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THE *CULEX PIPIENS* COMPLEX IN CONTINENTAL PORTUGAL: DISTRIBUTION AND GENETIC STRUCTURE

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ABSTRACT. Portugal is a southern European country that displays favorable ecological conditions for the establishment of West Nile virus (WNV) transmission cycles. Competent mosquito vector species are present throughout the country. Among the species with reported cases of WNV isolation in Portugal, *Culex pipiens* is the most ubiquitous and abundant mosquito. This species exhibits two biological forms with differences in host preferences. The molestus form has a greater tendency to feed upon humans and other mammals whereas the pipiens form prefers avian hosts. In northern latitudes, both forms are physically separated, with molestus occupying underground habitats and pipiens being found aboveground. However, the warmer climatic conditions of southern regions such as Portugal may favor the sympatric occurrence of both forms hence promoting interform hybridization. Genetic introgression between molestus and pipiens forms may result in a higher propensity for admixed populations to serve as bridge-vectors of WNV between humans and birds. Here we revise our present knowledge on the distribution, role in WNV transmission and genetic structure of the *Cx. pipiens* complex in continental Portugal. We focus on recent findings of sympatric molestus and pipiens populations that display considerable levels of hybridization and discuss the epidemiological repercussions of this occurrence.

KEY WORDS *Culex pipiens*, molestus form, hybridization, WNV transmission, Portugal

INTRODUCTION

The *Culex pipiens* complex comprises some of the most important mosquito vectors of disease with global distribution. Among the species of the complex, *Cx. pipiens* L. sensu stricto and *Cx. quinquefasciatus* Say are the most ubiquitous species in temperate and tropical regions, respectively. Intraspecific heterogeneity in *Cx. pipiens* s.s. populations has raised controversial opinions among taxonomists and evolutionary biologists on whether this taxon should be further subdivided. Two morphologically identical biological forms, denoted molestus Forskål and pipiens, have been recognized based on ecological and physiological traits. The molestus form is stenogamous (*i.e.* mates in confined spaces, < 0.1 m^3; Clements 1999), autogenous (can oviposit without a blood meal), homodynamic (remains active during winter), and mammophilic (prefers to feed on mammals, including humans). In contrast, the pipiens form is eurygamous (mates in open spaces), anautogenous (oviposition requires a blood meal), heterodynamic (undergoes winter diapause), and ornithophilic (feeds predominantly on birds) (Harbach et al. 1984, 1985).

In the northern latitudes of Europe, Russia and Northeastern USA, the molestus form is found in underground habitats whereas the pipiens form lives at the surface (Byrne and Nichols 1999, Vinogradova 2000, Huang et al. 2008). This habitat discontinuity may act as a barrier to gene flow between forms. However, in Southern European and Mediterranean regions, populations of both forms have been found at the surface (Chevillon et al. 1995, Fonseca et al. 2004, Gomes et al. 2009). This sympatric occurrence in aboveground habitats is likely to promote hybridization between molestus and pipiens (Fonseca et al. 2004, Kent et al. 2007, Vinogradova and Shaikevich 2007). Populations with intermediate behavior between the two forms have been described in the Mediterranean region. In the south of France, stenogamous and homodynamic populations of *Cx. pipiens* were found without the capacity of laying eggs when deprived of a blood meal (Pasteur et al. 1977).

The heterogeneity found among populations of *Cx. pipiens* s.s. may have important epidemiological repercussions. Hybridization between mammophilic molestus and ornithophilic pipiens forms could result in a more opportunistic biting behavior of *Cx. pipiens* s.s. This could in turn favor the ability to accidentally transmit arboviruses that have enzootic cycles in avian species to

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mammalian hosts, including humans. This is the case with the West Nile virus (WNV). This member of the Japanese encephalitis virus serocomplex is the most widely distributed arthropod-borne Flavivirus in the world (Briton 2002). Although the WNV burden on human health may not be as severe as that imposed by Dengue or Yellow Fever flaviviruses, it is clearly a non-negligible and frequently underestimated infection. The WNV natural cycle is complex, involving enzootic transmission between mosquitoes and avian species as reservoirs or amplifying hosts while mammals, including humans, behave as accidental dead-end hosts.

The incidence of WNV infections in Portugal remains relatively unknown. Serological surveys of WNV-specific neutralizing antibodies carried out from the 1960s to the 1980s, both in humans and horses (Filipe et al. 1973, Filipe and Andrade 1990), suggested a low transmission level of the virus. Similar results were obtained by serological surveys conducted in 1999–2002 and 2004–2010 that revealed seroprevalences of 12%–20% in birds and around 3% in horses (Formosinho et al. 2006, Barros et al. 2011). Of the 10 bird species with records of anti-WNV antibodies in Portugal (Formosinho et al. 2006), two (*Circaetus gallicus* Gmelin and *Ciconia ciconia* L.) exhibit migration routes between Africa and Europe (Mullarney et al. 2003). In humans, WNV seropositive cases have been detected on two occasions, in 2004 and 2010 respectively (Connell et al. 2004, Barros et al. 2011, Fig. 1). The most recent case, however, was not confirmed by neutralization tests. Of the 38 mosquito species recorded in Portugal (Ribeiro and Ramos 1999), WNV has been so far isolated from three species. Of these, *Cx. pipiens* s.l. is the most widely distributed and abundant mosquito in the territory.

Located on the extreme southwestern edge of Europe, continental Portugal experiences a Mediterranean climate with mild winters and warm/dry summers. As reported in other regions in southern latitudes, the climatic conditions of the country may favor the aboveground sympatric co-existence of molestus and pipiens forms and hence promote the occurrence of interform hybridization. In this context, the present review aims at providing an update on the present knowledge about the distribution and genetic structure of the *Cx. pipiens* complex in continental Portugal.

Distribution, species composition and role in WNV transmission

Culex pipiens s.l. is the most abundant and widespread mosquito in continental Portugal, being present in all 18 districts of the country from sea level to 1600m of altitude (Ribeiro et al. 1999, Almeida et al. 2008). It is the predominant species in most coastal areas from the north to the centre of the country, ranging from 60% to 80% of the mosquitoes collected; it comes second to *Culex theileri* Theobald or *Aedes (Ochlerotatus) caspius* (Pallas) in southern coastal areas dominated by marshlands, wetlands or rice fields, ranging from 20% to 40% of mosquitoes collected. In inland areas, it alternates between the first and second most common species with *Anopheles maculipennis* Meigen s.l., ranging from 20% to 60% depending on the sampled area and collection method (Almeida et al. 2008, Almeida et al. 2010).

Culex pipiens s.l. densities vary according to district, with overall means around 18 specimens/trap/night in Centers for Disease Control and Prevention (CDC) light traps baited with CO_2 and 55 specimens/collector/hour in resting collections (Almeida et al. 2008). It is also collected by human baited landing captures but at lower densities compared to other sampling methods (*ca.* 6 females/collector/hour). This species occurs all-year round but displays much lower indoors resting densities (*ca.* 1 specimen/collector/hour) during the winter. Its seasonal dynamics is characterized by increasing densities at the beginning of spring (April–May) reaching a peak in July and decreasing again after October–November (Almeida et al. 2010).

Of the species belonging to this complex, *Culex pipiens* s.s. and *Culex torrentium* Martini, have been identified in Portugal but the latter has been recorded only in the hilly regions of central and northern areas, not beyond 1200m of altitude (Ribeiro et al. 1999, Ribeiro et al. 2002, and Fig. 1). These species were identified based on morphological characters of the male genitalia. More recently, molecular identification (Smith and Fonseca 2004) of *Cx. pipiens* s.l. mosquitoes from three lowland regions revealed the presence of only *Cx. pipiens* s.s. (Fig. 1).

Until today WNV has only been isolated serendipitously from mosquito vectors in Portugal (Fig. 1). The virus was first isolated in southern Portugal, in the early 1970s, from a pool of *An. maculipennis* s.l. collected close to the Roxo dam (Filipe 1972), and again in 1996 from a pool of *An. atroparvus* van Thiel collected in the area of the Tejo estuary (Fernandes 1998). A 3-years survey initiated in 2000 involving detection of WNV by reverse transcriptase – polymerase chain reaction and cell culture, did not disclose any WNV strain in mosquitoes (*ca.* 30,000 adult specimens analyzed) collected over the whole continental territory (Galão et al. 2002). In the summer of 2004, the Irish National Disease Surveillance Centre confirmed WNV infection in two Irish bird-watchers, which had been acquired in the southernmost Portuguese province of the Algarve (Connell et al. 2004, Fig. 1). Subsequent mosquito surveys implemented in risk areas such as wetlands and bird sanctuaries

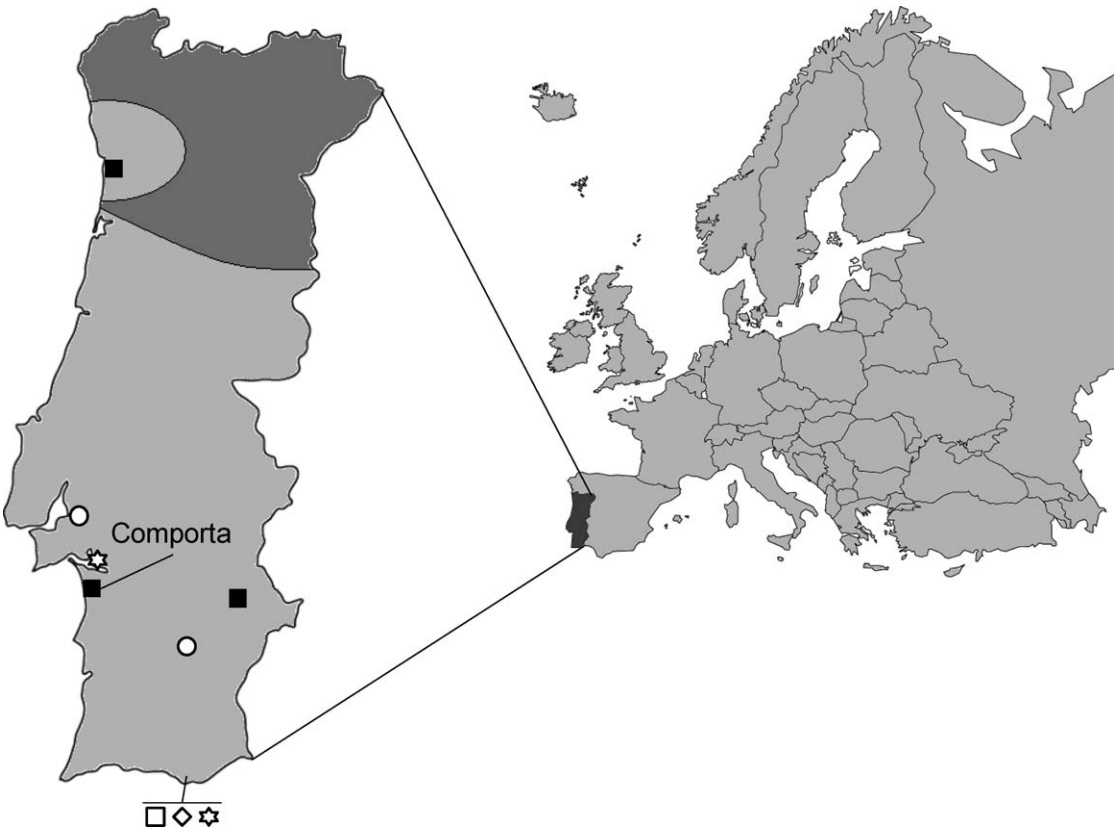

Fig. 1. Map of Portugal showing the distribution of the *Culex pipiens* complex members and the location of WNV infections detected in both mosquitoes and humans. Light gray area: *Cx. pipiens* s.s.; dark gray area: *Cx. pipiens s.s.* and *Cx. torrentium*; black squares: *Cx. pipiens* s.s. identified by molecular assay; white square: isolation of WNV in *Cx. pipiens* s.l.; white circles: isolation of WNV in *An. maculipennis* s.l.; white diamond: isolation of WNV in *Cx. univittatus* s.l.; white star: seropositive cases of WNV in humans. Comporta: study area where sympatric molestus and pipiens aboveground populations were determined.

allowed the detection of WNV RNA in wild caught mosquitoes in 4 out of 46 pools of unfed *Culex pipiens* s.l. (2,062 mosquitoes) and in 2 out of 4 pools of *Culex univittatus* Theobald (45 mosquitoes) captured near the putative area where the human infections had been acquired (Esteves et al. 2005).

POPULATION STRUCTURE AND HYBRIDIZATION

The first report on behavioral heterogeneities in *Cx. pipiens* s.s. populations from continental Portugal dates from the 1980's. An autogenous/stenogamous population, typical of the molestus form, was recorded based on the analysis of adults reared from larvae collected in urban surface habitats in the periphery of Lisbon (Ribeiro et al. 1983). More recently, insectary experiments on F1 progenies from field caught gravid females disclosed molestus and pipiens fractions of a *Cx. pipiens* s.s. population collected in the region of Comporta (Gomes et al. 2009, Fig. 1). These fractions were determined by analyzing autogeny and stenogamy traits, which were highly correlated, and families displaying intermediate phenotypes were also recognized. In addition, there was an association between form-specific alleles at a diagnostic locus located in a flanking region of microsatellite CQ11 (Bahnck and Fonseca 2006) and the phenotypes used to characterize molestus and pipiens forms (Fig. 2). These results provided evidence for the sympatric occurrence of molestus and pipiens forms in aboveground habitats in Portugal and of hybridization between them.

Genetic analysis based on microsatellite loci confirmed the expectation of hybridization between molestus and pipiens in the Comporta region (Gomes et al. 2009). Bayesian clustering and admixture analyses based on multilocus individual genotypes revealed distinct genetic backgrounds for molestus and pipiens forms and interform hybridization rates of 8–10% (Gomes et al. 2009). Introgression between forms seemed to be asymmetric, with more molestus

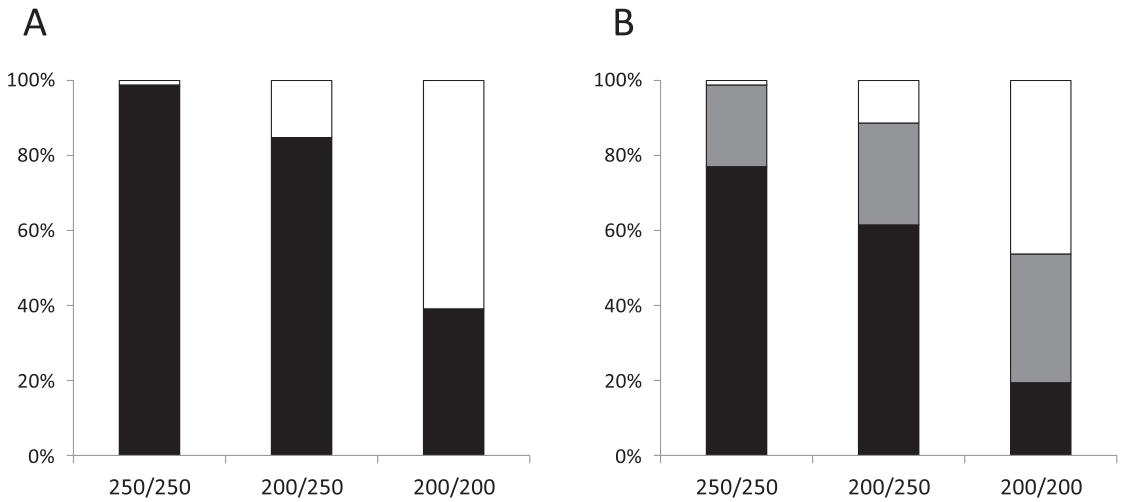

Fig. 2. Association between the CQ11 flanking region diagnostic marker (Bahnck and Fonseca 2006) with autogeny and stenogamy traits. X-axis: genotypes at the CQ11 flanking region. 250/250: homozygous for the 250bp allele associated with the molestus form; 200/200: homozygous for the 200bp allele associated with the pipiens form; 200/250: heterozygous genotype (putative hybrid). A: association with autogeny. Black bars: relative proportion of genotyped females belonging to autogenous F1 families (*i.e.* in which oviposition occurred without a blood meal); white bars: proportion of genotyped females belonging to non-autogenous F1 families (*i.e.* oviposition did not occur without a blood meal). B: association with stenogamy measured by insemination rates (IR) at each F1 family. Black bars: females belonging to F1 families with 100% IR; White bars: females belonging to F1 families with 0% IR (*i.e.* no inseminated females); gray bars: females belonging to families displaying $<0\%IR<100\%$. The methodological procedures of the insectary experiments with F1 *Cx. pipiens* s.s. families are described in Gomes et al. (2009).

genes being introgressed into the pipiens form. This asymmetry may relate with the different mating strategies adopted by the forms. In sympatric conditions, stenogamous molestus males are more likely to mate with both pipiens and molestus females than eurygamous pipiens males that display specialized swarm-based mating behavior (Downes 1969, Gomes et al. 2009). More recently, evidence of hybridization between Northern European molestus and pipiens populations was found in the Netherlands, using the CQ11 flanking region diagnostic marker (Reusken et al. 2010). Interestingly, this finding was based on the analysis of mosquitoes sampled underground. Cases of hybridization between molestus and pipiens forms have also been identified in some areas of the USA and often associated with intermediate behavioral traits (Fonseca et al. 2004, Kilpatrick et al. 2007, Huang et al. 2009, Kothera et al. 2010).

One of the consequences of molestus and pipiens introgressive hybridization may be the acquisition of a more opportunistic biting behavior in admixed populations, increasing the risk of acting as bridge vectors in WNV transmission. Evidence supporting this hypothesis comes from studies in the USA, where admixed populations appear to have a higher propensity to bite both bird and human hosts (Fonseca et al. 2004, Kilpatrick et al. 2007, Huang et al. 2009). However, factors other than genetic predisposition, such as host availability, can also affect host choice. The decrease of the bird populations at the end of the summer can lead to an increase of bites in mammals (including humans) by mosquito populations that during the summer fed almost entirely on avian hosts (Kilpatrick et al. 2007).

Entomological surveys carried out in Comporta in 2010 did not reveal any particular association between molestus and pipiens genetic backgrounds (determined by microsatellites) and ornithophilic/mammophilic host preferences (B. Gomes unpublished data). Apart from humans, a variety of mammalian and avian domestic animals is present in this rural area. Nevertheless, the great majority (97%) of the *Cx. pipiens* s.s. blood feeds determined by blood meal enzyme linked immunosorbent assay in females collected indoors resting were made on avian hosts. However, this result could have been influenced by the fact that over 98% of blood fed females analyzed were caught resting inside chicken coops, in spite of these constituting only 59% of all animal shelters surveyed. In these surveys, there was a tendency for collecting more molestus individuals resting inside animal shelters whereas pipiens predominated in outdoors collections made by CDC light traps and landing catches (B. Gomes unpublished data). A higher endophilic behavior of the molestus form population in surface habitats, associated with human-made constructions, suggests a higher synanthropic propensity compared to the sympatric population of the pipiens form.

CONCLUSIONS

Continental Portugal is in a geographic region with considerable potential for the establishment of transmission cycles of arboviral infections. The presence of trans-Saharan migratory bird species that are competent amplification hosts for the virus along with competent bridge-vectors such as *Cx. pipiens* s.l., *Cx. univittatus* s.l. and *An. maculipennis* s.l. creates the conditions required for the introduction/circulation of WNV and accidental transmission to mammal hosts including humans. Of the mosquito vectors in which WNV infections have been detected in the country *Culex pipiens* s.s. deserves particular attention. There is now solid evidence for the sympatric occurrence in the country of both molestus and pipiens forms in aboveground habitats and that under these conditions introgressive hybridization occurs between forms. As in other cases of sympatry between partially isolated taxa (*e.g.,* Weetman et al. 2011), this provides exceptional opportunities to study the evolutionary relationships and the biological mechanisms underlying genetic isolation between these forms. Furthermore, the possibility of hybridization potentiating the capacity of these vector populations to act as WNV bridge vectors begs for additional studies in order to further characterize bio-ecological traits of medical importance such as biting activities and blood feeding preferences.

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