Annals of the Entomological Society of America Advance Access published April 23, 2015

Systematics

Molecular Systematics and Origin of the Mediterranean Sea rock-pool Mosquitoes of the *Aedes mariae* (Diptera: Culicidae) Complex

VALENTINA MASTRANTONIO,[1,2] DANIELE PORRETTA,[1,3] ROMEO BELLINI,[4] GIUSEPPE NASCETTI,[2] and SANDRA URBANELLI[1]

Ann. Entomol. Soc. Am. 1–7 (2015); DOI: 10.1093/aesa/sav031

ABSTRACT Mosquitoes belonging to the *Aedes mariae* complex, including *Aedes mariae* (Sergent & Sergent), *Aedes zammitii* (Theobald), and *Aedes phoeniciae* (Coluzzi & Sabatini), are among the few animals inhabiting the extreme habitat of sea rock pools. Morphological analysis of these species and crossing experiments conducted in laboratory and natural conditions inferred the occurrence of three taxa with a parapatric distribution along the coasts of the Mediterranean Basin. To date, however, genetic characterization of the three species within the *Ae. mariae* complex and an assessment of their interspecific differentiation are lacking. In this study, we used both nuclear (i.e., 14 allozymic loci) and mitochondrial genetic markers (i.e., sequences of the cytochrome oxidase I and II genes) to genetically characterize the three species belonging to the complex. Then, we used mitochondrial markers to infer phylogenetic relationships between the species and estimate the time to most recent common ancestor (TMRCA). The allozymic and mitochondrial markers showed the occurrence of three distinct gene pools, namely, *Ae. mariae*, *Ae. zammitii*, and *Ae. phoeniciae*. The TMRCAs for the entire in-group were estimated to have occurred during the early Pleistocene (i.e., mean node age of 1.739 million years ago). An important role of Pleistocene climatic changes could be suggested in the origin of the species of the *Ae. mariae* complex and in shaping their pattern of intraspecific genetic diversity.

KEY WORDS *Aedes mariae* complex, sibling species, genetic characterization, mitochondrial DNA, allozymes

There are ~3,500 species of mosquitoes, including important vectors of human and animal diseases, that are found throughout the world, except in places that are permanently frozen (Clements 1999, Epis et al. 2014). Their geographic distribution is largely based on the availability of suitable aquatic sites for larval growth. The larvae of most species live in freshwaters, but ~5% of mosquito species live in brackish or saline waters (Clements 1999, Porretta et al. 2007). The salinity of these latter habitats is liable to fluctuate widely, decreasing as a result of rain or increasing through evaporation. Thus, rock pools along the marine littoral are considered extreme habitats because their salt concentration may range from virtually fresh water to 250% seawater (87.5 ppt; Rioux 1958, Clements 1999). Only a few mosquito species live in this habitat under such severe conditions, including *Opifex fuscus* (Hutton 1992) in New Zealand, *Aedes* (*Halaedes*) *australis* (*Haledes australis*) (Erichson, 1842) in Australia, *Aedes* (*Finlaya*) *togoi* (Theobald) in Canada and Eurasian regions and the mosquitoes of the so-called *Aedes mariae* complex (Coluzzi and Sabatini 1968) that inhabit the rock pools along the coasts of the Mediterranean Basin.

The first evidence of the existence of a complex of sibling species within *Aedes mariae* (*Acartomya mariae*), (Sergent and Sergent 1903) was discovered by Coluzzi and Sabatini (1968); on the basis of slight morphological differences, they recognized the occurrence of three taxa, named *Aedes mariae*, (Sergent & Sergent, 1903), *Aedes zammitii* (*Acartomya zammitii*) (Theobald, 1903), and *Aedes phoeniciae* (*Acartomya phoeniciae*) (Coluzzi and Sabatini, 1968). They have been suggested to have a contiguous distribution along the Mediterranean Basin, with *Ae. mariae* distributed in the western Mediterranean; *Ae. zammitii* along the central and eastern Mediterranean coasts; and *Ae. phoeniciae* along the coasts of Israel, Lebanon, Syria, Cyprus, and south-east Turkey (Coluzzi and Sabatini 1968, Coluzzi et al. 1974). No sympatric areas were found to exist among these taxa (Coluzzi et al. 1974). These three species were recently considered belonging to the genus *Acartomyia* (Reinert et al. 2009).

Reproductive isolation between members of the *Ae. mariae* complex has been investigated subsequently under laboratory conditions and in nature (Coluzzi and Sabatini 1968). Crossing experiments conducted in the

[1] Department of Environmental Biology, University of Rome "La Sapienza", Italy.
[2] Department of Ecological and Biological Sciences, Tuscia University, Viterbo, Italy.
[3] Corresponding author, E-mail: daniele.porretta@uniroma1.it.
[4] Agriculture Environment Centre "G. Nicoli", Crevalcore, Italy.

© The Authors 2015. Published by Oxford University Press on behalf of Entomological Society of America. All rights reserved. For Permissions, please email: journals.permissions@oup.com

laboratory revealed that both hybrid males and females between *Ae. phoeniciae* and the other two members of the complex were sterile, while only the F1 hybrid males produced from a crossing of *Ae. mariae* and *Ae. zammitii* were sterile (Coluzzi and Sabatini 1968).

The possibility of hybridization in nature was investigated by translocation experiments between *Ae. mariae* and *Ae. zammitii* (Coluzzi and Bullini 1971, Urbanelli et al. 2014). In 1970, an initial experiment was conducted by translocating *Ae. zammitii* into the geographic range of *Ae. mariae* at a site along the Tyrrhenian coast (Coluzzi and Bullini 1971). A hybridization rate of ~2% was found, showing the occurrence of efficient reproductive isolation mechanisms. The different heights of the mating swarms above the ground have been suggested as an important premating isolating mechanism (*Ae. mariae* mating swarms remain near the rock, while *Ae. zammitii* swarms are generally found at a height of 2 m above the rocks; Bullini and Coluzzi 1980; Urbanelli et al. 2014). After ~2 yr, the introduced species disappeared from the release area (Coluzzi and Bullini 1971). In 1986, a second translocation experiment was conducted along the Adriatic coast, where *Ae. mariae* individuals were translocated into the geographic range of *Ae. zammitii.* The introduced species has persisted and spread into the area along a transect of ~20 km. Over the following 25 yr (i.e., ~200 generations), the hybridization rate between the two species and the composition of mating swarms were monitored (Urbanelli et al. 2014). These field data showed that *Ae. mariae* and *Ae. zammitii* have remained as distinct taxa in sympatry, even though complete reproductive isolation has not occurred and hybridization persists between them at rate of ~5% (Urbanelli et al. 2014).

Population genetic studies on the *Ae. mariae* complex have been limited to the analysis of the only allozymic locus, phosphoglucomutase (Pgm) (Coluzzi and Bullini 1971). More recently, six allozymic loci have been identified between *Ae. mariae* and *Ae. zammitii* (Urbanelli et al. 2014), but data on the genetic characterization of all species within the complex, and assessments of intra- and interspecific genetic variation and the phylogenetic relationships among them are lacking.

In this study, we used both nuclear and mitochondrial genetic markers to genetically characterize the three species belonging to the *Ae. mariae* complex. Then, we used mtDNA markers to infer phylogenetic relationships and estimate the time to most recent common ancestor (TMRCA) of the three species within the *Ae. mariae* complex. Finally, we discuss the genetic differentiation observed in relation to the known pattern of reproductive isolation among the species and propose hypotheses to be tested in future studies.

Materials and Methods

Sampling. Twelve populations from the geographic range of *Ae. mariae* (five populations), *Ae. zammitii* (five populations), and *Ae. phoeniciae* (two populations) were analyzed (Fig. 1; Supp Table 1 [online only]). The *Ae. mariae* populations of Circeo, Scauri, and Scilla, and the *Ae. zammitii* populations of Peschici, Baia dei Campi, and Heraklion have been investigated at allozymic loci in our previous study (Urbanelli et al. 2014). We included these data in this study and analyzed ex novo at both allozymes and mitochondrial DNA 15 individuals that were stored at −80°C for each population. All other populations have not been previously investigated. The population's codes, geographic origin

Fig. 1. Map showing the sampling localities of *Ae. mariae*, *Ae. zammitii*, and *Ae. phoeniciae* populations. White lines show the neighbor of geographic distribution of the three species (Coluzzi et al. 1974). Inserted images show sea rock pools from Putignano, Italy (photo Alessandra Spanò), and a female individual of *Ae. mariae* from Circeo, Italy (photo Valentina Mastrantonio).

Table 1. Discriminative allozymic loci between *Ae. mariae*, *Ae. zammitii*, and *Ae. phoeniciae*

Locus	*Ae. mariae*	*Alleles Ae. zammitii*	*Ae. phoeniciae*
Adk	90, 100, 105, 112	90, 100, 105	92, 96
Pgm	100, 106, 110	90, 92, 96	98, 106, 114
Mdhp-1	82, 87, 92, 100	102, 105, 109	102, 107
Phi	95, 100, 106	104, 108, 112	100, 106, 110
Mpi	95, 100, 102	104, 108, 112	97, 104, 108
Odh	100	78, 88, 95	78, 88, 97
Hbdh	85, 90, 100, 110, 115	90, 100, 105	107
Sod-1	100	100	85
Ca-1	96, 100	88, 92, 102	94

Ae. mariae was discriminated by the loci *Mdhp-1, Mpi* and *Odh*; *Ae. zammitii* by the loci *Pgm*, and *Phi*; *Ae. phoeniciae* by the loci *Adk*, *Hbdh*, and *Sod-1*; the locus *Ca-1* was discriminating of all the three species.

and date of collection of the mosquitoes used in the work are listed in Supp Table 1 [online only].

Individuals of *Ae. mariae*, *Ae. zammitii*, and *Ae. phoeniciae* were collected as larvae in sea rock pools, brought in the laboratory, identified on the basis of the morphological keys of Coluzzi and Sabatini (1968) and reared to adults. Adults were kept in cages and fed with a sugar solution for 5 d, then frozen (−80°C) and stored for subsequent genetic analysis.

Laboratory Procedure and Data Analyses.

Allozymes. Fourteen allozymic loci were analyzed: adenylate kinase (*Adk*, EC 2.7.4.03); phosphoglucomutase (*Pgm*, EC 5.4.2.2); malate dehydrogenase (NADP+) (*Mdhp-1* EC 1.1.1.40); phosphohexose isomerase (*Phi*, EC 5.3.1.9); mannose phosphate isomerase (*Mpi*, EC 5.3.1.8); glutamate-oxaloacetate transaminase (*Got-1* EC 2.6.1.1); isocitrate dehydrogenase (*Idh-1*, EC 1.1.1.42); octanol dehydrogenase (*Odh*; EC 1.1.1.73); phosphogluconate dehydrogenase (*6Pgd*, EC 1.1.1.44); hydroxybutyrate dehydrogenase (*Hbdh*, EC 1.1.1.30); superoxide dismutase (*Sod-1*, EC 1.15.1.1); malate dehydrogenase (*Mdh-1*, EC 1.1.1.37); α-glycerophosphate dehydrogenase (α-*Gpdh*, EC 1.1.1.8); carbonic anhydrase (*Ca-1*, EC 4.2.1.1). The electrophoretic procedure and the criteria used to name numerically the alleles at each locus are reported in detail in Urbanelli et al. (1996).

Linkage disequilibrium across loci was tested using the linkdos algorithm (Garnier-Géré and Dillman 1992) into GENEPOP version 3.1 (Raymond and Rousset 1995) and significance was assessed by Fisher's exact tests. The same program was used to estimate allele frequencies, the observed heterozygosity, Nei's (1978) unbiased estimate of the expected heterozygosity and deviations from the Hardy–Weinberg equilibrium. For multiple tests, the significance threshold (5%) was corrected by applying the Bonferroni correction (Rice 1989).

Occurrence of distinct gene pools within the *Ae. mariae* complex was investigated using factorial correspondence analysis (Lebart et al. 1984), as implemented in the Genetix 4.05 software (Belkhir et al. 1996-2004), which does not use prior information about groups of individuals. Then, analysis of molecular variance (AMOVA) was performed to partition the total genetic variance into components due to differences among groups, among populations within groups, and within populations. Three groups were defined including the individuals of *Ae. mariae*, *Ae. zammitii*, and *Ae. phoeniciae*, respectively. Fixation indices were computed by variance components and their significance was tested using a nonparametric permutation approach as implemented in ARLEQUIN 3.01 (Excoffier et al. 2005).

Genetic divergence between species was estimated by Standard Nei's (1978) genetic distances using the Genetix 4.05 software (Belkhir et al. 1996–2004).

Mitochondrial DNA. Total DNA was extracted from the homogenate of single individuals used for allozymic analyses by using standard cetyltrimethyl ammonium bromide protocol (Green and Sambrook 2012) and used as template for polymerase chain Reaction (PCR) amplifications. Fragments of the mitochondrial genes cytochrome oxidase I (COI) and cytochrome oxidase II (COII) genes were amplified and sequenced.

PCR amplifications of mitochondrial gene fragments were carried out using the primers pairs *mzp*-COI-F 5′-TTTTCGGAGTTTGATCAGGAA-3′; *mzp*-COI-R5′-TTCAGGATGTCCAAAGAATCAA-3′; *mzp*-COII-f 5′-CACAAATTTCTGAACATTGACCA-3′; and *mzp*-COII-R 5′-GAAAATGGCAACATGAGCAA-3′ (Porretta et al. 2007). PCR cycling procedure was: 95° C for 5 min followed by 34 cycles at 93°C for 1 min, 57° C (for COI), 55°C (for COII) for 1 min, 72°C for 1 min 30s, and a single final step at 72°C for 10 min.

PCR sequences were obtained using ABI PRISM 3700 DNA sequencer by Macrogen Inc. (www.macrogen.com) (accessed 13 April 2015). All individuals were double sequenced using both forward and reverse primers to check for consistency. Sequences were edited and aligned using the software CHROMAS 2.31 and CLUSTALX 2.0, respectively. All sequences were deposited in GenBank (accession number KM592029–KM592074).

The COI and COII gene sequences were concatenated for all the subsequent analyses (partition–homogeneity test with 1,000 replicates, as implemented in the software PAUP* 4b10, $P > 0.05$; Farris et al. 1994). Polymorphisms of both nucleotide and amino-acidic sequences were assessed using the software MEGA 5.0 (Tamura et al. 2011). The same software was used to calculate mean net p-distance between haplotypes and groups of haplotypes and synonymous sequence divergence estimates using Nei and Gojobori's (1986) method.

Phylogenetic relationships were inferred by neighbor joining (NJ), maximum parsimony (MP), and Bayesian inference (BI) methods. The mosquitoes *Aedes* (*Ochlerotatus*) *detritus* (*Ochlerotatus detritus*) (Haliday, 1833) and *Aedes* (*Ochlerotatus*) *atropalpus* (*Georgecraigius atropalpus*) (Coquillet, 1902) were used as outgroups (GenBank accession number KM258326.1 and AF425845.1, respectively). NJ analysis was performed in PAUP* 4b10 (Swofford 2003). The evolution model used in NJ analysis was set following the model inferred by jModelTest 0.1.1 using the Akaike information criterion. MP was performed in PAUP (Swofford 2003) using a heuristic search option, with tree bisection and reconnection (TBR) branch

swapping and random sequence addition, with 10 random addition replicates. The robustness of the inferred NJ and MP tree topologies was assessed by the nonparametric bootstrap method with 1,000 replicates. BI was performed using MrBayes 3.2.2 (Ronquist et al. 2012). Bayesian posterior probability (BPP), i.e., the frequency of nodal resolution, was estimated using Markov chain Monte Carlo sampling approach with five million generations, saving one tree every 100 generations. The run was stopped when the average standard deviation reached 0.005. The initial 25% of the samples was discarded as burn-in and majority consensus tree was generated by the 50% remaining combined trees. FigTree 1.4 software (Rambaut, 2012) was used to visualize consensus tree.

TMRCA of the main mtDNA lineages was estimated by using BEAST v1.7.2 (Drummond and Rambaut 2007). Following explorative analyses, Markov chain Monte Carlo chains were run for 20 million generations, sampling every 1,000th generation, with Yule process prior, random starting tree, uncorrelated lognormal relaxed molecular clock, substitution rate of 0.0115 substitutions–site–lineage–Myr based on the divergence rate of 2.3% per million years (Brower 1994). This substitution rate has been recently used in several other mosquito species (Chen et al. 2011; Morgan et al. 2010, 2011; Porretta et al. 2012), and has been shown to correlate well with independent historical evidence. Models of sequence evolution were determined using jModelTest, as described above. Convergence was assessed using Tracer 1.5. Maximum clade credibility tree was generated using the TreeAnnotator software and visualized using FigTree 1.4.

Results

Allozymes. Among the studied loci, 13 were found polymorphic among the studied populations (*Adk*, *Pgm*, *Mdhp-1*, *Phi*, *Mpi*, *Got-1 Idh-1*, *Odh*, *6Pgd*, *Hbdh*, *Sod-1*, and *α-Gpdh*, *Ca-1*), whereas the locus *Mdh-1* was found monomorphic for the same allele in all samples (allele frequencies are available upon request to authors). No linkage disequilibrium was found among loci (Fisher's exact tests $P > 0.05$). Significant departures ($P < 0.05$) from the expected genotype frequencies under Hardy–Weinberg equilibrium were found in the Oludeniz and Castellorizo localities (*Ae. zammitii*) at the locus *Pgm* and *Ca-1*, respectively, and in the Scilla locality (*Ae. mariae*) at the locus *6Pgd*. Any significance was detected after Bonferroni correction. Nine discriminative allozymic loci between *Ae. mariae*, *Ae. zammitii*, and *Ae. phoeniciae* were found (Table 1): *Ae. mariae* was discriminated by the loci *Mdhp-1*, *Mpi* and *Odh*; *Ae. zammitii* by the loci *Pgm*, and *Phi*; *Ae. phoeniciae* by the loci *Adk*, *Hbdh*, and *Sod-1*; the locus *Ca-1* was discriminating of all the three species.

Factorial analysis of correspondences revealed that *Ae. mariae*, *Ae. zammitii*, and *Ae. phoeniciae* individuals form three different clusters (Supp Fig. 1 [online only]), with the first three FCA axes explaining 33% of the variability. The AMOVA results indicated that variation among groups explained the highest percentage of variability (74.82%, $P < 0.001$), followed by variation within populations (22.82%, $P < 0.001$; Supp Table 1 [online only]).

The Nei' (1978) average pairwise genetic distances between *Ae. mariae* and *Ae. zammitii* and *Ae. phoeniciae* were 0.555 and 0.975, respectively; between *Ae. zammitii* and *Ae. phoeniciae* was 0.789 (Table 2).

Mitochondrial DNA. An alignment of 594 and 474 bp was obtained for the COI and COII genes, respectively (data not shown). By concatenating the COI and COII gene sequences 36 haplotypes were found (12, 17 and 7 in *Ae. mariae*, *Ae. zammitii* and *Ae. phoeniciae*, respectively; Supp Table 1 [online only]) identified by 69 nucleotide substitutions (62 synonymous and 7 nonsynonymous), 56 of which were parsimony informative. In Supp Table 1 [online only] mitochondrial haplotypes found in each locality are shown. Mean net p distance between *Ae. mariae* and *Ae. zammitii* was 0.028; between *Ae. zammitii* and *Ae. phoeniciae* as well as between *Ae. mariae* and *Ae. phoeniciae* was 0.026. Synonymous sequence divergence estimates using Nei and Gojobori's (1986) method are shown in Table 2 and ranged from 0.109 to 0.116.

The best-fit evolution model for our data set was GTR+I with the proportion of invariable sites $I = 0.917$. The phylogenetic relationships among the haplotypes found are shown in Figure 2. Tree topologies were identical between NJ, MP and BI trees at all well supported nodes, so we showed only the tree obtained by the BI method. Three main clades were found corresponding to the *Ae. mariae*, *Ae. zammitii* and *Ae. phoeniciae* individuals, respectively. Within species, several supported subclades (BPP > 0.90 and bootstrap proportions of > 70%) were found, some of them were found in specific localities or geographic areas: the *Ae. mariae* haplotypes m1-m2 were found only in Llanca and Capo Teulada localities (i.e. along the costs of Spain and western Sardinia), the haplotypes m10-m12 were found only in Scilla; the *Ae. zammitii* haplotypes z10-z14 were found only in Crete, while the haplotypes z15-z17 were characteristics of Turkey (Supp Table 1 [online only]; Fig. 2). TMRCA estimates for the main mtDNA clades are shown in the Supp Fig. 2 [online only]. The TMRCAs for the entire ingroup and the main clades were estimated to have occurred in the early Pleistocene (mean node age 1.739 million years ago).

Table 2. Average pairwise Nei' (1978) genetic distances (below the diagonal) and net average Nei-Gojobory p-distance (up the diagonal) between *Ae. mariae*, *Ae. zammitii*, and *Ae. phoeniciae*

	Ae. mariae	*Ae. zammitii*	*Ae. phoeniciae*
Ae. mariae	****	0.116	0.109
Ae. zammitii	0.555	****	0.114
Ae. phoeniciae	0.975	0.789	****

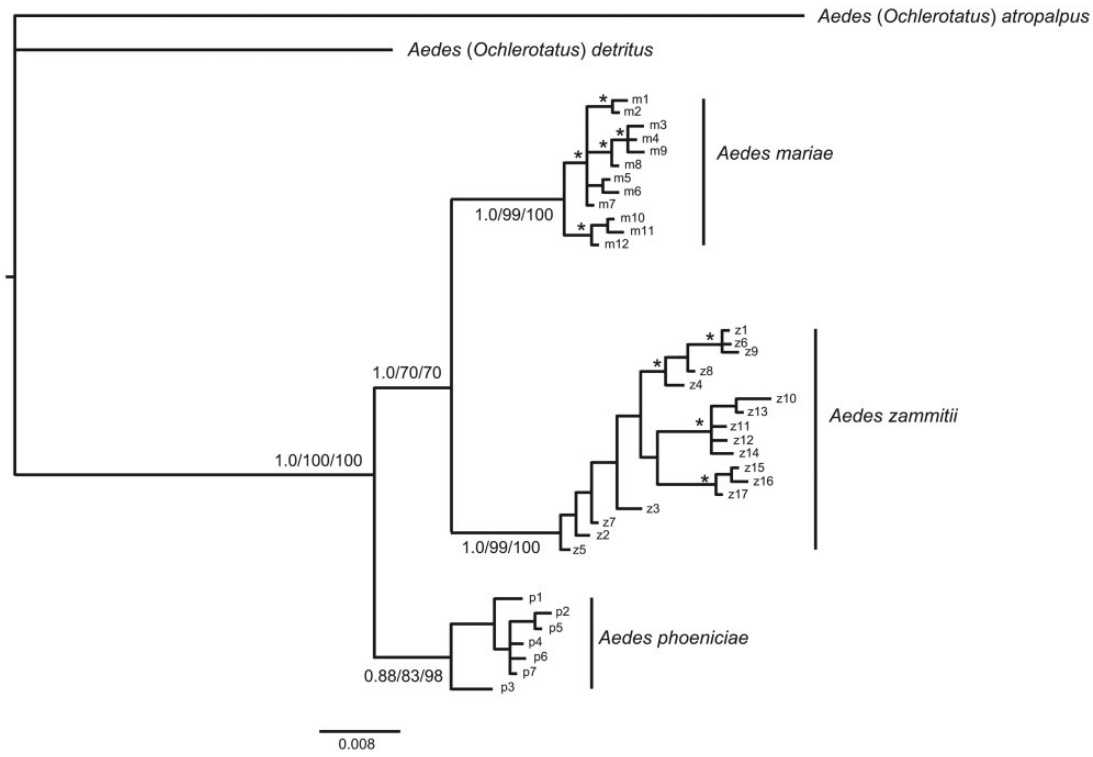

Fig. 2. Bayesian mtDNA gene tree for *Ae. mariae* complex species. Numbers above node indicate BPP and bootstrap values for MP and NJ trees, respectively. Asterisks refer to nodes supported by BPP >0.90 and bootstrap >70%.

Discussion

Morphological analysis of the *Ae. mariae* complex inferred the occurrence of three taxa with a parapatric distribution along the coasts of the Mediterranean Basin (Coluzzi and Sabatini 1968). The allozymic and mitochondrial markers used in this study showed the occurrence of three distinct gene pools, corresponding to *Ae. mariae*, *Ae. zammitii*, and *Ae. phoeniciae*. Allozymic markers showed the occurrence of three groups of individuals discriminated by several diagnostic loci (Supp Fig. 1 [online only]; Table 1; Supp Table 2 [online only]). Likewise, mtDNA analyses showed the occurrence of three main lineages that corresponded to the three groups of individuals defined by the allozymic markers (Fig. 2). The set of markers used in this study allowed us to genetically characterize the three members of the complex and could be a useful tool for further investigating their genetic structure and geographic distribution. The parapatric distribution of the species along the coasts of the Mediterranean Sea has been inferred by morphological analysis and polymorphism at the Pgm allozymic locus (Coluzzi and Sabatini 1968; Coluzzi and Bullini 1971). Morphology and the use of only one locus could have led to an oversight with regard to species recognition and, therefore, the possible occurrence of sympatric areas. Putative areas to be investigated are the gulfs of Catania and Gela in Sicily that separate *Ae. zammitii* from *Ae. mariae*, and the sandy coast of the gulf of Antalya, Turkey, that divides *Ae. zammitii* from *Ae. phoeniciae* (Fig. 1; Coluzzi et al. 1974).

The *Ae. mariae* complex has been suggested to have originated during the Plio-Pleistocene by fragmentation of the geographic range of an ancestor with a circum-Mediterranean distribution (Coluzzi et al. 1974). Our estimates of the TMRCA by mtDNA markers support an origin of the complex during the Pleistocene (Supp Fig. 2 [online only]). Notably, we found groups of haplotypes geographically localized with a more recent origin in both *Ae. mariae* and *Ae. zammitii* (Fig. 2; Supp Table 1 [online only]), which could underlie the occurrence of further fragmentation events. During the Pleistocene, drastic climatic and geographic changes occurred in the Mediterranean Basin. For instance, the coast line advanced and retreated repeatedly during glacial and interglacial phases, which led to the formation and disappearance of land bridges between the Mediterranean peninsulas (e.g., the Italian Peninsula and Balkans) and between the islands and the mainland (e.g., between Sardinia, Corsica, and Tuscan Archipelago Islands; Carrión et al. 2003, Horn et al. 2006, Médail and Diadema 2009, Habel et al. 2009, Porretta et al. 2011). For some continental species, these land bridges would have favored population connectivity during glacial phases (Porretta et al. 2011, 2012, Bisconti et al. 2011). For species with a strictly coastal distribution, they could have led to fragmentation of their distribution with consequent allopatric genetic divergence between groups of populations

(Urbanelli 2002, Urbanelli and Porretta 2008, Antonini et al. 2010, Audisio et al. 2010, Porretta and Urbanelli 2012). Our genetic data, therefore, support an important role of Pleistocene climatic changes in the origin of the species of the *Ae. mariae* complex and suggest a role in shaping the pattern of intraspecific genetic diversity. Future phylogeographic studies using a more extensive sampling of populations across the Mediterranean Basin, and mitochondrial and nuclear gene sequencing data could allow us to test this hypothesis.

Under the model of allopatric speciation, reproductive isolation would evolve mainly as a byproduct of genetic divergence in isolated populations (Coyne and Orr 2004). Comparative analyses across diverse taxa that have related the magnitude of reproductive isolation (as assayed by hybrid sterility and inviability) on genetic divergence have shown the occurrence of some notable patterns as follows: 1) reproductive isolation is positively correlated with genetic divergence between pairs of species; in this context, natural selection would play a major role in the evolution of postzygotic reproductive isolation between allopatric populations (Coyne and Orr 2004) and, as proteins are more subject to the action of natural selection than silent DNA polymorphisms, a pattern of higher correlation between genetic divergence and reproductive isolation at allozymic rather than mtDNA markers has to be expected (Fitzpatrick 2002); 2) hybrid sterility tends to evolve sooner than hybrid inviability; and 3) hybrid sterility and inviability appear early in heterogametic sex (i.e., Haldane's rule).

The *Ae. mariae* complex conforms to these expectations. When we look at the pattern of reproductive isolation between the species complex in relation to the observed genetic differentiation, genetic divergence at allozymic markers is concordant with the pattern of reproductive isolation between the species. Indeed, pairwise Nei's (1978) genetic distance estimates between *Ae. phoeniciae*, *Ae. mariae*, and *Ae. zammitii* showed that *Ae. phoeniciae* (which is fully reproductively isolated from all other members of the complex) is the more differentiated species. Similarly, mtDNA phylogenetic inferences showed a tighter relationship between *Ae. mariae* and *Ae. zammitii* than with *Ae. phoeniciae* (Fig. 2). Furthermore, synonymous sequence divergence estimates using Nei and Gojobori's (1986) method yielded divergence estimates for all species between 0.109 and 0.116, regardless of the reproductive isolation between them. Second, in the cross between *Ae. mariae* and *Ae. zammitii*, F1 hybrid males were sterile but did not show reduced viability (Coluzzi and Sabatini 1968), which is in accordance with the expectation that hybrid sterility tends to evolve sooner than hybrid inviability. Finally, the males are the heterogametic sex and are sterile according to Haldane's rule (Coluzzi and Sabatini 1968).

The *Ae. mariae* complex is one of the few animal groups able to live in the sea rock pools. In this study, for the first time, we genetically characterized the three members of the complex. A Pleistocene origin of the complex is suggested, as well as some interesting sparks to be further investigated are proposed. Therefore, future studies are welcome to explicitly test these hypotheses. In particular, the *Ae. mariae* complex could be a good study system for future studies aimed to investigate how the climatic cycles of the quaternary have affected the distribution of species distributed along the Mediterranean Basin.

Supplementary Data

Supplementary data are available at *Annals of the Entomological Society of America* online.

Acknowledgments

We thank Pina Sallicandro, Emanuele De Vito, and Florinda Sacco for their help in field and laboratory work; Alessandra Spanò for technical assistance; Editage (www.editage.com) for the linguistic revision.

References Cited

Antonini, G., P. Audisio, E. Mancini, A. De Biase, C. Tronci, G. Rossetti, and M. Trizzino. 2010. Molecular phylogeography of two Italian sibling species of *Calobius* (Coleoptera, Hydraenidae, Ochthebiinae) inhabiting Mediterranean marine rock-pools. Mar. Biol. 157: 371–381.

Audisio, P., M. Trizzino, A. De Biase, G. Rossetti, E. Mancini, and G. Antonini. 2010. Molecular and morphological evidence of a new sibling species of *Calobius* (Coleoptera: Hydraenidae) of the *C. quadricollis* complex from peninsular Italy. Ital. J. Zool. 77: 29–37.

Belkhir, K., P. Borsa, L. Chikhi, N. Raufaste, and F. Bonhomme. 1996–2004. GENETIX 4.05, logiciel sous Windows TM pour la génétique des populations. Laboratoire Génome, Populations, Interactions, CNRS UMR 5000, Université de Montpellier II, Montpellier (France).

Bisconti, R., D. Canestrelli, P. Colangelo, and G. Nascetti. 2011. Multiple lines of evidence for demographic and range expansion of a temperate species (*Hyla sarda*) during the last glaciation. Mol. Ecol. 20: 5313–5327.

Brower, A. V. 1994. Rapid morphological radiation and convergence among races of the butterfly *Heliconius erato* inferred from patterns of mitochondrial DNA evolution. Proc. Natl. Acad. Sci. USA 91: 6491–6495.

Bullini, L., and M. Coluzzi. 1980. Ethological mechanisms of reproductive isolation in *Culex pipiens* and *Aedes mariae* complexes (Diptera: Culicidae). Mon. Zool. Ital. 14: 99–100.

Carrión, J. S., E. I. Yll, M. J. Walker, A. J. Legaz, C. Chaín, and A. López. 2003. Glacial refugia of temperate, Mediterranean and Ibero-North African flora in south-eastern Spain: new evidence from cave pollen at two Neanderthal man sites. Glob. Ecol. Biol. 12: 119–129.

Chen, B., P. M . Pedro, R. E. Harbach, P. Somboon, C. Walton, and R. K. Butlin. 2011. Mitochondrial DNA variation in the malaria vector *Anopheles* minimus across China, Thailand and Vietnam: Evolutionary hypothesis, population structure and population history. Heredity 106: 241–252.

Clements, A. N. 1999. Mating, pp. 360–400. In The Biology of Mosquitoes Vol II, Chapman & All, London, United Kingdom.

Coluzzi, M., and A. Sabatini. 1968. Divergenze morfologiche e barriere di sterilità nel complesso *Aedes mariae* (Diptera Culicidae). Riv. Parass. 29: 49–70.

Coluzzi, M., and L. Bullini. 1971. Enzyme variants as markers in the study of precopulatory isolating mechanisms. Nature 231: 445–446.

Coluzzi, M., A. Sabatini, L. Bullini, and C. Ramsdale. 1974. Nuovi dati sulla distribuzione delle specie del complesso mariae del genere *Aedes*. Riv. Parass. 35: 321–330.

Coyne, J. A., and H. A. Orr. 2004. Speciation. Sinauer Associates, Sunderland, MA.

Drummond, A. J., and A. Rambaut. 2007. Beast: Bayesian evolutionary analysis by sampling trees. BMC Evol. Biol. 7: 214.

Epis, S., D. Porretta, V. Mastrantonio, F. Comandatore, D. Sassera, P. Paolo Rossi, C. Cafarchia, D. Otranto, G. Favia, C. Genchi, et al. 2014. ABC transporters are involved in defense against permethrin insecticide in the malaria vector *Anopheles stephensi.* Parasit Vectors 7: 349.

Excoffier, L., G. Laval, and S. Schneider. 2005. Arlequin (version 3.0): An integrated software package for population genetics data analysis. Evol. Bioinform. 1: 47–50.

Farris, J. S., M. Kallersjo, A. G. Kluge, and C. Bult. 1994. Testing significance of incongruence. Cladistics 10: 315–319.

Fitzpatrick, B. M. 2002. Molecular correlates of reproductive isolation. Evolution 56: 191–198.

Garnier-Gere, P., and C. Dillman. 1992. A computer program for testing pairwise linkage disequilibria in subdivided populations. J. Hered. 83: 239.

Green, M., and J. Sambrook. 2012. Molecular cloning: A laboratory manual, 4th Ed, Cold Spring Harbor Lab Press, Cold Spring Harbor, NY.

Habel, J. C., P. Dieker, and T. Schmitt. 2009. Biogeographical connections between the Maghreb and the Mediterranean peninsulas of southern Europe. Biol. J. Linn. Soc. 98: 693–703.

Horn, A., G. Roux-Morabito, F. Lieutier, and C., Kerdelhue. 2006. Phylogeographic structure and past history of the circum-Mediterranean species *Tomicus destruens* Woll. (Coleoptera: Scolytinae). Mol. Ecol. 15: 1603–1615.

Lebart, L., A. Morineau, K. M. Warwick. 1984. Multivariate descriptive statistical analysis: correspondence analysis and related techniques for large matrices. Wiley & Sons, New York, NY.

Médail, F., and K., Diadema. 2009. Glacial refugia influence plant diversity patterns in the Mediterranean Basin. J. Biol. 36: 1333–1345.

Morgan, K., Y. M. Linton, P. Somboon, P. Saikia, V. Dev, D. Socheat, and C. Walton. 2010. Inter-specific gene flow dynamics during the Pleistocene-dated speciation of forest-dependent mosquitoes in Southeast Asia. Mol. Ecol. 19: 2269–2285.

Morgan, K., S. M. O'loughlin, B. Chen, Y. M. Linton, D. Thongwat, P. Somboon, M. Y. Fong, R. Butlin, R. Verity, A. Prakash, et al. 2011. Comparative phylogeography reveals a shared impact of pleistocene environmental change in shaping genetic diversity within nine *Anopheles* mosquito species across the Indo-Burma biodiversity hotspot. Mol. Ecol. 20: 4533–4549.

Nei, M. 1978. Estimation of average eterozygosity and genetics distance from a small number of individuals. Genetics 89: 583–590.

Nei, M., and T. Gojobori. 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol. Biol. Evol. 3: 418–426.

Porretta, D., D. Canestrelli, R. Bellini, G. Celli, and S. Urbanelli. 2007. Improving insect pest management through population genetic data: a case study of the mosquito *Ochlerotatus caspius* (Pallas). J. App. Ecol. 44: 682–691.

Porretta, D., D. Canestrelli, S. Urbanelli, R. Bellini, F. Schaffner D. Petric, and G. Nascetti. 2011. Southern crossroads of the Western Palaearctic during the Late Pleistocene and their imprints on current patterns of genetic diversity: insights from the mosquito *Aedes caspius*. J. Biol. 38: 20–30.

Porretta, D., V. Mastrantonio, R. Bellini, P. Somboon, and S. Urbanelli. 2012. Glacial History of a Modern Invader: Phylogeography and Species Distribution Modelling of the Asian Tiger Mosquito *Aedes albopictus.* PloS ONE 7: e44515.

Porretta, D., and S. Urbanelli. 2012. Evolution of premating reproductive isolation among conspecific populations of the sea rock-pool beetle *Ochthebius urbanelliae* driven by reinforcing natural selection. Evolution 66: 1284–1295.

Raymond, M., and F. Rousset. 1995. GENEPOP 1.2: population genetics software for exact tests and ecumenicism. J. Heredity. 86: 248–249.

Reinert, J. F., R. E., Harbach, and I. J. Kitching. 2009. Phylogeny and classification of tribe Aedini (Diptera:Culicidae). Zool. J. Linn. Soc. 157: 700–794.

Rambaut, A. 2012. Figtree version 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

Rice, W. R. 1989. Analysing tables of statistical tests. Evolution 43: 223–225.

Rioux, J. A. 1958. Les culicids du "Midi" méditerranéen. Encyclopédie entomologique, XXXV. Lechevalier, Paris.

Ronquist, F., M. Teslenko, P. van der Mark, D. Ayres, A. Darling, S. Höhna, B. Larget, L. Liu, M. A. Suchard, and J. P. Huelsenbeck. 2012. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61: 539–542.

Swofford, D. L. 2003. PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods), Version 4. Sinauer Associates, Sunderland, MA.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28: 2731–2739.

Urbanelli, S., P. Sallicandro, E. De Vito, E. Colonnelli, and L. Bullini. 1996. Molecular reexamination of the taxonomy of *Ochthebius* (*Calobius*) (Coleoptera: Hydraenidae) from the Mediterranean and Macaronesian Regions. Ann. Entomol. Soc. Am. 89: 623–636.

Urbanelli, S. 2002. Genetic divergence and reproductive isolation in the *Ochthebius* (*Calobius*) complex (Coleoptera: Hydraenidae). Heredity 88: 333–341.

Urbanelli, S., and D. Porretta. 2008. Evidence of reinforcement of premating isolation between two species of the genus *Ochthebius* (Coleoptera: Hydraenidae). Evolution 62: 1520–1527.

Urbanelli, S., D. Porretta, V. Mastrantonio, R. Bellini, G. Pieraccini, R. Romoli, G. Crasta, and G. Nascetti 2014. Hybridization, natural selection and evolution of reproductive isolation: a 25-yr survey of an artificial sympatric area between two mosquito sibling species of the *Aedes mariae* complex. Evolution 68: 12490.

Received 24 September 2014; accepted 11 March 2015.