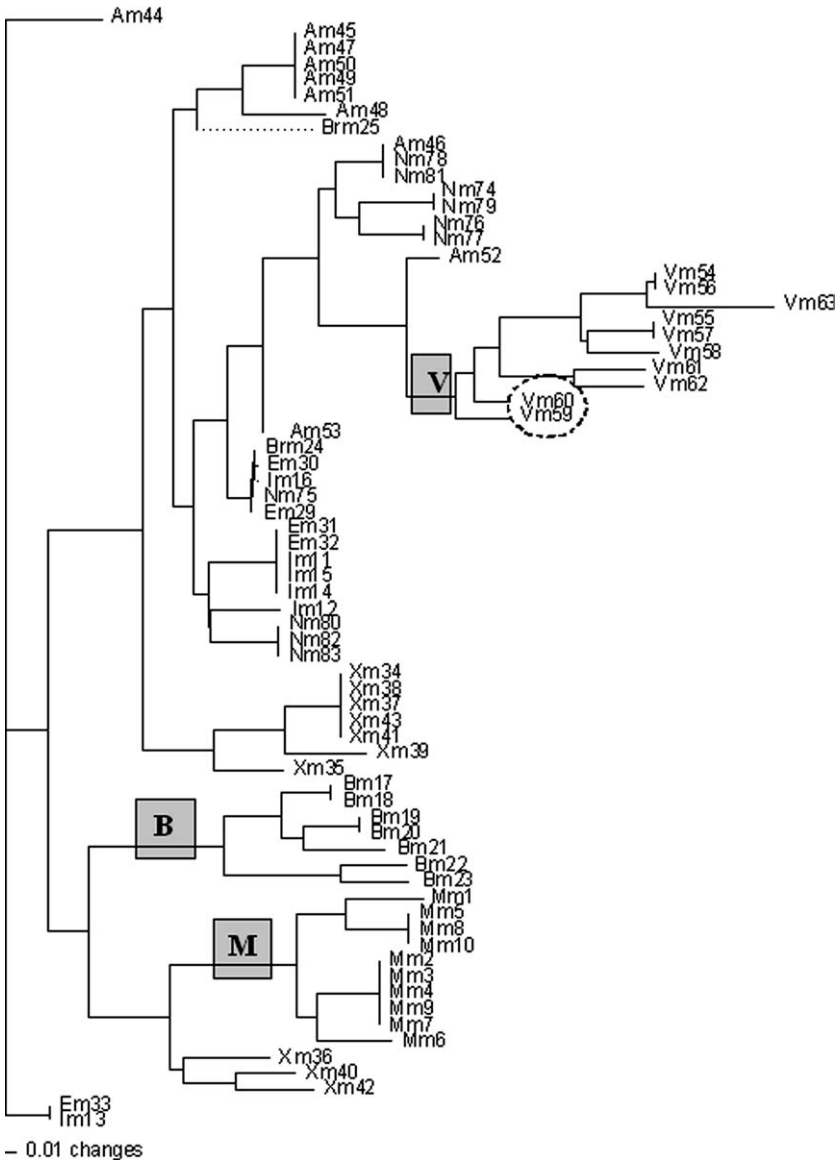

Fig. 9. Neighbourhood-joining dendrogram of male *Wohlfahrtia* based on external body characters only. Letters in bold in shaded boxes indicate where all members of a taxon were recovered in a monophyletic clade (*magnifica*, *bella* and *vigil* only). All specimens of *W. vigil* inside the dashed oval were from western North America. All other *W. vigil* were from eastern North America or Europe. A, *W. aschersoni*; B, *W. bella*; Br, *W. brunnipalpis*; E, *W. erythrocera*; I, *W. indigens*; M, *W. magnifica*; N, *W. nuba*; V, *W. vigil*; X, *W. monegrosensis*.

axis for both sexes of this species than for other species, especially along component 1, which lends support to the suggestion that specimens of this taxa potentially belong to more than one species (Figs 11 and 12). All taxa in the left side of the *W. vigil* 'envelopes' were from western North America, whereas all taxa in the right side of the envelopes were from eastern North America or Europe. Support for this geographical clustering also comes from the NJ trees (Figs 9 and 10).

Phylogenetic analysis. All 23 characters (body and genitalia of both sexes) for each taxon were used in a single cladistic analysis: where intraspecific variation was recorded, characters were scored as multistate characters. An MP heuristic search with all characters given equal weight generated a single most parsimonious tree of 36 steps (Fig. 13) with an ensemble consistency index (CI) of 0.667 and a retention index (RI) of 0.6. The tree gives strong support to the hypothesis that *W. magnifica* and *W. vigil* are sister taxa (bootstrap support 74%) and places the new species, *W. monegrosensis*, as a sister taxon to this grouping, but the support for this was weaker (bootstrap support 37%). Most of the branches have very weak support, particularly the basal ones (Fig. 13).

Because genitalia provide the distinguishing characters between species, these characters were analysed in isolation. Only the female genitalia characters showed a pattern of synapomorphies that support some phylogenetic relationships amongst species, but the support was weak throughout (Fig. 14).

Molecular analysis. The PCR primers amplified either a fragment containing the 3′ terminal 273 nucleotides of *cytochrome b* or, when the DNA was more degraded, two overlapping segments of the same sequence. In the latter treatment, the identity of 14 nucleotides near the midpoint could not be determined and this region (positions 163–176) of the alignment was then

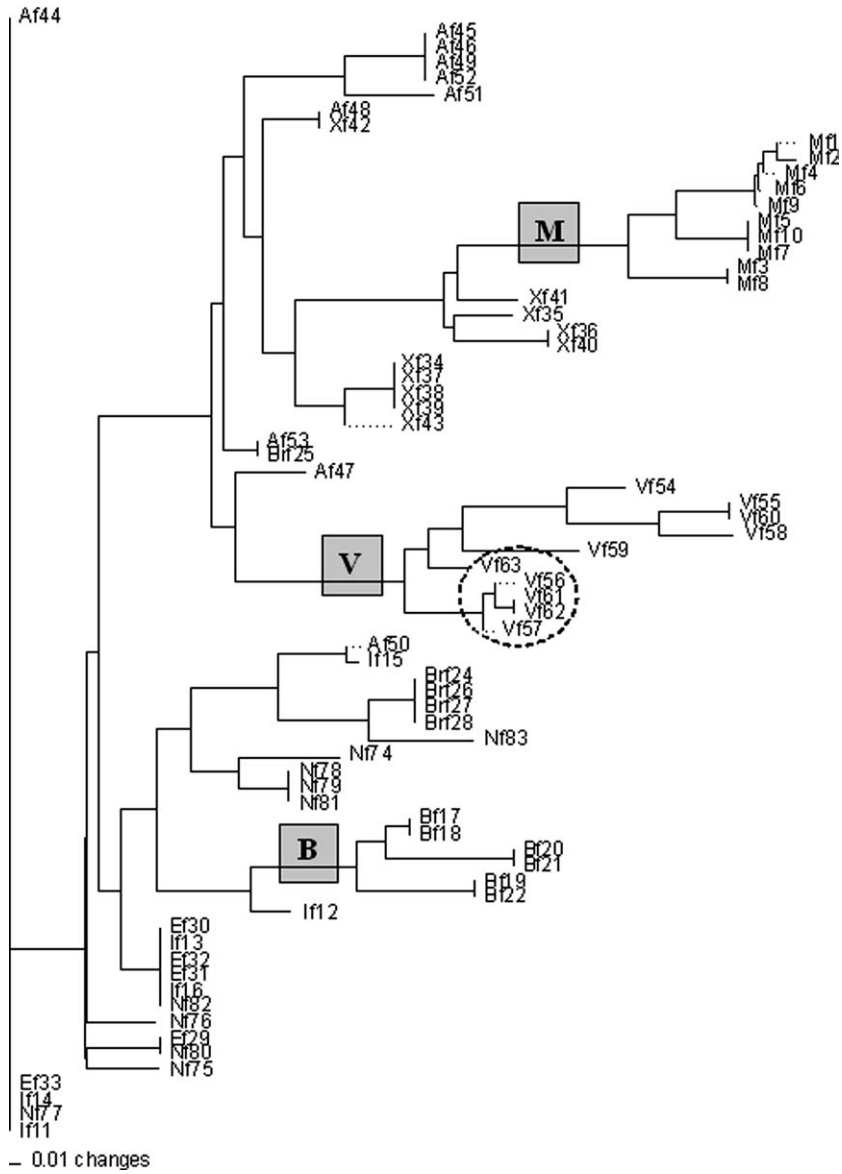

Fig. 10. Neighbourhood-joining dendrogram of female *Wohlfahrtia* based on external body characters only. Letters in bold in shaded boxes indicate where all members of a taxon were recovered in a monophyletic clade (*magnifica*, *bella* and *vigil* only). All specimens of *W. vigil* inside the dashed oval were from western North America, except for Vf61, for which only the description 'Nearctic' exists as locality data. All other *W. vigil* were from eastern North America or Europe. A, *W. aschersoni*; B, *W. bella*; Br, *W. brunnipalpis*; E, *W. erythrocera*; I, *W. indigens*; M, *W. magnifica*; N, *W. nuba*; V, *W. vigil*; X, *W. monegrosensis*.

excluded from the comparative analysis. Only a single haplotype (= unique mitochondrial DNA sequence) was discovered for most species, but two haplotypes were recorded for *W. magnifica* and *W. vigil s.l.* Sequences have been deposited in GenBank under accession numbers FJ379598–FJ379604.

Pairwise differences between haplotypes ranged from 1.3% to 6.7% intraspecifically (based on a 149-bp sequence in geographical populations of *W. magnifica* and *W. vigil s.l.*) and from 9.4% to 15.6% interspecifically (based on a 259-bp sequence in all species; Tables 5 and 6).

The pairwise genetic distances between the haplotype of the new species and those of the other *Wohlfahrtia* species were comparable with those between most pairs of the species characterized (Table 6), which supports its description as a morphological species. Using the haplotype of *Wohlfahrtia aschersoni* (Enderlein) or *W. bella* (Macquart) as an outgroup, an MP analysis of an alignment of the first 149 bp of the *cytochrome b* fragment was performed using a branch-and-bound search with 1000 bootstrap replicates; this gave a 50% majority-rule consensus tree (Fig. 15) that supported an ingroup containing the haplotype of the new species (CB3_mone1001), *W. magnifica* and *W. vigil s.l*, with the latter two as sister species represented by geographical pairs of haplotypes. For *W. magnifica*, western (Spain/France) and eastern (Hungary/Romania/Italy/Israel/Iraq) lineages were recognized, differing by just two nucleotides, a 0.77% divergence. For *W. vigil*, only the first half of the *cytochrome b* fragment from the single North American specimen (Wv3) was sequenced. However, this 149-bp sequence was shown to have 10 nucleotide differences compared with samples with haplotype vigi1001 from Europe (Table 4). This equates to a difference of 6.7% between the European and North American haplotypes of *W. vigil*. The sister-species relationship of *W. magnifica* and *W. vigil* was strongly supported in the consensus tree produced by an MP analysis of the 259-bp sequence

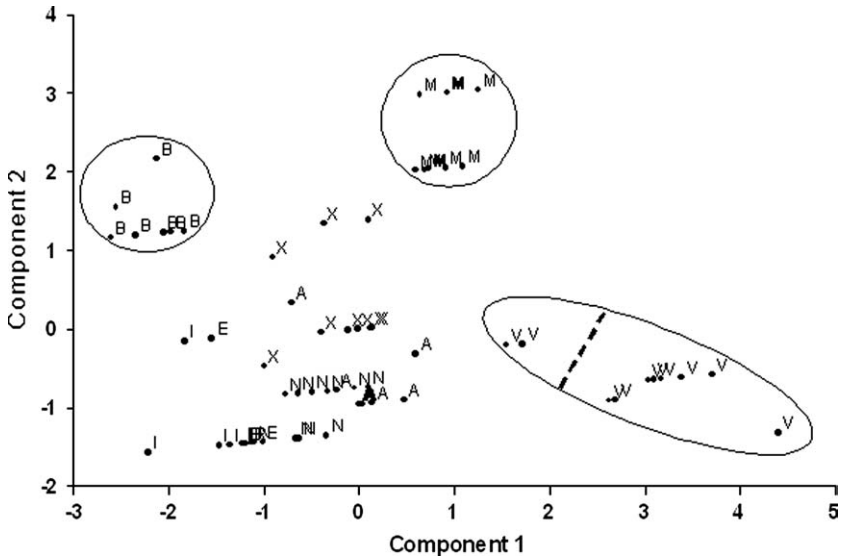

Fig. 11. Principal component analysis of male external body characters with antenna gap weighting (component 1 = 36%; component 2 = 27%). All specimens of *W. vigil* to the left of the dashed line were from western North America. All other *W. vigil* were from eastern North America or Europe. A, *W. aschersoni*; B, *W. bella*; E, *W. erythrocera*; I, *W. indigens*; M, *W. magnifica*; N, *W. nuba*; V, *W. vigil*; X, *W. monegrosensis*.

of *cytochrome b*, but this did not resolve the relationships among the other three species.

Discussion

Morphological analysis confirmed that only genitalia characters are diagnostic for most species of *Wohlfahrtia*. However, this study demonstrated that males and females of the economically important myiasis-causing species *W. magnifica* can be distinguished from all the other species examined using only external morphological characters. The NJ and PCA methods of statistical analysis used here grouped specimens on the basis of their overall similarity. This does not provide reliable evidence concerning evolutionary relationships, but it is useful when determining whether specimens comprise one or more distinct morphological forms. Evidence from both the molecular analysis of *cytochrome b* (Fig. 15) and the morphological analysis of body and genitalia characters (Fig. 13) support the hypothesis that *W. magnifica* and *W. vigil* are sister species, and the newly described species, *W. monegrosensis*, is closely related. Poor phylogenetic resolution based on morphology alone was not unexpected because the characters selected were taken from identification keys which, by their nature, favour species-specific characters. Hence several characters were autapomorphic, having derived states unique to a single taxon.

The analysis of molecular sequence data demonstrates that there are two geographically separated haplotypes of *W. magnifica*, with diagnostic characters at two nucleotide positions. Thus there is a haplotype in Western Europe (CB3_magn1002, Table 4) and another found elsewhere in Europe and eastwards to the Middle East (CB3_magn1001, Table 4). The genetic divergence does not appear to have resulted in

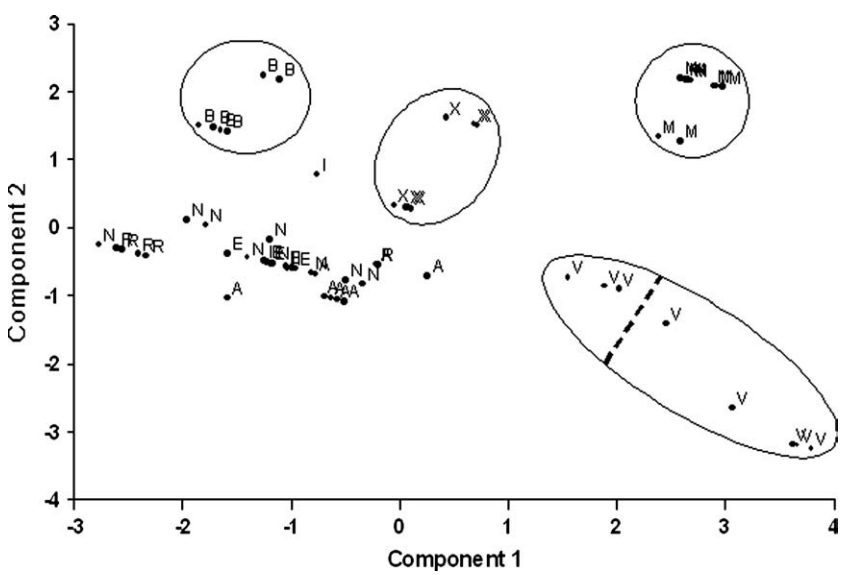

Fig. 12. Principal component analysis of female external body characters with antenna gap weighting (component 1 = 43%; component 2 = 24%). All specimens of *W. vigil* to the left of the dashed line were from western North America. All other *W. vigil* were from eastern North America or Europe. A, *W. aschersoni*; B, *W. bella*; E, *W. erythrocera*; I, *W. indigens*; M, *W. magnifica*; N, *W. nuba*; V, *W. vigil*; X, *W. monegrosensis*.

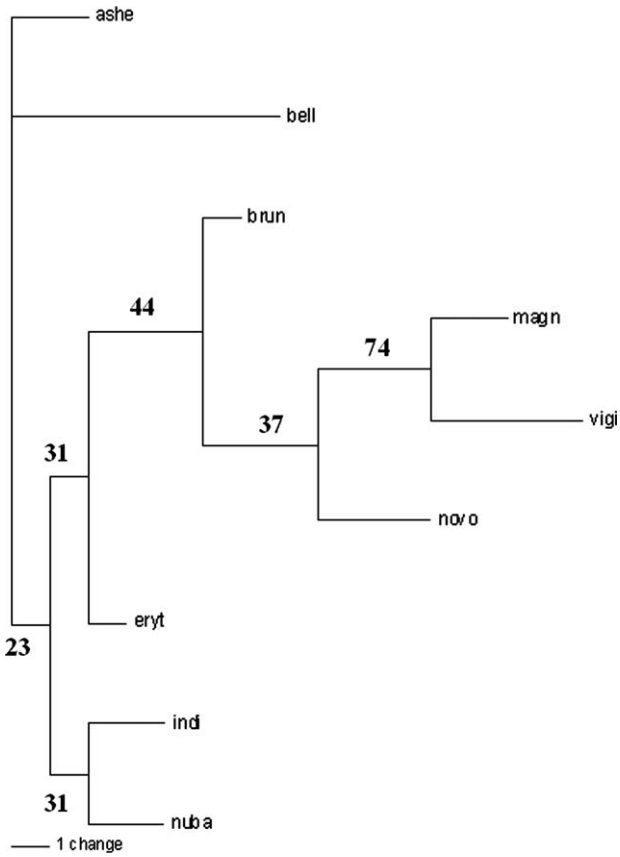

Fig. 13. Cladogram of relationships amongst species of *Wohlfahrtia* based on all morphological characters of males and females given by PAUP* (with heuristic searches). Numbers in bold are bootstrap support values for the clades indicated. ashe, *W. aschersoni*; bell, *W. bella*; brun, *W. brunnipalpis*; eryt, *W. erythrocera*; indi, *W. indigens*; nuba, *W. nuba*; magn, *W. magnifica*; vigi, *W. vigil*; novo, *W. monegrosensis*.

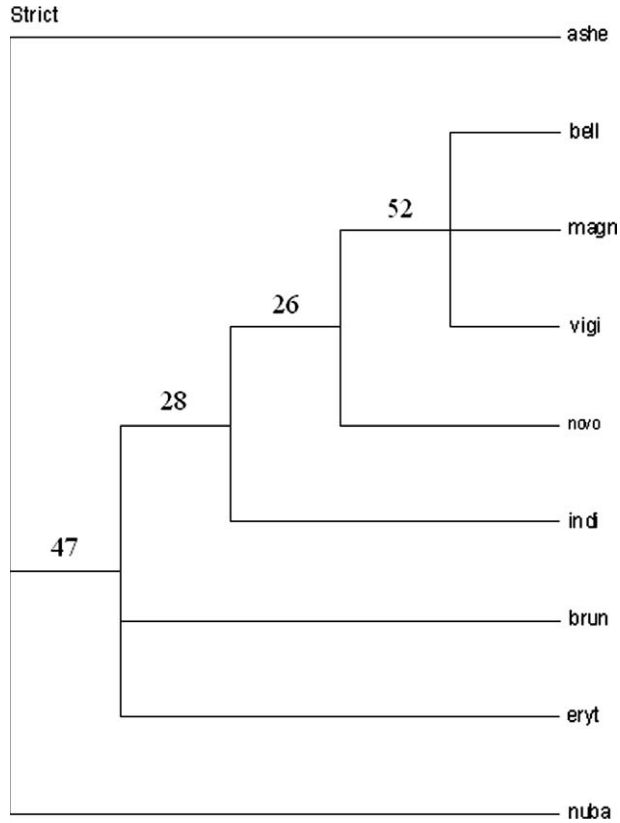

Fig. 14. Cladogram of relationships amongst species of *Wohlfahrtia* based on female genitalia characters, given by PAUP* (with heuristic searches). Percentages on branches are bootstrap confidence values. ashe, *W. aschersoni*; bell, *W. bella*; brun, *W. brunnipalpis*; eryt, *W. erythrocera*; indi, *W. indigens*; nuba, *W. nuba*; magn, *W. magnifica*; vigi, *W. vigil*; novo, *W. monegrosensis*.

differing pathogenicity. Strains from the Iberian peninsula appear to be just as aggressive and invasive as those from elsewhere (Hall & Farkas, 2000).

There is presently no information on the biology of *W. monegrosensis* and this study has examined only one-third of the described species of *Wohlfahrtia*. However, the morphological and molecular analyses in this study suggest that *W. monegrosensis* is closely related to *W. magnifica* and *W. vigil* (Figs 13 and 15), both of which have parasitic larvae, although whether *W. vigil* is obligate or facultative is not certain (Table 1). Whether or not *W. monegrosensis* is also parasitic and, if so, facultative or obligate, remains to be determined. However, if it is parasitic then it does not appear to be an important species in terms of causing myiasis in larger livestock. Identification of larvae from cases of sheep myiasis in the region of discovery of *W. monegrosensis* showed 96% of cases (45/47) were caused by *W. magnifica* (M.J.R. Hall, N.P. Wyatt, J. Lucientes Curdi and J.A. Castíllo Hernandez, unpublished data, 1995). The Los Monegros region where the species was discovered and after which it is named is in the central part of the Ebro Valley, to the east of Zaragoza. It has an arid climate with only 200–400 mm of rainfall per year, frequent northwest or southeast winds that are very desiccating and high summer temperatures (to $>$ 40 °C). Intensive cereal cultivation on the flatter lands, which are planted and left as bare gypsum soils in alternate years, has left the climax vegetation of juniper woods (*Juniperus thurifera*) only on the hillsides and hilltops. Wild rabbits are abundant in the area and, if *W. monegrosensis* is parasitic, they represent a potential host.

Although our analysis was limited by the degraded DNA from North American specimens of *W. vigil*, it shows a $>$ 6% difference between the sequence structures of New World and Old World *W. vigil*. This was based on specimens from Europe and a specimen from New Mexico in western North America. Morphology also suggested that there might be two groups of *W. vigil*, one in western North America, the other including specimens from eastern North America and Europe. This combined evidence strongly supports the need for a thorough re-examination of the synonymy of *W. vigil* to determine whether it is indeed a single species or a complex of two or three species or subspecies (i.e. with either New World and Old World taxa, or two taxa in North America, one of

Table 5. Alignment of the variant nucleotides of *cytochrome b* of the 259-bp haplotypes of selected species (Table 4). The two haplotypes of *Wohlfahrtia magnifica* (CB3_magn1001, CB3_magn1002) were characterized by nucleotide changes only at positions 84 and 126.

	1111111111111111111111111122222222222222
	1122223334455566778889901112223344445567777788999901112223335555
Haplotype	3495812780362824707281476951271360212450993567878036981450370240149
CB3_asch1001	ATTATTCTCCATTTTATTAACAACCATCTTTAAGATCTCTATTAACATCTACCATTACCCACTCCC-
CB3_mone1001	ATAACTCTTAGCTTTAATTTCAACCTCTTCTAAGATCTTTATCAACATTAATTATCATCTGATCCCC
CB3_bell1001	ATTATTCTCTATCACAACTACATTCTTTTTTTAGACCTCCGTTAACACCAATTTCTATTTATTCTTC
CB3_vigi1001	ACATTACCTAATCTCCTTTATTATTTCCATAAAATTCTTCACCGGTTTTACTTTTTACCTATTTTTT
CB3_magn1001	GTAATTTTTAATTACCCTTTCAATTTTTATTCAGACTATTACTAACATCTACTTTCCTCTATCTTTT
CB3_magn1002	GTAATTTTTAATTACCCTTTCAGTTTTTATTCGGACTATTACTAACATCTACTTTCCTCTATCTTTT

which is also found in the Palaearctic region, or three taxa, two in North America and one in the Palaearctic region). In addition to the morphological and molecular evidence for diversity within *W. vigil*, there appear to be differences in its larval biology associated with location, first highlighted by Salem (1938). Hence, in North America, *W. vigil* has been recorded as an obligate, myiasis-causing parasite of mink, fox, rabbit, cat, dog (Gassner & James, 1948; Strickland, 1949; Greve, 1968), ducklings (Wobeser *et al.*, 1981), rodents (Morrison, 1937; Boonstra, 1976; Craine & Boonstra, 1986; Schorr & Davies, 2002) and humans, especially infants, in whom it typically causes a furuncular myiasis (Ford, 1936; Rich & Knowlton, 1937; O'Rourke, 1954; Haufe & Nelson, 1957; Stabler, 1961; Stabler *et al.*, 1962; Dong, 1977; Eads, 1979). Both described forms of *W. vigil* in North America have been reported to cause myiasis, *W. vigil* in the east and *W. opaca* in the west (Gassner & James, 1948), but the majority of the cases reported above (12/16) were caused by the western form. In the Palaearctic region, *W. vigil* has only once been reported to cause myiasis in mammals (traumatic myiasis in a human infant; Grunwald *et al.*, 1998), but the accuracy of this identification is unclear. By contrast, its larvae have been reported as cutaneous and cavity parasites of frogs and toads (Cepelák and Artamonov, in Povolný & Verves, 1997). It is perhaps not surprising that there should be a genetic divergence between these geographically separate populations of *W. vigil* because they are neither synanthropic nor pests of widely dispersed livestock and, therefore, are unlikely to be moved around to any great extent by human activity. Future studies will concentrate on more robustly addressing the unresolved questions surrounding the identity of *W. vigil* and its synonyms.

Table 6. Uncorrected genetic distances ('p') between pairs of 259-bp haplotypes of *cytochrome b* for selected species (Table 4).

Taxon code		1	2	3	4	5
CB3_asch1001	1	–				
CB3_mone1001	2	0.09399	–			
CB3_bell1001	3	0.10555	0.11923	–		
CB3_vigi1001	4	0.15632	0.14615	0.15000	–	
CB3_magn1001	5	0.12890	0.12308	0.11538	0.13462	–
CB3_magn1002	6	0.13660	0.13077	0.11923	0.14231	0.00769

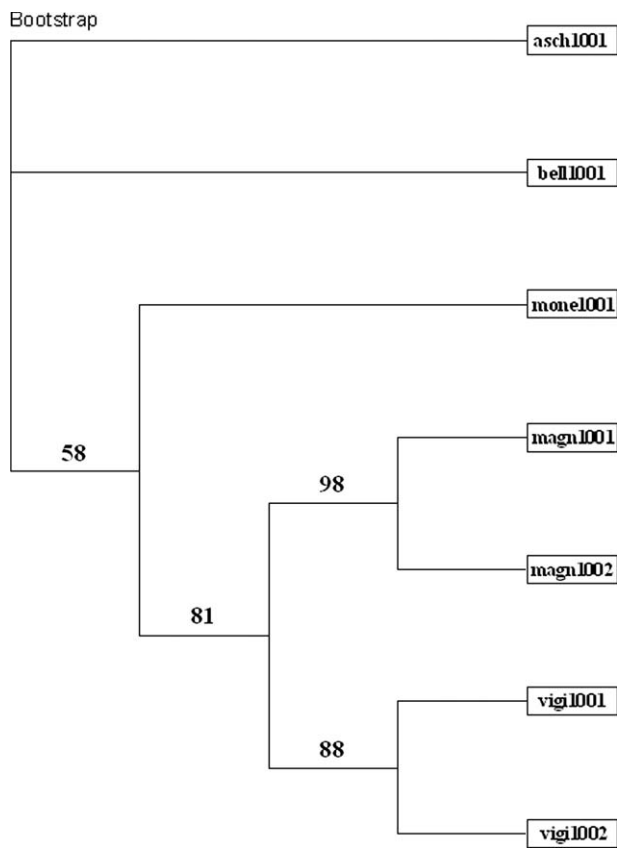

Fig. 15. 50% majority-rule consensus cladogram obtained by a maximum parsimony analysis of an alignment of the 149-bp *cytochrome b* haplotypes of *Wohlfahrtia* species. A branch-and-bound search with 1,000 bootstrap replicates was used and the percentages on branches are bootstrap confidence values (asch1001 = *W. aschersoni*; bell1001 = *W. bella*; magn1001, magn1002 = *W. magnifica*; vigi1001, vigi1002 = *W. vigil*; mone1001 = *W. monegrosensis* – see Table 4).

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Conflicts of interest

All authors declare no conflicts of interests.

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Molecular genetic analysis of populations of Wohlfahrt's wound myiasis fly, *Wohlfahrtia magnifica*, in outbreak populations from Greece and Morocco

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Abstract. Wohlfahrt's wound myiasis fly, *Wohlfahrtia magnifica* (Schiner) (Diptera: Sarcophagidae), is the most important cause of traumatic myiasis in the southern Palaearctic region. Larval stages are obligate parasites and the wounds caused by infestations are very similar to those caused by Old and New World screwworm flies. During the last decade, *W. magnifica* appears to have expanded its range to parts of northern and central Morocco, and to Crete, Greece. Specimens of *W. magnifica* were collected in Morocco and Crete either as larvae (preserved in 80% ethanol) or as adults (dry-pinned). Comparison specimens were collected in Spain, Hungary and mainland Greece. A DNA fragment containing the 3′ 715 base pairs of the mitochondrial *cytochrome b* gene was amplified by polymerase chain reaction from each of 132 larvae or adults of *W. magnifica* and the amplicons were directly sequenced and analysed phylogeographically. Twelve *cytochrome b* haplotypes were detected. All haplotypes from Morocco belonged to a lineage that included specimens from the Iberian peninsula, and restricted mixing of central and northern populations in Morocco was demonstrated. *Cytochrome b* haplotyping combined with an analysis of larval size provided clear evidence of multiple infestations of hosts in all geographical areas, with one quarter of wounds containing larvae from two to at least four females. More than 80% of specimens from Crete contained a haplotype predominating in mainland Greece and Hungary. Our survey indicated that wohlfahrtiosis was more widespread in northern and central Morocco than previously recorded by government veterinarians. However, the prevalence of wohlfahrtiosis was low ($< 1\%$). The high genetic diversity of Moroccan populations is consistent with longterm endemicity, rather than recent introduction. Crete showed a higher prevalence of wohlfahrtiosis ($\leq 15\%$) and less genetic diversity of *W. magnifica*, which is consistent with a recent introduction. The western and eastern Mediterranean lineages may have been isolated in different Pleistocene ice-age refugia, from which there has been limited post-glacial dispersal.

Key words. Sarcophagidae, *Wohlfahrtia magnifica*, *cytochrome b*, Mediterranean basin, mitochondrial DNA, post-glacial dispersal, Greece, Hungary, Morocco, Spain.

Introduction

The flesh fly, *Wohlfahrtia magnifica*, is an important cause of traumatic myiasis in livestock throughout the Mediterranean basin and eastwards into China, including the steppe regions of continental Europe (Hall & Farkas, 2000). It occurs along much of what is known as the Eurasian ruminant street or road (Slingenbergh *et al.*, 2004), an avenue of trade which runs

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from Europe through Turkey, Iraq and Iran into Asia, along which a considerable amount of livestock trade occurs. Its distribution in Iran and Iraq partly overlaps with that of the Old World screwworm (OWS) fly, *Chrysomya bezziana* Villeneuve, with which it can potentially be confused because of similarities in larval morphology and wound characteristics (Hall, 2004).

Female *W. magnifica* are attracted to wounds or natural body orifices of their hosts, where they deposit first instars. Sheep are a major host, but other livestock including poultry (Farkas *et al.*, 2001), wildlife (Rosen *et al.*, 1998) and even humans (Iori *et al.*, 1999; Lmimouni *et al.*, 2004) can be affected. In Europe, *W. magnifica* has been a particular problem for breeds of sheep introduced in Romania and Hungary, such as Merino, Romney and Corriedale, which are very susceptible to infestation (Lehrer & Verstraeten, 1991; Farkas *et al.*, 1997). Newly deposited larvae immediately start to feed on the host's cutaneous and underlying tissues, causing serious damage. These wounds become increasingly attractive to females (Hall *et al.*, 1995) and, therefore, more flies arrive and deposit their larvae, which enlarge the wound still further. Infestations of livestock can lead to death if left untreated. Even if death does not result, wohlfahrtiosis is a major animal welfare problem, causing pain and suffering. The genitalia of both sexes are a major site of infestations, which can lead to problems in the reproductive ability of hosts (e.g. sheep [Farkas & Hall, 1998] and camels [Hadani *et al.*, 1989; Valentin *et al.*, 1997]).

Recent molecular studies have shown that there are at least two genetic lineages of *W. magnifica*, one in Spain and France and the other in all countries studied to the east of these (Hall *et al.*, 2009). The distribution of the species is dynamic, possibly in part because of movements of infested animals. Since 1999, *W. magnifica* has been recorded on the island of Crete, Greece, where it has caused significant health and welfare problems in livestock, particularly sheep and goats, which form the basis of the local dairy industry (Sotiraki *et al.*, 2003, 2005a, 2005b). Its status as a pest in Morocco has also increased since 2001, when outbreaks were first reported in the northern province of Al-Hoceima, which borders the Mediterranean Sea (El Abrak *et al.*, 2002).

Most cases in Morocco were found in livestock, but human cases have also been reported (Lmimouni *et al.*, 2004; Tligui *et al.*, 2007). It was initially thought that cases may have been caused by larvae of OWS, *Ch. bezziana*, but they were confirmed as *W. magnifica* (M.J.R. Hall, unpublished report to the Food and Agriculture Organization of the United Nations, GFAGAHP RA213A6622010, 2002). It is probable that a similar misidentification occurred in Algeria, where a case of myiasis caused by *Ch. bezziana* was reported in a young shepherd who had not travelled outside the region (Abed-Benamara *et al.*, 1997). Such potential confusion confirms the need for a thorough taxonomic study of all fly species that can produce obligate traumatic myiasis in humans and livestock.

At least two publications have claimed that the distribution of *W. magnifica* included Morocco (James, 1947; Verves, 1986). James (1947) wrote that the records in his review were prepared either from published records or from specimens present in the collection of the United States National Museum, or were determined by the author from other sources. He did not specifically state where his information on the distribution of *W. magnifica* came from. At least four other species of *Wohlfahrtia* are reported to occur in Morocco: *Wohlfahrtia bella* (Macquart), *Wohlfahrtia nuba* (Wiedemann), *Wohlfahrtia indigens* Villeneuve and *Wohlfahrtia trina* (Wiedemann) (Verves, 1986). *Wohlfahrtia magnifica* is known to occur in mainland Greece (Verves, 1986), but until 1999 there are no records, to our knowledge, of its presence in Crete. In addition, neither farmers nor veterinarians on Crete were familiar with wohlfahrtiosis until 1999 (Sotiraki *et al*, 2003).

The objectives of the work reported here were to characterize genetically the populations of *W. magnifica* sampled in outbreak situations in Greece and Morocco, using mitochondrial DNA markers, in order to assess their geographical ranges and discover any evidence for exchange of flies within the region.

Materials and methods

Surveys in Crete and mainland Greece

Field sampling was undertaken on Crete in August 2001, May and July 2002, and July and September 2003 during studies of fly behaviour and control (Sotiraki *et al.*, 2003, 2005a, 2005b). Samples from mainland Greece were either collected by the authors during the summers of 2005 and 2006 or were submitted by local veterinarians (Fig. 1).

Survey in Morocco

Field visits to Morocco were made in December 2001, July and September 2002 and May 2003 (Fig. 2). During each field trip, many smallholder farms and communal wells, used for watering of livestock, were visited. Local farmers and animal owners were questioned about myiasis and encouraged to present active cases, either on the day of first visit or on a revisit. All larvae were collected from each active infestation presented.

In July 2002 M.J.R. Hall and R. Farkas visited the Diptera collections of the Institut Scientifique, University Mohammed V, in Rabat, to look for any historical evidence of *W. magnifica* in Morocco. Eight specimens labelled as *W. magnifica* were studied, two of which were identified as *W. magnifica* (Verves, 1986). These did not have clear date labels, but their localities were indicated as Volubilis (possibly in 1978), the site of an old Roman town about 19 km north of Meknes, and Mazagan, the old name for the sea port of El-Jadida, about 90 km southwest of Casablanca.

In July 2002 sampling was concentrated on Al Hoceima Province in northern Morocco, but samples were also collected at Volubilis in central Morocco. In September 2002, sampling concentrated on the Maaziz-Meknes-Fes (including Volubilis) and Middle Atlas regions, with a 1-day visit to Al Hoceima Province. In May 2003 sampling was undertaken around Maaziz and in the High Atlas region.

Samples from Spain and Hungary

Samples of larvae from Spain (Zaragoza) and Hungary (Harkakotony) were collected during ongoing studies of wohlfahrtiosis.

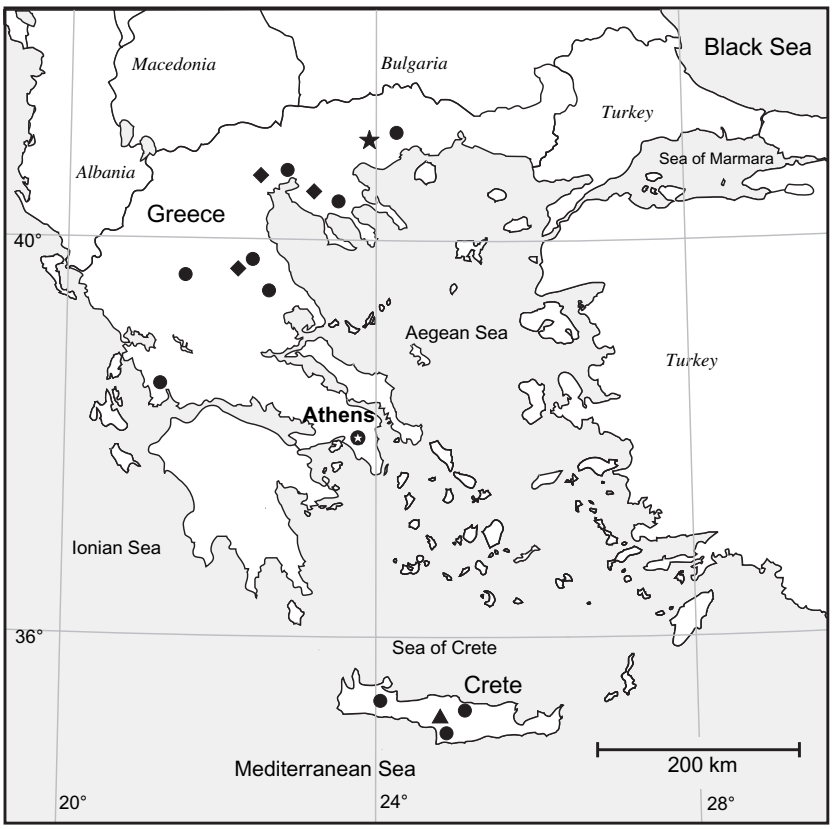

Fig. 1. Outline map of Greece showing approximate origins of specimens of each *cytochrome b* haplotype (●, CB_magn01; ▲, CB_magn10; ♦, CB_magn11; ★, CB_magn12).

Sampling, preservation methods and morphological identifications

Larvae were kept alive on moist tissue paper within plastic containers for 1–8 h and were then killed by immersion in boiling water for 15–30 s. This extended the larvae, enabled their preservation in 80% ethanol without tissue blackening (Adams & Hall, 2003) and had no negative impact on DNA extraction. At each site hand-netting was undertaken of adult flies settled on rocks, walls and other prominent objects in the environment. Most of these flies were males at 'waiting stations' or 'mating stations' (Guillot *et al.*, 1978; Hall *et al.*, 1995). Larvae were identified according to Zumpt (1965) and Spradbery (1991), and adults were identified according to Verves (1986) and Hall *et al.* (2009), following examination under a dissecting microscope at up to 50× magnification.

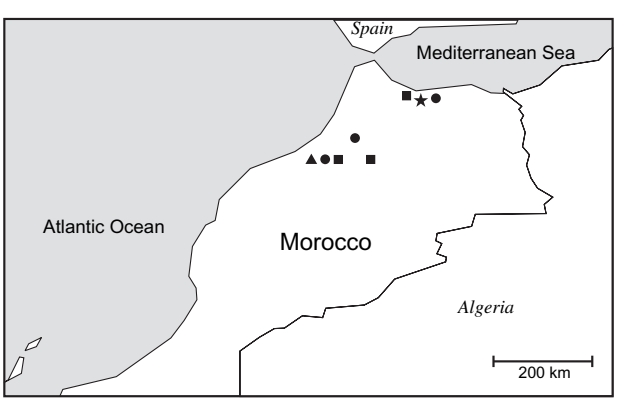

Fig. 2. Outline map of central and northern Morocco showing approximate origins of specimens of each *cytochrome b* haplotype (●, CB_magn02; ▲, CB_magn03; ■, CB_magn04; ★, CB_magn05/06/07).

DNA extraction, PCR amplification, sequencing and analysis

All steps used the protocols reported by Ready *et al.* (2009). DNA was extracted from either an abdominal tissue block of larvae (approximately from body segments 6–9) or from the thoracic muscle of adult flies. DNA was extracted from the tissues using the DNAzol® kit (Invitrogen Corp., Carlsbad, CA, U.S.A.; Chomczynski *et al.*, 1997), dissolved in 1× Tris-EDTA solution and stored short-term at 4 °C and long-term at − 20 °C. The 3′ terminal 715 bp of the mitochondrial *cytochrome b* gene (*cyt b*) was amplified by polymerase chain reaction (PCR), directly sequenced and analysed.

The PCR amplification was carried out using a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Inc., Foster City, CA, U.S.A.). Positive (voucher sample of the *cyt b* region) and negative (no DNA) controls were used. The size and quantity of the amplified DNA fragments were determined by fractionating the PCR products on 1.5–2.0% agarose gels along with DNA fragments of known sizes and quantities (Promega

PCR Markers G316A; Promega Corp., Madison, WI, U.S.A.). The DNA in the excised gel fragments was purified with glassmilk (Geneclean II®; MP Biomedicals, Solon, OH, U.S.A.) and each strand directly sequenced using one of the PCR primers (3.2 pmoles) and the ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit (Version 2.0; PE Applied Biosystems, Inc.). Purification of the extension products was carried out using the ethanol precipitation method. Sequences were resolved using the ABI 377 system. Sequences were edited and aligned with database sequences using Sequencher™ (Gene Codes Corp., Ann Arbor, MI, U.S.A.), all according to ABI protocols (PE Applied Biosystems, Inc.), and were identified and their relationships analysed using PAUP* software (Swofford, 2002).

The term 'adult equivalents' (AEs) was used when assessing haplotype frequency to indicate an individual adult or the offspring of one female. The use of AEs gives a minimum number of adults. This is because all larvae in a single wound with the same haplotype may derive from a single adult, but they may also derive from more than one. However, all specimens of the same haplotype in a wound were considered to be from just one adult unless they were obviously the product of two larvipositions, such as when mature third instars and second instars were found in the same wound, clearly indicating at least two larvipositions.

Results

Morphological identifications of larvae causing myiasis infestations and of adult flies

Of nine active cases sampled in Crete, all were exclusively infested by larvae of *W. magnifica*. Hosts sampled were sheep (8/9, 88.9%) and a dog (1/9, 11.1%).

Of 19 active cases of traumatic myiasis sampled in Morocco, 17 (89.5%) were infested exclusively by *W. magnifica*, one (5.3%) was infested jointly by *W. magnifica* and an unknown sarcophagid species and one (5.3%) was infested jointly by a *Lucilia s*pecies and *Chrysomya albiceps* (Wiedemann), but had previously been infested by *W. magnifica*, based on the deeply penetrating wounds. Thus, *W. magnifica* was involved in all cases seen. Hosts encountered were sheep (8/19, 42.1%), dogs (8/19, 42.1%), goats (2/19, 10.5%) and a horse (1/19, 5.3%). All *Wohlfahrtia* adults collected in Greece were of *W. magnifica*. In Morocco, adults of *W. bella* were also collected, but these were not sequenced.

For comparison with the outbreak populations, four larvae from Spain (Zaragoza; hosts: horse [1], unknown [1]), two larvae and three adults from Hungary (Harkakotony; host: pig [1]) and 45 larvae from the northern half of mainland Greece (north of 38°50′ N; hosts: sheep [9], goat [1], horse [1], dog [5], unknown [4]) were sequenced.

mtDNA haplotype diversity

In total, 12 mtDNA haplotypes (= unique sequence types) were identified based on analyses of the 3′ terminal 715 nucleotides of *cyt b* (Tables 1 and 2; GenBank accession numbers: FJ379605–FJ379616). The 16 specimens (all larvae) of *W. magnifica* from Crete belonged to one of two mtDNA haplotypes of the eastern Mediterranean lineage. Larvae of the rarer haplotype (CB_magn10) were recovered from two wounds, always in association with larvae of the common haplotype (CB_magn01). The 62 specimens (54 larvae, eight adults) of *W. magnifica* from Morocco were found to contain one of six mtDNA haplotypes (Tables 1 and 2) belonging to the *cyt b* lineage of the western Mediterranean (Spain, France) (Hall *et al.*, 2009) and they shared one haplotype with the sample from Spain (CB_magn02).

The western and eastern Mediterranean lineages differed by at least two nucleotide changes in 715 positions (Fig. 3), equivalent to a genetic distance of 0.28%. The greatest genetic distance was between haplotypes CB_magn12 (Greece) and CB_magn07 (Morocco), which showed seven nucleotide changes, equivalent to a 0.98% difference.

Spanish specimens belonged to three haplotypes, one shared with Morocco (Table 1). Hungarian specimens were of one haplotype (CB_magn01), which predominated in Crete and Greece (Table 1). The majority of specimens from mainland Greece belonged to this common haplotype of the eastern lineage of

Table 1. Numbers of individuals of *Wohlfahrtia magnifica* with each *cytochrome b* gene haplotype in different geographical locations and regions. Numbers in brackets are adult equivalents.

	Wohlfahrtia magnifica cyt b haplotype number (CB_magn01–CB_magn12)												
	Western Palaearctic region								Central and eastern Palaearctic region				
Geographical location	02	03	04	05	06	07	08	09	01	10	11	12	Total of all haplotypes
Central Morocco	24 (14)	2 (1)	6 (3)	–	–	–	–	–	–	–	–	–	32 (18)
North Morocco	3 (2)	–	12 (6)	11 (8)	3 (1)	1 (1)	–	–	–	–	–	–	30 (18)
Spain	1 (1)	–	–	–	–	–	1 (1)	2 (1)	–	–	–	–	4 (3)
Hungary	–	–	–	–	–	–	–	–	5 (4)	–	–	–	5 (4)
Mainland Greece	–	–	–	–	–	–	–	–	38 (21)	–	6 (4)	1 (1)	45 (26)
Crete	–	–	–	–	–	–	–	–	14 (9)	2 (2)	–	–	16 (11)
Total from all regions	28 (17)	2 (1)	18 (9)	11 (8)	3 (1)	1 (1)	1 (1)	2 (1)	57 (34)	2 (2)	6 (4)	1 (1)	132 (80)

Table 2. Variant nucleotides in an alignment of all *cytochrome b* haplotypes found. All 13 polymorphic characters were included in the parsimony analysis (Fig. 3) and four were parsimony informative.

	Nucleotide position														
Haplotype code	5 0	7 9	2 4 1	2 7 2	2 9 7	3 5 5	3 9 4	4 4 5	5 2 6	5 5 5	5 6 8	6 1 0	6 7 6	Country	Region
CB_magn02	C	A	T	G	C	T	T	G	G	T	G	T	T	Spain, Morocco	West
CB_magn03	C	A	T	G	C	T	T	A	G	T	G	T	T	Morocco	
CB_magn04	C	A	T	G	C	T	T	G	G	C	G	T	T	Morocco	
CB_magn05	C	A	G	G	C	T	T	G	G	T	G	T	T	Morocco	
CB_magn06	C	A	T	A	C	T	T	G	G	T	G	T	T	Morocco	
CB_magn07	C	A	T	G	C	T	T	G	G	C	G	T	C	Morocco	
CB_magn08	C	A	T	G	C	T	T	G	G	T	A	T	T	Spain	
CB_magn09	C	A	T	G	C	C	T	G	G	T	G	T	T	Spain	
CB_magn01	C	A	T	G	T	T	T	G	A	T	A	T	T	Hungary, Greece	East
CB_magn10	T	A	T	G	T	T	T	G	A	T	A	T	T	Greece (Crete)	
CB_magn11	C	A	T	G	T	T	C	G	A	T	A	T	T	Greece	
CB_magn12	C	G	T	G	T	T	T	G	A	T	A	G	T	Greece	

W. magnifica, but two rare haplotypes were also collected (CB_magn11 and CB_magn12).

Regional differences in genetic diversity in Morocco

There was a clear and statistically significant difference between the genetic composition of the central and northern faunas of Morocco ($\chi^2 = 13.29$, d.f. $= 5$, $P = 0.02$) (Fig. 4). In the central sampling area of Volubilis and Maaziz, the dominant *cyt b* haplotype was CB_magn02 (14/18 female equivalents, 77.7%). However, in the northern sampling area of Al Hoceima there were two co-dominant haplotypes, CB_magn04 (6/18, 33.3%) and CB_magn05 (8/18, 44.4%). Two haplotypes were shared between the regions, CB_magn02 and CB_magn04, but CB_magn02 was rare in the north and CB_magn04 was rare in the central region. Both sampling areas demonstrated unique or rare haplotypes: CB_magn03 in the central region, and CB_magn06 and CB_magn07 in the north.

In the central region of Morocco, most wounds (10/11, 90.9%) were infested with larvae of the dominant haplotype CB_magn02. Two of those wounds were co-infested with either haplotypes CB_magn03 or CB_magn04, and a further wound was found infested by larvae just of CB_magn04. In the northern region, half the wounds (4/8) were infested with haplotype CB_magn04 and the other half (4/8) with CB_magn05. No wounds contained both these haplotypes. Haplotypes CB_magn02 and CB_magn06 were found together in a single wound with haplotype CB_magn05. Haplotype CB_magn07 was detected from a single adult.

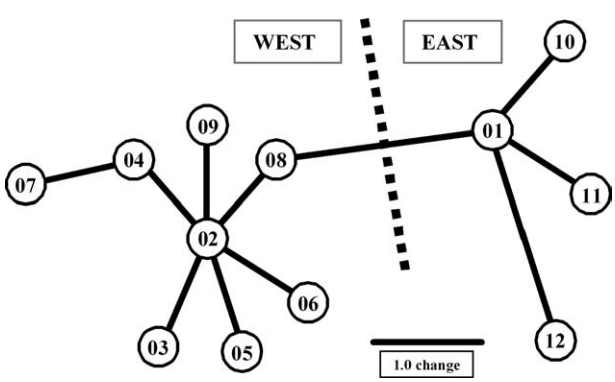

Fig. 3. Unrooted neighbour-joining tree generated by using untransformed genetic distances between the 12 *cytochrome b* haplotypes of *Wohlfahrtia magnifica* (CB_magn01–CB_magn12). The numbers of nucleotide differences between each haplotype are indicated by the branch lengths (see scale). Western haplotypes were found in Spain and Morocco; eastern haplotypes were found in Greece and Hungary.

Multiple infestations

The majority of wounds examined in all geographical regions (40/52, 76.9%) were infested by larvae of *W. magnifica* characterized by the same *cyt b* haplotype. Such cases could have been infested by larvae of two or more females sharing the same *cyt b* haplotype or, as only a small subsample of larvae in the wounds were analysed, by several females with more than one haplotype. However, despite the minimal sampling, there was clear evidence of multiple infestations of hosts, based on the detection of multiple haplotypes of larvae within a single wound, or of a single haplotype in larvae of such differing sizes or instars that they had clearly been deposited by different females. Thus, almost a quarter of wounds (12/52, 23.1%) contained larvae of at least two females and 7.7% (4/52) contained larvae of at least three females. The greatest number of female larvipositions detected in a single wound found in Morocco was four (1/52, 1.9%). The greatest number of *cyt b* haplotypes detected

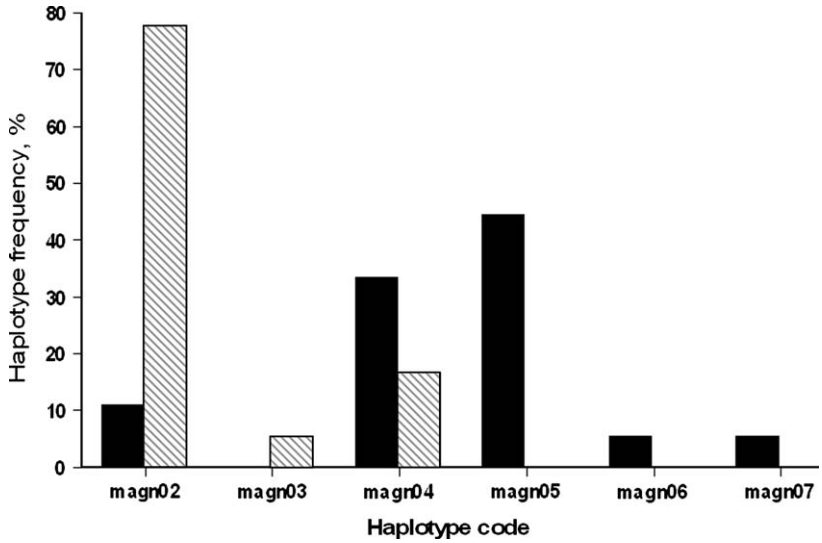

Fig. 4. Frequency (%) of each of the six *cytochrome b* haplotypes (CB_magn02–CB_magn07) of *Wohlfahrtia magnifica* found in northern Morocco (black bars; $n = 18$ adult equivalents) and central Morocco (cross-hatched bars; $n = 18$ adult equivalents).

in a single wound was three (CB_magn02, CB_magn05 and CB_magn06), found in a dog in Al Hoceima. Two or more *cyt b* haplotypes were detected in 15.4% of wounds (8/52).

Discussion

The analysis of the *cytochrome b* mtDNA haplotypes confirmed the presence of two lineages of *W. magnifica* reported by a preliminary analysis of a shorter region of the same gene (Hall *et al.*, 2009). The most likely hypothesis to explain the geographical distributions of the two lineages is that they were isolated in western and eastern Pleistocene glacial refugia and did not disperse far afterwards, maintaining allopatry. There is good evidence for post-glacial expansion of a number of Mediterranean species similar to that hypothesized here for *W. magnifica* (Schmitt, 2007). The Balkans and the Iberian and Italian peninsulas are recognized as important glacial refugia (Schmitt, 2007). In our study we did not have access to Italian specimens, but an earlier study using shorter sequences did link specimens from Italy with those from the eastern lineage (Hall *et al.*, 2009). A detailed survey for *W. magnifica* in central Western Europe, including the Alps and Pyrenees, which may act as barriers to spread, is necessary to determine the extent of expansion and possible areas of sympatry of the two lineages.

In addition to post-glacial expansion of Mediterranean species, Schmitt (2007) considered the expansion of continental and Arctic/Alpine species. Although it occurs in the Mediterranean region, *W. magnifica* is also considered to be a continental species, adapted to the open steppe regions of Europe and Asia, tolerant of hot and dry summers and able to diapause through cold winters (Portchinsky, 1916; Ternovoy, 1978; Valentin *et al.*, 1997). It is possible, therefore, that *W. magnifica* also occupied glacial refugia in the eastern Palaearctic region. The suggestion that extra-Mediterranean ice-age refugia were more common in Europe than previously considered deserves more investigation (Schmitt, 2007). *Wohlfahrtia magnifica* would be an ideal species with which to study this in view of its present Mediterranean and continental distribution, using a more complete phylogeographic analysis based on nuclear loci as well as mtDNA.

The haplotype profile in Crete was quite different to that in Morocco. Only two haplotypes were detected, of which one was predominant and the other was found in only 20% of AEs (Table 1). The predominant haplotype was identical to that found in greatest abundance in Hungary and mainland Greece. This result emphasizes the relative lack of genetic diversity in Eastern Europe, contrasting with that in Morocco. That the rare haplotype CB_magn10 was found only in Crete is not proof of an old and diverging population, but more probably reflects our under-sampling elsewhere, especially in the south of Greece, which should be a focus of further surveys. The prevalence of wohlfahrtiosis in sheep in Crete was up to 15% (Sotiraki *et al.*, 2003, 2005a, 2005b), which is much higher than the equivalent figure for Morocco and lends support to the hypothesis that this infestation represents a recent introduction. Farmers and veterinarians had no previous experience of wohlfahrtiosis on Crete and no specimens of *W. magnifica* were found in the collections of the Natural History Museum in Heraklion. The data show that the *W. magnifica* introduced to Crete came from somewhere in the central or eastern part of the species' range, but certainly not from the Iberian peninsula or Morocco. Early after its introduction it was speculated that the fly could have been introduced from Spain because Crete imported approximately 2000 live sheep from Spain each year in 1998 and 1999 for the tourist trade. Approximately 80% were slaughtered within 2 days of importation, but the remainder were slaughtered up to 1 month later (Z. Somaras, National Veterinary Services, Heraklion, Crete, personal communication, 2007). This time period would have enabled any undetected and untreated larvae on the imported sheep to mature, leave the host and, potentially, establish a breeding population of flies on Crete. However, our results show that the *W. magnifica* found in Crete had clearly not been imported from Spain and thus confirm the value of molecular genetic studies in evaluating the origin of invading species.

Despite the impression given by veterinarians in Morocco that wohlfahrtiosis was a new problem in the northern province of Al Hoceima, our molecular studies do not support this. Five different haplotypes were found in a relatively small geographical area (20×5 km) and three of those were unique to that region (Fig. 2). If there had been a recent introduction of *W. magnifica*, it would most likely have come from a single source, such as a consignment of infested animals, with one or, at most, two haplotypes, because infestations with more than two haplotypes were rare in our study (1/52, 1.9%). Hence a recent introduction would be unlikely to have shown the genetic diversity we found in Al Hoceima Province. Therefore, the situation in Al Hoceima appears most likely to be the result of resurgence from extant populations of *W. magnifica*, for reasons at present unknown. That we found *W. magnifica* in a museum collection in Morocco demonstrates that it has been present in the country for at least 30 years.

It is interesting that northern and central Morocco showed significantly different haplotype diversities. The most common haplotype in central Morocco (CB_magn02: 77.8%) was comparatively rare in northern Morocco (11.1%; Table 1). This suggests that there is limited genetic exchange between these two regions. The Rif Mountains, which reach over 1800 m above sea level, are a likely barrier to the movement of adult flies between these regions. In this regard, it is interesting to note that the greatest altitude at which we recorded cases of wohlfahrtiosis in Morocco was 360 m a.s.l. However, elsewhere in the distribution of *W. magnifica*, cases have been recorded at much greater altitudes, such as 1200 m a.s.l. in Mongolia (Valentin *et al.*, 1997) and up to 2600 m a.s.l. in the French Pyrenees (Ruíz Martínez & Leclercq, 1994). We were unable to find evidence of wohlfahrtiosis in the northern foothills of the Middle Atlas, at approximately 1000–1700 m a.s.l. Reasons for this cannot include altitude, unless the populations in Morocco are less adapted to high altitudes and the associated climate than populations in Mongolia and the Pyrenees. The cold winter temperatures at these altitudes would also not normally be a contra-indicator for *W. magnifica*, which endures a much harsher winter in Eastern Europe. The region had many large flocks of sheep and goats and appeared ideal for *W. magnifica*. It is possible that some unique aspect of animal husbandry in the region limits wohlfahrtiosis, but this needs further study and the apparent lack of the species at higher altitudes in Morocco is, at present, a mystery.

The sampling of Hall *et al.* (2009) was more widespread than the present survey in geographical terms, but it looked at only a 273-bp sequence. The present analysis looked at 715 bp, but only in populations from Morocco, Spain, Hungary and Greece. Clearly, a much broader survey for the longer sequence is needed throughout the distribution range of *W. magnifica* to fully explore its genetic diversity and post-glacial expansion. Reasons for the comparatively high diversity of *W. magnifica* in Morocco compared with other areas studied to date in the Mediterranean basin and Europe deserve further study as they could provide indicators for how the species has spread with human interventions. Thus, the commonality of haplotypes on Crete and in mainland Greece and Hungary (Table 1) may have resulted from more extensive trade over centuries within Europe compared with that in Morocco. In this connection, *W. magnifica* could be a useful tool for examining the potential spread of insect pests of animals along the Eurasian ruminant road (Slingenbergh *et al.*, 2004). It is possible that the degree of genetic diversity in any area may be linked to factors, such as animal trade, that can overcome natural barriers to pest dispersal.

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Conflicts of interest

All authors declare no conflicts of interests.

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Traumatic myiasis in dogs caused by *Wohlfahrtia magnifica* and its importance in the epidemiology of wohlfahrtiosis of livestock

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Abstract. In the province of Al Hoceima, northern Morocco, and on two farms in Hungary, dogs were inspected for the presence of traumatic myiasis. Nine and four infested dogs were found in Morocco and Hungary, respectively. All the larvae and adults reared from them in the laboratory were identified as *Wohlfahrtia magnifica* (Schiner) (Diptera: Sarcophagidae). To our knowledge, these are the first cases of wohlfahrtiosis in dogs to be reported in these countries. All infested animals lived close to livestock, where wohlfahrtiosis was endemic. Infested body sites included limbs (six cases), external genitalia (two), ears (three), nose (one) and neck (one). Developing larvae caused severe welfare problems and tissue destruction in most cases. Although the number of cases reported here is small, wohlfahrtiosis in dogs may be very important from an epidemiological perspective because farm and stray dogs can act as both reservoirs and carriers of this parasitic fly species. Therefore, education of dog owners concerning the risk factors in endemic regions is recommended in order to reduce the prevalence of wohlfahrtiosis in dogs and thereby in livestock. Both owners and veterinarians should pay regular attention to any wounds and to the natural orifices of dogs, especially during the fly seasons.

Key words. *Wohlfahrtia magnifica*, dogs, wohlfahrtiosis, Hungary, Morocco.

Introduction

Traumatic myiasis (= wound myiasis) describes the infestation of living humans or vertebrate animals with fly larvae which feed and develop in the cutaneous tissues of their hosts, causing a more or less severe traumatizing injury (Hall & Farkas, 2000). Larvae of several species of fly can cause this parasitic infestation of domestic animals; however, the main agents of traumatic myiasis are a few species of blowfly (Calliphoridae) and flesh fly (Sarcophagidae). The association of these larvae with the host has two main forms, either facultative or obligate. Larvae of the facultative myiasis-causing species usually develop on carrion or in other decaying organic materials, but their gravid females can also cause larval infestation of living hosts, usually at sites of previous wounding or other predisposing conditions. A smaller number of fly species cause obligatory traumatic myiasis because their larvae require living animals, including humans, for development.

Traumatic myiasis can lead to serious animal welfare and health problems and economic losses in livestock worldwide, but it can also occur in other domestic species, such as dogs. Traumatic myiasis of dogs is usually reported to be caused by larvae of *Lucilia sericata* Meigen or *Lucilia caesar* L. or other blowflies (Wetzel & Fischer, 1971; Rauchbach & Hadani, 1972; Azeredo-Espin & Madeira, 1996; Anderson & Huitson, 2004). Necrotic tissues and/or bacterial skin infections of dogs can attract female flies, which are capable of laying hundreds of eggs, and the sites offer a favourable

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environment for developing maggots. Dogs confined outdoors are most susceptible to fly strike, especially those which have faecal or urine-stained coats, draining wounds or inflamed tissues (e.g. otitis externa, pyometra or infected paraproctal glands).

Sarcophagid flies can also cause facultative myiasis in dogs: Principato *et al.* (1994) reported a rare case of an Italian dog with traumatic myiasis caused by larvae of *Sarcophaga haemorrhoidalis* (Fallen). Obligate traumatic myiasis-causing fly species can also infest dogs, but there have been only a few reported cases. The New World screwworm fly, *Cochliomyia hominivorax* (Coquerel), which is responsible for severe economic losses in livestock in the New World, has infested many local dogs in Brazil (Cramer-Ribeiro *et al.*, 2003). Larvae of the Old World screwworm fly, *Chrysomya bezziana* Villeneuve, occurring in Africa and Asia, was found in the wounds of dogs (Chemonges-Nielsen, 2003). The movement of these exotic myiasis-causing fly species, as well as the Tumbu fly (*Cordylobia anthropophaga* Blanchard), between continents in infested dogs carries a potential threat of introductions to Europe and other continents where they do not yet exist (Anon., 1987; Chermette, 1989; Fox *et al.*, 1992; Hendrix *et al.*, 1995; Dongus *et al.*, 1996; Chemonges-Nielsen, 2003; Ferroglio *et al.*, 2003). Wohlfahrt's wound myiasis fly, *Wohlfahrtia magnifica* (Schiner), which is one of the most important obligate traumatic myiasis-causing species in the Palaearctic zone, is widely distributed from the Mediterranean basin, through central and eastern Europe to northern Asia. Infestations with larvae of *W. magnifica* (wohlfahrtiosis) have been detected in many domesticated animal species (e.g. sheep, horse, cattle, pig and goose) (Hall & Farkas, 2000; Farkas & Képes, 2001; Farkas *et al.*, 2001). Although the occurrence of wohlfahrtiosis in dogs was mentioned almost a century ago (Portschinsky, 1916), few cases have been reported recently (Şaki, 2004; Ütük, 2006).

This paper addresses the particular problem of dogs as hosts of *W. magnifica* in Morocco and Hungary, with the objective of evaluating their importance in the epidemiology of wohlfahrtiosis of livestock.

Materials and methods

Field studies in Morocco

During 4–8 July 2002, a field survey was carried out in the province of Al Hoceima (provincial capital: Al Hoceima 35°15′ N, 03°56′ W), in northern Morocco, where an outbreak of wohlfahrtiosis had been detected in livestock in 2001. Nine farms, two major communal wells, used for drinking water for humans and livestock and for laundry, and a number of community centres (e.g. rural mosques) were visited and revisited. Farmers and local people were asked about maggot infestations of animals and all active and recent infestations were inspected. Several dogs were inspected on farms, as were others that were taken to the communal wells by their owners. On 10 September 2002 a further day was spent sampling the same part of Al Hoceima province.

Field studies in Hungary

In August 2005 two Hungarian farms were visited in the neighbourhoods of Üllő (47°23′ N, 19°20′ E) and Kiskunhalas (46°26′ N, 19°29′ E), located in the centre of the country, about 160 km from one another. Flocks of Hungarian Merino sheep, pigs, poultry and horses were kept at the farms. Wohlfahrtiosis had been diagnosed among grazing sheep and pigs kept outdoors on both farms. Twelve local dogs (three and nine on farms 1 and 2, respectively) were inspected for traumatic myiasis.

After clinical examination of the dogs, the data (breed, sex, age and body location of wounds having fly larvae) for the infested animals were recorded and photographs were taken. All larvae were removed using forceps, and the wounds were treated topically with a broad spectrum antibiotic spray and by injection with antibiotics. The majority of larvae were preserved in 80% ethanol. Some fully grown third instar larvae from a few cases were placed in sawdust for rearing to adults in the laboratory. Identifications were made based on a stereomicroscope study of larval or adult morphology according to standard identification keys (Zumpt, 1965; Spradbery, 1991).

Results

Morocco

Six active cases of traumatic myiasis were observed in the dogs inspected in July 2002. The wounds of two other dogs, which were reported to have been previously infested, were found to be free of larvae but purulent, as noted elsewhere in wounds freed of larvae (Farkas *et al.*, 1997). All the infested dogs were males of mixed breeding and their estimated ages were between 2 and 8 years. They were unchained and allowed to roam freely around the houses and surrounding area. Five dogs had only one wound, with a diameter in the range of 3–15 cm (Figs 1 and 2). The anatomical locations of the wounds were the penis (one dog, Fig. 3), legs (two dogs) and ears (two dogs, Fig. 1). The sixth dog had a 12–15-cm diameter tumour on the ventral aspect of his neck containing many distinct foci, each completely filled by developing larvae (Fig. 2). Larvae in any focus tended to be at the same stage of development. In September 2002 three further cases of wohlfahrtiosis were recorded in dogs (one male, two of unrecorded sex), all of which had single wounds on the rear limbs (paw, one dog, Fig. 4; leg, two dogs). In both months cases were recorded in the Al-Hoceima province neighbourhoods of Taguidit (35°08′ N, 04°10′ W; four cases), Aghbal (35°10′ N, 04°04′ W; three cases) and Tafensa (35°12′ N, 04°01′ W; two cases).

The larval masses in each wound (range 4–133 larvae) were composed mainly of third instars (in seven of nine cases), but one also contained second instars and one contained only first and second instars. Third instars were always found packed tightly together in the manner of screwworm larvae (*C. hominivorax* and *Ch. bezziana*), with their anterior ends buried deeply in the tissues and only their posterior segments containing the posterior respiratory spiracles exposed (Fig. 2). All the recovered larvae and the adults reared in the laboratory were identified

Fig. 1. Severe tissue damage to the ear of a male dog caused by 80 third instars of *Wohlfahrtia magnifica*, observed near Tafensa on 8 July 2002. Scale bar = 2 cm.

as *W. magnifica*. Wohlfahrtiosis caused severe welfare problems and tissue destruction in most cases (Figs 1–4). During our surveys, only four other active cases of wohlfahrtiosis were detected in Al Hoceima province, of which three were on sheep and one on a goat.

Hungary

Four of 12 dogs (33.3%) inspected on the two farms had traumatic myiasis. At farm 1, a 5-year-old male dog of Caucasian breed, kept outside, had developing fly larvae in the external ear canal of his left ear (Fig. 5). The owner reported that the dog vigorously shook its head and scratched at its ears. The hair was wet and malodorous around the infested ear canal. At farm 2, three of nine guard and shepherd dogs kept around the buildings and livestock had active wound myiasis. Two of them were female Kuvasz, a Hungarian breed, and the third was a male of mixed breeding. They were 1.5, 2.5 and 5 years old. Each dog had only one infested wound located, respectively, on the dorsal part of the nose (Fig. 6), on the hind leg or in the vulva region (Fig. 7). The animals were all noticeably preoccupied with their lesions, often trying to lick the wounded areas. The number of larvae found in a wound ranged from 11 to 26 and, as in Morocco, they exhibited screwworm-like behaviour. Moderate bleeding was observed at the wounds. All the second and third instar larvae found in the wounds, and the adults reared from them, were identified as *W. magnifica*. Many blowflies were observed in the environment around the infested dogs, but no larvae of these flies were noted on the dogs.

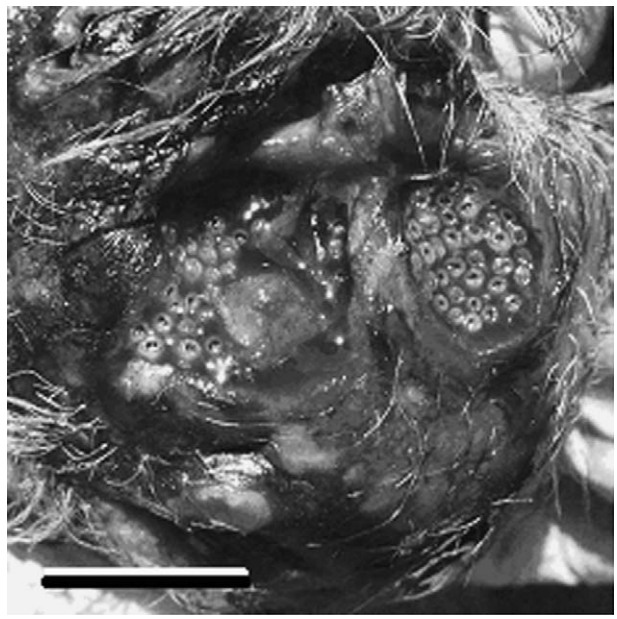

Fig. 2. Two distinct foci completely filled by developing larvae of *Wohlfahrtia magnifica* in a tumour on the ventral aspect of the neck of a male dog, observed near Aghbal on 6 July 2002. A total of 145 third instars were removed from the two foci. Scale bar = 4 cm.

Discussion

There are few reports of dogs infested by obligate traumatic myiasis-causing fly species, which, in nature, mainly infest livestock. These species require particular environmental and climatic conditions as adults, and the females usually only infest animals outdoors (Ruiz Martínez *et al.*, 1992). Therefore, stray dogs and dogs kept around livestock with obligate traumatic myiasis are probably more likely to become infested in rural than urban areas, especially if dog owners are negligent of their animals' health. Larval infestation is not always obvious to owners and, as it is impossible to inspect and treat stray animals, it is probable that many cases of infestations of dogs with larvae of obligate myiasis-causing fly species go unreported. This hypothesis is likely to be true for cases infested with the larvae of *W. magnifica*, one of the most important obligate myiasis-causing fly species in the Palaearctic zone (Hall & Farkas, 2000).

There are some published reports of wohlfahrtiosis of dogs, but these do not discuss its pathogenesis or epidemiological importance (Portschinsky, 1916; Zumpt, 1965; Şaki, 2004; Ütük, 2006). To our knowledge, these are the first cases of wohlfahrtiosis in dogs reported in Morocco and Hungary. We found wohlfahrtiosis in dogs of a range of breeds and ages, but more case data are needed to conclude the effect of these factors on its incidence. All infested dogs lived close to livestock, where wohlfahrtiosis was endemic. Infested body sites included limbs (six cases), external genitalia (two), ears (three), nose (one) and neck (one). Why were these hosts and particular sites infested?

The specific cues that attract gravid *W. magnifica* females to hosts and stimulate larviposition are still largely unknown, but odours are important (Hall *et al.*, 1995). Females can certainly

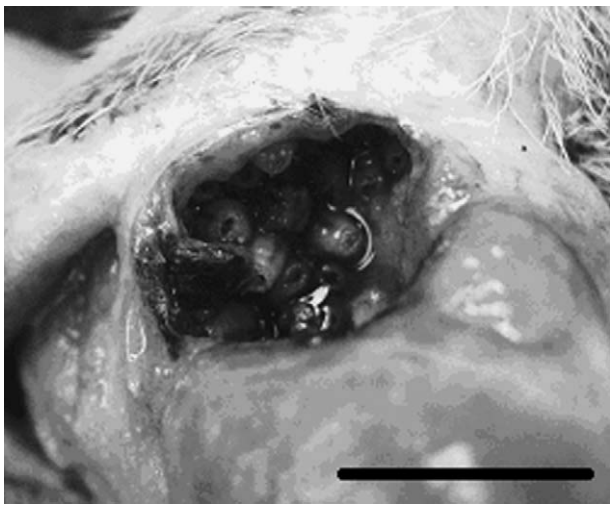

Fig. 3. Wohlfahrtiosis at the base of the penis of a male dog, observed near Aghbal on 8 July 2002. A total of 26 third instars were removed from the wound. Scale bar = 2 cm.

be attracted to sites of wounding for larviposition, as has been observed on other host species (Farkas & Képes, 2001; Farkas *et al.*, 2001). Therefore, it is likely that wohlfahrtiosis occurred mainly in male dogs as a consequence of inter-dog aggression, territorial behaviour and fights causing wounds. Over 80% of cases (nine of 11) for which host sex was recorded were in males, and all cases in Morocco were male. According to the owners of dogs in northern Morocco, each infestation appeared at the site of wounds, some of which had been caused by dog bites. Active infestations become highly attractive to gravid females (Hall *et al.*, 1995) and, therefore, multiple infestations with larvae at all stages of development frequently occur (Ruiz Martinez *et al.*, 1987; Farkas *et al.*, 1997).

It has also been reported that wounds are not a prerequisite for infestation by *W. magnifica*, the females of which can be attracted to healthy hosts without obvious predisposing conditions (Hall *et al.*, 1995). At these hosts they can infest seemingly undamaged sites, particularly natural body orifices. It has been suggested that unknown volatiles produced by microorganisms may be responsible for fly attraction and larviposition responses on healthy animals (Farkas *et al.*, 1997; Tóth *et al.*, 1998). Wohlfahrtiosis was recorded without any preliminary wounds in at least two infested dogs in Hungary. In one of these cases the untreated otitis externa may have been a predisposing factor. In the other case wohlfahrtiosis was found in the seemingly undamaged vulva of a female dog. It is hypothesized that unknown volatiles produced during changed physiological and/or pathological processes – related in one example to otitis externa and, in the other, to the breakdown of products of urine or vaginal discharges – may be responsible for the attraction of gravid females to the ear and vulva regions. The ears of dogs in Brazil were reported to be the site most infested with larvae of *C. hominivorax*, with otitis being the main predisposing condition leading to myiasis (Cramer-Ribeiro *et al.*, 2003).

Wohlfahrtiosis in dogs has been reported from Russia (Portschinsky, 1916), Turkey (Şaki, 2004; Ütük, 2006) and Israel (D. Zivotofsky, unpublished data, 2004). Compared with the numbers of *Wohlfahrtia* infestations in livestock, mainly sheep, the actual numbers of dog infestations are low. However, the number of dogs is also much lower than the numbers of sheep or other livestock in the endemic areas. Of the active cases detected in Morocco, a high proportion (nine of 13 cases, 69.2%) of

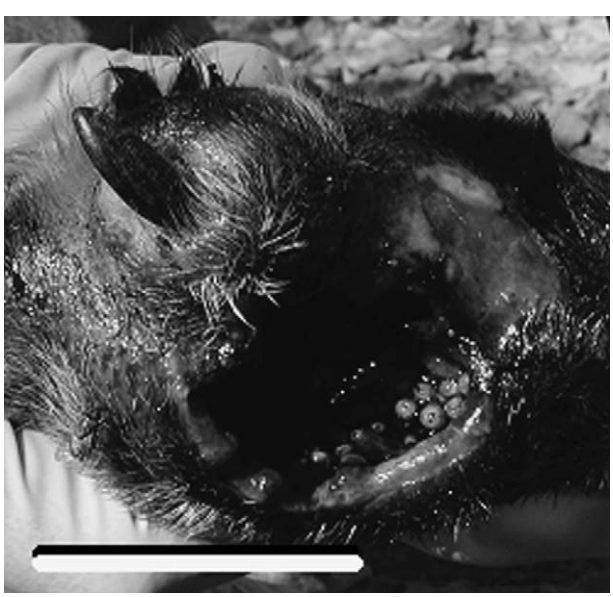

Fig. 4. Traumatic myiasis caused by *Wohlfahrtia magnifica* in the rear paw of a male dog, observed near Taguidit on 10 September 2002. A total of 133 second and, mainly, third instars were removed from the wound. During the treatment a gravid female of *W. magnifica* was captured on the wound as she prepared to larviposit. Scale bar = 5 cm.

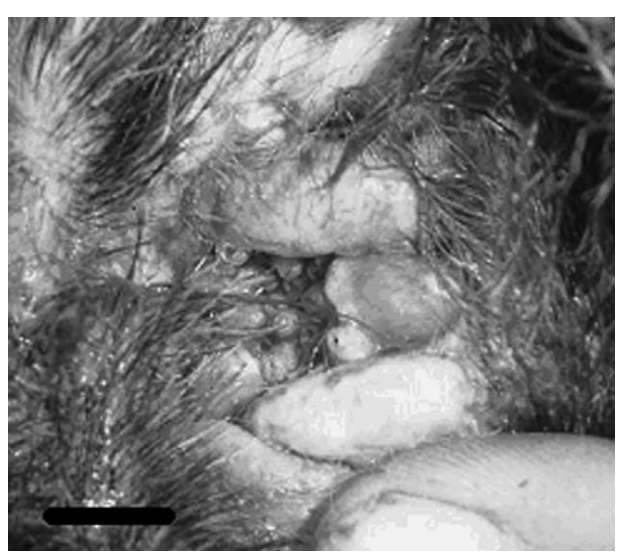

Fig. 5. Developing *Wohlfahrtia magnifica* larvae in the external left ear canal of a male dog in the neighbourhood of Üllő, observed on 8 August 2005. A total of 16 third instars were removed from the ear canal. Scale bar = 2 cm.

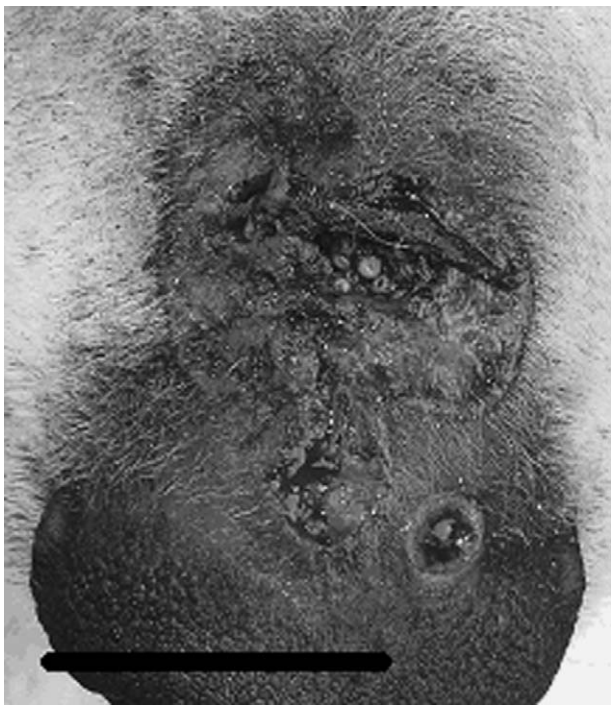

Fig. 6. Infested wound with 26 larvae (some second, but mainly third instar) of *Wohlfahrtia magnifica* located on the dorsal part of the nose of a female dog, observed near Kiskunhalas on 23 August 2005. Scale bar = 3 cm.

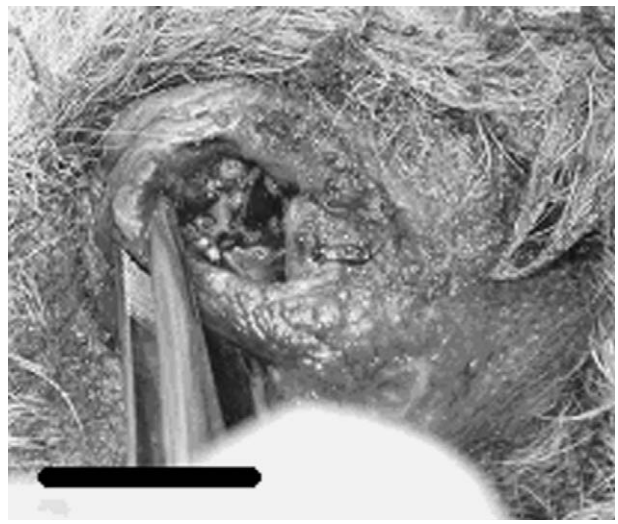

Fig. 7. Wohlfahrtiosis in the vulva region of a female dog, observed near Kiskunhalas on 23 August 2005. A total of 11 mainly second instars were removed from the wound. Scale bar = 2 cm.

infestations were found in dogs, which is especially high when numbers of dogs and livestock are compared.

Many farm dogs in both countries surveyed go untreated, either because the owners do not realize they are infested or cannot afford to treat the dogs, or because they are negligent. Although the numbers of cases reported here was small, wohlfahrtiosis in dogs may be very important from an epidemiological perspective because farm dogs and strays can act as both reservoirs and carriers of this parasitic fly species. When left untreated, wohlfahrtiosis infestations can produce mature larvae within 5 days (Hall & Farkas, 2000), which leave the wound, develop to adults and then pose a threat to local livestock and humans. Infested stray dogs are never treated and are a particular problem when they exist in large numbers, such as in Morocco, where a conservative estimate of more than 200 000 strays has been reported (Anon., 2000). Reservoir infestations in untreated dogs have been recognized as a problem in other myiasis situations. For example, in a collection of primarily New World screwworm myiasis cases in the urban Kingston area of Jamaica, dogs were found to be the most common hosts (Rawlins & Chen Sang, 1984). This was confirmed by survey during the subsequent campaign to eradicate screwworm on Jamaica using the sterile insect technique; of screwworm cases reported during 1999–2002, 94.6% of those in the Kingston–St. Andrew parish and 10–70% of those in other parishes originated from dogs (M. Vreysen, unpublished report to the International Atomic Energy Agency, 2002). One of the important complicating factors in this campaign was reported to be the urban 'hot spots' of incidence in unmanaged and stray dogs (Robinson *et al.*, 2000). It was concluded that the success of the programme required increased emphasis on the treatment of such hot spots (Robinson *et al.*, 2000). Dogs also have the potential to be vectors in the spread of wohlfahrtiosis if this disease is not taken into account when dogs are moved across international boundaries for leisure, sport, breeding and other purposes. There have been a number of cases of similar myiasis-causing fly species moving between continents in infested dogs (Anon., 1987; Chermette *et al.*, 1989; Fox *et al.*, 1992; Roosje *et al.*, 1992; Dongus *et al.*, 1996). The severity of risk for the establishment of this obligate myiasis-causing fly species depends on both location and the season of introduction.

Based on our findings, we conclude that wohlfahrtiosis in livestock cannot be completely controlled without the frequent inspection and treatment of local dogs. Preventing the spread of this myiasis-causing species is of great importance in veterinary medicine. Therefore, we recommend that dog owners are educated concerning the risk factors in endemic regions in order to reduce the prevalence of wohlfahrtiosis in dogs and, thereby, in livestock. Both owners and veterinarians should pay regular attention to any wounds and to the natural orifices of dogs, especially during the fly seasons. The role of dogs in the epidemiology of wohlfahrtiosis merits further attention, especially in countries with large populations of stray dogs.

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Conflicts of interest

All authors declare no conflicts of interests.

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Molecular characterization of esterase E3 gene associated with organophosphorus insecticide resistance in the New World screwworm fly, *Cochliomyia hominivorax*

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Abstract. The New World screwworm, *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae), is one of the most important myiasis-causing flies in South America. It is responsible for severe economic losses to livestock producers, mainly because it causes mortality in newborn calves and reductions in the quality of leather and in the production of milk and meat. The economic losses caused by myiasis, along with those caused by other internal and external parasites, are the main factors limiting meat production. In Brazil, *C. hominivorax* has been controlled by applying insecticides, particularly organophosphate (OP)-based compounds. However, the improper and continuous use of these chemicals can lead to the selection of OP-resistant strains. This, associated with the fast development of OP resistance in other myiasis-causing flies, shows the importance of investigating resistance in *C. hominivorax.* Based on the findings of previous studies, the objective of the current work was to isolate and sequence the E3 gene in *C. hominivorax.* Mutations at the positions (Gly137 and Trp251) responsible for conferring OP resistance in *Lucilia cuprina* and *Musca domestica* L. (Muscidae) were identified in *C. hominivorax*. In addition, the orthologous region in *C. hominivorax* contained motifs that are highly conserved among carboxyl/cholinesterases and contribute to the catalytic mechanism of the active site. The characterization of this gene in natural populations of New World screwworm can be an important tool for monitoring resistance to insecticides throughout its current geographic distribution. This will provide information for the selection and implementation of more effective pest management programmes.

Key words. Esterase, insecticide resistance, myiasis, organophosphorus, screwworm.

Introduction

Brazil has the largest commercial herd of cattle in the world, numbering approximately 200 million in total (Instituto Brasileiro de Geografia e Estatística, 2005). High levels of meat production require efficient sanitary control of animals in order to reduce costs and increase meat quality. The economic losses caused by internal and external parasites, such as myiasis-causing flies, are the main factors limiting meat production. The New World screwworm (NWS), *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae), is one of the most important myiasis-causing flies in the Neotropics (Hall & Wall, 1995). The female flies lay eggs on fresh wounds and the larvae penetrate the host tissues and create an open, growing lesion (Zumpt, 1965). This occurrence is responsible for severe economic losses to livestock producers, especially through mortality in newborn calves,

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and reductions in the quality of leather and in milk and meat production.

Eradication of NWS in North America and almost all of Central America has been achieved by using an area-wide integrated approach, including the release of sterile insects (Wyss, 2000). The current distribution of *C. hominivorax* covers part of the Caribbean region and South America, except Chile. In these countries, *C. hominivorax* has been controlled for several decades almost exclusively by insecticides, particularly organophosphate (OP)-based compounds, whose target site is the enzyme acetylcholinesterase. This, associated with the fast development of OP resistance in other myiasis-causing flies (Levot, 1995), shows the importance of investigating genetic resistance in *C. hominivorax.*

Insecticide resistance is assumed to be a pre-adaptive phenomenon because, prior to insecticide exposure, rare individuals that carry an altered genome already exist. This genome predisposes them to the development of different resistance mechanisms and allows them to survive the evolutionary selection pressure of insecticides (World Health Organization, 1957). Thus, resistance of insects to insecticides is an ongoing challenge to pest management, but, at the same time, represents an ideal context in which to study the process of microevolution and environmental adaptation (Li *et al.*, 2007). The major mechanisms of resistance described so far involve either metabolic detoxification of the insecticide before it reaches its target site, or changes in the sensitivity of target sites that abolish the insect's susceptibility to the insecticide. Alone, or in combination, these mechanisms confer resistance to all available classes of insecticide (Hemingway *et al.*, 2004). The most common metabolic resistance mechanisms involve esterases, glutathione S-transferases or monooxygenases.

Esterases have been implicated in both target sites and metabolic resistance to OP insecticides in 49 pest insects (revised in Oakeshott *et al.*, 2005). Several cases of metabolic resistance in the Hemiptera (aphids and whiteflies) and lower Diptera (mosquitoes) involve sequestration, in which an esterase that binds the pesticide with high affinity is over-expressed (in many cases because of gene amplification) to a degree that allows effective sequestration of the toxin. In higher Diptera (blowflies and houseflies), metabolic resistance has been associated with a structural change in the esterase that confers detoxification activity that allows the enzyme to degrade the pesticide.

Structural changes in esterase 3 (E3) have been characterized in some OP-resistant strains of two Calliphoridae, *Lucilia cuprina* (Newcomb *et al.*, 1997a) and *Lucilia sericata* Meigen (Hartley *et al.*, 2006), and one Muscidae, *Musca domestica* L. (Claudianos *et al.*, 1999). Decreased carboxylesterase activity has been observed in resistant individuals. These findings have been explained by a mutant ali-esterase hypothesis in which a structural mutation in a carboxylesterase results in a reduced ability to hydrolyze carboxylesterase substrates, but an acquired ability to hydrolyze OP substrates (Claudianos *et al.*, 1999).

Recently, there have been two single nucleotide changes reported in *C. hominivorax* at the same positions (Gly137 and Trp251) in the E3 gene (*Ch*α*E7*) that confer the two forms of OP resistance in *L. cuprina* and *M. domestica* (Carvalho *et al.*, 2006). A fragment that included the intron III (63 bp) and the exon region, corresponding to positions 359–831 in the *L. cuprina* α*E7* nucleotide sequence, as well both amino acid positions (137 and 251) related to OP resistance, was amplified and sequenced in *C. hominivorax*.

Based on previous studies, the objective of the current study was to isolate and sequence the entire coding region of the E3 gene in *C. hominivorax*, which is orthologous to that previously described for some Diptera species. The sequencing of the E3 gene in *C. hominivorax* allowed the characterization of mutants and wild alleles in this species. In addition, cDNA isolation will allow biochemical assays to be carried out in order to establish the associations of some mutations with insecticide resistance. The identification of resistant individuals in natural populations can be an important tool for surveying resistance to insecticides throughout a current geographic distribution. This will provide information for the selection and implementation of more effective pest management programmes.

Materials and methods

Samples

Larvae of *C. hominivorax* were obtained from the wounds of infested cattle in Caiapônia, Goiás, Brazil, in 2005. Collected larvae were reared to adults in the laboratory. Total RNA was extracted from larvae and adults using Trizol (Invitrogen, Inc., Carlsbad, CA, U.S.A.), according to the manufacturer's instructions. The cDNA was synthesized using the ImProm-II™ Reverse Transcription System Kit (Promega Corp., Madison, WI, U.S.A.), according to the manufacturer's instructions.

cDNA amplification

The total coding region of E3 was amplified using six primers (Fig. 1). Two of them had been previously described for *L. cuprina* (Newcomb *et al.*, 1997a): 7F1a (5′ – AGC TAA ATC CCG AAA CTA AAC – 3′) and 7R4 (5′ – CTG TKG ARC CNT ATC AGA C – 3′). The primers 7R3a (5′ – ATC CTT ATC ATT ATT TTC ACC C – 3′) and 7F4a (5′ – CTG TKG ARC CNT ATC AGA C – 3′) were designed from sequenced fragments amplified by 7F1a/7R4 in *C. hominivorax* (Carvalho *et al.*, 2006). The primers E3F (5′ – ATG AAT TTC AAI GTY AGY YWI ITG GAG – 3′) and 7R7 (5′ – TTG GTT ACA CTC TAA AAT AAA TC – 3′) were designed based on E3 nucleotide sequence alignment of *Lc*α*E7* of *L. cuprina*, *Md*α*E7* of *M. domestica*, *Dm*α*E7* of *Drosophila melanogaster* Meigen and *Hi*α*E7* of *Haematobia irritans* L. obtained from GenBank. The amplification conditions were standardized and the polymerase chain reaction (PCR) procedures were carried out in a Perkin-Elmer 9600 Thermal Cycler (Perkin Elmer, Inc., Waltham, MA, U.S.A.). The 15-μL PCR mix contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1 unit of *Taq* polymerase (Invitrogen, Inc.), 200 μM of each dNTP, 1.8 mM $MgCl_2$, 1 μM of each primer and 10–15 ηg of DNA. After an initial denaturing step of 3 min at 96 °C, 35 cycles were performed,

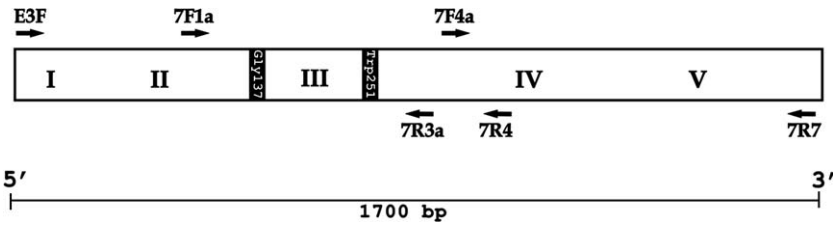

Fig. 1. Schematic view of cDNA from *Lucilia cuprina* (Newcomb *et al.*, 1997a), showing the mutation positions (Gly137 and Trp251) associated with organophosphate resistance. The roman algorisms represent the positions of introns.

each including 1 min at 95 °C, 1 min at 50 °C (or 52 °C, depending on the fragment) and 2 min at 72 °C, with a final step of 10 min at 72 °C to fully extend all amplicons.

Sequencing

Purified PCR products were cloned into pGEM-T plasmid vector (Promega Corp.). These vectors were used to transform *Escherichia coli* cells, which were plated on LB media with ampicillin (50 μg/mL) and X-Gal (0.8 mg in each plate). Plasmid DNA was isolated (Sambrook *et al.*, 1989) and the clones were sequenced using M13 direct and reverse primers. The reactions were run on an ABI 3.700 automated DNA sequencer (Applied Biosystems, Inc., Foster City, CA, U.S.A.). The nucleotide sequences of *C. hominivorax* were submitted to BLASTN (Altschul *et al.*, 1997) to search for similarities, and sequence alignments were performed using ClustalX (Thompson *et al.*, 1997). The predicted amino acid sequences were obtained using BioEdit (Hall, 1999).

Results

Amplification

The 7F1a/7R4 primer pair amplified a fragment of approximately 700 bp, the sequence of which was used to design the *C. hominivorax*-specific primers 7F4a and 7R3a. Furthermore, the 7F1a/7R3a primer pair was used for the PCR-restricted fragment length polymorphism (RFLP) technique in order to identify mutant individuals in natural populations of *C. hominivorax* (Carvalho *et al.*, 2006). The characterization of entire coding regions of the E3 gene in *C. hominivorax* was carried out in two steps. First, the E3F/7R3a primer pair amplified a fragment with approximately 840 bp, as expected from *L. cuprina* and *M. domestica* sequences (Newcomb *et al.*, 1997a; Claudianos *et al.*, 1999), corresponding to the 5′ portion of E3 cDNA in *C. hominivorax.* This region includes both site mutations (Gly137 and Trp251) possibly related to OP resistance in *C. hominivorax*.

The second step of the characterization, amplification of the 3′ portion, employed a semi-nested PCR technique. Because the amplification reactions using the primer pairs 7F1a/7R7 and 7F4a/7R7 did not amplify any cDNA fragment that was visible in agarose gel, products from the reaction using 7F1a/7R7 were used as a template for further amplification with the 7F4a/7R7 primer pair. This reaction amplified a fragment with approximately 750 bp, as expected. Therefore, the entire coding region of the E3 gene was amplified, allowing for further sequencing.

Sequence analysis

The amplification reaction of the 5′ portion of E3 cDNA in *C. hominivorax* resulted in a fragment with 831 bp, corresponding to the nucleotide positions 1–831 reported for the orthologous *L. cuprina* gene. The esterase E3 genes characterized in *L. cuprina* and *M. domestica* contain three introns inserted into the genomic DNA of this sequence. However, only intron III has been sequenced in *C. hominivorax* (Carvalho *et al.*, 2006).

Using semi-nested PCR, a 781-bp fragment was amplified (3′ portion), corresponding to positions 940–1721 of the *L. cuprina* nucleotide sequence (Newcomb *et al.*, 1997a). This region is predicted to contain the introns IV and V. The entire coding region of the E3 gene in *C. hominivorax* contains 1713 nucleotides. The cDNA consensus sequence from *C. hominivorax* showed 83% similarity with that from *L. cuprina*. The predicted amino sequence of the E3 gene showed 87.5% similarity with that from *L. cuprina* (U56636), 75.6% with *M. domestica* (AF133341), 73.2% with *H. irritans* (AF139082) and 63.7% with *D. melanogaster* (NM079537). The high identity scores suggest that *Chα E7* is orthologous to the E3 genes from these species.

Discussion

Amino acid substitutions in the active site of the protein have a higher probability of changing its function than changes elsewhere. Primary sequence comparisons between esterase E3 from *C. hominivorax* and acetylcholinesterase (AChE) from *D. melanogaster* (Harel *et al.*, 2000), the crystal structure of which has been elucidated, suggest that secondary and tertiary structural characteristics are well conserved. This conservation of active sites was confirmed by Harel *et al.* (2000), who compared the 3-dimensional structures of vertebrate AChEs and *D. melanogaster* AChE and showed that these 3-D structures are folded similarly and their active sites closely overlap.

The codon GGG at position 409–411 of the wild alleles of the *C. hominivorax* α7 nucleotide sequences translate to glycine. Mutation of this site in some alleles showed the substitution Gly137/Asp in the predicted protein sequence, which is associated with OP hydrolase activity in *L. cuprina* and *M. domestica* (Newcomb *et al.*, 1997b; Claudianos *et al.*, 1999).

In addition, as in *M. domestica*, some alleles that contained Gly137 showed another substitution, Trp251/Ser (data not shown), which is associated mainly with dimethyl OP hydrolase. Dimethyl OP-resistant strains of *L. cuprina* demonstrated the Trp251/Leu substitution (Campbell *et al.*, 1998). These findings are in accordance with those of Heidari *et al.* (2004), who used site-directed mutagenesis at position 251 and obtained synthetic E3 variants with improved OP hydrolase activity. However, no synthetic enzymes were found to have higher diethyl OP turnover rates than the Gly137/Asp enzyme.

The Gly137/Asp substitution in E3 changes the structure of the catalytic centre and converts a carboxylesterase to an OP hydrolase, conferring insecticide resistance. The Asp137 enzyme is more active on diethyl OPs, which is the main OP insecticide used for *C. hominivorax* control. The replacement of Trp251 by a smaller residue, such as Gly, Ser or Leu, confers a lower level of OP hydrolase activity, but has a much smaller effect on carboxylesterase activity (Newcomb *et al.*, 2005). This second mutation has greater OP hydrolase activity for dimethyl OPs and may be the basis of cross-resistance between OPs and pyrethroids (Heidari *et al.*, 2005).

The finding that resistance is conferred by replacement of the same amino acids in different species not only illustrates parallel evolution, but raises questions as to whether a mutation occurs once and then spreads, or whether the same mutation occurs in different geographic locations (Ffrench-Constant, 2007). These questions are not only of academic interest; their answers will also influence strategies for the management of insecticide resistance. The data for these different species constitute evidence for biochemically precise convergent evolution and suggest that the options for evolving esterase-based metabolic resistance to OPs are tightly constrained (Hartley *et al.*, 2006).

The cDNA of *Ch*α*E7* gene encodes a 570-amino acid protein, which contains candidates for all residues required to assemble motifs that are highly conserved among carboxyl/cholinesterases and that contribute to the catalytic mechanism of the active site (Cygler *et al.*, 1993). These include the 'nucleophilic elbow' (VFGESAG, residues 214–220), 'acid turn' (SYEG, residues 349–352) and 'histidine loop' (GVSHADELT, residues 468–476). These, respectively, contain the residues of the catalytic triad (S218, E351 and H471), which protrude into the active site gorge (Claudianos *et al.*, 1999). The 24 residues that Cygler *et al.* (1993) found to be invariant across 29 carboxyl/cholinesterases are conserved in *C. hominivorax* (Fig. 2). In addition, the predicted E3 protein of *C. hominivorax* is not thought to have a signal peptide. It has been suggested that, in the Diptera at least, it may be important for an OP-hydrolyzing enzyme to be distributed in the cytoplasm or a cellular organelle to confer metabolic resistance (Newcomb *et al.*, 1997a).

One strategy to delay or minimize resistance involves alternating use of different compounds. This approach is based on the assumption that frequencies of resistance to each compound will decline fairly rapidly in the absence of the compound (Georghiou, 1983), either because resistance is diluted by the immigration of susceptible individuals or as a result of natural selection against carriers of resistant alleles, or both. However, unless migration is unidirectional, which is unlikely, a decrease in resistance would have to depend on natural selection against the genotypes with resistant alleles (Roush & Mckenzie, 1987). Thus, it is important to determine whether resistant individuals of *C. hominivorax* present selective disadvantages that are sufficiently large to be useful in practical situations.

For *L. cuprina*, it is clear that the malathion-resistant mutations allow the enzyme to retain much of the carboxylesterase activity seen in susceptible forms, whereas the diazinon-resistant mutations abolish it (Heidari *et al.*, 2004). Furthermore, there is a clear fitness cost for the diazinon-resistant mutation in the absence of the insecticide, which is expressed in the form of developmental instability, but no such cost is documented for malathion-resistant mutations at position 251 (Batterham *et al.*, 1996).

This approach to investigating insecticide resistance in *C. hominivorax* has shown the substitution Gly137/Asp in some alleles, which is related mainly to resistance to diethyl OPs. This class of insecticides has been used widely for *C. hominivorax* control in several countries, including Brazil. The second mutation found in *C. hominivorax* (Trp251/Ser) is associated with resistance to dimethyl OPs, which is also used in insecticide formulations. However, this mutation can also confer pyrethroid hydrolase activity and may be the molecular basis of esterase-based cross-resistance between malathion (a dimethyl OP) and pyrethroids found in some species (Heidari *et al.*, 2005). Although pyrethroids have not yet been widely used to control *C. hominivorax*, it may be that the species will show a level of pre-adaptation to them on account of this mutation at site 251, as observed in *L. cuprina* by Hartley *et al.* (2006).

The recent advances in genome technology may contribute to the development of new antiparasitic drugs, using a more empirical approach than was previously possible (Gilleard *et al.*, 2005). Thus, the characterization of mutations in detoxifying enzymes like E3, responsible for conferring OP resistance, can yield information crucial to producing improved versions of existing insecticides used for *C. hominivorax* control. Irrespective of their structures, modes of action or methods of application, it is virtually certain that the chemical pesticides will select for resistance in target site populations and that such resistance will, at least in some cases, involve metabolic enzyme systems (Li *et al.*, 2007). A continuing challenge is to use what is known of molecular mechanisms of metabolic resistance in the design and implementation of environmentally sustainable management programmes to control NWS in its current distribution.

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Conflicts of interest

All authors declare no conflicts of interests.

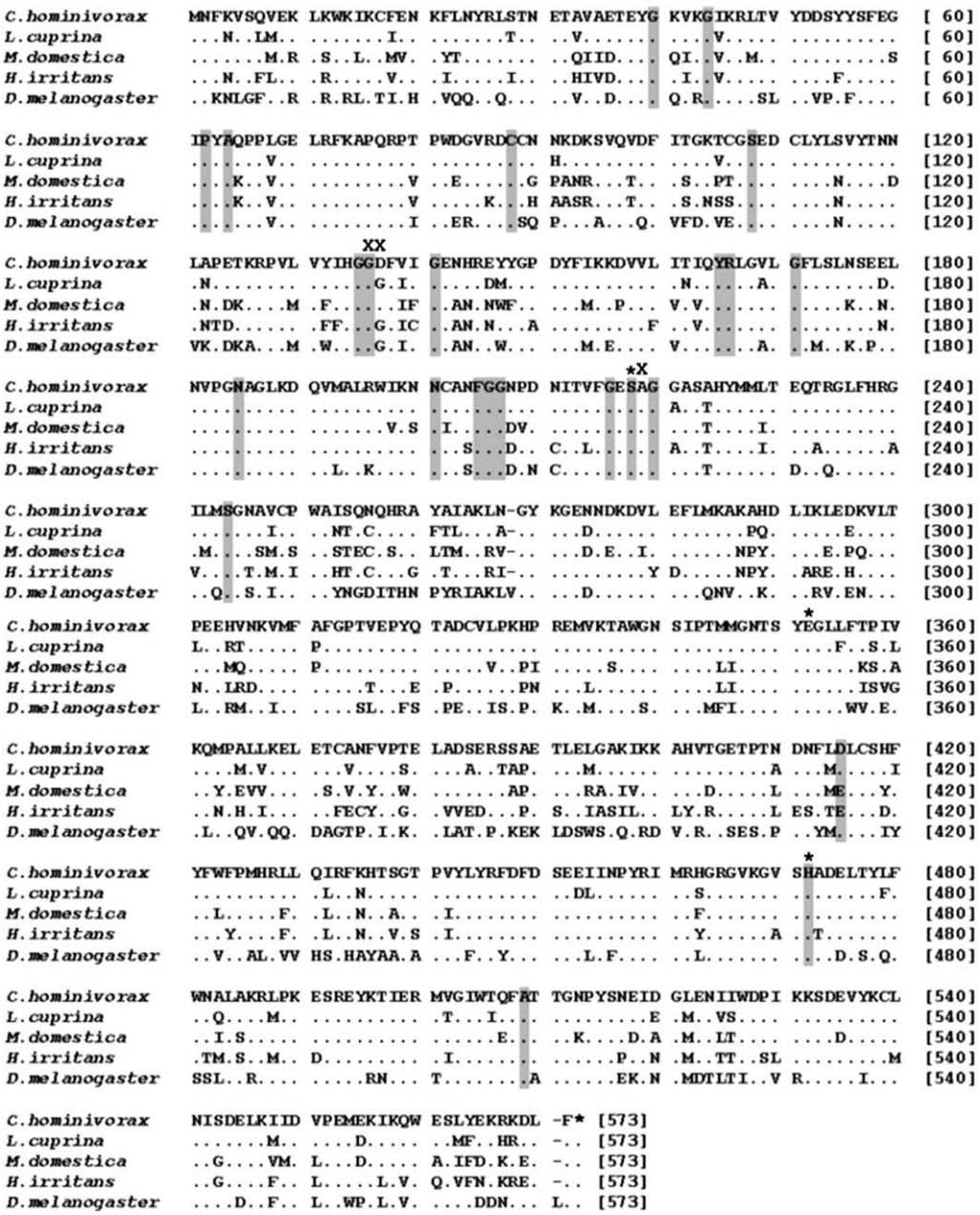

Fig. 2. Alignment of inferred amino acid sequences of *ChαE7* with three esterases involved in organophosphate resistance in *Lucilia cuprina* (U56636), *Musca domestica* (AF133341) and *Haematobia irritans* (AF139082), and an esterase from *Drosophila melanogaster* (NM079537). The conserved residues that Cygler *et al.* (1993) described for 29 carboxyl/cholinesterases are shaded. Residues predicted to form the catalytic triad are indicated with asterisk (*) and those contributing to the oxyanion hole are marked with a cross (X) below the sequence.

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Photographic map of the polytene chromosomes of *Cochliomyia hominivorax*

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Abstract. *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae) is one of the most important myiasis-causing flies and is responsible for severe economic losses to the livestock industry throughout the Neotropical region. A polytene chromosome map is an invaluable tool for the genetic analysis and manipulation of any species because it allows the integration of physical and genetic maps. *Cochliomyia hominivorax* has a diploid number of 12 chromosomes ($2n = 12$): five pairs of autosomes and one pair of sex chromosomes (XX/XY), which do not polytenize. We created a new photomap of the polytene chromosomes of *C. hominivorax* describing its five autosomes (chromosomes 2–6). Pupal trichogen cells, which have chromosomes with a high degree of polytenization, were used to elaborate this map. The photomap was made by comparing 20 different nuclei and choosing, for each chromosome segment, the region with the highest resolution. Thus, we present a new photomap of the five autosomes of this species, with a total resolution of 1450 bands.

Key words. *Cochliomyia hominivorax*, photomap, polytene chromosomes.

Introduction

A cytogenetic map is an invaluable tool for the genetic analysis and manipulation of any species, as it allows the integration of physical and genetic maps. The polytene chromosomes of Diptera have proved to be an especially favourable material because they allow for the identification of hundreds of bands in a single chromosome. Painter (1933) was the first to use them for preparing maps of *Drosophila* chromosomes using the squashing technique. In 1935, Bridges set up a system to assemble polytene chromosome maps. He divided the five main chromosome arms of *Drosophila melanogaster* into 100 divisions and two extra divisions for the small dot fourth chromosome. After successive revisions, he was able to distinguish a total of 2650 bands (e.g. Bridges, 1935, 1942; Zhimulev, 1996).

Polytene chromosome maps may be used to identify with precision chromosome rearrangements in order to differentiate geographical races and species, detect the action of natural selection and make phylogenetic inferences (Sperlich & Pfriem, 1986; Anderson *et al.*, 1991; Krimbas & Powell, 1992). They can also be used to carry out fluorescence *in situ* hybridization (FISH), to determine the exact location of nuclear genes and allow them to be cloned (Saunders *et al.*, 1989; Grushko *et al.*, 2004; Mahjoubi *et al.*, 2006).

The New World screwworm fly, *Cochliomyia hominivorax*, and the human bot fly, *Dermatobia hominis* (Linnaeus in Pallas), are considered the most important agents of traumatic myiasis in the Neotropics (Zumpt, 1965; Guimarães *et al.*, 1983). Larvae of *C. hominivorax* are obligate ectoparasites that can infest almost all warm-blooded vertebrates, particularly livestock. Because of the substantial economic losses caused by this species and its influence on the trade of live animals among infested and non-infested countries, an international effort has been devoted to its eradication from endemic areas of Central America (Wyss, 2000), as well as to preventing invasions into screwworm-free areas (Lindquist *et al.*, 1992). The identification of target populations and an understanding of their genetic variability can improve the effectiveness of eradication programmes.

A number of studies on the nuclear and mitochondrial genome of *C. hominivorax* have been carried out (Lessinger *et al.*, 2000;

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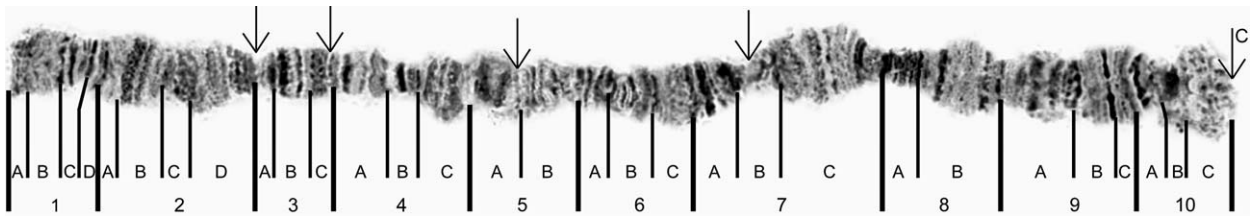

Fig.1. Photomap of chromosome 2L. Arrows show the weak spots. Arrow C points to the centromere.

Torres *et al.*, 2004; Lyra *et al.*, 2005; for a detailed review see: Azeredo-Espin & Lessinger, 2006). Although a preliminary photomap of the polytene chromosomes of this species was published (Dev *et al.*, 1985, 1986) with encouraging results, a photomap of higher resolution is required.

Materials and methods

Samples of *C. hominivorax* were collected primarily from Brazil and Argentina, where this species is highly abundant. Additional specimens were obtained from Venezuela and Uruguay and colonies were established in the laboratory. Because the salivary gland polytene chromosomes of *C. hominivorax* have a low degree of polyteny, the cytological preparations were made from trichogen cells from the scutellum of the pupae.

Methods were adapted from Dev *et al.* (1985) and Gariou-Papalexiou *et al.* (2002). Puparium were removed from 5-day-old pupae reared at 24 °C, and were fixed overnight at 4 °C in fresh Carnoy's fixative. To prepare the slides, the abdomen and first cuticular external layer of each pupa were removed in absolute ethanol. Then, the scutellum was dissected and transferred to a drop of acetic acid (60%) on a coverslip. The scutellum tissue was carefully separated from the second chitinous layer. A drop of lacto-acetic orcein stain was put on the material immediately and left for 5–10 min. A slide was then slowly placed on top of the coverslip, turned over and gently tapped to spread the chromosomes. The preparation was squashed under an absorbent paper and sealed with nail polish.

The slides were photographed with Cool SNAP-Pro (Colour) using a Nikon Eclipse E800 microscope (1000×). They were then digitized using the software Image Pro-Plus Version 4.1 and finally edited in Corel Photopaint Version 9 and Adobe Photoshop Version 7.0.1. The photomap of each chromosome was made by comparing at least 20 different nuclei. For each chromosome segment, the region which presented the highest degree of resolution was chosen to make the map.

Results

Cochliomyia hominivorax has a diploid number of 12 chromosomes ($2n = 12$): five pairs of autosomes and a pair of sex chromosomes (XX/XY), which do not polytenize (Dev *et al.*, 1985, 1986). Thus, a photomap of the polytene chromosomes of *C. hominivorax* must describe its five autosomes (chromosomes 2–6).

The polytene chromosomes of *C. hominivorax* are frequently found fragmented because of the presence of weak spots, as well as the centromere; these can be consistently mapped. The second chromosome is the longest and is metacentric, with seven weak spots. Its two arms, 2L and 2R, show 192 and 155 bands, respectively. The third chromosome is the second longest metacentric chromosome; its arms show 151 (3L) and 157 (3R) bands. It has few weak spots and large unfragmented pieces are frequently observed. The fourth chromosome shows the smallest number of weak spots – only two were observed and it can be seen intact. The left and right arms show 137 and 139 bands, respectively. Although the fifth chromosome is small, it shows eight weak spots. The left arm shows 138 bands and the right 134. The sixth chromosome is submetacentric and its left and right arms show 174 and 73 bands, respectively. It has five weak spots.

Each arm of the chromosomes has a characteristic band sequence and particular landmarks which allow them to be differentiated and identified. These landmarks are: distal and proximal endings; puffs, and weak spots. The divisions we used for this map are the same as those used in the previous map (Dev *et al.*, 1986).

Chromosome descriptions

Chromosome 2L (Fig. 1). The main landmark of this arm is its proximal region. This region begins with puff 8A followed immediately by two other puffs (8B and 9A), with a very thick, darkly stained band at the end. Two other characteristic regions

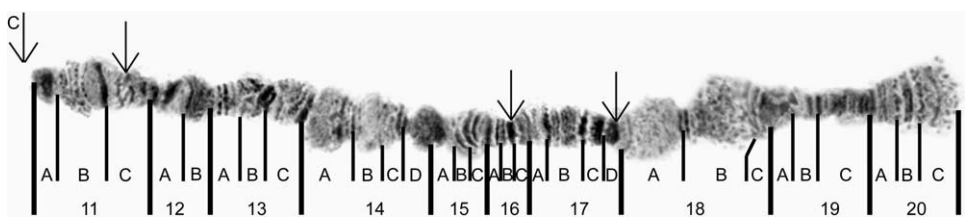

Fig.2. Photomap of chromosome 2R. Arrows show the weak spots. Arrow C points to the centromere.

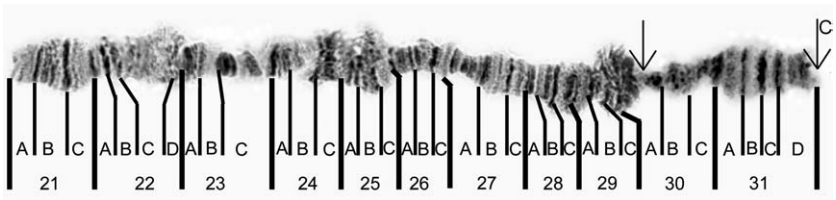

Fig. 3. Photomap of chromosome 3L. Arrows show the weak spots. Arrow C points to the centromere.

are: puff 4C, with a dark thick band, and division 10, with a rectangular big band and a crown of uncondensed chromatin (the centromeric region). There are four weak spots in this chromosome arm located at 2B–C, 3C, 4C and 7C.

Chromosome 2R (Fig. 2). The proximal region begins with a dark squared band, which is followed by an easily recognizable sequence of bands. Division 14 is a good landmark for this arm. There are two puffs in division 18 that are also very evident. Finally, the distal region is marked by a thick band in 19 and a series of bands in 20, which culminate in a larger, rectangular puff with a free end (20C). The three weak spots of this arm are in divisions 11C, 16B and 17D.

Chromosome 3L (Fig. 3). This arm is characterized by three particular regions: the distal region (21), a pear-shaped puff with three bands; puff 29C next to 30A, the only breakpoint on this arm, and a large puff in the proximal region with five bands (31A–31D). It is common to find fragments of this arm, including stretches from 21 up to 29C.

Chromosome 3R (Fig. 4). The proximal region of this arm has a series of puffs, two in division 32, one in 33 and one more in 34. They are split by a constriction in division 32D, immediately after four clear bands. Although it is rich in bands, this region is frequently observed with its structure uncondensed and disorganized. Two other segments can also be used as landmarks: puff 36 and the distal region (38, 39 and 40), which are both very rich in bands. There are two weak spots: in divisions 34B and 36C.

Chromosome 4L (Fig. 5). This arm is easily identified by the following landmarks: the distal region (41A–C), which shows evident bands, especially those from division 41C; the puff at 42B, just after the distal region, and the breakpoint located at 43C–D, which is frequently observed. The puffs located in division 46 and between 47C and 48C represent other useful landmarks. The second breakpoint is located in division 49C, but in many preparations whole stretches ranging from 43C to the centromere can be seen.

Chromosome 4R (Fig. 6). This arm is peculiar in that it does not have a breakpoint and thus is observed in one piece in most preparations. The main landmark, the proximal region, is made up of two similar puffs (52 and 53) split by a constricted region. Another landmark is the puff in division 56, which shows as a bright region between two darkly stained bands. There is also the distal region, division 60, which shows an oval puff with four evident bands in which the telomere dot is always present.

Chromosome 5L (Fig. 7). This chromosome arm can be identified by two landmarks: the distal region of division 61, which shows a very characteristic fan-like structure with clear bands, and the puff that begins in division 68C and runs to the centromeric division, 70C, where there are two dark bands with crowns of expanded chromatin. The latter is, perhaps, the best landmark of the chromosome. It has three weak spots (63C, 64C and 65C).

Chromosome 5R (Fig. 8). This chromosome arm shows a large number of weak spots, five in total, located in divisions 73A, 74A, 75A, 76B and 78C. However, the arm still presents a distinctive structure that allows its identification. Its main landmark is the proximal region, with two puffs, the first smaller and darker (71), and the second a bright region between thick dark bands (72). Another useful landmark is a large puff, rich in bands, that ranges from division 76C up to 78A. The distal region (80) is also a good landmark thanks to the tip, division 80C (a thick, dark band topped with a degranulated, clear, fan-like area), which follows a well-banded region (division 79).

Chromosome 6L (Fig. 9). This is the longest chromosome arm and it shows five weak spots (83A, 85C, 86C, 90C and 92A). Its main landmark is the distal region (81, 82 and 83), which has four puffs followed by characteristic bands. Other landmarks are: the puffs in divisions 86 and 87, which are very similar but are split by a constriction (87A); a large puff – which may appear unpaired in some preparations – between divisions 89A and 90C, and, finally, the proximal region of this

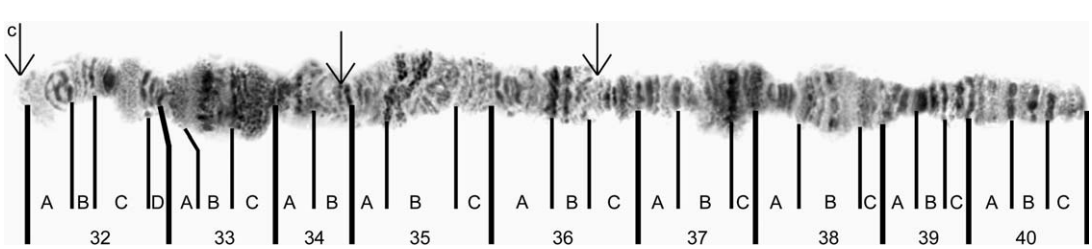

Fig. 4. Photomap of chromosome 3R. Arrows show the weak spots. Arrow C points to the centromere.

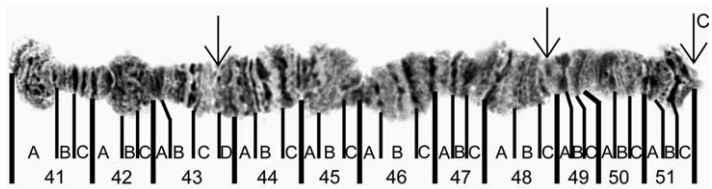

Fig. 5. Photomap of chromosome 4L. Arrows show the weak spots. Arrow C points to the centromere.

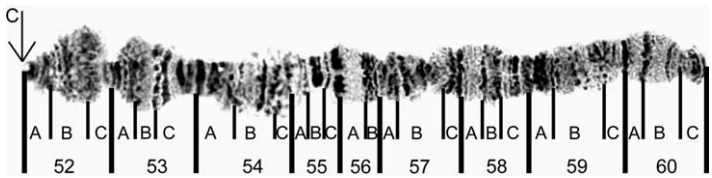

Fig. 6. Photomap of chromosome 4R. Arrows show the weak spots. Arrow C points to the centromere.

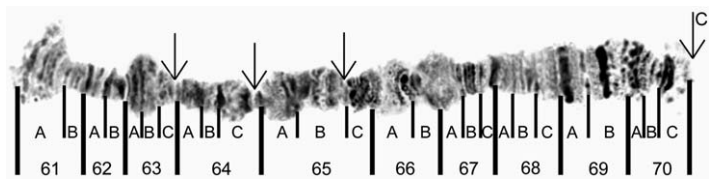

Fig. 7. Photomap of chromosome 5L. Arrows show the weak spots. Arrow C points to the centromere.

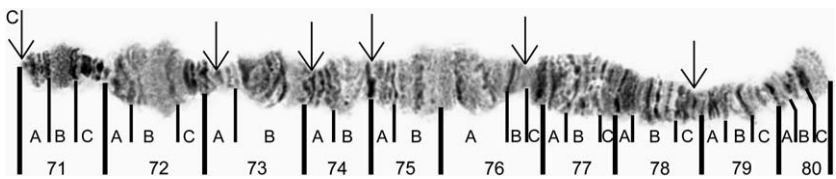

Fig. 8. Photomap of chromosome 5R. Arrows show the weak spots. Arrow C points to the centromere.

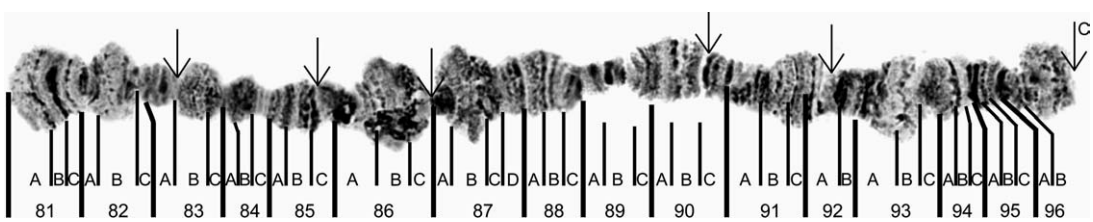

Fig. 9. Photomap of chromosome 6L. Arrows show the weak spots. Arrow C points to the centromere.

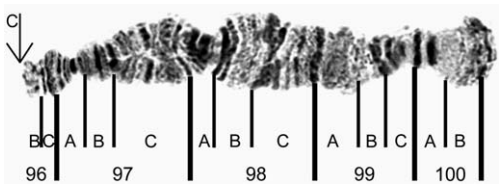

Fig. 10. Photomap of chromosome 6R. Arrows show the weak spots. Arrow C points to the centromere.

chromosome, which is not well banded and not very well structured.

Chromosome 6R (Fig. 10). This is the smallest chromosome arm. It is easily recognized by the four darkly banded puffs of which it is composed, and by the bands in divisions 97C, 98A–C and 99C–100A. The main landmark is the distal region, which looks like a rose bouquet. This arm shows no weak spot and is often found intact.

Discussion

Polytene chromosome maps usually have between 1500 and 5000 bands, depending on the technique used (Zhimulev, 1996). This photomap is just under the lower limit (1450 bands). As the sex chromosomes do not polytenize – they generally appear in preparations as uncondensed chromatin bodies – we expected the map to have fewer than the typical number of bands. Thus, this map can be considered to have a resolution comparable with those published in the Diptera literature. It represents an improvement over the preliminary map (Dev *et al.*, 1986), but it should be used as a step towards an enhanced map with even higher resolution.

The large number of weak spots (25) visible as constrictions or chromosome breaks in cytological preparations was of some interest. It is noteworthy that this was first pointed out by Dev *et al.* (1986), who observed approximately the same weak spots as we report in the current study. It may be worth investigating their origin and nature. Although these weak spots may only represent regions rich in heterochromatin, it is possible that they represent interesting genetic phenomena such as those recently found in *D. melanogaster* (for details, see: Zhimulev *et al.*, 2003; Belyaeva *et al.*, 2006; Gvozdev *et al.*, 2007).

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Conflicts of interest

All authors declare no conflicts of interests.

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Development and utilization of transgenic New World screwworm, *Cochliomyia hominivorax*

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Abstract. The New World screwworm (NWS), *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae), was the first insect to be effectively controlled using the sterile insect technique (SIT). Recent efforts to improve SIT control of this species have centred on the development of genetically transformed strains using the *piggyBac* transposon vector system. Eight transgenic strains were produced incorporating an enhanced green fluorescent protein (EGFP) marker gene under *polyubiquitin* regulation that has the potential for use as a genetic marking system for released males. The transgenic strains were genetically and phenotypically characterized, including for life fitness parameters and mating competitiveness. These characteristics were unique for each strain and thus some strains were deemed suitable for incorporation into SIT eradication programmes. The strain with the best attributes is designated 'CLAY'. Four of the strains, including CLAY, have been successfully cryopreserved so that their original characteristics should be unchanged when further evaluation is required. With the demonstration of efficient germ-line transformation in NWS, allowing production of fit and competitive transformants, it is now possible to consider further transgenic strain development to improve SIT that are currently being tested in other dipteran species. This includes strains that allow genetic marking with fluorescent proteins, genetic sexing by female lethality, male-specific fluorescent sorting and male sterility by testis-specific lethality. The SIT may also be improved upon by new strategies resulting in lethality of offspring of released insects using conditional lethal systems based upon temperature-dependent or dietary tetracycline regulation of lethal gene expression. Both the creation of new NWS transgenic strains and the ecological safety of their release will be enhanced by new vector systems that allow specific genomic targeting of vector constructs and their subsequent immobilization, ensuring transgene and strain stability.

Key words. *Cochliomyia hominivorax*, biological control, fluorescent proteins, *piggyBac* transformation, sterile insect technique, transgenic insects.

Introduction

Transposon-mediated germ-line transformation is possible in nearly 20 non-drosophilid insect species (Handler & O'Brochta, 2005), including the New World screwworm (NWS), *Cochliomyia hominivorax* (Coquerel) (Allen *et al.*, 2004a), which now allows the development of transgenic strains for functional genetic analysis as well as for improved and novel means of biological control. In particular, the sterile insect technique (SIT) (Knipling, 1955), which has been highly effective in eradication

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programmes, is especially amenable to improvement by transgenic strains (Robinson & Franz, 2000). These strains include those that provide fluorescent-protein (FP) marking to detect released insects, sexing for male-only strains by male-specific selectable markers or female-specific lethality, and, potentially, male sterility by testes-specific lethality. Lethality systems may be highly effective in eliminating a particular sex or tissue function, but must be highly and conditionally regulated so that strains may be reared under permissive conditions. Significant strides have been made in recent years to achieve this regulation based upon dietary components or ambient temperature conditions.

These same conditional lethality systems that may improve SIT can be extended to control insect viability, allowing the survival of populations reared under permissive conditions, but resulting in the death of offspring under non-permissive field conditions in the environment (Alphey, 2002; Handler, 2002). Such systems would not improve conventional SIT *per se*, but would, rather, provide a new means of 'sterilization'. Instead of a lack of fertility in released males, the lethality system would prevent the survival of their embryonic or larval offspring in the field. These systems, based upon temperature-dependent lethal genes or tetracycline-dependent lethal gene expression, have been described as autocidal biological control (ABC) (Fryxell & Miller, 1995) or release of insects with a dominant lethal (RIDL) (Thomas *et al.*, 2000; Alphey & Andreasen, 2002). These and similar concepts provide a new paradigm for how pest insect species may be biologically controlled, but, importantly, they depend upon the creation and release of transgenic strains which will pose biological and environmental challenges. It is the purpose of this paper to discuss the status of the transgenic strains in NWS, and the possibilities of developing new strains for improved biological control.

Materials and methods

Methods for transgenic strain development

Cochliomyia hominivorax presented unique challenges to transformation efforts. Firstly, the eggs of this insect are deposited by the female on a warm-blooded host. To collect eggs in culture, fresh ground meat warmed and maintained at body temperature must be presented to the adults. This was accomplished by placing warm ground beef on top of a container filled with warm water. The water container must be enclosed or the adults submerge and drown. The ground beef is supplemented with a small amount (200 μL) of warm aged blood. The eggs are deposited with an adhesive substance that must be removed. A method to remove the adhesive using NaOH has been described (Berkebile & Skoda, 2002).

DNA is microinjected into pre-blastoderm embryos and embryological development of *C. hominivorax* is complete after only 9 h under ideal conditions. Eggs are incubated in high humidity and high temperature (37 °C) conditions that simulate those of the warm-blooded host, resulting in rapid embryogenesis that narrows the window for injection. This effort, that resulted in the transformation of *C. hominivorax*, adjusted to rapid embryogenesis by a rapid microinjection process after thorough pre-injection preparations. Identification of alternative methods that delay early embryogenesis of *C. hominivorax*, such as limited exposure to cool temperatures, would be useful.

The larvae of *C. hominivorax* thrive as a mass in culture, whereas small numbers of larvae fail to survive. This posed a serious problem for transformation because few eggs hatch after microinjection. The successful transformation strategy used a maximum number of injected eggs per experiment to ensure that sufficient numbers of larvae would be available. Methods to ensure survival of small numbers of larvae would improve future transformation projects. Culture of the screwworm in the U.S.A. is permitted only in biological containment facilities.

Fitness parameters

Fitness parameters measured included egg production, egg hatch, average and total pupal weight, adult emergence and male: female ratio. These measurements were used to evaluate the overall fitness of each strain. Once transgenic *C. hominivorax* strains were considered to have established as stable colonies (by a minimum of five generations), fitness measurements were recorded. Transgenic strains were handled with some modified procedures. Because the verification of transgenic status is through visualization of the FP marker (PUbnlsEGFP) (Handler & Harrell, 2001b), each strain was screened under epifluorescence optics prior to pupation. The pupal and imaginal cuticles are completely opaque, making the screening of these stages impossible without destruction of the insect. For each transgenic strain, fully mature (crawler) third instar larvae were separated from rearing media and examined each generation. The procedure was usually performed on one day (day 5) during the development of each cohort of insects and unscreened pupae were discarded. Non-transgenic strains were allowed to pupate unmolested for several days; the pupae were collected on day 9 or 10. Thus, a much smaller proportion of pupae was collected for each generation of the transgenic colony, and a smaller number of transgenic adults were available to produce eggs.

Because of the handling differences between transgenic and non-transgenic *C. hominivorax* strains, certain fitness parameters were not comparable. Average pupal weight (100 pupae), egg hatch, adult emergence and male: female ratios were compared; the handling differences were assumed to have low impact on these factors. It is not known whether male and female insects pupate at different rates, but it was assumed that this was not the case.

Mating competence and competitiveness

The fluorescent marker in transgenic *C. hominivorax* strains provided a convenient mechanism for evaluating mating competitiveness in a cage containing wild-type females and equal numbers of wild-type males and homozygous transgenic males. A representative sample of eggs produced by the females was reared and the surviving larvae screened for fluorescence. Successful male mating was determined according to the

assumption that a homozygous transgenic male will father only heterozygous fluorescent offspring, whereas a wild-type male will have wild-type offspring.

Results

The first issues to resolve in the development of NWS transgenic strains for biocontrol concern whether routine and efficient germ-line transformation is possible and if the resulting transgenic insects are suitably viable and reproductively competitive. This has been well-demonstrated by the creation of eight transgenic strains using the *piggyBac* transposon vector system (Allen *et al.*, 2004a), several of which have viability parameters equal to or exceeding those of the host strain (Allen *et al.*, 2004b).

Transgenic strain development and fitness evaluation

Transgenesis technology allows recombinant DNA to introduce desired traits and characteristics into insects in order to improve their use in SIT programmes. The eight transgenic strains generated in the initial experiments were used to assess the fitness costs associated with genomic transgene integration. Because there were multiple unique integrations, and the marker protein was expected to be benign, transgenic strain quality characteristics could be compared with those of the parental strain (Allen *et al.*, 2004b; Allen & Scholl, 2005).

Some of the key issues involved in the mass release of sterile insects revolve around strain quality, including mating competence and competitiveness, of released insects. If transgenic insects are to be used in SIT, after sterilization and release they must be capable of flight, mate-seeking and mating behaviour that allows them to be reproductively competitive with wild-type individuals in the field. Within the mass rearing facility, the insects must maintain fertility and fecundity, resist infections and generally be productive. Therefore, it is critical to collect data and analyse multiple fitness traits or strain quality parameters of the transgenic insects (Table 1).

Here, we consider the phenotypes and fitness parameters of eight transgenic strains, with emphasis on four which have been cryopreserved (Leopold, 2007) and are available for further study. Strain designations were based on the first-generation transgenic parent (X, Y or Z) and the definitive area of phenotypic fluorescent expression (cuticle C, gut G, fat body F, or spiracle S). Letters were added to the two-letter designations to make pronounceable names, resulting in strains CLAY, CLIX, CLOX, COTY, FOLY, GARY, GIZA and SUEZ (Allen *et al.*, 2004a).

Cryopreserved transgenic strains

The CLAY transgenic strain had the brightest fluorescence based on visual inspection, with expression in epidermal cells beneath the cuticle spreading over the entire surface of the larva (Fig. 1). The salivary glands also expressed EGFP brightly, along with a structure at the anterior of the gut, the pharyngeal filter. The clarity of expression in this strain would make it the primary choice as a genetic marker. All strain quality characteristics measured for this strain scored better than for the parental strain except larval survival (Table 1). The only statistically significant scores were for fecundity and adult lifespan, both of which were higher than wild-type. This was the only strain tested for mating competition, and performance matched the non-transgenic parental strain. Furthermore, this strain was successfully cryopreserved and therefore may be available for use in mass production. The insertion site was amplified and sequenced (426 bases).

The CLIX and CLOX strains were derived from the same first-generation parent, but reared separately based on a perceived, but subtle, difference in EGFP expression. CLIX had a more uniform distribution of EGFP expression in the epidermal cells beneath the cuticle, like CLAY, and both strains expressed fluorescence in the salivary glands. CLOX expression beneath the cuticle was more restricted to the epidermal cells under the segmental spines. Southern blot analysis indicated that CLIX had a single transgene insert, whereas CLOX had two. Inverse polymerase chain reaction (PCR) identified a partial junction sequence from the CLIX strain, but not from CLOX. The CLIX strain was successfully cryopreserved, but the CLOX strain was lost. The CLIX strain did not have as favourable strain quality characteristics as several other strains, including CLOX (Table 1). CLIX larval survival was significantly lower than that of the control.

Table 1. Comparison of transgenic strains to parental strain (P95) of *Cochliomyia hominivorax*. Strain quality values are expressed as an absolute ratio to the control to indicate lower (negative value) or higher (positive value) quality.

Strain	Egg hatch	Larval survival	Pupal weight	Adult emergence	Fecundity	Adult lifespan	5′	3′
Control	0	0	0	0	0	0	NA	NA
CLIX	− 0.03	− 0.27*	− 0.09*	− 0.06	0.36*	0.01	No	Yes
CLOX	− 0.01	− 0.15	0.04	− 0.04	0.36*	0.04	No	No
CLAY	0.07	− 0.13	0.05	0.03	0.33*	0.18*	Yes	Yes
COTY	0.04	− 0.09	0.08*	0	0.24	− 0.04	No	Yes
FOLY	0.22*	− 0.11	0.08*	0.02	− 0.33*	− 0.17	Yes	Yes
GARY	0.03	− 0.11	0.01	− 0.06	− 0.21	− 0.09	Yes	Yes
GIZA	0.20*	− 0.21†	0.10*	0.05*	0.15	0.08	Yes	Yes
SUEZ	0.16*	0.03	0.09*	0.02	0.09	0.05	Yes	Yes

*Statistically significant.
†GIZA strain insert produces homozygous lethal.
NA, not applicable.

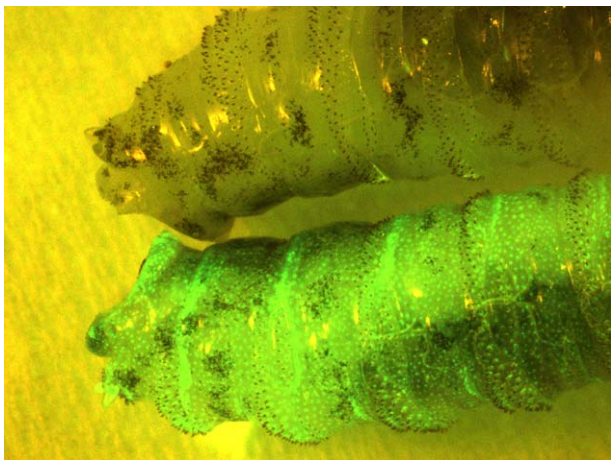

Fig. 1. Transgenic *Cochliomyia hominivorax* strain CLAY (lower) and wild-type parental strain P95 (upper) larvae under epifluorescence optics (green fluorescent protein/fluorescein isothiocyanate [GFP/FITC] filter). The posterior half of each specimen is shown. Vivid expression of green fluorescence from the PUbnlsEGFP marker gene is apparent in the transgenic strain.

The GIZA strain originated from one G1 male that produced many identical transgenic offspring. The transgenic offspring had brightly fluorescent salivary glands and a long section of fluorescent cells in the gut. No other fluorescent cells or tissues were immediately apparent, but, during screening of second-generation transgenics, it was noted that some larvae discarded as wild-type seemed to have fluorescent spots in the cells surrounding the posterior spiracles. These individuals were retrieved and used to found the SUEZ strain, and efforts were made to exclude individuals exhibiting the two combined fluorescent patterns from the GIZA strain. Thus two distinct strains were obtained. It was not possible to produce a homozygous GIZA strain, and further testing indicated that the transgene insert interrupted a vital gene, resulting in homozygous lethality. GIZA larval hatch rates were significantly higher than those of control, but the number of larvae surviving to pupation were significantly lower, and thus the homozygous lethal effect apparently occurs during larval development (Table 1). A pathology leading to larval death was never observed. Southern blots indicated a single insertion in this strain, and the insertion site was amplified and sequenced (407 bases; GenBank accession no. EU143852); the sequence translates to an uninterrupted peptide similar to a portion of *Drosophila melanogaster Myo28B* gene (expect = 2e-24, identities = 62/146 [42%], positives = 94/146 [64%], gaps = 17/146 [11%]) (Fig. 2). Clearly, this makes the strain inappropriate for release, but it was successfully cryopreserved.

This SUEZ strain, as discussed above, expressed EGFP only in the cells surrounding the spiracles. As the strain was selected, expression was more distinct and detectable around both the anterior and posterior spiracles. However, the strain was still difficult to use owing to limited fluorescence. This strain was not subjected to Southern blot analysis, but inverse PCR amplified insertion sites were sequenced. The strain quality characteristics compared favourably with those of controls, but the expression pattern precludes the use of this strain for release.

Non-cryopreserved strains

Three transgenic strains, in addition to CLOX, were not successfully cryopreserved and are therefore unavailable for further consideration and testing. We review some of their phenotypic attributes, which should be useful in the analysis and comparisons of future strains.

The FOLY strain expressed EGFP differently from the others in that it was somewhat diffuse in the posterior half of the maggot, corresponding primarily to fat body tissue and some cells of the hindgut. The quality of this strain was significantly lower than that of the parental strain in egg hatch, pupal weight and fecundity. GARY was another phenotypically distinctive strain exhibiting bright EGFP in the pharyngeal filter and a specific region of the midgut, as well as in several other tissues. Molecular analysis indicated a single transgene insert and the genomic insertion site did not appear to be a coding sequence. The quality characteristics of GARY were similar to those of the control strain, suggesting its suitability for programme use. COTY was isolated from the same mating that produced CLAY, FOLY and GARY, and may have been closely linked to FOLY. Expression of EGFP was limited to the epidermal cells under the segmental spines and was relatively faint. Salivary glands also expressed EGFP. The strain had a single transgene insert, which was partially sequenced, but had quality characteristics that compared favourably with the parental strain.

In summary, of the initial eight transgenic NWS strains created at the USDA-ARS Lincoln, Nebraska facility, only the CLAY, CLIX, GIZA and SUEZ strains were successfully cryopreserved and thus are available for further study. From initial studies, the CLAY strain is most suitable for use as a marked strain in SIT, and further evaluation and modifications of this strain will be considered.

Discussion

Transgenic strain mating competence and competitiveness

Mating competence and competitiveness will be factors critical to the success of a transgenic strain used for SIT. The fluorescent marker in transgenic *C. hominivorax* strains provided a convenient mechanism for evaluating mating competitiveness in a cage containing wild-type females and combined transgenic and wild-type males. A representative sample of eggs produced by the females was reared and the surviving larvae screened for fluorescence. A homozygous transgenic male will father only fluorescent offspring, and the wild-type male's offspring will all be wild-type. Comparisons of the transgenic strain CLAY and the P95 wild-type strain showed the CLAY transgenic males to be as competitive as P95 males.

There is concern that transgenic organisms could persist once released and generate hazards above and beyond those they were intended to control. This could occur by a failure in the

A

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G ATC ATA ACA ATA CAA AGA GGT TTT CGT AAA GTA TTA TTT AAA CGT TTC CTG GAT AAA
  Ile Ile Thr Ile Gln Arg Gly Phe Arg Lys Val Leu Phe Lys Arg Phe Leu Asp Lys

  TAT CGT AAA GCG GTA ATA CTA ATA CAA AAA ACT TGG AGA GGT TAT AGA GAA CGT AAA
  Tyr Arg Lys Ala Val Ile Leu Ile Gln Lys Thr Trp Arg Gly Tyr Arg Glu Arg Lys

  AAT TAT TTG GTG ATG CAT AAT GGA TTC CAT CGT TTG GCT GCC TCT GTG GCC TCA AGA
  Asn Tyr Leu Val Met His Asn Gly Phe His Arg Leu Ala Ala Ser Val Ala Ser Arg

  CAA TTA ACC TAT CGC TTT GGT TTA TTG AGA AAT CGC ATA AGT GGT TTG CAA GCT CAC
  Gln Leu Thr Tyr Arg Phe Gly Leu Leu Arg Asn Arg Ile Ser Gly Leu Gln Ala His

  TGC CGA GGT TAT TTG GTG AGA AAA GAA TTT AAA GCA AAA TAT GCT TTA AGA ATA GCA
  Cys Arg Gly Tyr Leu Val Arg Lys Glu Phe Lys Ala Phe Tyr Ala Leu Arg Ile Ala

  CGA GTG CGA GAA CTT AAA TTA TTG AGA ACA CAA GAA GAA GAA CAA TAT CGT AAG GCT
  Arg Val Arg Glu Leu Lys Leu Leu Arg Thr Gln Glu Glu Glu Gln Tyr Arg Lys Ala

  AAA GAA CGT AAT TGG AAA CAA CAT GCA GAA GAA AAT TAT CAA AAA CGT TTA AGA GGT
  Lys Glu Arg Aln Trp Lys Gln His Ala Glu Glu Aln Tyr Gln Lys Arg Leu Arg Gly

  ATT GAT C
  Ile Asp
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B

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GIZA     : 1   IITIQRGFRKVLFKRFLDKYRKAVILIQKTWRGYRERKNYLVMHNGFHRLAASVASRQLT
               I+TIQRG R+VLF+R++ +YR+A+I +Q+ WRG  +R+ Y VM  GFHRL A +A++QLT
DmMyo28B1: 745 IVTIQRGIRRVLFRRYMKRYREAIITVQRYWRGRLQRRKYQVMRQGFHRLGACIAAQQLT

GIZA     : 61  YRFGLLRNRISGLQAHCRGYLVRKEFKAKY-------------ALRIARVREL-KLLRTQ
              +F ++R R   LQA  RGYLVRK+F+ K                L++A+++E  +LLR Q
DmMyo28B1: 805 TKFTMVRCRTIKLQALSRGYLVRKDFQKKLLERRKQNQLKKEELLKLAKIKEAEELLRLQ

GIZA     : 107 EEEQYRKAKERNWKQHAEENYQKRLR 132
                 Q ++ KER  ++  E+  Q+  R
DmMyo28B1: 865 ---QLKEQKEREQREQQEKRLQEEQR 887

Score = 114 bits (285), Expect = 2e-24. Identities = 62/146 (42%), Positives
= 94/146 (64%), Gaps = 17/146 (11%).
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Fig. 2. (A) Nucleotide and translated sequence (5′–3′ with respect to logical translation; transgene insert is in reverse orientation) surrounding the GIZA transgene genomic insertion site. *Sau*3AI restriction sites are in bold, as is the *piggyBac* TTAA target site. (B) BLASTn comparison between translated GIZA sequence and *Drosophila melanogaster* myosin 28B1 (gi 24582547).

transgenic insect itself, for example, by mutation or remobilization of the transgene, or by a failure in the production system, as in the inadvertent release of fertile insects. Assessment of these risks requires baseline data establishing the relative fitness of transgenic insects compared with 'wild-type', laboratory-reared counterparts. A series of experiments and analyses to compare overall fitness and mating fitness between transgenic and parental strain (P95) wild-type insects indicated that there was little or no loss of fitness in most strains of transgenic NWS. Specific strains showed some fecundity and adult emergence costs, but these were probably associated with specific insertion sites rather than the existence of the transgene itself.

Improved strains for SIT

The efficient transformation of NWS, yielding transgenic organisms with strong fitness characteristics, is supportive of creating additional transgenic strains that can be used for SIT and other means of biological control. Routine testing and creation of NWS transgenics is, however, complicated by quarantine issues and the relatively difficult rearing of this species. Thus, it is advantageous to first consider transgene constructs that have been tested in other species for the same or similar purposes. As the *piggyBac* vector and *polyubiquitin*-regulated EGFP marker system used to transform NWS was originally created for and tested in Drosophila (Handler & Harrell, 1999) and tephritid flies (Handler & Harrell, 2001b), many of the advanced vectors tested in these species should be easily transferred for use in NWS, and the development and use of these systems will be discussed.

New marked strains and use of stabilized vectors

Currently, the CLAY strain marked with EGFP has several characteristics that make it the primary candidate for release as a marked strain in SIT. However, the creation of additional, new fluorescent-marked strains is important for backup purposes, different fluorescent markers can be used to distinguish between flies from different releases, and their use in new advanced vectors can simplify strain creation and provide for greater ecological safety (Wimmer, 2005). Therefore, developing new transgenic marked strains remains a high priority. Other FP colours distinguishable from EGFP and used as transgenic markers include red (DsRed), blue (CFP) and yellow (YFP) fluorescence, and their enhanced versions (Handler & Harrell, 2001a, Horn *et al.*, 2002). Their use, and use of EGFP strains intended for release, should be within new transposon vectors that can be immobilized post-integration, which ensures the stability of the transgene vector within the host genome. This is essential to maintain strain integrity and also to minimize concerns for ecological safety (Handler, 2004). These types of vectors, which depend on post-integration remobilization of vector terminal sequences, have been created and tested in several Drosophila

and tephritid species (Handler *et al.*, 2004; Dafa'alla *et al.*, 2006). Another vector system additionally allows targeting to specific genomic insertion sites (Horn & Handler, 2005). Vector stabilization by terminus remobilization in Drosophila was achieved by mating transformants carrying the initial unstabilized vector to a jumpstarter strain with a chromosomal source of *piggyBac* transposase (Handler *et al.*, 2004). Importantly, in tephritid species where jumpstarter strains are not available, terminus remobilization was achieved efficiently by direct *piggyBac* helper plasmid injection (A. Handler & R. Krasteva, unpublished data, 2007). This had the added advantage of producing stabilized vector strains in a single generation, and this approach will be taken with NWS, for which jumpstarter strains are also unavailable.

Sperm and testes markers

A priority for SIT is to determine whether trapped female flies have mated with released males. This may be determined by having sperm marked with an FP, which can be achieved by linking the FP to a sperm-specific promoter, from genes such as *β2-tubulin* (Fackenthal *et al.*, 1993). In Drosophila, we have shown that marked sperm from transgenic β2-tubulin:DsRed males can be detected in the seminal receptacle of mated females (A. Handler & R. Harrell, unpublished, 2005). Similar detection has been shown for the Caribbean fruit fly, *Anastrepha suspensa*, where β2-tubulin:DsRed-marked sperm is detected in the female spermathecae (G. Zimowska, N. Xavier & A. Handler, unpublished, 2006). There is also the potential to use marked sperm in sperm precedence studies when multiple matings occur. The Drosophila *β2-tubulin* promoter was tested in sperm marking in the Caribbean fruit fly, but heterologous function was not apparent based on visual detection of DsRed fluorescence (G. Zimowska, N. Xavier & A. Handler, unpublished, 2006). It was assumed that, despite the relatively high conservation of *β2-tubulin* genes, promoter function was more species-specific, and thus isolation of the *β2-tubulin* gene, and proximal genomic DNA, from Caribbean fruit fly would be necessary. The gene was therefore isolated by a direct PCR approach using degenerate primers, with inverse PCR used to sequence the proximal 5′ and 3′ genomic DNA, and approximately 1.5 kb of 5′ sequence was tested for promoter activity. This was confirmed by testes-specific DsRed fluorescence observed in the sperm tails and seminal material of Caribbean fruit fly males, and in the spermathecae of non-transformed mated females. In addition, PCR was able to specifically detect marked sperm from abdominal DNA preparations of individual mated females. Existing vectors could be tested initially for use in NWS, but it is likely that isolation of the NWS *β2-tubulin* promoter will be necessary, and this should be straightforward.

Sexing for male-only strains

A high priority for programme efficiency and cost-effectiveness is the ability to sex strains early in development and, ideally, to eliminate females by the first instar larval stage. Transgenic vectors allow this to be achieved in several ways. Firstly, fluorescent body-marked vectors that integrate into the male-specific Y-chromosome will only be expressed in males. Although Y-chromosomes often represent a relatively small percentage of total genomes in dipterans, routine transformation of these species typically results in some Y integrations, and we have created three Caribbean fruit fly male-fluorescent strains (A. Handler & R. Harrell, unpublished data, 2005). Importantly, fluorescent sorters that allow high-throughput sorting of insect embryos and larvae that express specific fluorescence are available and these may be used in sexing protocols (Furlong *et al.*, 2001). Sexing based on male-specific fluorescence may also be achieved by testes-specific fluorescence using *β2-tubulin*-regulated fluorescence, which has been demonstrated in a mosquito species (Catteruccia *et al.*, 2005; Smith *et al.*, 2007). However, detection may not be feasible until the third instar larval stage, resulting in suboptimal efficiency.

A third transgenic approach towards sexing is the use of sex-specific lethality systems. These require sex-specific regulatory control systems to drive the expression of a conditional lethal system and, ideally, lethality should occur early in development. For many dipterans the ideal sex-specific regulatory system regulates sex determination gene expression, which is initiated in early embryogenesis and functions throughout development (Handler, 1992). This is based on a sex-specific transcript splicing system that has alternative male and female 3′ intron splice sites. A translational stop codon is specifically revealed in the longer male transcript, which results in a truncated, non-functional polypeptide. Use of a downstream 3′ splice site in females results in deletion of the stop codon within the intron. Several sex determination genes in Drosophila utilize this system, and cognates of the *transformer* (*tra*) and *doublesex* (*dsx*) genes are known to exist in other dipterans, and the *transformer* splicing system has been tested in several species (Saccone *et al.*, 2002). The *tra* alternative splicing cassette can be placed in-frame within a variety of conditional lethal systems, including the tet-off suppression system or several temperature-dependent lethal genes.

Sterile male strains

Currently, male sterility is achieved by pupal irradiation which, although it effectively disrupts germ-line chromosomes, also creates somatic damage that can adversely affect fitness. There are also considerable insect handling costs involved in irradiation and the production of suitable irradiators is becoming more limited. Thus, developing a genetic means of specifically sterilizing males is a high priority, which, potentially, can be efficiently achieved using recombinant constructs already described for other objectives. One of these involves having the testis-specific *β2-tubulin* promoter drive a conditional lethal system. A caveat for this approach is that production or transfer of the male seminal peptides that inhibit subsequent matings in females should not be disrupted.

Organismal lethal strains

A logical extension of conditional lethality systems that kill a specific sex or destroy a specific tissue for use in SIT is lethality

limited to insects early in development under non-permissive conditions. This could allow survival under mass rearing, with early death of the progeny of released insects after they have mated with wild insects. This can be achieved in several ways. One is by temperature-dependent lethality resulting from a dominant temperature-sensitive lethal gene or temperature-sensitive mutations of toxin subunit genes. One system based on a cold-sensitive allele of the Drosophila *Notch* gene, which would cause lethality at temperatures of ≤ 20 °C, has been described as autocidal biological control (ABC) (Fryxell & Miller, 1995). We have tested a converse system resulting in temperature-dependent lethality at high temperatures. The DTS-5 mutant allele from *Drosophila melanogaster*, which causes larval or pupal death at 29 °C (Saville & Belote, 1993), was tested by transforming it into *Ceratitis capitata*. Several *C. capitata* lines homozygous for the Drosophila DTS-5 transgene exhibited 90–95% lethality by the larval or pupal stage at 30 °C. DTS-5 is a point mutation in a highly conserved 20S proteasome subunit (Saville & Belote, 1993), and efforts to improve lethality in tephritids centre around isolating and mutagenizing the DTS-5 cognate, as well as another DTS point mutation, DTS-7 (Smyth & Belote, 1999). These conditional lethal mutations would be particularly useful in tropical and subtropical insects, and their high conservation suggests that they can be similarly isolated and mutagenized for use in NWS.

Conditional lethality may also be regulated by gene expression systems that are either turned on or turned off by a chemical supplement to the diet. Foremost among these systems is the tet-off system based on the tetracycline operon from *Escherichia coli*, where tetracycline, or an analogue, suppresses gene expression (Gossen *et al.*, 1993). A mutated tet-on version of this system acts conversely in that tetracycline is needed to promote gene expression. The tet-off system was originally tested in Drosophila as a female-specific lethality system, using the yolk protein promoter active in female fat body to ultimately drive expression of the *hid* cell death gene (Heinrich & Scott, 2000; Thomas *et al.*, 2000). Another version of this system, using embryonic promoters, resulted in both male and female lethality in Drosophila during early development in the absence of tetracycline (Horn & Wimmer, 2003). These approaches are being tested in *C. capitata* (Schetelig *et al.*, 2007) and could be extended to NWS if suitable embryonic promoters, such as cognates from the *serendipity* or *nullo* genes, were available. Potentially, the allele of the *hid* cell death gene used in Drosophila could also confer lethality in other dipterans.

Conclusions

The New World screwworm was the first insect to be successfully eradicated using the SIT. In an effort to improve future SIT programmes, research was initiated to develop transgenic insect technology. The first transformation experiments produced eight transgenic strains incorporating an EGFP marker that could be used, potentially, to identify released insects. Each stable strain had at least a single copy of the transgene incorporated into a unique genomic location and had unique genetic, phenotypic and strain quality characteristics. The original lines were produced in the ARS Biosecure facility in Lincoln, Nebraska, and the subsequent closure of this laboratory resulted in the cryopreservation of the transgenic screwworm germplasm. This was successful for four of the eight transgenic strains, one of which, CLAY, has attributes as a fluorescent marked strain that have led it to be considered for use in SIT, to unambiguously identify released insects from wild pests.

Desirable traits for further improvement of SIT for NWS include the development of male-only strains by conditional female lethality, or sexing achieved by fluorescent sorting of males with vector insertions in the male-specific Y chromosome. It may also be possible to sort males by testis-specific expression of FPs driven by promoters such as those for the *β2-tubulin* gene. These strains could also be used for sperm marking to identify females that have mated with released males and the *β2-tubulin* promoter could additionally be used with lethal genes to confer conditional male sterility.

Strain improvements for SIT using transgenesis are achievable; however, the ultimate goal for pest management using this technology is the creation of new strains that are conditionally lethal in early development. Systems have already been tested in Drosophila and tephritid species based on dominant temperature-sensitive lethality and tet-off regulation of cell death gene expression. As the transgenic conditional lethal strains were created using the same *piggyBac* vector system successfully tested in NWS, it is highly likely that similar strains can be created for NWS. This should be a high priority of future research.

Conflicts of interest

All authors declare no conflicts of interests.

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Current status of the New World screwworm *Cochliomyia hominivorax* in Venezuela

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Abstract. The geographical distribution and seasonality of the New World screwworm (NWS), *Cochliomyia hominivorax* Coquerel (Diptera: Calliphoridae), were monitored through the use of sentinel animals as part of a co-ordinated programme involving veterinarians and farmers, as well as undergraduate students and teachers from veterinary colleges in Venezuela. This surveillance activity made it possible to collect NWS egg masses or larvae from all 23 states in the country and to determine that the rainy season has a strong positive influence on the number of cases of myiasis caused by *C. hominivorax* in dogs. In addition, efforts were made to obtain the co-operation of the public health service in order to document the extent of human myiasis in the western–central region of Venezuela. Preliminary results revealed 241 cases over a 7-year period, with cases reported in infants as well as in elderly people. Larvae causing myiasis, other than *C. hominivorax*, were collected from primary myiasis in rabbit (*Lucilia eximia* [Wiedemann]), dog (an unidentified sarcophagid species), birds (*Philornis* sp.) and wild mice (*Cuterebra* sp.). The economic impact of NWS in Venezuela has not been calculated in terms of loss of milk and meat production, damage to hides or death of animals. Control costs (e.g. cost of larvicides) have been estimated at US$ 2 m per year. Control of myiasis in animals is achieved through the use of chemical compounds, mainly organophosphorus (OP) compounds, macrocyclic lactones and, more recently, a foamy spray based on spinosad. Concerns about insecticide resistance to OP compounds have been raised.

Key words. *Cochliomyia hominivorax*, geographical distribution, human myiasis, seasonality, Venezuela.

Introduction

The New World screwworm (NWS), *Cochliomyia hominivorax*, causes myiasis in man and warm-blooded animals. It is a Neotropical species, and its former geographical distribution ranged from the southern U.S.A. to northern Argentina and throughout the Caribbean basin. Economic losses caused by NWS stem from the death of animals, as well as decreases in both milk and meat production (Vargas-Terán *et al.*, 2005). Additionally, the cost of treatments must be considered in order to assess the actual economic impact of this pest in livestock. Cases in humans represent another consideration (Powers *et al.*, 1996; Couppie *et al.*, 2005).

Chemical control has been the most important tool for controlling myiasis in Venezuela. Some issues, such as increased cost of production, residues in milk and meat, environmental damage, undesirable effects on non-target species and the rise of resistance have prompted the search for new control strategies. Use of the sterile insect technique (SIT) for NWS has represented a milestone in pest control. Through the integrated use of SIT, the pest has been eradicated from both North and Central America (Wyss, 2000). Sterile insects were also used for the eradication of an outbreak in Libya, North Africa (Lindquist *et al.*, 1992).

The lack of reliable data on screwworm-associated losses in Venezuelan livestock limits a full appreciation of the current

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status of the pest in the country. However, complaints from cattlemen are very frequent. The country is not self-sufficient in milk or beef production and the presence of this pest certainly worsens this situation.

Venezuela is located at the most northern tip of South America, and has common borders with Brazil, Colombia and Guyana. Any expansion of the use of SIT for the suppression and eradication of NWS in these countries will demand reliable information on geographical distribution and population dynamics, including genetic variation within and among populations (Vargas-Terán *et al.*, 2005).

The goals of the present study in Venezuela were to: (a) investigate the geographical distribution of NWS; (b) determine the seasonality of the pest in the western–central region; (c) investigate the range of hosts affected by myiasis, and (d) relate the presence of other flies causing myiasis to NWS.

Materials and methods

Geographical distribution

Monitoring of NWS was accomplished through the use of trapping. Use of hand-nets and rotting liver was shown to be a reliable method for estimating adult populations of this pest in Central America (Parker & Welch, 1991). Other approaches include the use of sentinel animals and baited traps, such as the classical wind-oriented trap (WOT). Baited traps rely on the use of animal tissues (liver) or chemical attractants (swormlure) (Thomas & Chen, 1990; Parker & Welch, 1992; Phillips *et al.*, 2004).

The present studies were carried out using WOTs baited with different substrates, such as fresh or rotted beef liver, rotted beef spleen, rotted beef brain, fresh chicken liver and fresh ground beef.

Additional samples were collected from several regions of the country with the invaluable help of veterinarians engaged in the project, who obtained material from infected hosts (mainly dogs and cattle). In order to obtain good collaboration, many short visits were made and a concise explanation given on the objectives of the project and on the best ways of collecting and sending samples. Small plastic vials containing dry sawdust were used by veterinarians for sending larval samples. Egg masses were sent in small plastic boxes containing wet cotton. Samples were received at the laboratory within 24 h of shipment.

Indirect sampling

Several field trials to evaluate the efficacy of new drugs against natural infestations utilized newly castrated beef cattle as sentinels to assess the presence of the pest in two regions of the country. The first experiment (Experiment 1) was conducted in the Aroa region (10°26′07″ N, 68°53′99″ W), 45 miles west of the Caribbean Sea. In this trial, 56 males were castrated and returned to pasture. The second experiment (Experiment 2) was performed in 2006 in the Encontrados region (09°03′60″ N, 72°13′88″ W), 36 miles east of the Colombian border. In this experiment, a total of 89 males were castrated and returned to pasture. A third experiment, using both newborn calves and post-parturient cows as sentinels, was conducted in the Los Llanos region (8°29′28″ N; 69°50′02″ W).

Seasonality

Because of the ineffectiveness of liver-baited WOTs for monitoring adult populations, a retrospective study was performed with the participation of three small animal clinics from Barquisimeto City and the Small Animal Hospital of the Veterinary College at the Universidad Centroccidental Lisandro Alvarado (UCLA) in the same city. A review of the files for 1999–2006 allowed us to determine the monthly incidence of cases during that period.

Human infestations

Information on human infestations was collected from primary health centres and from two university hospitals located in two cities. The centres were located in three states in the region: Falcon, Lara and Yaracuy. The fact that the condition ‘myiasis’ is not listed on the forms that are completed in health centres obviously results in an underestimation of the actual number of cases.

Results

Geographical distribution

Wind-oriented traps at different heights were not effective for NWS and many calliphorids other than *C. hominivorax* were caught. The most prevalent species were *Chrysomya albiceps* (Wiedemann), followed by *Chrysomya megacephala* L. In total, 38 samples of *C. hominivorax* were collected from different places throughout the country and from all 23 states.

Indirect sampling

In Experiment 1, 48 (85.7%) of the sentinel cattle were found to have at least one egg mass (see Muniz *et al.*, 1995a). In Experiment 2, 45 cattle (≈ 50%) were found to have at least one egg mass around the wound (A. J. Coronado, unpublished data, 2006). In Experiment 3, 40% and 8% of calves and cows, respectively, developed myiasis (see Muniz *et al.*, 1995b).

Seasonality

Monthly averages of myiasis cases are shown in Fig. 1. There was an increase in the number of cases that occurred during May–July, coinciding with the main rainy season in the region. Similar results have been reported from other countries (Amarante *et al.*, 1992; Madeira *et al.*, 1998). The exact role of the rainy season in the number of myiasis cases is unknown.

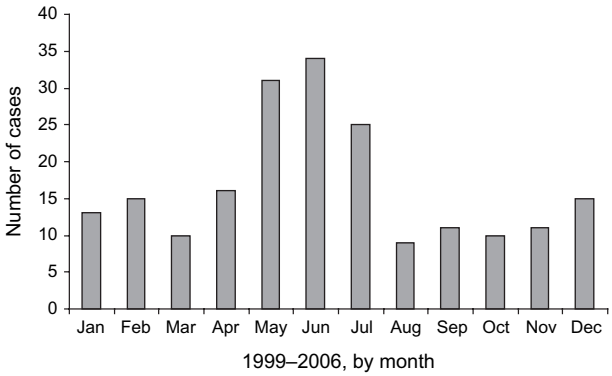

Fig. 1. Monthly distribution of New World screwworm cases in dogs in Barquisimeto City, Venezuela (1999–2006).

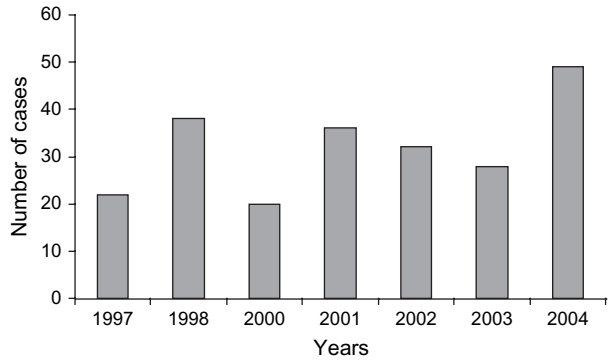

Fig. 2. Human myiasis cases in the western–central region of Venezuela, 1997–2004.

Host range and human infestations

A total of 241 cases were registered during a 7-year period (Fig. 2). Patient ages ranged from newborn to 91 years, with most cases occurring in children aged 0–6 years (Fig. 3). The infestations were associated with several risk factors, such as extreme poverty, lice infestation, diabetes, skin vesicular diseases (chicken pox) and mental illness. Unfortunately, two elderly people died as a consequence of massive infestation by larvae of *C. hominivorax*.

Discussion

From an economic point of view, cattle are undoubtedly the most important hosts for *C. hominivorax*. Although first instars may affect healthy tissues of bodily orifices, the presence of small wounds is the most important factor in the epidemiology of myiasis. These include small lacerations in the external genitalia of cows caused during calving or in the navel in newborn calves, and wounds caused by wire fences, sharp wood or animal management techniques (e.g. de-horning, branding, castration). Injuries caused by other ectoparasites, such as the tropical cattle ticks *Boophilus microplus* Canestrini and *Amblyomma cajennense* (F.), also predispose cattle to myiasis. Papillomas of viral origin also have a strong relationship with the presence of the pest in the field, mainly in the teats of heifers. The presence of *Cochliomyia macellaria* (F.), which causes a facultative myiasis, has been incriminated as a factor that exacerbates the pathological condition.

Dogs are frequently affected by NWS and represent the second most important host in Venezuela. Overall, 60% of infested wounds in dogs are located in the head, suggesting that fighting could be associated with myiasis in this host, as in cats (de Almeida *et al.*, 2007). Other domestic animal hosts include sheep, pigs, horses, goats and cats. Information collected at the Barquisimeto City Zoo shows that most cases in wild animals are seen among felids.

Infestation in humans is a growing concern and deserves special consideration. Two cases of human myiasis caused by *C. hominivorax* have been reported recently in Aragua State, in the central region (de Moissant *et al.*, 2004a, 2004b). Human cases of myiasis were recorded in the emergency rooms of public hospitals from the western–central region of Venezuela. However, these data were not available in the epidemiological register at the Federal Health Ministry and had to be collected from records kept in individual health centres.

Other flies causing myiasis

The species *L. eximia*, *Sarcophaga* sensu lato, *Dermatobia hominis*, *Oestrus ovis*, *Cuterebra* spp. and *Philornis* spp. have been detected in domesticated animals, as well as in wild animals, as primary agents of myiasis in Venezuela. *Lucilia eximia* larvae were recovered from a wounded domesticated rabbit. Primary myiasis by larvae of a species of *Sarcophaga* sensu lato was observed in a 12-year-old Yorkshire terrier bitch; larvae were recovered from a tumour close to the vulva. Adult flies of *L. eximia* and *Sarcophaga* sensu lato were obtained from these two cases (A. J. Coronado, unpublished data, 2005).

The recent introduction into the country of new species from the Old World, specifically three species of the genus *Chrysomya*, seems to be affecting the native fauna. Larvae of the species *Ch. albiceps* (Wiedemann) are predators that attack larvae of the native species *C. macellaria* F. as well as those of the recently introduced species *Chrysomya putoria* Wiedemann and *Ch. megacephala* (Faría & Godoy, 2001). During the present work, most of the *L. eximia* larvae present in guinea-pig carcasses were attacked by *Ch. albiceps* larvae.

Populations of the native species, *C. macellaria*, seem to be declining in the New World (Guimarães *et al.*, 1978). In the present work, we found a marked reduction in numbers of this species compared with data reported in the region previously (Osorio, 1981). There was a marked predominance of the *Chrysomya* species over *C. macellaria*, demonstrated by low numbers of the latter species by contrast with the huge numbers of *Ch. albiceps* observed.

Chemical control

Therapeutic and prophylactic measures to limit the damage caused by NWS in domesticated animals and, to a lesser extent,

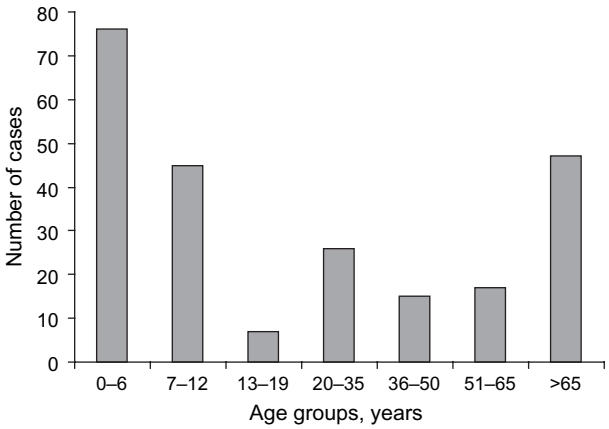

Fig. 3. Age distribution of human myiasis cases in Venezuela.

in wild captive animals in Venezuela rely on the use of chemical compounds. There are some 20 commercial formulations of organophosphorus (OP) and synthetic pyrethroids available on the Venezuelan market. These are formulated as sprays, powders and ointments. Macrocyclic lactones, such as ivermectin and doramectin, have been used as both control and preventative substances, with doramectin showing superior results to ivermectin in both applications. Recently, spinosad, a derivative from *Saccharopolyspora spinosa*, was introduced onto the Venezuelan market.

There is concern about the development of resistance (Carvalho *et al.*, 2009) to OP compounds. In one case larvae treated in the wound with a spray formulation containing Dichlorvos were able to successfully pupate and complete adult emergence (Coronado & Triana, 1998).

The future of the pest in the country

Cochliomyia hominivorax is clearly widespread in Venezuela and presents a serious threat to the livestock industry, as well as to the human population. Further research is required to determine the genetic relationships between local Venezuelan populations of NWS and those of nearby regions in the Caribbean, as well as in neighbouring South American nations. This will be a precursor to the integration of Venezuela into region-wide control strategies.

The huge experience gained in other countries in the Americas and Africa in eradicating *C. hominivorax* has established the absolute necessity of involving the official sector in any strategies initiated as part of a campaign of suppression or eradication of NWS. Another, but no less important, consideration is that any programme to be launched in the area must involve more than one country because of the absence of natural barriers.

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Conflicts of interest

All authors declare no conflicts of interests.

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Mark–recapture estimates of recruitment, survivorship and population growth rate for the screwworm fly, *Cochliomyia hominivorax*

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Abstract. Pradel model mark–release–recapture estimates of survivorship, ϕ, recruitment, f, and the rate of density-independent population growth, λ, are presented for eight mark–recapture studies of the screwworm *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae) from Costa Rica, totalling 19 573 released and 4476 recaptured flies. Corroborative estimates of survivorship and the rate of population growth based on an extensive review of the literature are also reported. Weighted-mean $\pm$ standard error of the mean (SEM) mark–release–recapture estimates of survivorship, recruitment and the rate of population growth were $\phi = 0.798 \pm 0.008$, $f = 0.193 \pm 0.008$ and $\lambda = 1.005 \pm 0.002$, respectively. Population doubling time was estimated from λ at 139 days. Estimates of ϕ and λ from the literature both exceeded those calculated by mark–recapture methods and estimates of population doubling times were consequently shorter.

Key words. *Cochliomyia hominivorax*, Calliphoridae, Diptera, Insecta, MARK, mark–recapture, Pradel, screwworm.

Introduction

The New World screwworm (NWS), *Cochliomyia hominivorax*, is a myiasitic fly injurious to livestock and human health (Steelman, 1976; Spradbery, 1993; Powers *et al.*, 1996; Wyss, 2000). *Cochliomyia hominivorax* was eradicated from the U.S.A., Mexico, Central America, Curacao, Puerto Rico and the U.S. Virgin Islands by the U.S. Department of Agriculture, Animal Plant Health Inspection Service, Screwworm Eradication Program, by an integrated approach, incorporating the release of sterile insects (Wyss, 2000). An eradication programme is currently underway in Jamaica, but the screwworm remains extant in the rest of the Caribbean and in South America.

Per capita adult rates of recruitment and survivorship, and the rate of density-independent population growth (hereafter f, ϕ and λ, respectively), are key life history parameters required for predicting population dynamics, for modelling the efficacy of sterile insect technique (SIT) control programmes and for estimating the rate of spread of potential new outbreaks. New World screwworm mortality rates have been estimated in the laboratory (e.g. Crystal, 1967a, 1967b; DeVaney & Garcia, 1975) and death rates can be inferred from the results of some outdoor cage (Davis & Camino, 1968) and field (Thomas & Chen, 1990) studies. Recruitment rates can be calculated from laboratory estimates of life history parameters (references included in the Appendix). However, to date, no field estimates of NWS recruitment or population growth rates have been published.

Wildlife biologists commonly measure life history parameters for field populations by mark–release–recapture techniques (Jolly, 1965; Seber, 1965, 2002; Pradel, 1996), in which animals are sequentially captured, marked and released and recaptured. Survivorship, recruitment and population growth rates can then be estimated from the rates of addition and loss to the marked population through time. Increasingly, these methods are used by entomologists to estimate life history parameters as well (Anholt *et al.*, 1978; Stoks, 2001; Schtickzelle *et al.*, 2003; Joyce *et al.*, 2004; Nowicki *et al.*, 2005). This paper reports f, ϕ and λ estimates calculated by the Pradel method (Pradel, 1996) for eight fertile NWS mark–release–recapture studies carried out at two locations in Costa Rica. A total of 19 573 flies were captured,

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marked and released and 4476 were recaptured (datasets are described in detail in Materials and methods) in the eight surveys. The current study also investigates whether f, ϕ and λ varied among tropical premontane wet forest and tropical dry forest life zones, the wet and dry seasons, or male and female flies. Next, corroborative ϕ and λ estimates from an extensive review of the literature on NWS life history parameters are presented. Finally, calculation of doubling times was carried out for NWS outbreak populations under density-independent growth conditions from both mark–recapture and published parameter estimates.

Materials and methods

Datasets

In each of the eight mark–release–recapture studies, flies of both sexes were captured manually in sweep nets at four to seven observation stations baited with one of three attractants: (a) rotting beef liver allowed to age at ambient temperature for approximately 1 week; (b) Merino sheep infested with NWS or (c) Swormlure IV (SWL IV), a synthetic, volatile, liquid screwworm attractant (Mackley & Brown, 1984) deployed in a 150-mL bottle with a cotton dental wick refilled at weekly intervals following the methods of Broce (1980). Flies were then marked by gluing numbered plastic bee tags (Chr. Graze KG, Weinstadt, Germany) to the scutum, released and then recaptured at one of the observation sites (potentially including the site of initial capture and release). Table 1 gives the: (a) location of each study; (b) mean latitude and longitude of observation sites; (c) dates of sampling; (d) the Holdridge life zone (Holdridge, 1947, 1967; Holdridge *et al.*, 1971); (e) season (wet or dry); (f) attractants used at observation stations; (g and h) total numbers of flies of each sex marked and released, and recaptured, respectively, and (i) publications providing additional details on methods.

Seven of the eight datasets were previously analysed by Matlock *et al.* (1993). Dates in Table 1 disagree with those in Table 1 of Matlock *et al.* (1993). Here, we report the dates of the first and last days of observations analysed, which represents a more accurate accounting than that presented in the earlier paper. The numbers of flies released in Table 1 differed slightly from those reported in Table 1 of Matlock *et al.* (1993). A small number of flies were reported as both males and females during their mark–recapture history. These flies were included in the analysis by Matlock *et al.* (1993), but have been omitted from the current analysis. The numbers of flies recaptured in Table 1 also differ from those reported in Matlock *et al.* (1993). Recaptures made on the same day as the initial capture, mark and release were counted by Matlock *et al.* (1993), but eliminated from the current analysis because parameters were estimated on a 1-day timescale (see below). In addition, the reduction in the number of flies released in the current study caused a concomitant reduction in the number of recaptures.

Statistical analyses

Mark–release–recapture analyses were conducted with the public domain software Program MARK (White & Burnham, 1999) available at http://www.warnercnr.colostate.edu/~gwhite/mark/mark.htm. Remaining statistical analyses were conducted in SAS Version 9.1 (SAS Institute Inc., Cary, NC, U.S.A.). For each study, estimates of daily recruitment f, survivorship ϕ, population growth rate λ, and the daily *per capita* probability of recapture of marked flies, p, were calculated by fitting the Pradel mark–release–recapture model to data (Pradel, 1996). All parameters were estimated for a 1-day timescale. The Pradel model defines recruitment rate, f, as the number of new individuals added to the population, per individual, per day and population growth rate, λ, as the ratio $\lambda = N_{t+1}/N_t$, where N_t and N_{t+1} are the population sizes at days t and $t+1$, respectively. Parameters were fit separately for males and females and were assumed to be constant (time and age invariant) within each dataset, but to vary among datasets. The Pradel model cannot fit age-specific survival rates (Pradel, 1996; Franklin, 2001). Although evidence from laboratory studies suggests that survivorship may be age-specific (Spates & Hightower, 1967, 1970; Davis & Camino, 1968; Milward de Azevedo *et al.*, 1992), survivorship can be closely approximated by constant survival rates. Furthermore, field estimates for Studies 1–3 (Parker & Welch, 1992 [Fig. 7]; Parker *et al.*, 1993 [Fig. 8]) are consistent with constant survivorship. Thus, Pradel estimates assuming age-independence of ϕ should be approximately valid. Recapture rates and the sex ratios of flies captured vary among attractants (Parker & Welch 1991a, 1991b). Two attractants were used simultaneously in four of the eight mark–recapture studies analysed (Table 1). The Pradel model is only able to fit one recapture probability per sex and thus it was not possible to estimate separate recapture probabilities for the different attractants. Hence, the estimated recapture probabilities represent averages over the different attractants used in each study. To fit all four parameters, two equivalent formulations of the Pradel model (parameterized in terms of f, ϕ and p, and λ, ϕ and p, respectively) were fit.

Goodness-of-fit

The Pradel model assumes that different individuals in the marked population are statistically independent and that f, ϕ, λ and p are homogenous (i.e. do not vary among individuals). Violation of these assumptions or biologically unrealistic model structure can cause the model to fail to adequately fit the data (Burnham *et al.*, 1987; Lebreton *et al.*, 1992). The Cormack–Jolly–Seber mark–recapture model (Cormack, 1964; Jolly, 1965; Seber, 1965) is related to the Pradel model but is formulated in terms of parameters ϕ and p alone. Adequate goodness-of-fit of the Cormack–Jolly–Seber model (under the same assumptions of independence and homogeneity described above) implies proper fit for the Pradel model also, because the lack of fit of the Pradel model is a function of the recaptures portion of the likelihood, which is equal to the likelihood of the Cormack–Jolly–Seber model. Thus, in Program MARK, goodness-of-fit of the Pradel model is assessed by evaluating the goodness-of-fit of the proxy Cormack–Jolly–Seber model.

The record of captures and recaptures for each individual recorded in a mark–recapture dataset constitutes its encounter

Table 1. Mark–release–recapture datasets.

Site/Study	Position*	Dates	Holdridge life zone†	Season	Attractant	Flies marked	Flies recaptured	Publications
EJN‡								
1	10°19.399′ N 85°8.447′ W	23/01/1989; 19/03/1989	Tropical dry forest	Dry	Liver	♀ 1809; ♂ 190	♀ 1009; ♂ 18	Parker & Welch (1992); Matlock *et al.* (1996) Study 1§
2	10°20.093′ N 85°8.344′ W	01/09/1989; 30/10/1989	Tropical dry forest	Wet	Liver and sheep	♀ 2269; ♂ 165	♀ 646; ♂ 7	Parker *et al.* (1993); Matlock *et al.* (1996) Study 2§
3	10°20.105′ N 85°8.296′ W	23/01/1990; 23/03/1990	Tropical dry forest	Dry	Liver and sheep	♀ 1149; ♂ 136	♀ 483; ♂ 19	Parker *et al.* (1993); Matlock *et al.* (1996) Study 3§
4	10°19.180′ N 85°7.929′ W	18/04/1990; 11/01/1991	Tropical dry forest	Wet	Liver	♀ 3582; ♂ 289	♀ 656; ♂ 23	Matlock *et al.* (1996) Study 4§
Montezuma¶								
5	10°39.970′ N 85°3.779′ W	22/10/1991; 27/01/1992	Tropical premontane wet forest	Wet	Liver and SWL IV	♀ 1323; ♂ 298	♀ 268; ♂ 23	Matlock *et al.* (1996) Study 5§
6	10°39.936′ N 85°3.765′ W	27/01/1992; 03/04/1992	Tropical premontane wet forest	Dry	Liver and SWL IV	♀ 485; ♂ 205	♀ 70; ♂ 25	
7	10°40.413′ N 85°4.006′ W	06/04/1992; 11/12/1992	Tropical premontane wet forest	Wet	Liver	♀ 4380; ♂ 729	♀ 703; ♂ 39	Matlock *et al.* (1996) Study 6§
8	10°40.098′ N 85°3.673′ W	11/01/1993; 31/05/1993	Tropical premontane wet forest	Dry	Liver	♀ 2097; ♂ 467	♀ 441; ♂ 46	Matlock *et al.* (1996) Study 7§

*Mean latitude and longitude of observation stations.
†See text for references.
‡Enrique Jimenez Nuñez Experiment Station of the Costa Rican Ministry of Agriculture, 21 km south of Cañas, Guanacaste Province, Costa Rica.
§Study number in Matlock *et al.* (1996).
¶Hacienda Montezuma, 3 km southwest of Rio Naranjo, Guanacaste Province, Costa Rica.

history. This encounter history is typically represented as a binary string of 1s (for captures and recaptures) and 0s (for samples where the animal was not observed), where the number of digits in the string is equal to the total number of samples collected (Burnham *et al.*, 1987; Lebreton *et al.*, 1992). For example, the encounter history '010101' represents an animal captured for the first time in the second sample of a six-sample survey, recaptured in the fourth and sixth samples, but not observed in the odd-numbered samples. The maximum number of parameters, k, that can be fit with a mark–recapture dataset is equal to s, the number of unique encounter histories observed. Models for which $k = s$ are said to be saturated (full rank) (Burnham *et al.*, 1987; Lebreton *et al.*, 1992), whereas for $k < s$ (as is true for the models fit in this study), models are classified as unsaturated or reduced.

Lack of fit of unsaturated models is measured by the Deviance:

$$\text{Deviance} = 2\ln L_{sat}(\hat{\theta}_{sat}) - 2\ln L(\hat{\theta})$$

where $L_{sat}(\hat{\theta}_{sat})$ and $L(\hat{\theta})$ are the likelihoods of the saturated and unsaturated models, respectively, where both likelihoods are evaluated at the maximum likelihood estimates of their respective parameter vectors $\hat{\theta}_{sat}$ and $\hat{\theta}$ (Lebreton *et al.*, 1992). Provided the reduced model is structurally sound biologically, the Deviance is asymptotically distributed central χ^2 for large sample sizes, with $v = v_{sat} - k$ degrees of freedom (d.f.), where $v_{sat} = s - w$ is the d.f. associated with the saturated model, w is the number of samples in which at least one animal was caught and marked for the first time and k is the number of parameters fit in the reduced model. When the parameters are fit for more than one group (e.g. males and females), the log-likelihoods and d.f. are tallied independently for each group and summed.

The variance inflation factor, $\hat{c}$, is defined as:

$$\hat{c} = \frac{\text{Deviance}}{v}$$

If the assumptions of structural adequacy, statistical independence and homogeneity of recapture and survival rates are met, $\hat{c} = 1$, otherwise $\hat{c} > 1$. Thus, $\hat{c}$-values ≈ 1 imply satisfactory model fit, whereas $\hat{c} > 3$ implies a significant lack of fit between model and data (Lebreton *et al.*, 1992). We estimated $\hat{c}$ using the median $\hat{c}$ simulation procedure contained within Program MARK; $\hat{c}$-values from five repetitions of median $\hat{c}$ procedure with 49 design points and three replicates at each design point were averaged and the standard error calculated as SE $= s_{\hat{c}}/\sqrt{5}$, where $s_{\hat{c}}$ is the mean standard deviation (SD) of the $\hat{c}$ estimates for the five repetitions.

Parameter adjustment

If $\hat{c}$ is > 1, the variances of parameter estimates will be inflated by a factor $\hat{c}$. Hence, the SE of parameter estimates should be adjusted to SE $=$ SE$(\hat{\theta})\sqrt{\hat{c}}$ (Lebreton, 1992). Thus, the SE of parameter estimates f, ϕ, λ and p were adjusted by multiplying by $\sqrt{\bar{\hat{c}}}$, where $\bar{\hat{c}}$ is the average of the median $\hat{c}$-values generated by the median $\hat{c}$ procedure.

Meta-analysis

To investigate whether the estimates f, ϕ and λ varied between sexes or were influenced by Holdridge life zone or the season in which the data were collected, the parameter estimates from the eight datasets were analysed with factorial, weighted analysis of variance, the weight for each observation being $1/\text{SE}^2$, where SE is the standard error of the parameter estimate calculated by MARK (Table 2, discussed in Results and Discussion). A simple additive model was fit, because insufficient d.f. were available to estimate all interaction terms. A second weighted ANOVA was conducted to determine whether recapture probabilities, p, varied with sex, season or type of attractant (liver, liver + sheep, liver + SWL IV).

Results and Discussion

Parameter estimates, SEs and 95% confidence intervals (CIs) are displayed in Table 2. Median $\hat{c}$ measures of goodness-of-fit are displayed in Table 3. All median $\hat{c}$-values were ≈ 1, suggesting that the Pradel model adequately fit the data. Parameter SEs were adjusted by the mean median $\hat{c}$ estimates, $\bar{\hat{c}}$.

Meta-analysis

Holdridge life zone and sex had no significant effects on f, ϕ and λ. However, as the numbers of males released and recaptured were much smaller than those for females, statistical power for detecting differences between sexes was limited. The effect of season on ϕ was nearly significant ($F_{1,13} = 3.48$, $0.05 < P < 0.1$), with daily survivorship being 3% greater in the wet than the dry season, corresponding to extension of mean lifespan by approximately 1 day during the wet season. Recapture probability, p, varied with sex ($F_{1,13} = 18.17$, $P < 0.001$) and season ($F_{1,13} = 14.66$, $P < 0.005$) and the combination of the two variables explained 74% of the variation in p. Females were nearly six times as likely to be recaptured as males (Table 2) and flies were three times as likely to be recaptured in the dry than the wet season (weighted $p \pm$ SE: dry 0.153 ± 0.036); wet 0.048 ± 0.013). Attractant had no significant impact on recapture probability ($F_{2,13} = 0.45$, $P > 0.05$). It is difficult to assess differences among the three attractants, however, because sheep and SWL IV were always used in combination with liver. Weighted mean parameter estimates (the weighting factor being 1SE^2 as in weighted ANOVA), SEs and 95% CIs are presented in Table 2. Sexes were combined for weighted mean parameter estimates of f, ϕ and λ; separate weighted means are presented for males and females for p.

Comparison with published life history parameter estimates

Recruitment rate, survivorship and population growth are interrelated by the equation: $\lambda = \phi + f$ (Pradel, 1996). Hence, comparisons with published life history parameters are restricted to ϕ and λ. The Appendix gives estimates of the 10 parameters needed to estimate λ: ϕ_e, ϕ_l and ϕ_p, the probability of surviving the egg, larval and pupal phases, ϕ_a, the adult daily

Table 2. Pradel model parameter estimates.

Parameter	Study	Sex	Estimate	Standard error	95% lower confidence interval	95% upper confidence interval
ϕ	1	♀	0.801	0.005	0.791	0.811
		♂	0.761	0.045	0.662	0.838
	2	♀	0.822	0.006	0.809	0.834
		♂	0.772	0.094	0.544	0.906
	3	♀	0.762	0.008	0.745	0.778
		♂	0.821	0.042	0.725	0.889
	4	♀	0.801	0.006	0.789	0.813
		♂	0.759	0.042	0.667	0.832
	5	♀	0.700	0.015	0.670	0.728
		♂	0.783	0.044	0.685	0.857
	6	♀	0.700	0.028	0.643	0.752
		♂	0.709	0.045	0.613	0.790
	7	♀	0.819	0.006	0.807	0.829
		♂	0.879	0.018	0.838	0.911
	8	♀	0.749	0.010	0.730	0.767
		♂	0.766	0.031	0.701	0.821
	Weighted $\bar{x}$*		0.798	0.008	0.780	0.815
f	1	♀	0.166	0.005	0.156	0.176
		♂	0.201	0.045	0.127	0.304
	2	♀	0.175	0.006	0.162	0.187
		♂	0.208	0.094	0.079	0.444
	3	♀	0.213	0.008	0.197	0.230
		♂	0.120	0.041	0.060	0.227
	4	♀	0.207	0.006	0.195	0.219
		♂	0.249	0.042	0.175	0.340
	5	♀	0.284	0.015	0.257	0.314
		♂	0.197	0.044	0.125	0.297
	6	♀	0.283	0.028	0.232	0.341
		♂	0.274	0.045	0.194	0.371
	7	♀	0.187	0.006	0.176	0.198
		♂	0.126	0.018	0.094	0.167
	8	♀	0.254	0.010	0.236	0.273
		♂	0.245	0.031	0.190	0.310
	Weighted $\bar{x}$*		0.193	0.008	0.175	0.211
λ	1	♀	0.967	0.002	0.964	0.970
		♂	0.962	0.005	0.951	0.971
	2	♀	0.996	0.001	0.992	0.998
		♂	0.979	0.005	0.966	0.988
	3	♀	0.975	0.002	0.971	0.979
		♂	0.942	0.007	0.926	0.954
	4	♀	1.008	0.0003	1.008	1.009
		♂	1.008	0.001	1.006	1.010
	5	♀	0.984	0.001	0.982	0.986
		♂	0.980	0.002	0.975	0.984
	6	♀	0.984	0.002	0.978	0.988
		♂	0.983	0.004	0.974	0.989
	7	♀	1.005	0.0002	1.005	1.006
		♂	1.005	0.0005	1.004	1.007
	8	♀	1.003	0.001	1.002	1.004
		♂	1.011	0.001	1.008	1.013
	Weighted $\bar{x}$*		1.005	0.002	1.001	1.008

Continued

Table 2. Continued.

Parameter	Study	Sex	Estimate	Standard error	95% lower confidence interval	95% upper confidence interval
p	1	♀	0.234	0.006	0.222	0.247
		♂	0.038	0.012	0.020	0.069
	2	♀	0.100	0.005	0.091	0.110
		♂	0.012	0.008	0.003	0.044
	3	♀	0.329	0.012	0.307	0.353
		♂	0.033	0.012	0.016	0.069
	4	♀	0.078	0.004	0.071	0.086
		♂	0.035	0.011	0.019	0.063
	5	♀	0.163	0.012	0.140	0.188
		♂	0.034	0.010	0.019	0.061
	6	♀	0.102	0.016	0.075	0.137
		♂	0.081	0.021	0.048	0.134
	7	♀	0.061	0.003	0.056	0.067
		♂	0.011	0.002	0.007	0.017
	8	♀	0.130	0.008	0.116	0.146
		♂	0.049	0.011	0.032	0.075
	Weighted $\bar{x}$	♀	0.099	0.023	0.045	0.153
		♂	0.017	0.005	0.005	0.028

*Sexes combined because there was no significant difference between ♀♀ and ♂♂.

survivorship, t_e, t_l, t_p, t_n and t_g, the durations of the egg, larval, pupal and nulliparous (preoviposition) phases and gonotrophic cycle, respectively, and m, the clutch size.

Published estimates of mean and median longevity from sources in the Appendix were converted to daily survivorship probabilities by assuming constant mortality. Under this assumption, longevity (age at the time of death), x, is exponentially distributed:

$$f(x) = \mu e^{-\mu x} \quad (1)$$

with mean $\bar{x} = \frac{1}{\mu}$ and median $x_{\frac{1}{2}} = \frac{\ln(2)}{\mu}$. Survivorship to age x is then given by $\exp\left(-\frac{x}{\bar{x}}\right)$ and $\exp\left(-\ln(2)\frac{x}{x_{\frac{1}{2}}}\right)$, and daily survivorships become $\exp\left(-\frac{1}{\bar{x}}\right)$ and $\exp\left(-\frac{\ln(2)}{x_{\frac{1}{2}}}\right)$, respectively. When survivorships, l_x, were reported for periods of $x > 1$ day, they were converted to a 1-day timescale by taking $\phi_a = (l_x)^{\frac{1}{x}}$. No attempts were made to convert SEs or other variability measures to the daily timescale.

Table 3. Median $\hat{c}$ goodness-of-fit statistics for Pradel model.

Study	Median $\hat{c}$	SEM
1	1.069	0.004
2	1.373	0.005
3	1.419	0.005
4	1.009	0.005
5	1.013	0.005
6	1.012	0.006
7	1.033	0.003
8	1.146	0.004

SEM, standard error of the mean.

Survivorship

Using field data on the age structure of an NWS adult population, Thomas & Chen (1990) estimated $\phi_a = 0.813$, which was similar to the Pradel estimates in Table 2. Estimates of ϕ_a for cage (Davis & Camino, 1968) and laboratory studies (remaining references in the Appendix) all exceeded 0.9, being universally higher than the Pradel estimates (Table 2). Three (mutually compatible) explanations for the differences between the field and laboratory cage estimates are: (a) marking tags were lost and/or burdened marked flies, increasing mortality; (b) flies in the field were lost to emigration in addition to mortality, and (c) the adult flies experienced higher mortality in the field than in either the laboratory or cages (e.g. as a result of predation or other sources of mortality not experienced in confinement). Thomas & Chen's (1990) ϕ_a estimate was based on age structure determination by pteridine and involved no marking. Thus, loss of tags cannot explain the agreement between these authors' results and the mark–recapture estimates reported here. In a separate analysis, data for Studies 1–8 were fit to a diffusion model which simultaneously estimates the effects of mortality and emigration separately (R. B. Matlock & R. Skoda, in preparation). Mean (95% CIs) of survivorships for this analysis were: ♀♀ 0.947 (0.910–0.986); ♂♂ 0.886 (0.851–0.922). These estimates are higher than those in Table 2, suggesting that emigration may account for the difference between the Pradel

and laboratory cage results. Predation or other sources of mortality may also have diminished survivorship. Thomas (1991) reported that screwworm adults in field cages were under constant threat from ants, especially while feeding on nectar.

Population growth rate

The λ value estimated by the Pradel model is the growth rate of the adult age class, which is not equal to the growth rate of the entire population unless the population is at stable age distribution. Once the age distribution is stationary, all age classes (and the population as a whole) grow at the same rate, λ. Insect populations with long growing seasons are more likely to be at stable age distribution (Taylor, 1979). Thus, stable age distribution is a reasonable assumption for NWS, which is active throughout the year in tropical climates and has no diapause period. Under stable age distribution, λ may also be calculated from Euler's equation:

$$\sum_{x=0}^{\infty} \lambda^{-x} l_x m_x = 1 \tag{2}$$

where l_x and m_x are the probability of survival to age x and the mean fecundity at age x, respectively. To estimate λ for NWS females from parameter values in the Appendix, we make the following assumptions: (a) that daily adult survivorship takes the constant value ϕ_a throughout adulthood, including the preoviposition period; (b) that fecundity $m_x = ihm/2$, where i is the probability the female has mated and is inseminated, h is the probability she finds a suitable host and m is the constant, mean clutch size ($m/2$ being the number of female offspring per oviposition), and (c) that females oviposit at a fixed time interval following the initial oviposition, t_g being the length of the gonotrophic cycle. Given these assumptions, equation (2) becomes:

$$\sum_{x=0}^{\infty} \lambda^{-\left(t_e+t_l+t_p+t_n+t_g x\right)} \phi_e \phi_l \phi_p \phi_a^{t_n} \phi_a^{t_g x} ih \frac{m}{2} = 1 \tag{3}$$

Equation (3) sums to:

$$\frac{\phi_e \phi_l \phi_p \phi_a^{t_n} ih \dfrac{m}{2} \lambda^{t_g - \left(t_e + t_l + t_p + t_n\right)}}{\lambda^{t_g} - \phi_a^{t_g}} = 1, \tag{4}$$

λ being given by the single real root of (4). To calculate numerical estimates of λ from equation (4) and parameter values in the Appendix, we make the following assumptions with regard to parameter ranges:

1. Egg development time, t_e, is 1 day (Laake *et al.*, 1936; Smith, 1960; Baumhover, 1966; Davis & Camino, 1968) and survivorship through the egg phase is $0.9 \leq \phi_e \leq 1.0$ (Laake *et al.*, 1936; Baumhover, 1966; Davis & Camino, 1968; Taylor, 1988; Taylor & Mangan, 1987; Friese, 1992).
2. Based on estimates for larvae reared on wounds in sheep (Laake *et al.*, 1936; Davis & Camino, 1968; Rubink, 1987; Thomas & Pruett, 1992), the larval period, t_l is 5–7 days and survivorship through the larval phase is $0.5 \leq \phi_l \leq 0.9$.
3. Pupal development time and survivorship are strongly temperature-dependent and estimates in the Appendix were highly variable. Assumed ranges for prepupal (crawl-off) + pupal development time and survivorship are: 6 days $\leq t_p \leq$ 10 days and $0.2 \leq \phi_p \leq 0.99$.
4. Adult daily survivorship ranges from: $0.8 \leq \phi_a \leq 0.98$ (range of estimates in the Appendix).
5. The proportion of females mated in field studies is generally high (Guillot *et al.*, 1977a, 1977b; Parker & Welch, 1991a; Parker *et al.*, 1993). Parker & Welch (1991a) reported that it was 100% for gravid females. Thus, we assume that $i = 1$ for females that survive through the nulliparous phase.
6. The length of the preoviposition (nulliparous) period, t_n, is 4–7 days, commensurate with tropical temperatures (Krafsur *et al.*, 1979; Thomas & Chen, 1990; Thomas, 1993).
7. The length of the gonotrophic cycle, t_g is 3 days, consistent with estimates in tropical forest habitats (Thomas & Mangan, 1989; Parker & Welch, 1991b; Parker *et al.*, 1993).
8. The probability of host location, h, cannot be estimated from current published literature. Therefore, we assume $h = 1$ (i.e. that hosts are always found).
9. Mean clutch size, $m = 200$, based on Thomas & Mangan's (1989) estimate for egg masses oviposited on wounds in tropical forest habitat in Mexico and Belize, similar to the habitats in Studies 1–8.

Estimates for λ from Table 2 ranged from 0.942 to 1.011, including both growing and declining populations, the weighted mean estimate being $\lambda = 1.005 \pm 0.002$. Under density-independent growth conditions such as those likely to prevail during outbreaks in screwworm-free territory, this would correspond to a doubling time of $t_2 = \ln(2)/\ln(\lambda) = 139$ days. By contrast, the estimate of λ from (4) for the least favourable parameter values for population growth specified in the assumed ranges 1–9 was 1.05, implying a population doubling time of 14 days. This was substantially greater than even the largest 95% CI for the Pradel model estimates in Table 2, 1.013 (♂♂, Study 8), with a doubling time of 54 days. Parameter values typical of the screwworm production facility in Mexico are: $\phi_e = 0.9$ (Taylor, 1988; Friese, 1992; Taylor & Mangan, 1987), $t_e \leq 1$ (all references in the Appendix), $\phi_l = 0.9$ (Taylor & Mangan, 1987), $t_l = 6$ days (Taylor & Mangan, 1987; Taylor *et al.*, 1991), $\phi_p = 0.95$ (Taylor & Mangan, 1987; Taylor, 1988), $t_p = 9$ days (Thomas, 1989), $\phi_a = 0.95$ (Peterson *et al.*, 1983, 1987), $t_n = 6$ days (Thomas, 1993), $t_g = 3$ days (Thomas & Mangan, 1989; Parker & Welch, 1991b; Parker *et al.*, 1993) and $m = 250$ (Thomas, 1993). For these values of the parameters $\lambda = 1.25$, corresponding to a potential doubling time of 3 days under optimal conditions.

Assuming no net movement of the populations in Studies 1–8, errors in ϕ and f resulting from emigration and immigration should tend to cancel one another out, affecting the Pradel model estimate of $\lambda = \phi + f$ less than that of ϕ. Hence, unlike adult survivorship, emigration seems less likely to explain the differences between Pradel and equation (4) estimates of λ. Many of the laboratory estimates of survivorship in the Appendix were calculated for near optimal conditions and probably overestimate rates in the field. Thomas (1989) reported, for

example, that larvae that pupated in soil exposed to direct sunlight experienced 77% mortality. Development times are also typically shorter under constant temperature than in natural fluctuating thermal environments. Both could have contributed to the disagreement between the Pradel and equation (4) estimates. The least justifiable of assumptions 1–9 is that host location is certain. At stable age distribution, the proportion of the adult population that is of reproductive age, c_r, is given by:

$$c_r = \frac{\phi_e \phi_l \phi_p \sum_{x=t_n}^{\infty} \lambda^{-t_e - t_l - t_p - x} \phi_a^x}{\phi_e \phi_l \phi_p \sum_{x=0}^{\infty} \lambda^{-t_e - t_l - t_p - x} \phi_a^x} = \left(\frac{\phi_a}{\lambda}\right)^{t_n}$$

If the probability of finding a host, $h \approx 1$, then most females of reproductive age should be parous. In this case, c_r should be approximately equal to the proportion of parous females in the population. Taking $\lambda = 1.005$ (Table 2) and $0.8 < \phi_a < 0.9$ (Table 2, and Matlock and Skoda, in preparation), then $0.25 < c_r < 0.52$. Parker & Welch (1991a, 1991c, 1992) and Parker *et al.* (1993) reported parity fractions at Enrique Jiminez Nuñez Experiment Station, the site of Studies 1–4, in the range of 31–50%, which is consistent with this estimate. Female screwworm reabsorb eggs when they are unable to oviposit (Adams & Reinecke, 1979). Parker & Welch (1991a) reported that 2.9–6.8% of females contained eggs undergoing reabsorption, suggesting that the number of females that failed to find hosts was small. Thus, other life history traits, such as pupal survival, may be more likely to account for differences between Pradel estimates and equation (4).

Doubling times for λ estimates in Table 2 ranged from 54 to 139 days, whereas the maximum doubling time calculated from the life history parameters in the Appendix was 14 days. Thus, it seems very plausible that screwworm outbreak populations could double in 1–2 months or faster. Given the difficulty of detecting sparse outbreak populations, the presence of undetectable phases in the life-cycle (e.g. pupae), and the likely inexperience with screwworm in countries where NWS is introduced (e.g. Libya; Krafsur & Lindquist, [1996]), screwworm populations could easily double several times before discovery, underscoring the need for effective quarantine measures to protect livestock industries worldwide from the catastrophic costs of screwworm introduction.

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Conflicts of interest

All authors declare no conflicts of interest.

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Appendix. Screwworm, *Cochliomyia hominivorax*, life history parameters.

Parameter	Source	Value	Notes
Proportion eclosing (ϕ_e)	Crystal (1967a)	0.873[1], 0.86[2], 0.887[3], 0.858[4], 0.851[5], 0.82[6]	[1]Fig. 3; [2]Fig. 4; [3]Fig. 5; [4]Fig. 6; [5]Fig. 7; [6] p 447
	Devaney & Garcia (1975) Table 2	0.886[1], 0.903[2], 0.922[3], 0.919[4], 0.864[5], 0.863[6], 0.936[7]	Strain: [1]OMS; [2]NMS1; [3]NMS2; [4]NMS; [5]PRN; [6]CTX; [7]RF; 27 ± 2° C, 46–82% RH
	Friese (1992) Table 2	0.923	
	McInnis *et al.* (1983) Table 3	0.920[1], 0.829[2]	[1,2]Means for two strains
	Taylor (1988) Table 1	Mean ± SD: 0.896 ± 0.0221[1], 0.913 ± 0.0155[2], 0.832 ± 0.0577[3], 0.885 ± 0.0283[4]	Diet: [1]Water lock; [2–4]Carageenan 25%, 37%, 50%
	Taylor & Mangan (1987) Table 1	Mean ± SD: 0.873 ± 0.0652[1], 0.853 ± 0.0538[2]	Diet: [1]Meat, [2]Gel
Egg development time (t_e)	Baumhover (1966) p 242	16 h	
	Davis & Camino (1968) Table 1	8–12 h	For temperatures, see pupal development time
	Laake *et al.* (1936) p 4	11–21.5 h[1], 9.2 h[1], 13.9 h[3]	[1]On wounds; [2,3]100% RH, 37.2° C and 28.9° C
	Parman (1945) p 73	8–24 h	
	Smith (1960) p 1112	12–14 h	
Larval survivorship (ϕ_l)	Thomas & Pruett (1992) Table 1	0.630 ± 0.142[1], 0.585 ± 0.131[2], 0.618 ± 0.110[3], 0.559 ± 0.028[4]	In wounds on sheep. Mean ± SD of four replicates each of four treatments: 25[1], 50[2], 100[3] and 200[4] larvae per host. Survival measured after 4 days of infestation
	Laake & Smith (1939) p 340	0.820[1], 0.797[2], 0.806[3], 0.902[4]	In wounds in cavies (presumably guinea pigs). [1]Initial infestation; [2]first re-infestation; [3]second re-infestation; [4]third re-infestation
	Milward de Azevedo *et al.* (1992) Table 4	0.520	38° C
	Rubink (1987) p 602	0.050–0.950[1], 0.035–0.985[2]	Larvae reared in wounds in sheep. [1]0–19 larvae per host; [2]23–193 larvae per host
	Taylor & Mangan (1987) Tables 1 and 4	0.632[1], 0.880[2]	Diet: [1]Meat, [2]Gel. Calculated as: $l_L = \frac{n}{1{,}350 \times l_E \times 9}$ where n is number of larvae in Table 4, 1350 is number of larvae in 50 mg eggs, l_E is the proportion of eggs (see above) and 9 is number of lines examined

Continued

Appendix. Continued.

Parameter	Source	Value	Notes
Larval development time (t_l)	Baumhover *et al.* (1966) p 545	4–6 days	
	Davis & Camino (1968) Table 1	7 days	In wounds in sheep. See notes for adult survivorship for approximate minimum ambient temperatures
	Hightower *et al.* (1971) p 1475	3.75–5.04 days (90.1–120.9 h)	Horsemeat diet; 27 ± 2° C
	Laake *et al.* (1936) p 24	4.3–7.3 days (103.8–174.5 h)[1], 3.4–10.0 days (82–239 h)[2]	[1]Sheep; [2]cattle
	Milward de Azevedo *et al.* (1992) Table 2	6.35 ± 0.63	38°C
	Rubink (1987) p 602	4.97 ± 0.25 days[1], 5.70 ± 0.57 days[2]	Mean ± SD of median crawl-off time for larvae reared in wounds in sheep. [1]0–19 larvae per host; [2]23–193 larvae per host
	Taylor & Mangan (1987) Table 3	6.0 ± 1.1 (143.2 h)[1], 6.5 ± 0.9 (156.5 h)[2], 6.5 ± 0.9 (154.8 h)[3]	Reared on artificial diets: [1]Meat; [2]Gel G-400; [3]Gel G-100. 35–39 °C, 70–75% RH
	Taylor *et al.* (1991) p 5	5.18 ± days (124.4 h)[1], 5.27 days (126.4 h)[2]	Reared on artificial diets: [1]hydroponic; [2]water lock; 35–39 °C, 70–75% RH
Pupal survivorship (ϕ_p)	Baumhover (1963) p 475	0.955[1], 0.960[2], 0.937[3]	[1]32.2 °C, 85% RH; [2]32.2 °C with 2-inch sand cover; [3]26.7 °C, 85% RH Prepupal + pupal period
	Cardoso *et al.* (1992) Table 2	0.588[1], 0.55[2], 0.638[3], 0.813[4], 0.775[5], 0.325[6], 0.613[7]	Emergence rates for seven pupation substrates. Prepupal + pupal period
	Deonier (1945) p 93, Table 3	0.360[1], 0.460[2], 0.220[3], 0.880[4], 0.380[5], 0.660[6], 0.200[7], 0.220[8], 0.700[9]	[1–3]Range soil, 9.5 °C, 11.8 °C, 12.8 °C; [4–6]cultivated soil, 13.3 °C, 11.9 °C, 12.4 °C; [7–9]manure, 10.2 °C, 11.8 °C, 12.4 °C. Prepupal + pupal period
	Flitters & Benschoter (1971) p 66, Table 1	0.17[1], 0.07[2], 0.00[3]	[1]p 66, $\overline{T}$ = 12.2 °C; [2]p 66, $\overline{T}$ = 10.0 °C; [3]Table 1. $\overline{T}$ = 6.5 °C. Pupal period only
	Friese (1992) Table 1	0.919	
	Hightower *et al.* (1971) Table 4	0.934	27 °C, pupal period only
	McGinnis *et al.* (1983) Table 3	Mean ± SD: 0.923 ± 0.0061[1], 0.935 ± 0.0112[2], 0.918 ± 0.0044[3], 0.895 ± 0.0156[4], 0.827 ± 0.0393[5], 0.797 ± 0.0720[6], 0.800 ± 0.0682[7], 0.780 ± 0.0597[8]	[1–8]Means for eight selected strains, pupal period only
	Milward de Azevedo *et al.* (1992) Table 4	0.889	25 °C, 60% RH. Pupal period only
	Taylor (1988) Table 1	0.988[1], 0.960[2], 0.959[3], 0.949[4]	Diet: [1]water lock; [2–4]Carageenan 25%, 37%, 50%. Pupation in sawdust
	Taylor & Mangan (1987) Table 1	0.909[1], 0.942[2]	Diet: [1]Meat; [2]Gel
	Taylor *et al.* (1991) Table 1	0.930[1], 0.864[2], 0.961[3], 0.956[4]	[1]Hydroponic (experimental); [2]hydroponic (production); [3] water lock 2g; [4]water lock 3g. 30.0 °C, 60% RH, pupal period only
	Thomas (1989) Table 2	0.915[1], 0.229[2], 0.597[3], 0.753[4]	[1]Indoors (control); [2]exposed, $\overline{T}_{min}$ = 20.2 °C, $\overline{T}_{max}$ = 33.7 °C; [3]semi-shade; [4]shade, $\overline{T}_{min}$ = 22.2 °C, $\overline{T}_{max}$ = 32.5 °C. Pupal period only

Continued

Appendix. Continued.

Parameter	Source	Value	Notes
Pupal development time (t_p)	Cardoso *et al.* (1992) Table 2	Mean (95% CI): 9.29 (9.03–9.55) days, 9.26 (8.97–9.55) days, 9.19 (8.99–9.39) days, 9.65 (9.15–10.15) days, 9.25 (8.9–9.6) days, 10.14 (9.74–10.54) days, 9.38 (8.79–9.97) days	Pupation time for seven pupation substrates. 27.0 °C, 65 ± 10% RH
	Davis & Camino (1968) Table 1, Fig. 1	12 days[1], 12 days[2], 12 days[3], 9 days[4], 9 days[5]	[1–3]See corresponding notes for adult survivorship for approx. minimum ambient temperatures; [4,5]greenhouse 5–8 °C warmer than ambient temperatures[1–3]
	Deonier (1945) p 93, Table 3	34 days[1], 50 days[2], 47.5 days[3], 39 days[4], 59.5 days[5], 44 days[6], 36.5 days[7], 47.5 days[8], 41.5 days[9]	See notes for pupal survivorship
	Laake *et al.* (1936) p 19	5.93 days (142.2 h)[1], 31.7 days (760.4 h)[2]	[1]34.4 °C; [2]15.0 °C
	Milward de Azevedo *et al.* (1992) Table 4	8–10 days	25 °C
	Parman (1945) p 70	Approximate development times: 10 days[1], 15 days[2], 30 days[3]	[1]23.9 °C; [2]21.1 °C; [3]15.5 °C
	Smith (1960) p 1111	6.50–9.34 days	26.7 °C, 50–60% RH, pupal period only
	Thomas (1989) p 323	8–9 days	See notes for pupal survivorship
Adult survivorship (ϕ_a)	Adams (1979) Fig. 2	0.965 ± 0.000135	Weighted mean of daily survivorship between 12.8 °C and 37.8 °C (survivorship was nearly constant over this temperature range), weighted by number of flies assayed
	Baumhover (1965) p 545	♂♂ 0.950, ♀♀ 0.980	74% and 46% survivorship @ 15 days, 26.7 °C for ♀♀ and ♂♂caged alone, corresponding to ♀♀ $\phi = 0.74^{\frac{1}{15}} = 0.980$ and ♂♂: $\phi = 0.46^{\frac{1}{15}} = 0.950$
	Crystal (1967b) Table 1	0.957[1], 0.966[2], 0.976[3], 0.957[4], 0.975[5], 0.980[6]	[1–3]♂♂mixed sexes, unisexual, individual, respectively; [4–6]♀♀mixed sexes, unisexual, individual, respectively. 27 °C, 60% RH; calculated from mean longevity (see text)
	Davis & Camino (1968) Fig. 1A–C	0.965 ± 0.0111[1], 0.917 ± 0.0163[2], 0.905 ± 0.0225[3]	ϕ estimated by fitting model: $l(t)=\phi^t$ with non-linear regression, where $l(t)$ is survivorship to time t. [1]Fig. 1A. $\overline{T}_{min}$ = 14 °C. Approximate $F_{1,6}$ = 57.83; P < 0.0005. [2]Fig. 1B. $\overline{T}_{min}$ = 13.9 °C. Approximate $F_{1,6}$ = 87.56; P < 0.0001. [3]Fig. 1C. $\overline{T}_{min}$ = 16.9 °C. Approximate $F_{1,6}$ = 63.74; P < 0.0005
	Devaney & Garcia (1975) Table 1	♂♂: 0.900[1], 0.914[2], 0.913[3], 0.930[4], 0.934[5], 0.943[6], 0.938[7]; ♀♀: 0.904[1], 0.908[2], 0.911[3], 0.924[4], 0.940[5], 0.930[6], 0.944[7]	Strain: [1]OMS, [2]NMS1, [3]NMS2, [4]NMS, [5]PRN, [6]CTX, [7]RF. 26.5 ± 1.5 °C, 20–68% RH; calculated from mean longevity (see text)

Continued

Appendix. Continued.

Parameter	Source	Value	Notes
	Hightower & Garcia (1972) Table 1 Controls	♂♂: 0.944, 0.944, 0.939; ♀♀: 0.953, 0.958, 0.956	33 °C; calculated from mean longevity (see text)
	Milward de Azevedo *et al.* (1992) Figs 7–10	0.911[1], 0.934[2], 0.924[3], 0.945[4]	ϕ estimated by fitting model: $l(t)=\phi^t$ with non-linear regression, where $l(t)$ is survivorship to time t. [1]Fig. 7: ♂♂, 27 °C, 60% RH; [2]Fig. 8: ♀♀, 27 °C, 60% RH; [3]Fig. 9: ♂♂, 25 °C, 60% RH; [4]Fig. 10: ♀♀, 25 °C, 60% RH
	Peterson *et al.* (1983) Table 1	0.973[1], 0.944[2]	[1]Fertile ♂♂; [2]sterile ♂♂. $\overline{T}_{min}$ = 24.4 °C. $\overline{T}_{max}$ = 27.4 °C. 54.6–74.1% RH; calculated from median longevity (see text)
	Peterson *et al.* (1987) p 132	0.965[1], 0.964[2]	[1]Fertile ♀♀; [2]fertile ♂♂. 27 °C, 65% RH, honey and water; calculated from median longevity (see text)
	Spates & Hightower (1967) Figs 1 and 2	♂♂: 0.931[1], 0.944[2], 0.947[3], 0.951[4], 0.957[5], 0.962[6]; ♀♀: 0.968[1], 0.968[2], 0.974[3], 0.976[4], 0.978[5], 0.979[6]	Six strains: [1]BR, [2]KC, [3]PR, [4]MC, [5]SC, [6]FL. BR is significantly different from the rest
	Spates & Hightower (1970) p 1382	0.961[1], 0.976[2], 0.962[3], 0.976[4]	♂♂: [1]Lab strain, [2]wild-type; ♀♀ [3]lab strain, [4]wild-type. 27 °C, 60% RH; calculated from median longevity assuming constant survivorship (see text)
	Thomas & Chen (1990) p 1426	0.813	Field estimate based on age structure. $\overline{T}_{min}$ = 22 °C (range 20–24 °C). $\overline{T}_{max}$ = 33 °C (range 32–37 °C)
Length of preoviposition period (t_n)	Adams (1979) Table 2	37.9 days (909 h)[1], 3.4 days (81 h)[2]	[1]15.6 °C; [2]32.2 °C
	Adams & Reinecke (1979) Table 4 (Stage 10)	6.33 days (152 h)[1], 3.33 days (80 h)[2]	[1]24 °C; [2]30 °C
	Hammack (1991) p 189	5 days	25 °C, 50% RH
	Hightower *et al.* (1972) Table 1	Mean ± SD: 4.8 ± 1.2 days, mode 4 days[1]; mean ± SD: 11.1 ± 1.1 days, mode 8 days[2]	[1]Lab strain; [2]wild-type. 24–26 °C, 35–60% RH
	Krafsur *et al.* (1979) p 477	17 days[1], 6 days[2,3], 8 days[4], 4 days[5]	Strain 001: [1]15 °C, [2]25 °C, [3]30 °C; [4]strain CTX, 25 °C; [5]Florida strain, 25 °C
	Laake *et al.* (1936) p 20	5–10 days	
	Thomas & Chen (1990) p 1425	4 days[1], 7 days[2]	[1] $\overline{T}_{min}$ = 22 °C (range 20–24 °C); [2] $\overline{T}_{max}$ = 33 °C (range 32–37 °C)
	Thomas (1993) Table 1	4–6 days[1], 8–10 days[2]	[1]30 °C; [2]22 °C
Length of gonotrophic cycle (t_g)	Hightower *et al.* (1972) p 229	5.4 days[1], 3.2 days[2], 3.0 days[3]	[1]Between first and second oviposition, wild-type; [2]between first and second oviposition, lab strain; [3]between succeeding ovipositions, both strains; 24–26 °C, 35–60% RH; 36–38 °C oviposition substrate
	Parker & Welch (1991b) p 1470 Parker *et al.* (1993) Fig. 10B	Mean 3.3 days (range 2–4 days) Mode 3 days (range 1–7 days)	♀♀ visiting wounded sheep. Wet season: 25.4 °C, 78% RH; dry season: 27.9 °C, 60.4% RH

Continued

Appendix. Continued.

Parameter	Source	Value	Notes
	Thomas & Mangan (1989) Fig. 2	3 days	
Clutch size (*m*)	Crystal & Meyners (1965) p 215	200–250	27 °C, 60% RH; 37 °C oviposition substrate
	Devaney & Garcia (1975) Table 2	290[1], 236[2], 255[3], 260[4], 214[5], 241[6], 266[7]	Strain: [1]OMS, [2]NMS1, [3]NMS2, [4]NMS, [5]PRN, [6]CTX, [7]RF. 27 ± 2 °C, 46–82% RH. Oviposition substrate 35 °C
	Laake *et al.* (1936) p 5	10–393	
	Milward de Azevedo *et al.* (1992) Table 4	227.44 ± 10.85	
	Spates & Hightower (1970) Fig. 5	307 ± 1.59[1], 331 ± 1.88[2]	[1]Laboratory strain; [2]wild-type
	Thomas (1993)	285.2 ± 17.0[1], 283.3[2], 277.7[3], 225.8[4], 223.3[5], 149.3[6], 203.2[7]	[1]p 1468, wound-reared blue-curly mutant laboratory strain; [2,3]p 1469, 5- and 6-day-old wound-reared Belize strain, Table 2, 22 °C; [4]Guatemala strain; [5]Belize strain, Table 3, 30 °C; [6]Guatemala strain; [7]Belize strain
	Thomas & Mangan (1989) Table 3	199.7 ± 6.0	On wounds on sheep

Numerical footnotes in the Value column apply only to the Notes to the immediate right within the same row. All parameters are for fertile screwworm flies unless otherwise stated. Daily survivorships are reported for adults. Survivorships and development time for the larval and pupal phases are for the complete life stages. When stipulated, survivorship for the prepupal (crawl-off) period is combined with the pupal stage; otherwise the prepupal (crawl-off) survivorship is not reported. Variability estimates associated with means are standard errors unless otherwise stipulated.

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Determination by HPLC fluorescence analysis of the natural enantiomers of sex pheromones in the New World screwworm fly, *Cochliomyia hominivorax*

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Abstract. Bioassays of six racemic synthesized candidate sex pheromone compounds against male New World screwworm *Cochliomyia hominivorax* (Coquerel) flies showed that the most potent bioactivity was found with 6-acetoxy-19-methylnonacosane and 7-acetoxy-15-methylnonacosane compared with four other isomeric acetoxy nonacosanes and a larger aliphatic ketone. As all these methyl-branched compounds have two asymmetric carbons and four possible enantiomers, characterization of the natural enantiomers was essential. All four enantiomers for the two most bioactive isomers of the natural sex pheromone were synthesized for bioassay. Hydrolysis and derivatization of these enantiomers with different fluorescent reagents was followed by column-switched high-performance liquid chromatography. The use of two linked, reversed-phase columns of different polarity held at sub-ambient temperatures allowed good separation of each enantiomer. This analysis applied to natural material was successful, as (6*R*,19*R*)-6-acetoxy-19-methylnonanocosane, and (7*R*,15*R*)- and (7*R*,15*S*)-7-acetoxy-15-methylnonanocosane were detected in extracts of recently colonized female flies.

Key words. Pheromones, screwworm.

Introduction

The identification of the sex pheromone of the New World screwworm (NWS), *Cochliomyia hominivorax* (Coquerel) (Diptera: Calliphoridae), has been a long-standing challenge. Mackley & Broce (1981) found that sexual stimuli could be removed from female adults by solvent washing and could be restored by application of the extracts onto clean decoys. Their efforts to identify this sex pheromone were continued with the separation of a polar bioactive fraction obtained from open-column liquid chromatography (LC) and high-performance LC (HPLC). It contained 16 components, of which 13 were 29-carbon secondary alcohol acetates with acetoxy positions from 5 to 8. Six of these had a methyl branch internal to the chain in selected positions. There was about 0.8 μg of the mixture present per female in these isolates. Curiously, there was much less of this material on the cuticle of NWS females after some time in colony (Pomonis *et al.*, 1993). Despite repeated efforts, no natural pheromonal materials could be individually isolated for bioassay and the absolute activity of the individual isomers, as well as the stereochemistry of enantiomeric pairs, remained unknown. The sex pheromone of NWS was putatively defined by derivatization and gas chromatography-mass spectrometry (GC-MS) analysis as a mixture of acetate derivatives of 29-carbon secondary alcohols, seven of which had a single methyl branch at position 15 or 19, and six had no methyl branch (Pomonis *et al.*, 1993). Subsequent analysis by LC, HPLC and GC-MS of many different strains of NWS females showed that female flies from longterm colonies produced little of the presumed sex pheromone materials (G. Pomonis, personal communication, 2001).

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Old colony females from Chiapas and Jamaica produced much less pheromone than wild females from Belize and Libya (*c.* 200×). When too little of these pheromones were present, wild and colony males would not attempt to actively mate with these 'pheromone-depleted' females. No synthetic compounds were available at that time for further study.

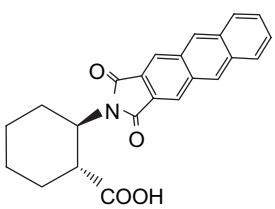
Six compounds were selected for synthesis as racemic mixtures (Furukawa *et al.*, 2002), and two were found to be highly bioactive, with 6-acetoxy-19-methylnonacosane showing full copulatory responses in 50% of the test male subjects at 1.1 μg. Similarly, 7-acetoxy-15-methylnonacosane yielded the same 50% response at 4-μg and 6-μg treatment on a decoy, although a few full copulatory responses were obtained with some other compounds (Carlson *et al.*, 2007). Four stereoisomers of these two compounds were then synthesized for dose–response studies. Bioassay results showed that 50% full copulatory attempts were calculated for a minimum dose of 0.2 μg of (6*R*)-acetoxy-(19*S*)-methylnonacosane, although the other three stereoisomers of this compound showed strong (50%) pheromone activity at higher treatment levels of 0.5 μg, and the bioactivity of treatments was indistinguishable at 1–6 μg (Mori *et al.*, 2004a, 2004b). All the methylnonacosane acetoxy compounds have two asymmetric carbons and four possible enantiomers. We describe the characterization of the natural enantiomers using column-switching HPLC of fluorescent derivatives and the full complement of available synthetic enantiomers.

Materials and methods

Chemicals

Both (1*R*,2*R*)- and (1*S*,2*S*)-2-(2,3-anthracenedicarboximido)-cyclohexanecarboxylic acids (**1**, Fig. 1) were prepared by the method previously reported (Mori, 2003). Methanol and acetonitrile were used as purchased (HPLC grade; Kanto Chemical Co., Tokyo, Japan), as was tetrahydrofuran (THF) (HPLC grade; Wako Pure Chemical Ind., Osaka, Japan). Other reagents and solvents used were purchased from Wako Pure Chemical Industries, Yokyo Kasei Organic Chemicals (Tokyo, Japan) and Sigma-Aldrich Japan (Tokyo, Japan).

Authentic samples including four enantiomerically active isomers of 19-methylnonacosane-6-ol, and four isomers of 15-methylnonacosane-7-ol were synthesized by Mori *et al.* (2004a, 2004b).



(1R,2R)-2-(2,3-anthracenedicarboximido)-cyclohexane carboxylic acid (**1**)

Fig. 1. Structure of reagent.

Wild NWS larvae were collected from wounds at seven unrecorded locations in Jamaica from 29 October 2002 to 4 September 2005 and transported to the U.S. Department of Agriculture Midwest Livestock Insect Unit (Lincoln, NE, U.S.A.) to initiate a colony. After 7 months, mature flies from this colony were collected in batches of 200 over several weeks, frozen and transported frozen to the Center for Medical, Agricultural and Veterinary Entomology (Gainesville, FL) in May 2004. A crude extract of 1400 females was prepared in ethyl acetate, the solvent was removed, and a large portion was sent to Japan for analysis.

HPLC instruments

The column-switching HPLC system was composed of two pumps, a six-port valve device, two analytical columns and a fluorescence detector (Fig. 2). The HPLC pumps were two Jasco PU-980 models (Jasco Corp., Tokyo, Japan) equipped with a Rheodyne 7125 sample injector (Perkin Elmer, Inc., Waltham, MA, U.S.A.) and a 20-μL sample loop. The six-port valve device was a PT-8000 (Tosoh Corp., Tokyo, Japan). The fluorescence detector was a Jasco FP-920 (Jasco Corp.) and the integrator was a Chromatocorder 12 (System Instruments, Inc., Tokyo, Japan). Column temperatures were lowered to 0 °C in an ice bath for the first column and to − 30 °C in an acetone bath, using Cryocool CC100-II (Neslab Instruments Inc., Portsmouth, NH, U.S.A.), for the second column.

*Derivatization procedure of a natural and synthetic sample with reagent **1***

An aliquot of ethyl acetate extract of female flies was dissolved in 5 mL methanol and a catalytic amount of sodium methoxide was added before the mixture was stirred overnight at room temperature to hydrolyze the acetate. After removing the solvent under a stream of dry N_2, the residue was dissolved in 20 mL of ethyl acetate and washed three times with water. The solvent was removed under a stream of N_2 and the residue was dried under reduced pressure. The residue was then dissolved in 0.3 mL of toluene and acetonitrile (1 : 1 v/v). The solution was divided into two portions; one

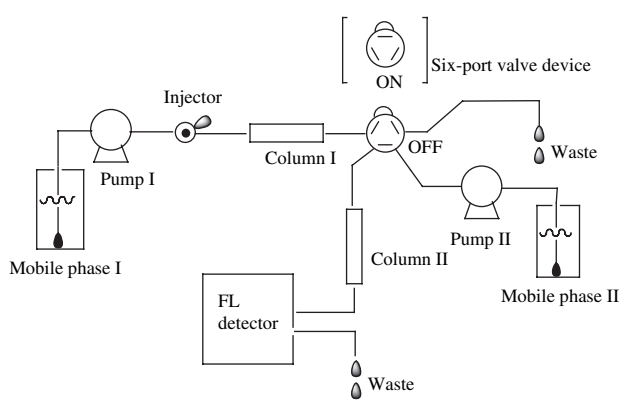


Fig. 2. Liquid chromatography–liquid chromatography system diagram.

was derivatized with (1*R*,2*R*)-**1** and the other with (1*S*,2*S*)-**1**. About 10 mg of optical pure reagent **1**, a catalytic amount of 4-dimethylaminopyridine and about 20 mg of 1-ethyl-3-(3-dimethylamino) carbodiimide hydrochloride were added to the sample solution and the mixture was held overnight at room temperature. After derivatization, an aliquot was loaded onto a silica gel thin-layer chromatography plate (10 cm length, Silicagel 60 F_{254}, Art-5744; Merck KGaA, Darmstadt, Germany) and developed with toluene/ethyl acetate (20 : 1 v/v). The target spot was collected and packed into a Pasteur pipette, which was eluted with 0.3 mL ethyl acetate. This eluate was used for HPLC analysis. The same procedure was used with all synthetic enantiomers of 6-acetoxy-19-methylnonacosane and 7-acetoxy-15-methylnonacosane to produce the derivatives.

HPLC separation

The derivatives were first separated on a Develosil ODS-A-3 column (3 μm, 4.6 × 150 mm) (Nomura Chemical Co., Aichi, Japan) at 0 °C, eluted with acetonitrile/tetrahydrofuran (2 : 1, v/v) at 0.6 mL/min. Then, only the target fraction was introduced onto the second column through a six-port valve. The timing of column switching was recorded in minutes for proper repetition. The second column used was a Develosil C30 UG-3 (3 μm, 4.6 mm × 150 mm), which was eluted with acetonitrile/methanol/tetrahydrofuran/n-hexane (4 : 8 : 21 : 12, v/v/v/v) at 0.4 mL/min and − 30 °C. A different mobile phase of acetonitrile/methanol/tetrahydrofuran/n-hexane (4 : 8 : 21 : 9, v/v/v/v) at 0.4 mL/min and − 20 °C was used for (7*R*,15*S*)- and (7*R*,15*R*)-15-methylnonacosane-7-yl (1*R*,2*R*)-**1** derivatives and their enantiomers (Table 1). The fluorescence intensities of these derivatives were monitored using excitation at 298 nm and emission at 462 nm. Sensitivity was linear in the range of 100 femtomoles with synthetic derivatives, with natural derivatives detected in the range of 10–20 femtomoles.

Results

As pheromone components were acetates of branched alkyl alcohols, the ethyl acetate extract was first subjected to methanolysis. Then the sample was divided into two portions to be derivatized with both enantiomers of reagent **1**, respectively, to check both possibilities. If a component was detected by derivatization with (1*R*,2*R*)-**1**, the corresponding (1*S*,2*S*)-**1** derivative, which had a different retention time, should have been detected. If only one of them was detected, the peak was not derived from the target compound.

It was possible to separate each isomer of the known C29 compounds on the C30 column (Mori *et al.*, 2004a). However, the sample extract contained too many other compounds to separate and detect the pheromones with a single-column system. As a result, a column-switching LC–LC system was applied to improve resolution. On the first column system, all eight possible isomers of 19-methylnonacosane-6-ol and 15-methylnonacosane-7-ol were separated into four peaks, depending on their positions of hydroxyl groups and their stereochemistry (Fig. 3). Here, the (1*R*,2*R*)-**1** derivatives of (6*S*,19*S*)- and (6*S*,19*R*)-alcohol and (1*S*,2*S*)-**1** derivatives of (6*R*,19*R*)- and (6*R*,19*S*)-alcohol were eluted for 34.6–37.0 min. The (1*R*,2*R*)-**1** derivatives of (6*R*,19*R*)- and (6*R*,19*S*)-alcohol and (1*S*,2*S*)-**1** derivatives of (6*S*,19*S*)- and (6*S*,19*R*)-alcohol were eluted for 40.0–44.0 min. The (1*R*,2*R*)-**1** derivatives of (7*S*,15*S*)- and (7*S*,15*R*)-alcohol and (1*S*,2*S*)-**1** derivatives of (7*R*,15*R*)- and (7*R*,15*S*)-alcohol were eluted for 52.4–55.6 min. The (1*R*,2*R*)-**1** derivatives of (7*R*,15*R*)- and (7*R*,15*S*)-alcohol and (1*S*,2*S*)-**1** derivatives of (7*S*,15*S*)- and (7*S*,15*R*)-alcohol were eluted for 46.4–49.6 min. Only the target fraction, which contained a pair of stereoisomers, was introduced into the second column by switching the six-port valve. By changing the timing of the valve switching, it was possible to detect each pair of stereoisomers.

Figure 4 shows HPLC chromatograms of 19-methylnonacosane-6-ols. Authentic samples were detected at 53.0 min (peak a = [1*R*,2*R*]-**1** derivative of [6*S*,19*R*]-alcohol and its enantiomer), 67.4 min (peak b = [1*R*,2*R*]-**1** derivative of [6*S*,19*S*]-alcohol and its enantiomer), 81.8 min (peak c = [1*R*,2*R*]-**1** derivative of [6*R*,19*R*]-alcohol and its enantiomer) and 86.3 min (peak d = [1*R*,2*R*]-**1** derivative of [6*R*,19*S*]-alcohol and its enantiomer), respectively. On chromatograms C and D, both (6*S*,19*S*)- and (6*S*,19*R*)-alcohol derivatives were not detected. By

Table 1. Timing of valve device switching.

Timing of switching (valve device ON)	Alcohols	
	(1*R*,2*R*)-**1** derivatives	(1*S*,2*R*)-**1** derivatives
34.6–37.0 min	(6*S*,19*S*) and (6*S*,19*R*)	(6*R*,19*S*) and (6*R*,19*R*)
40.0–44.0 min	(6*R*,19*S*) and (6*R*,19*R*)	(6*S*,19*S*) and (6*S*,19*R*)
46.4–49.6 min	(7*R*,15*S*) and (7*R*,15*R*)	(7*S*,15*S*) and (7*S*,15*R*)
52.4–55.6 min	(7*S*,15*S*) and (7*S*,15*R*)	(7*R*,15*S*) and (7*R*,15*R*)

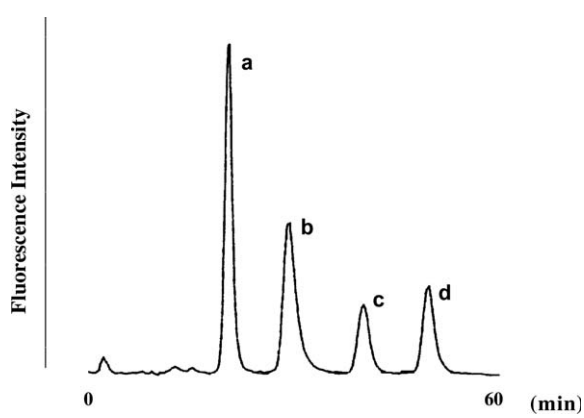


Fig. 3. High-performance liquid chromatography (HPLC) chromatogram of (1*R*,2*R*)-**1** derivatives of authentic samples on the first column. Derivatives were separated on a Develosil ODS-A-3 at 0 °C eluted with acetonitrile/tetrahydrofuran (2 : 1, v/v) at 0.6 mL/min. Peaks a, b, c and d represented a mixture of (6*S*,19*S*) and (6*S*,19*R*) derivatives, a mixture of (6*R*,19*R*) and (6*R*,19*S*) derivatives, a mixture of (7*R*,15*R*) and (7*R*,15*S*) derivatives, and a mixture of (7*S*,15*S*) and (7*S*,15*R*) derivatives, respectively. HPLC chromatograms of authentic samples and an ethyl acetate extract (1).

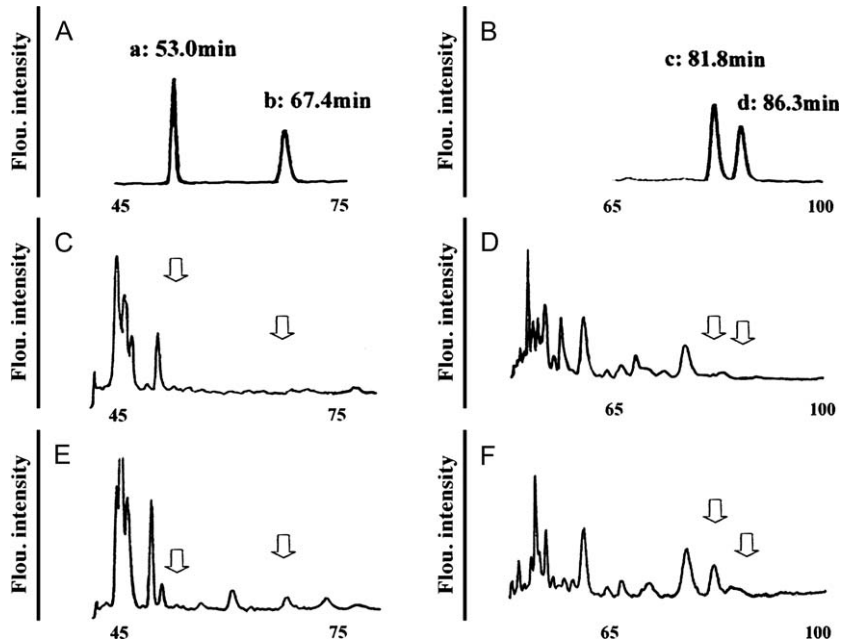


Fig. 4. The valve was switched at 34.6 min and 37.0 min on chromatograms A, C and E, and at 40.0 min and 44.0 min on B, D and F. Chromatograms A and B showed authentic samples, C and F showed (1*R*,2*R*)-**1** derivatives, and D and E showed (1*S*,2*S*)-**1** derivatives of the ethyl acetate extract. Peaks a, b, c and d represented (1*R*,2*R*)-**1** derivative of (6*S*,19*R*)-alcohol and its enantiomer, (1*R*,2*R*)-**1** derivative of (6*S*,19*S*)-alcohol and its enantiomer, (1*R*,2*R*)-**1** derivative of (6*R*,19*R*)-alcohol and its enantiomer and (1*R*,2*R*)-**1** derivative of (6*R*,19*S*)-alcohol and its enantiomer, respectively.

contrast, (6*R*,19*R*)-alcohol derivatives were detected on both chromatograms E (at 67.7 min) and F (at 81.9 min), whereas (6*R*,19*S*)-alcohol derivatives were not detected. Thus, these results showed unequivocally the existence of (6*R*,19*R*) isomer and the absence of other three isomers in the ethyl acetate extract.

Figure 5 shows HPLC chromatograms of 15-methylnonacosane-7-ols. As (7*R*,15*R*)-15-methylnonacosane-7-yl (1*R*,2*R*)-**1** derivatives and its enantiomer had much longer retention times, the separation of (7*R*,15*S*)- and (7*R*,15*R*)-15-methylnonacosane-7-yl (1*R*,2*R*)-**1** derivatives were performed at a higher column temperature (− 20 °C) to reduce retention times. Authentic samples were detected at 77.0 min (peak a = [1*R*,2*R*]-1 derivative of [7*S*,15*S*]-alcohol and its enantiomer), 84.6 min (peak b = [1*R*,2*R*]-1 derivative of [7*S*,15*R*]-alcohol and its enantiomer), 69.8 min (peak c = [1*R*,2*R*]-1 derivative of [7*R*,19*S*]-alcohol and its enantiomer) and 99.0 min (peak d = [1*R*,2*R*]-1 derivative of [6*R*,19*R*]-alcohol and its enantiomer), respectively.

Conclusions

On chromatograms C and D, (7*S*,15*S*)- and (7*S*,15*R*)-alcohol derivatives were not detected. By contrast, both (7*R*,15*R*)- and (7*R*,15*S*)-alcohol derivatives were detected on both chromatograms

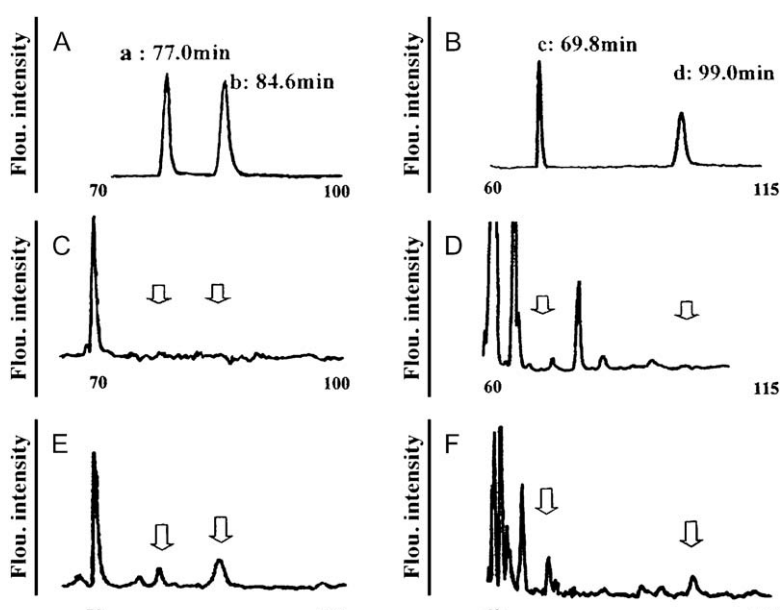


Fig. 5. High-performance liquid chromatography (HPLC) chromatograms of authentic samples and an ethyl acetate extract (2). The valve device was switched at 52.4 min and 55.6 min on chromatograms A, C and E, and at 46.4 min and 49.6 min on B, D and F. Chromatograms A and B showed authentic samples, C and F showed (1*R*,2*R*)-**1** derivatives, and D and E showed (1*S*,2*S*)-**1** derivatives of the ethyl acetate extract. Peaks a, b, c and d were (1*R*,2*R*)-**1** derivative of (7*S*,15*S*)-alcohol and its enantiomer, (1*R*,2*R*)-**1** derivative of (7*S*,15*R*)-alcohol and its enantiomer, (1*R*,2*R*)-**1** derivative of (7*R*,19*S*)-alcohol and its enantiomer, and (1*R*,2*R*)-**1** derivative of (7*R*,15*R*)-alcohol and its enantiomer, respectively.

E (at 77.0 min and 84.5 min) and F (at 69.8 min and 98.6 min). Thus, these results showed unequivocally the existence of (7*R*,15*R*) and (7*R*,15*S*) isomers in the ethyl acetate extract, thus proving the existence of at least these enantiomers in female flies. Proof of the presence of other structures of the same molecular weight containing methyl branches or the acetoxy function at other locations is not possible without the synthesis of all enantiomers of each, but this is an unlikely prospect at this time. The samples and authentic synthetic standards available were too small an amount to weigh and it was difficult to estimate the amount of sample derivatized. As the most acid derivatives of this reagent had 10^{-15} to 10^{-14} mole levels of detection limits (signal : noise ratio = 2) and the peaks detected here had a signal : noise of about 6^{-10}, the amounts detected were about several 10^{-14} mole levels.

The LC–LC method removed most interfering substances by switching them away in the first column and the resulting chromatograms were very simple. The synthetic samples were repeatedly injected to ensure that peak assignments were correct. Although each derivatized natural sample by each enantiomer of the reagents was analysed once, the results obtained by using both enantiomers of the reagents, respectively, did not contradict one another.

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Conflicts of interest

All authors declare no conflicts of interests.

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