Journal of Heredity, 2025, **116**, 241–254
https://doi.org/10.1093/jhered/esaf005
Advance access publication 30 January 2025
Original Article

American Genetic Association

OXFORD

Original Article

Genetic structure of the northern house mosquito (Diptera: Culicidae) in a WNV-susceptible area

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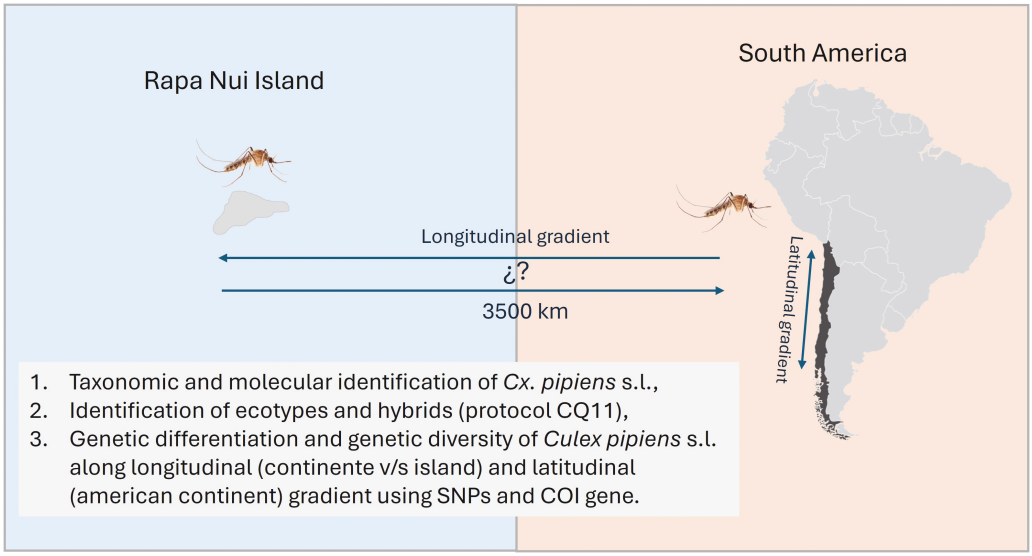
Corresponding Editor: Warren Booth

Abstract

Mosquitoes from the *Culex pipiens* complex are found worldwide and have been the focus of numerous studies due to their role as vectors of human pathogens. We investigated the population genetic structure of *Cx. pipiens* s.l. by analyzing single nucleotide polymorphisms (SNPs) and the *COI* gene, focusing on the genetic grouping signals of the ecotypes *pipiens* and *molestus*. Our analysis revealed no genetic association between the ecotypes and the SNPs, suggesting that the classification is based on ecological traits rather than genetic factors. Using data from 2,641 SNPs and 164 genotypes, our Bayesian clustering analysis categorized the populations into six distinct genetic groups, distinguishing the Rapa Nui island population from those on the American mainland. The estimated effective migration rates showed low levels of migration between island and continental populations, with significant migration occurring only among populations between 29° S and 33° S. Genetic differentiation between Rapa Nui island and the American continent was observed. In contrast, no significant differentiation was noted in other regions. The haplotype network suggests a possible migration pathway from that area of the South Pacific. In addition, demographic history analysis indicates a relatively recent founder effect of *Cx. pipiens* s.l. on the island. Geographic isolation and variations in genetic makeup can limit the spread of pathogens regionally and globally. However, these same factors can also promote specialization and enhance adaptation to new ecological niches, thereby improving the ability of pathogens to function as effective vectors in isolated environments.

Received September 4, 2024; Accepted January 28, 2025

Graphical Abstract



Key words: *Culex pipiens* s.l., gene COI, genetic structure, genotype, phenotype, SNPs

Introduction

Mosquitoes are significant vectors for viruses that impact human health, including filarial worms and protozoa that cause diseases such as yellow fever, lymphatic filariasis (elephantiasis), and malaria. These vectors indirectly cause more morbidity and mortality among humans than any other group of organisms (Harbach 2012). The *Culex pipiens* complex (Diptera: Culicidae) is the primary vector of human pathogens, including the West Nile virus (WNV), St. Louis encephalitis virus, Japanese encephalitis virus, and lymphatic filariasis (Andreadis 2012). Since its isolation in Uganda in 1937, WNV has become a significant public health burden worldwide (Andreadis 2012; Osorio et al. 2012). WNV is maintained in a natural transmission cycle between birds and *Culex* mosquitoes, with incidental infection in humans, horses, and other mammals (Erazo et al. 2024). The rapid spread of WNV primarily results from the mobility of its hosts, which are birds capable of carrying the virus over long distances, and the presence of viable mosquito vectors helps to maintain the disease in circulation (Erazo et al. 2024). The mosquitoes of the *Cx. pipiens* complex are ubiquitous species and are therefore available as a bridging host once the infected birds enter a virus-free habitat and are fed upon by the mosquitoes to begin the transmission cycle (Ciota and Kramer 2013). For this reason, understanding new areas' transmission dynamics and susceptibility to WNV requires knowledge of the available mosquito vectors' population structure and demographic processes (e.g. active dispersion, gene flow, and genetic diversity) (Ferraguti et al. 2023). In *Cx. pipiens* sensu lato (s.l.), several factors make this task difficult, such as its not-yet-clarified taxonomy at the morphotype and subspecies level and the hybridization between its members (Aardema et al. 2020).

The *Cx. pipiens* complex is formed by five recognized taxa that are almost morphologically indistinguishable: *Cx. pipiens* s.l. (Linnaeus), *Cx. quinquefasciatus* (Say), *Cx. australicus* (Dobrotworsky and Drummond), *Cx. globocoxitus* (Dobrotworsky), and Cx. *pipiens* pallens (Linnaeus) (Harbach 2012; Aardema et al. 2020). In addition, *Cx. pipiens* has two ecologically distinct forms: *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus*. The degree of separation between these taxonomic entities is still the subject of discussion because they are morphologically identical but have different preferences in behavioral and physiological requirements; the form "*pipiens*" has been described as anautogenic, ornithophilic, heterodynamic, and eurygamous, while the form "*molestus*" has been described as autogenic, mammophilic, homodynamic, and stenogamous (Fonseca et al. 2004; Farajollahi et al. 2011; Epstein et al. 2021). Most studies recognize these ecotypes as valid entities for inferring diversity patterns, but there is no evidence of a clear genetic separation between them (Asgharian et al. 2015; Shaikevich et al. 2016; Vogels et al. 2016).

The hybridization process (mating between genetically different populations or taxa resulting in gene flow between them) is frequent among the members of the *Cx. pipiens* complex (Dobrotworsky 1967). The hybridization between "*pipiens*" and "*molestus*" mosquitoes has been proposed as a mechanism for generating bridge vectors that exhibit intermediate feeding behavior and potentially drive WNV epidemics in urban areas (Fonseca et al. 2004; Kilpatrick et al. 2007; Ciota and Kramer 2013). Because of this, accurately identifying and distinguishing between the "*pipiens*" and "*molestus*" mosquitoes, as well as their hybrids, is crucial for understanding the vector competence and transmission potential of WNV in susceptible areas (Ferraguti et al. 2023).

After its first detection in the Americas (USA) in 1999, WNV spread southward into the Caribbean Basin and Latin America, where its public health impact remains poorly understood, and surveillance systems are unprepared to track its spread (Komar and Clark 2006). WNV has been reported in horses and birds throughout almost all South American countries (Morales et al. 2006; Díaz et al. 2008; Osorio et al. 2012; Mazzei et al. 2011; Coello-Peralta et al. 2019; Da Silva et al. 2020; Lorenz and Chiaravalloti-Neto 2022). The likelihood of transmission to humans in these regions remains high (Komar and Clark 2006). In addition, WNV has been detected in several islands of the American continent, e.g. the Dominican Republic, Jamaica, Puerto Rico, Cuba,

Guadeloupe, the island of Trinidad, and the British Virgin Islands (Geffroy et al. 2021).

Islands are more susceptible to invasion by non-indigenous mosquitoes because they lack natural competitors and predators that control populations in their native ecosystems (Lounibos 2002). Islands are generally depauperate in species richness, trophic complexity, and functional diversity relative to comparable mainland ecosystems, resulting in little biotic resistance to invaders and high availability of empty niches (Russell et al. 2017). Increased trade, tourism, and transportation are significant vectors of the invasion of non-indigenous mosquitoes into oceanic islands (Russell et al. 2017).

Rapa Nui (Easter Island), located 3,510 km from the American continent (Mieth and Bork 2010), has suffered from repeated outbreaks of dengue fever due to the entry of *Aedes aegypti* in the year 2000 (Canals et al. 2012). Dengue cases continue to arise, and efforts to eradicate this vector have been unsuccessful. Nowadays, *Ae. aegypti* show high population densities on the island. *Cx. pipiens* s.l. is also present in Rapa Nui, making it an area highly susceptible to WNV entry.

For this reason, we identified the ecotypes and hybrids present in Rapa Nui island and explored the population structure of *Cx. pipiens* s.l. among Rapa Nui and the American continent using high-resolution markers (single nucleotide polymorphisms—SNPs) to evaluate the possible geographic barriers for gene flow in this mosquito. In addition, we evaluated haplotypic differentiation in *Cx. pipiens* s.l. from Rapa Nui island by comparing them with global sequences available in GenBank to infer the haplotypic diversity of the *COI* gene and the phylogeographic pathways of arrival of *Cx. pipiens* s.l. to Rapa Nui island.

All these analyses will help us understand the composition of the mosquito vectors present in areas free of WNV, prepare for the virus's possible arrival, and understand the dynamics of invasive species in insular ecosystems.

Considering the first records of *Culex* on the island (Fuentes 1914) and previous studies regarding other cosmopolitan insect species that have arrived on the island, we predicted significant population structure between the island and the American continent, and consequently low genetic diversity, low effective population sizes, and low migration rates among continental and insular populations of *Cx. pipiens* s.l.

Materials and methods

Mosquito collection

About 1,000 larvae and adults of *Cx. pipiens* s.l. were sampled from nine continental sites and Rapa Nui island (Fig. 1). Mosquito larvae were sampled between November 2021 and November 2022 from different breeding sites, including urban and suburban cemeteries and wetlands in rural areas (Table 1) by a dipping method using plastic dippers. All larvae collected were transported in containers with water from the site where they were collected. Larvae were transferred to 300-cc plastic containers (randomly, with 50 larvae per container) prepared with demineralized water, and fish food was added daily. Emergent adults were transferred using a mouth aspirator, killed in a deep freezer, and identified under a stereoscopic microscope to 20× using the keys of Rossi and Almirón (2004), Andreadis et al. (2005), and González et al. (2015, 2016). Adults of *Cx. pipiens* s.l. were sampled from below- and aboveground habitats in rural areas (Melipilla and Puaun sites) using a handheld vacuum cleaner and were identified following the guidelines described above (Table 1). We selected 20 individuals from each continental site (180) and 26 individuals from Rapa Nui (the total number of individuals collected on the island) to perform molecular analysis associated with the determination of ecotypes and hybrids (protocols based on loci CQ11 and acetylcholinesterase-2, detailed below), and to conduct an analysis based on SNP markers. Of the 206 individuals considered for analysis, we obtained DNA from 180, as shown in Table 1. To infer the haplotypic diversity of the *COI* gene, we selected a subset of 24 specimens of continental samples and seven individuals from Rapa Nui (four individuals collected in November 2022 and three individuals collected in April 2024). We could not analyze all the individuals of Rapa Nui collected in 2022 (26) because the DNA was damaged after obtaining the sample for SNPs, and we had to collect new individuals from the island.

Identification of ecotypes

Bahnck and Fonseca (2006) developed an assay based on polymorphisms in the flanking region of a microsatellite locus (CQ11) to identify ecotypes (*pipiens* and *molestus*) and their hybrids. We applied this protocol to the 180 individuals from which we could extract DNA and from which material for SNP analysis was also obtained. Following Bahnck and Fonseca (2006), polymerase chain reaction (PCR) was carried out using the pipCQ11R, molCQ11R, and CQ11F primers. The PCR assay was standardized for a 20-µL reaction volume. Reactions contained 0.12 µL Taq, 0.5 µL BSA, 0.5 µL $MgCl_2$, 2.4 µL dNTP, 1.3 µL buffer, 0.5 µL primer pipCQ11R for biotype *pipiens*, 0.75 µL primer molCQ11R for biotype *molestus*, 0.75 µL primer CQ11F, 2 µL genomic DNA, and 11.2 µL free water. The PCR was performed with an initial denaturation at 94 °C for 5 min, followed by 40 cycles at 94 °C for 30 s, 54 °C for 30 s, and 72 °C for 40 s, with a final extension at 72 °C for 5 min. Amplified fragments were visualized on 1% agarose gel; a single DNA fragment of 200 bp corresponds to the ecotype *pipiens*, a single DNA fragment of 250 bp corresponds to the ecotype *molestus*, and individuals with both fragments were considered hybrids.

Although *Cx. quinquefasciatus* has not been described in the studied areas, we also applied the protocol of Smith and Fonseca (2004) based on a nuclear locus (acetylcholinesterase-2) to differentiate *Cx. pipiens* s.l. from *Cx. quinquefasciatus*. DNA fragment sizes visualized by agarose gel electrophoresis showed that all individuals were *Cx. pipiens* s.l. Information about the protocol and PCR reactions used can be provided upon request.

SNP analysis

DNA/SNP sequencing.

A small piece of the thorax of each of the 180 mosquitoes was extracted and subjected to massive sequencing at Dart Diversity Arrays Technology Pty Ltd. (DArT; Canberra, Australia). DNA was digested with the restriction enzymes SbfI and PstI following Kilian et al. (2012). Fragments > 200 bp were ligated with an 8-bp barcode and amplified by PCR. The PCR products were standardized and sequenced on a HiSeq 2500 (Illumina Inc, San Diego, USA). Sequences generated from each lane were processed using proprietary DArT PL analytical pipelines. The resulting SNP data were

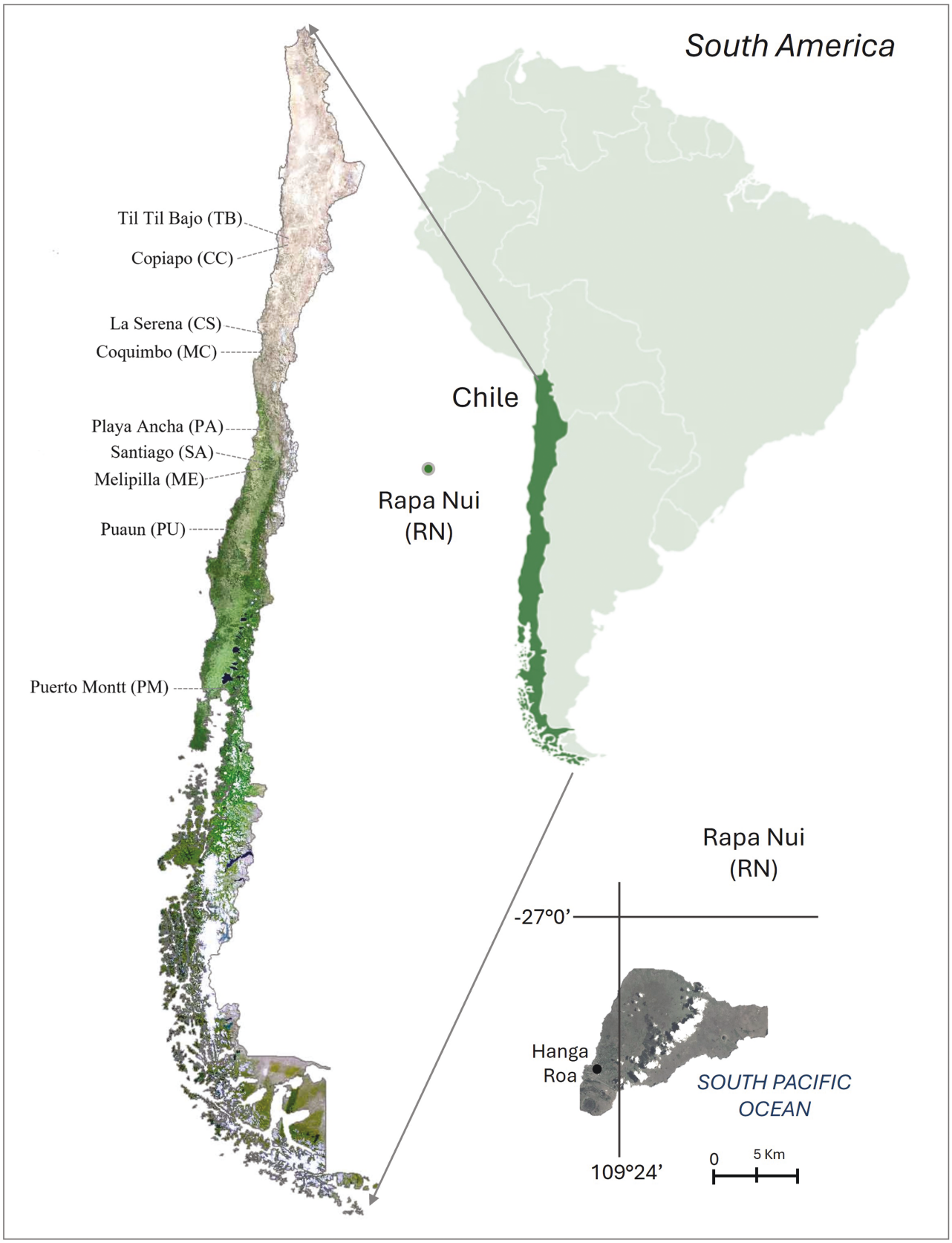


Fig. 1. Mosquito collection sites in continental Chile and Rapa Nui (modified from GISgeography.com).

Table 1. Summary of sampling sites (ID, location, sampling date, and sample type) and *Culex pipiens* s.l SNPs data (sample size before and after filtering, observed heterozygosity (H_O), expected heterozygosity (H_E), inbreeding coefficient (F_{IS}), and Queller and Goodnight (1989) estimator of relatedness (QGE), at each study site). * Significant relatedness values ($P < 0.05$).

Sample site (ID)	Geographic coordinates	Sampling date (day/month/year)	Sample type and habitat	Initial sample	Sample after filtering	H_O	H_E	F_{IS}	QGE	lynchrd	ritland
Til Til Bajo, Copiapó (TB)	27°22'43" S, 70°18'13" W	26/11/2021	Larvae collected from two suburban ponds	18	18	0.115	0.138	0.191	−0.060	−0.060	−0.067
Cementerio Municipal de Copiapó (CC)	27°22'18" S, 70°20'16" W	29/11/2021	Larvae were collected from three vases located throughout the cemetery	20	18	0.103	0.094	−0.062	−0.032*	−0.060	−0.060
Cementerio Parque La Foresta, La Serena (CS)	29°55'26" S, 71°11'16" W	08/12/2021	Larvae were collected from five vases located throughout the cemetery	20	19	0.143	0.159	0.126	−0.055	−0.057	−0.061
Cementerio Municipalde Coquimbo (MC)	29°58'03" S, 71°19'50" W	09/12/2021	Larvae were collected from seven vases located throughout the cemetery	18	18	0.140	0.154	0.118	−0.060	−0.060	−0.065
Cementerio de Playa Ancha (PA)	33°01'39" S, 71°38'45" W	12/03/2022	Larvae were collected from three vases located throughout the cemetery	11	10	0.148	0.167	0.160	−0.114	−0.114	−0.122
Cementerio Bajos de Mea, Santiago (SA)	33°36'50" S, 70°36'09" W	17/11/2021	Larvae were collected from two vases located throughout the cemetery	20	17	0.146	0.149	0.057	−0.062	−0.065	−0.065
Los Maitenes, Melipilla (ME)	33°49'49" S, 71°23'11" W	20-22/11/2021	Adults in a rural sewer	18	18	0.106	0.128	0.199	−0.049	−0.061	−0.070
Puaun, Ñuble (PU)	36°22'50" S, 72°48'41" W	25-28/12/2021	Adults inside a rural home	16	16	0.099	0.111	0.132	−0.065	−0.068	−0.074
Cementerio Parque Puerto Montt (PM)	41°28'26" S, 72°55'41" W	24/01/2022	Larvae were collected from six vases located throughout the cemetery	18	17	0.136	0.128	−0.036	−0.040*	−0.065	−0.063
Hanga Roa, Rapa Nui (RN)	27°09'02" S, 109°25'55" W	31/08–30/09/2022	Larvae from different artificial in urban sites	21	13	0.039	0.049	0.229	−0.087	−0.088	−0.097

filtered using the dartR library in R statistical software, retaining (a) only one SNP in reads containing two or more SNPs, (b) SNPs with a read depth > 5 or < 150, and (c) SNPs with a > 99% reproducibility score, an index provided by DArT PL that indicates the proportion of replicate technical samples with a consistent marker score (reproducible result). Monomorphic SNPs had > 15% missing data, and SNPs with a minimum allele frequency of < 1% were removed, as well as samples with > 20% missing data. All SNPs showing signs of selection were removed to avoid potential bias in estimating differences among sampling sites. The approach used here was the relationship between F_{ST} and heterozygosity implemented in the Fsthet library (Flanagan and Jones 2017) in R software. Finally, SNPs with significant departures from Hardy–Weinberg equilibrium for one or more sites were removed using the dartR library in R. SNPs with significant linkage disequilibrium > 0.5 in all sampling sites using PLINK 2.0 software (Chang et al. 2015). Genetic diversity at each sampling site was described using expected heterozygosity (H_E), observed heterozygosity (H_O), and inbreeding coefficient (F_{IS}) estimated using the dartR library, and the Queller and Goodnight (1989) estimator of relatedness (QGE) was estimated with the related library (Pew et al. 2015) in R software. The QGE considers allele frequencies, eliminates a downward bias for small sample sizes, and allows estimation of relatedness for subsets of population samples. The QGE was estimated per sampling site, and the level of relatedness was statistically tested using Identix software (Belkhir et al. 2001). The significance of QGE was tested using a permutation test (1,000 permutations) implemented in the same software. Furthermore, the lynchrd (Lynch & Ritland 1999) and ritland (Ritland 1996) indexes, both accounting for inbreeding on the estimate of relatedness, were estimated using Coancestry software (Wang 2011).

Population genetic analysis.

Population genetic structure was estimated using the SNP database after removing all SNPs showing a selection signal. Three population genetic methods were used with the following data: (a) principal coordinate analysis (PCoA) to describe the distribution of individuals in multivariate space using the dartR library in R, (b) pairwise F_{ST} calculated using the dartR library in R, and (c) estimation of the most probable number of genetic clusters (K) using the Bayesian approach implemented in STRUCTURE v 2.3.4 (Pritchard et al. 2000). The admixture model and correlation of the allele frequencies were used as input. The procedure was performed three times for each K between 1 and 11 with a burn-in of 100,000 iterations and an after-burn-in of 200,000 iterations. The probability of each K value was estimated using the delta K described by Evanno et al. (2005) and implemented in Structure Selector online software (Li and Liu 2018).

Migration analysis.

Estimating Effective Migration Surfaces (EEMS) software (Petkova et al. 2016) was used to visualize gene flow patterns among the sampling sites. EEMS is based on the stepping-stone model, in which individuals migrate locally between subpopulations (demes), and migration rates can vary by location (Petkova et al. 2016). EEMS uses Markov chain Monte Carlo (MCMC) and outputs a visualization of the posterior mean for effective migration and a measure of genetic diversity for every spatial position of the focal habitat. Regions with relatively low effective migration can be interpreted as having reduced gene flow over time, whereas those with relatively high migration can have elevated gene flow (Marcus et al. 2021). EEMS was run with the complete set of 2,641 SNPs using 500 demes and three independent chains of 5,000,000 MCMC iterations with a burn-in of 1,000,000, and sampling was performed every 9,999 iterations. The proposed variances were adjusted considering acceptance rates ranging from 10% to 40%. The results were plotted using the rEEMSplots2 package (https://github.com/dipetkov/reemsplots2) in R. Note that the results for the effective migration rates are in a $\log_{10}$ scale (denoted as log(m) in the plot) relative to the overall migration rate in the habitat. Thus, a log(m) = 1 represents an effective migration ten times greater than the average, and a log(m) = −1 corresponds to an effective migration ten times lower than the average.

Demographic analysis.

We used Stairway Plot 2 (Liu and Fu 2020) to infer the demographic history of *Cx. pipiens* s.l. Stairway Plot 2 is a model-free method that does not require whole-genome sequencing data or a reference genome. A folded site frequency spectrum was generated per sampling site using the function *gl.sfs* from the dartR library. We used a mutation rate of $4.85e^{-9}$ described for another mosquito (Kent et al. 2024) and a generation time of 0.122 years (or 44.7 days) described for *Cx. pipiens fatigans* (Gómez et al. 1977).

COI gene analysis

A subset of 31 specimens of *Cx. pipiens* s.l. (24 specimens from continental samples and 7 individuals from Rapa Nui) were selected to infer the haplotypic diversity of the *COI* gene and phylogeography using barcoding. Each mosquito was homogenized, and DNA was extracted using the salt extraction protocol described by Aljanabi and Martinez (1997). For PCR, amplification was conducted using primers targeting the mtDNA gene (658-bp fragment of *COI*). The primer pair used was LCO1490_F (5' GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198_R (5' TAA ACT TCA GGG TGA CCA AAA AAT CA-3') (Folmer et al. 1994). Reactions were made to a 25-µL volume containing 2 µL DNA (50 ng/µL), 2.5 µL 10× PCR buffer (Invitrogen), 1.6 µL $MgCl_2$ (50 mM) (Invitrogen), 2 µL dNTPs (2.5 mM) (Invitrogen), 14.6 µL water, 1 µL forward and reverse primers (50 ng/µL), and 0.3 µL Taq DNA polymerase (Invitrogen). The procedure started at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s, and a final step of 72 °C for 5 min. Amplifications were verified in 1% agarose gels, and the Pontificia Universidad Católica de Chile sequencing service performed sequencing. A 549-bp fragment of the *COI* was amplified. A GenBank BLAST analysis of the sequences obtained was run through the NCBI website (ncbi.nlm.nih.gov). The obtained sequences were deposited in GenBank under accession numbers PQ112595 to PQ112602.

In addition to our sequences, we downloaded most of the *Cx. pipiens* s.l. worldwide sequences from GenBank. Sequences were filtered by country and length (> 600 bp) using SeqIO in Biopython (Cock et al. 2009) and were edited and aligned using BioEdit v. 7.2 (Hall 1999). The haplotype network was constructed using the median-joining network algorithm in PopART version 1.7 (Bandelt et al. 1999; Leigh and Bryant 2015).

Results

Ecotypes

Microsatellite CQ11 was successfully amplified in 153 of the 180 studied specimens (Table 2). This locus showed

Table 2. Number of ecotypes and hybrids determined at each sampling site. N.D. indicates not determined or not amplified.

Site	Ecotypes			N.D.	Number of samples
	"Molestus"	Hybrids	"Pipiens"		
TB (Til Til Bajo)	11	6	0	1	18
CC (Copiapó)	13	4	0	3	20
CS (La Serena)	7	5	4	4	20
MC (Coquimbo)	4	9	3	2	18
PA (Playa Ancha)	4	3	4	0	11
SA (Santiago)	10	6	0	4	20
ME (Melipilla)	10	6	0	2	18
PU (Puaun)	9	3	2	2	16
PM (Puerto Montt)	9	3	4	2	18
RN (Rapa Nui)	13	0	0	8	21
Total	90	45	17	28	180

the presence of both alleles (200 and 250 bp), and both homozygotes and the heterozygous genotype, in the samples. Of these, 58.8% were identified as ecotype *molestus*, 11.1% were classified as ecotype *pipiens*, and 29.4% were classified as hybrids. A total of 18.3% of the samples were not classified because of the absence of bands or because the bands were unclear in the agarose gel.

Ecotype *molestus* was present in all sampling sites, including below- and aboveground sites, whereas ecotype *pipiens* was present only in aboveground sites. In particular, the ecotype *pipiens* was observed only in coastal sites located < 10 km from the coast, and only the ecotype *molestus* was identified on Rapa Nui island. All individuals identified in Puaun, a rural location, were females collected after biting humans and were classified as *molestus*, *pipiens*, and hybrids (Table 2).

Population genetic analysis based on SNPs

Considering the genome size of 559,749Mb described by Liu et al. (2023), the length of the sequences of 70 bp and a mean of 1,023,187 reads per individual obtained in our study, the genome coverage of our study was 0.013%. From the 180 collected individuals, 97,424 raw SNPs were obtained. After removing low-quality and outlier SNPs, 2,641 SNPs and 164 individuals were retained for analysis. The sample sizes before and after filtering are presented in Table 1. Playa Ancha showed the highest observed and expected heterozygosity values (0.148 and 0.167, respectively), while Rapa Nui Island showed the lowest (0.039 and 0.049, respectively), as expected. Rapa Nui had the highest inbreeding coefficient (F_{IS}, 0.229), indicating a significant degree of inbreeding among the collected mosquitoes (Table 1). The QGE index does not detect significant values of relatedness in all sampling sites except for Copiapo (QGE = −0.032, $P < 0.05$) and Puerto Montt (QGE = −0.040, $P < 0.05$). The lynchrd and ritland indexes that account for inbreeding showed negative values (Table 1), suggesting low relatedness in the sampling sites used in the study.

The first two principal coordinates of the PCoA explained 28% of the total variance (Fig. 2). The first component (17.9% of the variance) separated Rapa Nui from continental localities. In contrast, the second component (10.1% of the variance) separated Puerto Montt (the southernmost sampled site) from the other localities in Chile (Fig. 2a). The same analysis showed no evidence of differentiation between ecotypes and their hybrids (Fig. 2b). These results were corroborated by the Bayesian method implemented in STRUCTURE software and the Delta *K* estimated with the Evanno method, which found $K = 6$ to have the highest ln (*K*) value (mean LNP[*K*] = −190,219) (Fig. 3, Supplementary Table S1). These genetic groups (from north to south) correspond to 1: Rapa Nui (RN); 2: Copiapó (CC); 3: Til Til Bajo, La Serena (CS), Coquimbo (MC), and Playa Ancha (PA); 4: Santiago (SA); 5: Melipilla (ME) and Puaun (PU); and 6: Puerto Montt. Pairwise F_{ST} values among populations were statistically significant ($P < 0.001$) and showed higher values in Rapa Nui (Table 3).

EEMS showed apparent qualitative differences between the estimated effective migration rates, reflecting the underlying simulation truth. Migration rate estimates obtained with EEMS indicated high gene flow in continental sites in the country's center and low gene flow among northern and southern sites. Similarly, this analysis suggests null migration between continental Chile and Rapa Nui island (Fig. 4).

The demographic analysis inferred by Stairway Plot 2 (Fig. 5) suggested that the *Cx. pipiens* s.l. population on Rapa Nui island has had the lowest effective population size (~800 individuals) for at least 300 years. A similar scenario was observed in Santiago and Puaun, with constant effective population size over time (3,000 and 1,800 individuals, respectively). In contrast, La Serena, Coquimbo, Playa Ancha, and Melipilla showed an increase in the effective population

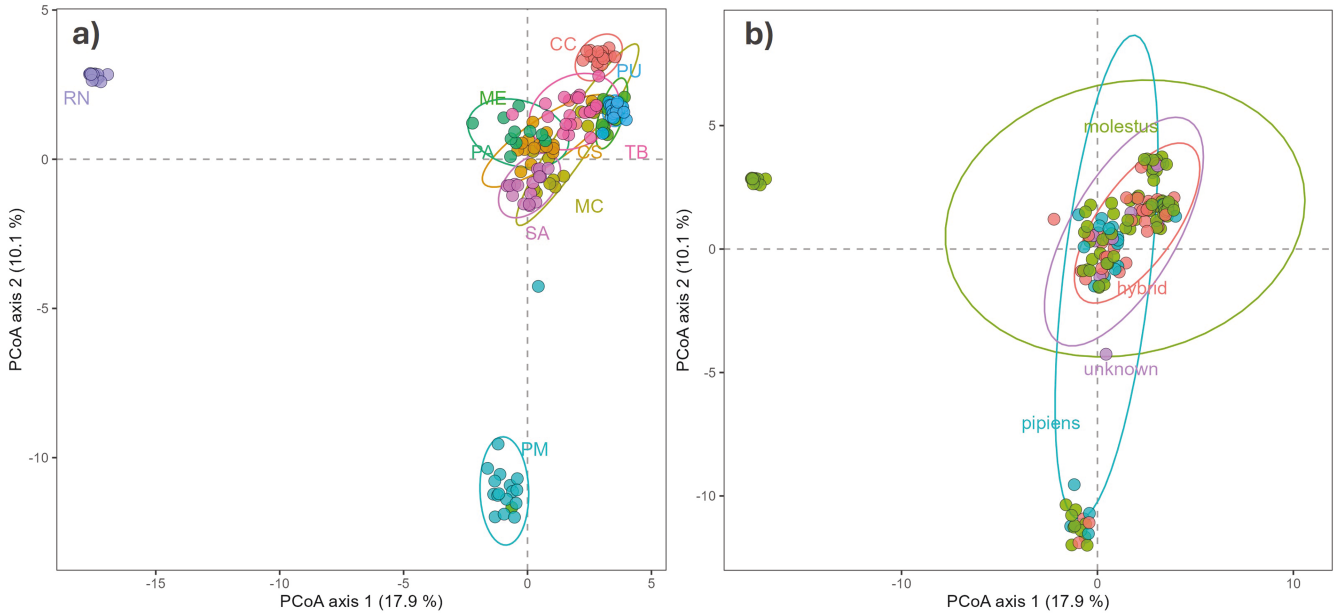


Fig. 2. Principal coordinate analysis (PCoA) based on 2,641 SNPs among the 164 *Culex pipiens* s.l. genotypes. a) Color differentiation by location site, and b) color differentiation by ecotype (*pipiens*, *molestus*, and hybrids). The first two coordinates of the PCoA explained 28% of the total variation.

Fig. 3. The population structure of *Culex pipiens* s.l. inferred using STRUCTURE software for $K = 1$ to $K = 11$ of the 164 individuals analyzed after filtering SNPs. A single vertical line represents each individual, a square represents each sample site, and the colors show the estimated individual proportions of cluster membership. The collection sites are indicated at the top of the graph, and the codes are listed in Table 1.

Table 3. Pairwise F_{ST} (below diagonal) and corrected *P*-value (above diagonal) values between populations based on SNPs.

F_{ST}	CC	MC	CS	ME	PU	PA	PM	RN	SA	TB
CC	–	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
MC	0.192	–	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
CS	0.189	0.067	–	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
ME	0.177	0.092	0.092	–	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
PU	0.247	0.153	0.150	0.108	–	<0.001	<0.001	<0.001	<0.001	<0.001
PA	0.209	0.090	0.069	0.097	0.164	–	<0.001	<0.001	<0.001	<0.001
PM	0.425	0.290	0.280	0.309	0.373	0.281	–	<0.001	<0.001	<0.001
RN	0.677	0.557	0.525	0.616	0.659	0.519	0.628	–	<0.001	<0.001
SA	0.277	0.176	0.158	0.189	0.248	0.150	0.305	0.559	–	<0.001
TB	0.131	0.078	0.074	0.065	0.129	0.075	0.307	0.568	0.166	–

Abbreviations: CC (Copiapo), MC (Coquimbo), CS (La Serena), ME (Melipilla), PU (Puaun), PA (Playa Ancha), PM (Puerto Montt), RN (Rapa Nui), SA (Santiago), and TB (Til Til Bajo).

size approximately 800 years ago, with Playa Ancha showing a particularly significant increase from ~1,000 to ~6,000 individuals. Finally, the effective population sizes of Copiapó and Puerto Montt decreased over the last 100 years.

Genetic variation and haplotype network based on *COI* variability

A total of 45 haplotypes were obtained from 540 individuals of *Cx. pipiens* s.l. through GenBank and this study for a 497-bp segment of *COI* (Supplementary Table S2). The haplotype network created using the median-joining algorithm did not show a configuration structured by continent or country (Fig. 6). Instead, the most frequent haplotypes were shared in most countries. For example, haplotype 5, the most abundant, was observed in individuals from Europe, South America, Asia, and Oceania. However, we also observed 34 single haplotypes along its entire distribution, of which five were present in continental Chile (haplotypes 25 to 29).

Fig. 4. Effective migration rates in *Culex pipiens* s.l. for ten collection sites were estimated using EEMS software. Log(m) denotes the effective migration rate on a $\log_{10}$ scale relative to the overall migration rate throughout the habitat. The blue colors represent areas where effective migration is higher than average, while brown colors represent areas where effective migration is lower than average. Maps were drawn using the library rEEMSplots2 implemented in R software.

In Supplementary Table S2, we indicate the number of sequences used by the country, the GenBank accession number of each sequence, and the genetic diversity evaluated by the number of polymorphic sites (S), number of haplotypes (h), haplotype diversity (H_d), and nucleotide diversity (P_i) of the *COI* gene per country with at least two sequences. Continental Chile showed a relatively high number of polymorphic sites and haplotype diversity, whereas Rapa Nui showed the lowest haplotype and nucleotide diversity values.

Discussion

Understanding the limits of gene flow in mosquitoes from the *Cx. pipiens* complex is essential for optimizing control strategies against potential disease outbreaks transmitted by these vectors. Nowadays, *Cx. pipiens* s.l. is found globally in almost all temperate regions (Vinogradova 2000). Its tolerance to human-altered environments has greatly facilitated its global distribution in natural and anthropized areas (Farajollahi et al. 2011). Despite its high dispersal and adaptive capacity, this species can exhibit spatial geographic structure.

When a species is genetically structured, subpopulations within the species may have differences in allele and haplotype frequencies due to various factors, such as genetic drift, natural selection, or reproductive isolation (Ellegren and Galtier 2016). This implies that subpopulations do not mix homogeneously, and there are genetic "barriers" that may be geographic (isolation by distance) or biological (e.g. differences in reproductive behavior). In this study, we reinforce the hypothesis of isolation by distance inferred previously in mosquito vectors and provide evidence of the founder effect of *Cx. pipiens* s.l. in the highly isolated Rapa Nui island.

We also identified the ecotypes and hybrids in these regions to evaluate the vector competence and transmission potential of WNV in these vulnerable areas. Although the molecular analyses did not reveal genetic differentiation between the two ecotypes, we believe their ecological and physiological preferences might provide insights into how these mosquitoes could respond to a potential WNV outbreak on the island and the continent.

Our comparison of the haplotypic differentiation in Rapa Nui island with global sequences available in GenBank showed that vector and associated disease control efforts should now focus on those connection routes from Polynesian islands.

Ecotypes and hybrids present in the American continent and Rapa Nui island

We successfully applied the protocol of Bahnck and Fonseca (2006) to identify ecotypes (*Cx. pipiens pipiens* and *Cx. pipiens molestus*) and hybrids within *Cx. pipiens* s.l. This protocol enabled us to identify *pipiens* and *molestus* ecotypes and hybrids across continental Chile while noting the sole presence of the anthropophilic ecotype *molestus* on Rapa Nui island.

In South America, the described members of the *Cx. pipiens* complex include *Cx. pipiens* s.l., *Cx. quinquefasciatus*, and hybrids between the two species (Cardo et al. 2018). Concerning ecotypes "*pipiens*" and "*molestus*," both have been identified in the southernmost distribution, with a significant percentage of hybrids between them (Cardo et al. 2020, 2024; Alvial et al. 2024). The hybridization between these ecotypes has been identified as a major factor influencing WNV transmission (Ciota and Kramer 2013). This is mainly due to differences in feeding habits and habitat preferences among the two ecotypes (Ferraguti et al. 2023). The *pipiens* ecotype feeds more on birds and is typically associated with rural environments, whereas the *molestus* ecotype prefers mammals, including humans, and is more commonly found in urban settings (Fonseca et al. 2004).

Ferraguti et al. (2023) developed an epidemiological model, "SEIR" (Susceptible–Exposed–Infectious–Recovered), to

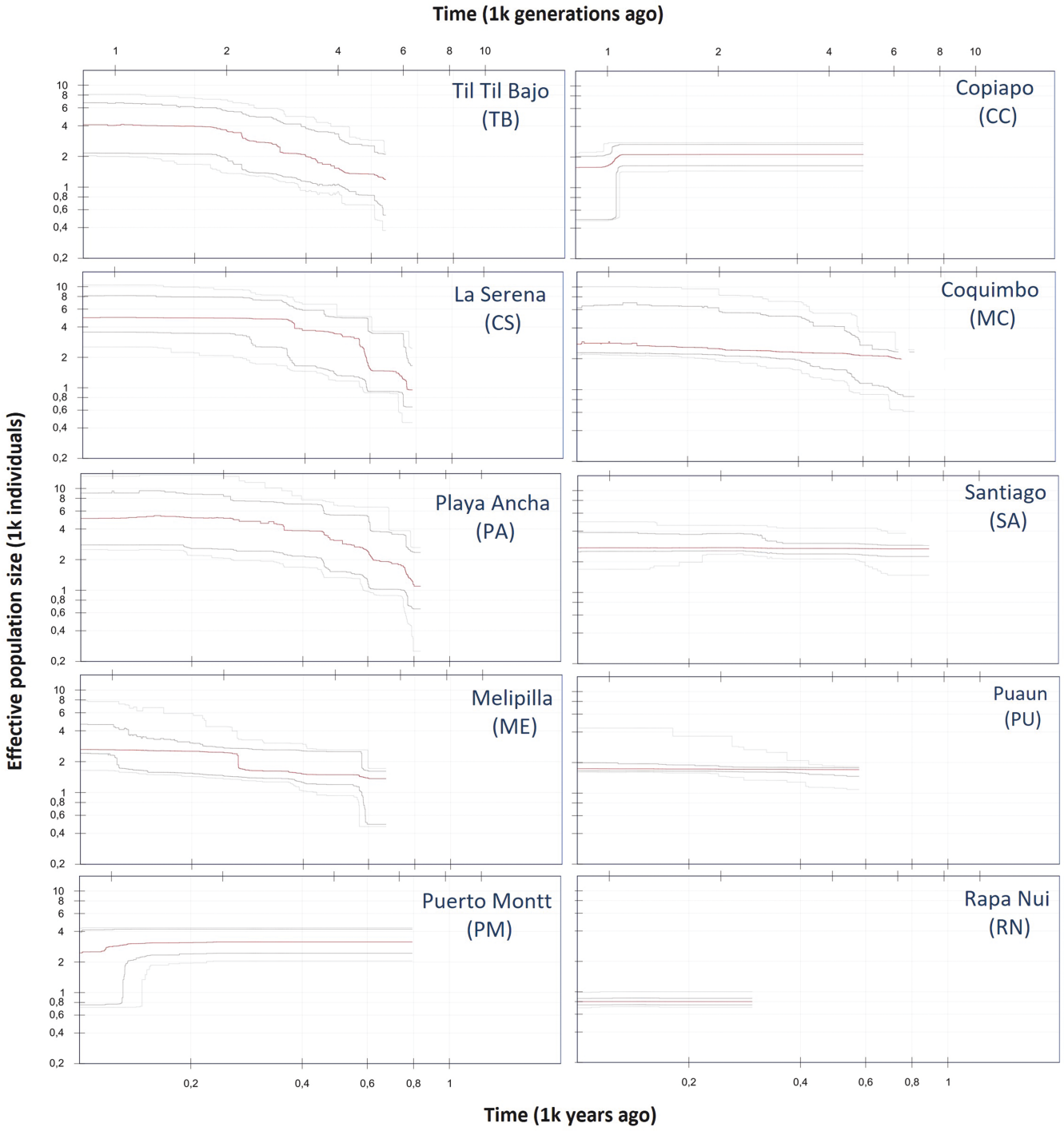


Fig. 5. The demographic history of *Culex pipiens* s.l. inferred using stairway plot 2 in the ten populations. The central line represents the estimation of the effective population size, and the two black lines delineate the 95% confidence interval of the estimation.

identify which life-history traits have the most significant impact on the invasion of WNV into susceptible communities. They modeled three eco(types) of vectors (*Cx. pipiens* f. *pipiens*, *Cx. pipiens* f. *molestus*, and their hybrids) and two vertebrate hosts (birds and humans), and examined how variations in feeding preferences and transmission rates affected WNV transmission in different habitats and seasons. They discovered that the feeding preferences of vectors and transmission rates were the factors that most significantly influenced the risk of WNV invasion. They also found that the *pipiens* ecotype acted as the primary vector in all habitats, especially in habitats with abundant bird populations or rural habitats. At the same time, hybrids are limited in increasing the risk of WNV transmission to humans across different habitats. However, they can enhance the risk of virus invasion when interacting with either *pipiens* or *molestus* mosquitoes in natural or urban environments (Ferraguti et al. 2023). According to this study, any ecotype (*pipiens*, *molestus*, and hybrids) and the presence of migratory birds increase the susceptibility of a WNV-free area to the virus.

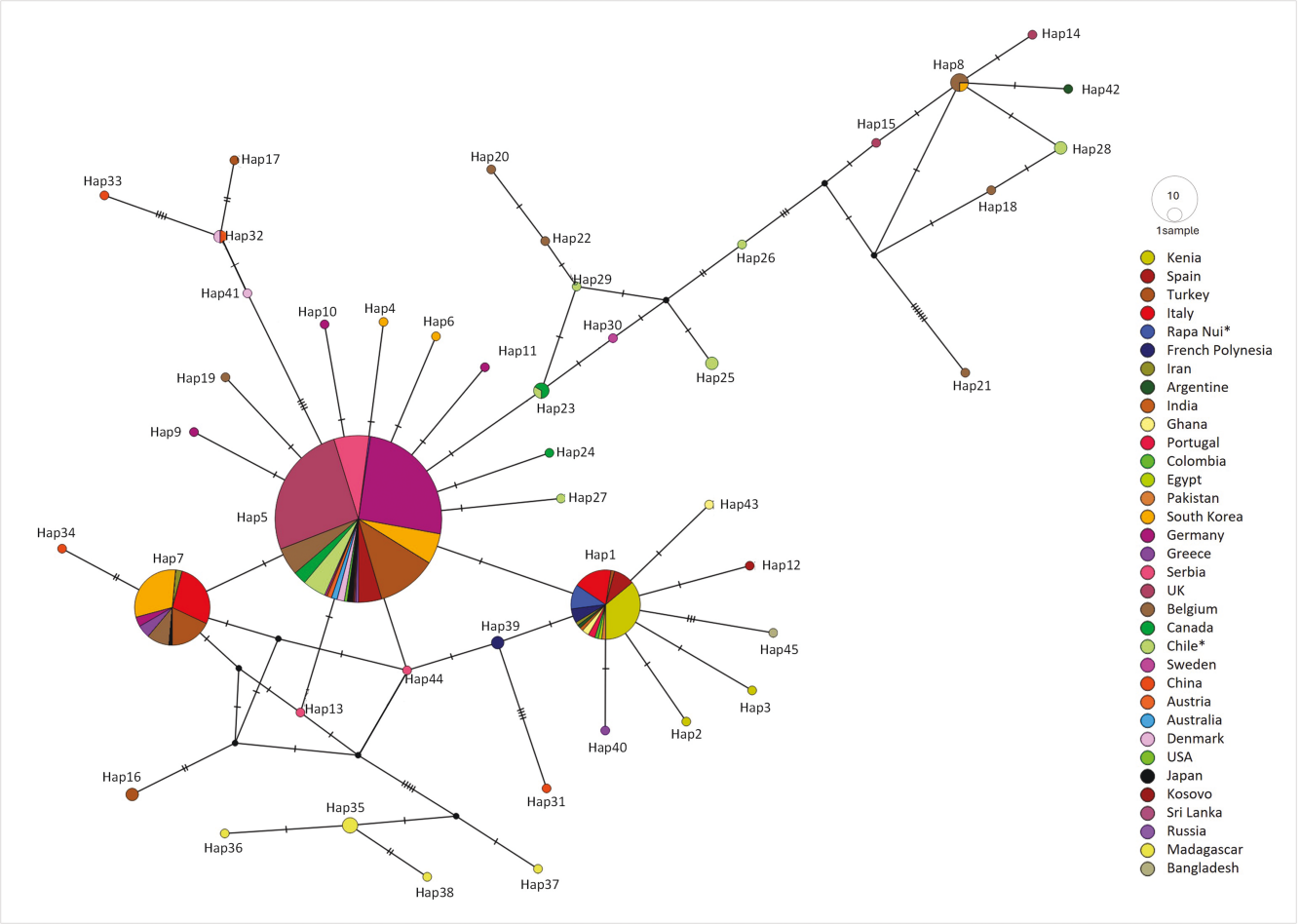


Fig. 6. Haplotype network of *Culex pipiens* s.l. populations containing our sequences and the sequences available in GenBank. Circles represent haplotypes, and dashed lines indicate additional nucleotide differences between haplotypes. Colors represent the countries listed in the legend, and the size of the circles indicates the number of individuals with that haplotype. The new sequences created in this study are marked with asterisks.

As mentioned in the "Results" section, PCoA based on SNP loci did not provide evidence for a valid separation of these ecotypes, perhaps explained by the high percentage of hybrids found in all continental locations (~30%). Consequently, we can infer that these ecotypes are not genetically valid entities (at least at this latitude). Rather, ecotype *molestus* seems to be an ecological or phenotypical variation of *Cx. pipiens* s.l.

The idea of an independent monophyletic origin of *Cx. p. pipiens* and *Cx. p. molestus* is sustained by Fonseca et al. (2004) and Yurchenco et al. (2020), who studied and compared microsatellites and nuclear genomes of above- (ecotype *pipiens*) and belowground populations (ecotype *molestus*) from different locations worldwide. They described higher hybridization rates in North America than in the Old World. Therefore, hybridization in members of the *Cx. pipiens* complex implies that reproductive isolation between them is incomplete and that genetic differentiation would be ineffective in places with more significant hybridization by sympatry, like South America.

Phylogeographic inferences based on haplotype diversity of the *COI* gene

The *COI* variability did not show haplotypes shared between the American continent and Rapa Nui island. We observed the globally most abundant haplotype in all continental populations. In contrast, we found one haplotype on Rapa Nui island, which was shared with populations in Africa, Europe, French Polynesia, and other locations in America. These findings suggest that the *Cx. pipiens* s.l. population on Rapa Nui island was introduced from Polynesia in the early 1900s, with the first documented records of this species occurring in 1914 (Fuentes 1914). A historical connection between Polynesia and Rapa Nui island has been proposed for several cosmopolitan species likely linked to human movement (Campos and Peña 1973; Alvial et al. 2018). However, the pathways for the entry of culicid vectors have remained unclear until now. It is crucial for vector control and disease management efforts to concentrate on these connection routes from Polynesian islands.

We observed low haplotype diversity at multiple locations when we compared sequences from 540 individuals—509 sourced from GenBank and 31 generated in this study. However, the lowest haplotype and nucleotide diversity was observed on Rapa Nui island. This low diversity may be linked to several factors, including its relatively young geological age (ranging from 0.24 to 0.11 Mya), its small size (171 km²), and mainly the island's isolation, which suggests an evolutionary process influenced by founder effects, as indicated by the SNP analyses discussed below.

Population structure based on SNP analysis

In continental Chile (between 29° S and 33° S), we detected low gene flow between northern (TB, CC, CS, and MC) and southern populations (PU and PM), which could be related to isolation by distance. Specifically, EEMS models showed low migration rates in sites separated by a distance greater than 500 km. This confirms the proposal by Cui et al. (2007) that mosquitoes of the *Cx. pipiens* complex requires a considerable distance (500–1,000 km) to show isolation by distance, irrespective of subspecies (*Cx. pipiens* s.l., *Cx. quinquefasciatus*, and *Cx. pipiens pallens*) or geographic location. Genetic differentiation was most pronounced in the context of continent and island populations, and all evidence obtained from SNP loci indicated a clear geographic isolation of *Cx. pipiens* s.l. on Rapa Nui island. In effect, Bayesian clustering analysis revealed two main genetic groups: the insular and continental populations of *Cx. pipiens* s.l.

In this context, the Pacific Ocean has been a geographic barrier to the genetic exchange of non-indigenous organisms between populations on the American continent and Rapa Nui island, highlighting a closer connection with other Polynesian islands. As a result, our findings indicate high inbreeding but not a significant level of relatedness among insular individuals, as reflected by the F_{IS} and QGE coefficients. In addition, the demographic analysis revealed that the population on Rapa Nui island has had the lowest effective population size—of approximately 800 individuals—for at least the past 300 years. A similar pattern was observed in another cosmopolitan insect on the island, the odonate *Pantala flavescens*, which showed high inbreeding values, low effective population size, and low migration rates to and from the American continent (Alvial et al. 2018). This implies that *Cx. pipiens* s.l. and other cosmopolitan species on the island are undergoing the foundation process. Establishing species in new environments (or remote islands) involves sampling from the parent population. Genetic drift can become significant since the genes represented in the founding population are only a small subset of the original population. This may reduce genetic diversity, making the population more vulnerable to environmental changes and diseases (Bataille et al. 2009; Habel and Zachos 2013; Gillespie 2024).

Islands typically have fewer species, reduced trophic complexity, and lower functional diversity compared to similar mainland ecosystems. This results in limited biological resistance to invaders and creates numerous unoccupied ecological niches (Russell et al. 2017). For instance, *Aedes aegypti* was first detected on Rapa Nui island in 2000, and, to date, it has been responsible for the continuous outbreak of classic dengue on the island (Canals et al. 2012). Efforts to control the vector have been unsuccessful by climate, population growth, and human behavior (Fica et al. 2016).

Studies on the genetic structure of mosquito vectors indicate that regions with low genetic diversity and limited gene flow are less susceptible to the spread of viruses. This is because such conditions affect the species' resilience to significant population declines, which can occur due to events like vector eradication measures (for example, the application of insecticides) (Fouet et al. 2017). Then, although the presence of *Cx. pipiens* s.l. on the island increases its vulnerability to the entry of viruses associated with this vector; the genetic characteristics of the population provide insight into the most effective control strategies.

In conclusion, geographic isolation and genetic differentiation would have positive and negative effects on the vectorial capacity of non-indigenous mosquitoes. On the one hand, they may reduce their ability to disperse pathogens regionally or globally (continental context). On the other hand, they may also encourage specialization and adaptation to new ecological niches, potentially increasing their efficacy as vectors in specific local contexts (insular conditions).

The findings of this study will enhance our understanding of the demographic and population characteristics of mosquito vectors that may affect the spread of viruses in continental and insular locations. In addition, this research will contribute to developing effective control and management measures for non-indigenous mosquitoes in areas susceptible to WNV.

Supplementary material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/.

Acknowledgments

I want to thank F. Henriquez-Tucki for assisting with the sampling on Rapa Nui Island. I also appreciate the collaboration of the Oficina de Zoonosis y Control de Vectores from the División de Políticas Públicas Saludables y Promoción del Ministerio de Salud in Chile, who provided specimens of *Culex pipiens* s.l. from Rapa Nui Island. Thank you to B. Tubin for helping create the map of collection sites, S. Britto for providing support during the laboratory analyses, and R. Lazo for editing the English language version.

Author contributions

Ingrid Alvial (Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Writing—original draft, Writing—review & editing), Noemi Rojas-Hernández (Formal analysis, Methodology, Software, Writing—review & editing), Nicolas Guerra (Formal analysis, Methodology, Software), David Véliz (Conceptualization, Formal analysis, Methodology, Supervision, Writing—review & editing), Christian González (Conceptualization, Investigation, Methodology, Writing—review & editing), Laura Pérez (Funding acquisition), and Hugo Benítez (Conceptualization, Visualization, Writing—review & editing)

Funding

This work was supported by Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) through grant Postdoctorado Number 3210081. DV thanks Anid Anillo ATE220044.

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