

# HYBRIDIZATION, NATURAL SELECTION, AND EVOLUTION OF REPRODUCTIVE ISOLATION: A 25-YEARS SURVEY OF AN ARTIFICIAL SYMPATRIC AREA BETWEEN TWO MOSQUITO SIBLING SPECIES OF THE *Aedes mariae* COMPLEX

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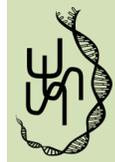
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Natural selection can act against maladaptive hybridization between co-occurring divergent populations leading to evolution of reproductive isolation among them. A critical unanswered question about this process that provides a basis for the theory of speciation by reinforcement, is whether natural selection can cause hybridization rates to evolve to zero. Here, we investigated this issue in two sibling mosquitoes species, *Aedes mariae* and *Aedes zammitii*, that show postmating reproductive isolation (F1 males sterile) and partial premating isolation (different height of mating swarms) that could be reinforced by natural selection against hybridization. In 1986, we created an artificial sympatric area between the two species and sampled about 20,000 individuals over the following 25 years. Between 1986 and 2011, the composition of mating swarms and the hybridization rate between the two species were investigated across time in the sympatric area. Our results showed that *A. mariae* and *A. zammitii* have not completed reproductive isolation since their first contact in the artificial sympatric area. We have discussed the relative role of factors such as time of contact, gene flow, strength of natural selection, and biological mechanisms causing prezygotic isolation to explain the observed results.

**KEY WORDS:** Longitudinal studies, maladaptive hybridization, premating isolation mechanisms, reinforcing natural selection, translocation experiments.

Natural selection may have a major role in speciation when it affects mating choice among populations. This may occur when natural selection, in response to local adaptation, acts on traits that affect mating choice or on those genetically correlated with

them (ecological speciation) (Nosil 2012). Natural selection may also act against maladaptive hybridization between co-occurring taxa after secondary contact (speciation by reinforcement) (Coyne and Orr 2004). Although ecological speciation and speciation



by reinforcement are distinct processes with different starting conditions, natural selection promotes the (even rapid) evolution of reproductive isolation among populations in both processes, leading to speciation (Coyne and Orr 2004; Yukilevic and True 2006; Hendry et al. 2007; Svensson and Gosden 2007; Nosil 2012; Servedio et al. 2013).

In this study, we focused on the action of natural selection against maladaptive hybridization. Under speciation by reinforcement, when individuals belonging to divergent populations come into contact and hybridize, natural selection may act against unfit hybrids (i.e., inviables, steriles, or individuals with lower ecological fitness than parental species), or against hybridization itself (e.g., as consequence of reduced survival or fertility of females) (Servedio and Noor 2003). In both cases, it would favor the divergence in mating traits to avoid interbreeding, leading to reproductive isolation between populations (Dobzhansky 1937; Howard 1993; Servedio and Noor 2003; Coyne and Orr 2004; Ritchie 2007). However, some questions remain unanswered (Abbott et al. 2013), although substantial consensus about the plausibility of reinforcement has been achieved after years of strong scepticism (Coyne and Orr 2004), and recently some studies have been performed to investigate the effects of reinforcement among conspecific populations (Lemmon 2009; Porretta and Urbanelli 2012; for reviews Ortiz-Barrientos et al. 2009; Pfenning and Pfenning 2009; Hoskin and Higgie 2010; Smadja and Butlin 2011). A critical question is whether natural selection can cause hybridization rates to evolve to zero, leading to complete reproductive isolation (Servedio 2011; Bank et al. 2012).

Theoretical studies have shown that several factors may slow or limit the completion of reproductive isolation. These include gene flow among sympatric and allopatric populations, which can erode premating adaptations that evolve in sympatric zones (Servedio and Noor 2003), or the strength of natural selection against hybridization, which decreases with decreasing hybridization rate (Coyne and Orr 2004). The occurrence of the cost of choosiness (Britch et al. 2001; Coyne et al. 2002; Servedio 2011), a weak linkage disequilibrium between the genes that cause reproductive isolation and the genes under selection (Servedio and Noor 2003), the action of sexual selection (Ritchie 2007; Svensson and Gosden 2007; Servedio 2011) and the mate preference mechanism (Bank et al. 2012) are other factors that can constrain the completion of reproductive isolation by reinforcement. On the other hand, the likelihood or extent of this process may be increased by combined effect of natural and sexual selection (Ritchie 2007; van Doorn et al. 2009), by mutational step sizes of assortment (e.g., mutations that cause a complete isolation in a single step), or the effect size of the mutation (e.g., mutation that allows females to completely discriminate between conspecific and heterospecific males) (Servedio 2011; Bank et al. 2012).

Empirical studies have shown that, in some cases, hybridization ceased completely after reinforcement (e.g., the beetles *Ochthebius quadricollis/O. urbanelliae*, Urbanelli and Porretta 2008); in other cases, reinforcement is thought to have reduced hybridization, which however persists as in *Drosophila persimilis/D. pseudoobscura* (Noor 1995), flycatchers (Sætre et al. 1997), sticklebacks (Rundle and Schluter 1998), spadefoot toads (Pfennig 2003), walking-stick insects (Nosil et al. 2003), and the green-eyed tree frog *Litoria genimaculata* (Hoskin et al. 2005).

In this study, we investigated the action of natural selection against hybridization on reproductive isolation between the sea rock-pool mosquitoes *Aedes mariaae* and *Aedes zammitii*. These mosquitoes are sibling species, distributed along the Mediterranean coast (*A. mariaae* is distributed along the western Mediterranean coast, while *A. zammitii* inhabits the central and eastern coasts) (Coluzzi and Sabatini 1968; Coluzzi et al. 1974). Their geographic ranges are contiguous and no natural sympatric areas are known (Coluzzi et al. 1974). In 1970 Coluzzi and Bullini (1971) created an artificial sympatric area along the Tyrrhenian coast, but the introduced species (*A. zammitii*) disappeared after 2 years. In 1986 a new artificial sympatric area was created along the Adriatic coasts, where *A. mariaae* individuals were translocated to the geographic range of *A. zammitii*. These mosquito species are a good study system to investigate this issue. First, reproductive isolation between the species is not complete; they may hybridize under laboratory conditions (Coluzzi and Sabatini 1968), and in the field (Coluzzi and Bullini 1971). Second, postmating isolation barriers occur between the species. Crossing experiments conducted in the laboratory have revealed that the F1 females, as well as backcrossed males and females, are vigorous and fertile, while the F1 hybrid males are sterile (Coluzzi and Sabatini 1968). Third, premating mechanism of isolation is known to occur between the species. In allopatric areas, the mating swarms of *A. mariaae* and *A. zammitii* occur at different heights (*A. mariaae* swarms at approximately 50 cm above rocks, whereas *A. zammitii* swarms at 2 m above the rocks). Heterospecific swarms were found in sympatric areas (i.e., some *A. zammitii* were found in low swarms and some *A. mariaae* in high swarms) (Coluzzi and Bullini 1971). Because mosquitoes mate in flight, the height of the mating swarm is therefore an incomplete mechanism of premating isolation that could theoretically be reinforced by natural selection against hybridization. Fourth, because *A. mariaae* was translocated in the geographic range of *A. zammitii*, no gene flow may occur between allopatric and sympatric populations of *A. mariaae* that could counteract the effects of natural selection against hybridization in the sympatric area (Servedio and Noor 2003; Mallet 2005).

Here, we report a 25-year survey of the artificial sympatric area between *A. mariaae* and *A. zammitii* to understand whether

natural selection against hybridization can lead to zero hybridization between the two species. We sampled individuals from 1986 to 2011 and (i) analyzed the composition of mating swarms to investigate possible changes in this mating trait and (ii) used genetic markers to estimate the hybridization rate between the two species across time.

## Materials and Methods

### TRANSLOCATION EXPERIMENT

*Aedes mariaae* and *A. zammitii* are morphologically indistinguishable species (Coluzzi and Sabatini 1968). In previous studies, they were genetically recognized by polymorphism at the only allozymic locus Phosphoglucumutase (Pgm) (Coluzzi and Bullini 1971). Therefore, before the translocation of *A. mariaae*, we analyzed allopatric populations of *A. mariaae* and *A. zammitii* to find further allozymic diagnostic loci. Allopatric populations analyzed included also the *A. mariaae* populations used for translocation (Circeo and Scauri) and the *A. zammitii* population where *A. mariaae* was introduced (Baia dei Campi) (Supplementary Table S1) (Suppfile1A for details).

In 1986, to form an artificial sympatric area between *A. mariaae* and *A. zammitii* more than 20,000 larvae and pupae of *A. mariaae* were collected at two localities, Circeo and Scauri (Tyrrhenian sea) and released at Baia dei Campi (Adriatic sea) (Supplementary Table S1). The zone chosen for the experiment included some rock-pools (about 10–12) located at the end of a rocky-shore bordering a sand beach. The density of *A. zammitii* in this locality was previously decreased by collecting and removing larvae and pupae in the rock-pools to avoid the predominance of indigenous taxon, taking into account the existence in the zone of adult females and quiescent eggs of *A. zammitii* (Coluzzi and Bullini 1971).

### REPRODUCTIVE ISOLATION WITHIN THE ARTIFICIAL SYMPATRIC AREA

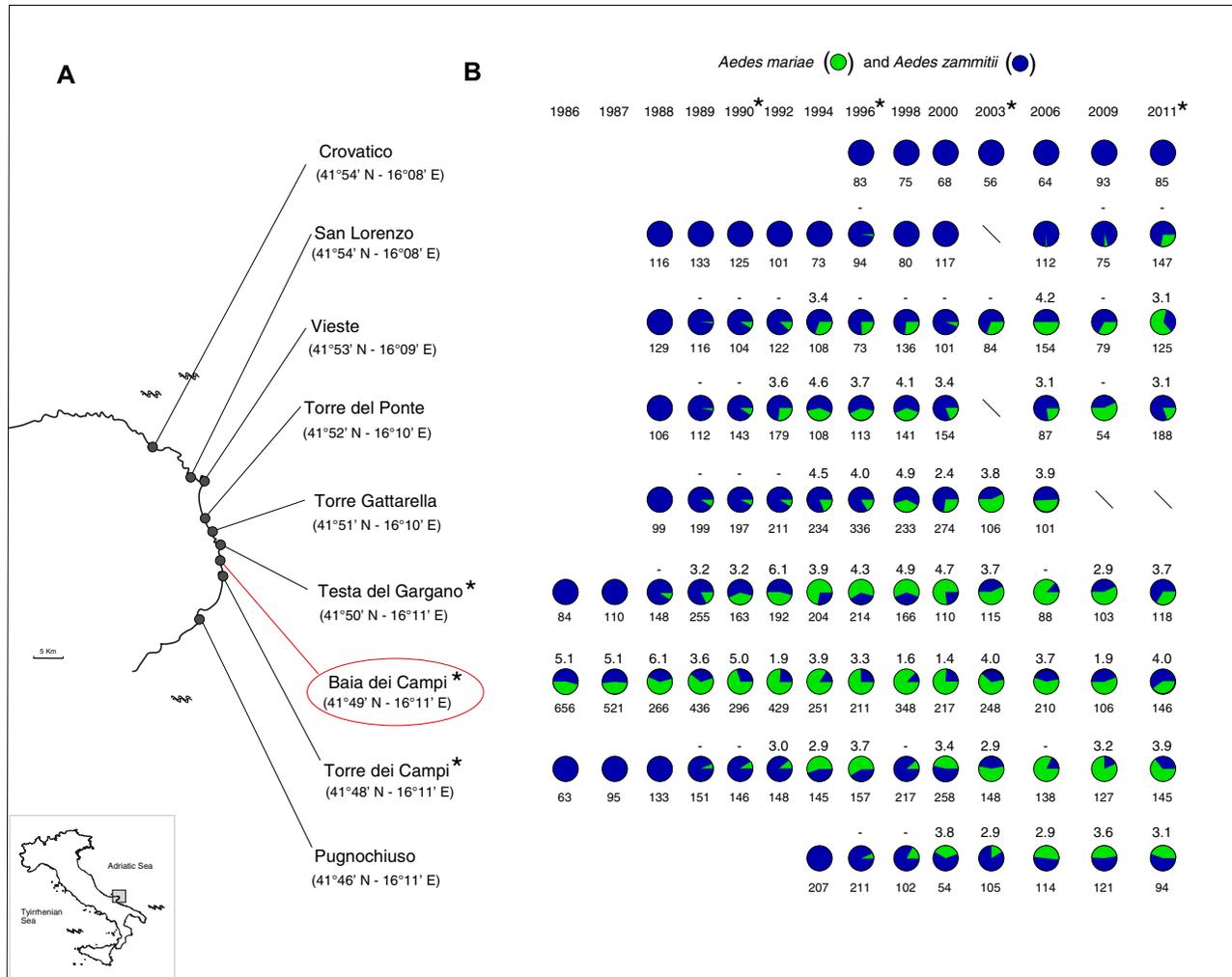
**Mating swarm composition.** In mosquitoes, mate occurs in air where males form mating swarms to attract females and possibly mate in flight (Clements 1999; Becker et al. 2010). In both *A. mariaae* and *A. zammitii*, swarms are formed above rocks at sunset and sunrise. They differ in the height: *A. mariaae* swarms just close to the rocks (low swarm), whereas *A. zammitii* swarms at a height of about 2 m above the rocks (high swarm) (Bullini and Coluzzi 1980; Urbanelli pers. obs.). This distribution without intermediate swarms was also observed when the two species were put in sympatry by Coluzzi and Bullini (1971). They observed also heterospecific (i.e., mixed) swarms. Here, we analyzed the composition of mating swarms collected in the years 1990, 1996, 2003, and 2011 from three localities of the sympatric area (Baia

dei Campi, Testa del Gargano, and Torre dei Campi, Fig. 1). Sampling of mating swarms was carried out on rock pools at the two times of day when they are formed: just after dawn and before sunset. A swarm is recognized by the fact that it is composed by mosquitoes that fly in a cohesive stationary cloud. Swarming mosquitoes were collected by an insect net and care was taken to not disperse the mosquitoes before they could be caught. Mosquitoes from each swarm were then frozen in dry ice and kept in separate 50 ml tubes. Allozymic markers were used to analyze mating swarm composition by identification of males and females collected. The swarms were referred as “homospecific” when included only individuals belonging to *A. zammitii* or *A. mariaae*, and “heterospecific” when included individuals of both species. The  $\chi^2$  test as implemented in Biostat 2009 was used to test differences among years of the frequency of heterospecific swarms.

**Hybridization survey.** Sampling over the course of the 25 years following the release (from 1986 until 2011) was conducted as follow: from 1986 to 2000, sampling was conducted every one/two years and more sampling campaigns were conducted during the reproductive season of each year (June–October); from 2003 to 2011, samplings were conducted more sporadically (2003, 2006, 2009, and 2011) with one-two sampling campaigns in the year. During each sampling year, we visited the localities where *A. mariaae* was observed in the previous sampling and the neighbor sites to examine whether changes in its geographic distribution occurred (Fig. 1). Mosquitoes were collected as larvae, brought in the laboratory, reared to adults and stored for subsequent genetic analysis (Porretta et al. 2012).

Our survey of genetic variation at allozymic markers showed the occurrence in the two species of fixed alternative alleles at six allozymic loci (see Results section). To determine the power of these markers to identify parental and hybrid individuals, data simulations were performed using the software hybridlab version 1.0 (Nielsen et al. 2006) (see Suppfile1B). NewHybrids (Anderson and Thompson 2002) was then used to analyze the individuals collected in the sympatric area. Runs with  $10^6$  Monte Carlo Markov Chain (MCMC) sweeps following a burn-in period of  $10^5$  were performed. A posterior probability  $>0.95$  was used as a threshold for assigning individual to a specific genotypic class (parental, F1, F2, and first-generation backcrosses).

The hybridization rate through time was evaluated (1) by analyzing the variation of proportion of sites exhibiting hybrids. Because the presence or absence of F1 hybrids in each site is a categorical variable (F1 hybrids either occur or did not occurred), we used logistic regression model; (2) by analyzing the variation of the proportion of hybrids through time. Because F1 hybrids proportion may be affected by the relative frequency of the two species, we used the departure from random mating, measured



**Figure 1.** (A) Map showing the area along the Adriatic Sea coasts into the geographic range of *Aedes zammitii* where *A. mariae* was translocated in 1986. The localities sampled across years are shown. The site of release (Baia dei Campi) is shown in the red oval. (B) Occurrence of parental individuals (*A. mariae* in green and *A. zammitii* in blue) and F1 hybrids. The numbers below the pies show the number of individuals assayed in each locality each year; the numbers above the pies show the percentage of F1 hybrids found. Asterisks show the localities and years of sampling of mating swarms.

as heterozygote deficiency, and its variation through time using a maximum likelihood approach. The observed data were fitted with the trinomial:

$$AA = p^2 + p(1 - p)F$$

$$AB = 2p(1 - p)(1 - F)$$

$$BB = (1 - p)^2 + p(1 - p)F$$

where AA, AB, and BB are the proportions of individuals observed of *A. zammitii*, F1 hybrids and *A. mariae*, respectively, and  $F$ , measuring the heterozygote deficiency, is a function of time ( $F = at + b$ ). The parameters  $a$ ,  $b$ , and  $p$  were then estimated. The null hypothesis  $a = 0$  (i.e., no  $t$  dependence of  $F$  parameter) was tested using the Wald test and the likelihood ratio

test. The analyses were performed using data from single sites across time and aggregate data from all sites. Only the sites where both species occurred were used (see Suppfile2 for details). All analyses were performed in R 2.6.2 (R Development Core Team (2012)).

## Results

### TRANSLOCATION EXPERIMENT AND DIFFUSION OF *A. mariae*

Ten loci (*Adk*, *Pgm*, *Phi*, *Mpi*, *Got-1*, *Odh*, *Hbdh*, *6Pgd*, *Me-1*, and *Ca-1*) out of the 14 studied were found to be polymorphic among the allopatric populations studied (allelic frequencies are available under request to authors). No significant departures (5%)

from the expected genotype frequencies under Hardy–Weinberg equilibrium were found in allopatric populations and no significant linkage disequilibria were observed across loci. Six discriminative loci (*Pgm*, *Me-1*, *Phi*, *Mpi*, *Odh*, and *Ca-1*) (Supplementary Table S2) were found between the two species with alternative alleles that have been used to identify all the *Aedes mariae* and *A. zammitii* individuals analyzed in the sympatric area.

Since 1986, *A. mariae* diffused from the site of the release (Baia dei Campi) into the neighboring sites, and in 2011 it was present along a transect of about 20 km, where the two species are syntopic. In all sites where *A. mariae* arrived, it not only persisted but also increased its relative abundance across years (Fig. 1). South of Pugnochiuso locality (Fig. 1) there are unsuitable habitats for the mosquitoes larval development such as sand stretches, and rocky shores with steep slope, which limited the diffusion of both species in these part of coast.

#### REPRODUCTIVE ISOLATION WITHIN THE SYMPATRIC AREA

**Mating swarms composition.** A total of 126 mating swarms (3333 individuals) have been collected from three sites sampled in the sympatric area (Baia dei Campi, Testa del Gargano, and Torre dei Campi) (Fig. 1) in the four sampling years (1990, 1996, 2003, 2011) (Supplementary Tables S3 and S4). Mating swarms had a mean of 26 ( $\pm 5$  SD) mosquitoes and were composed mostly of males ( $92\% \pm 4\%$ ) with few females, as expected for mosquitoes mating swarms (Bullini and Coluzzi 1980; Clements 1999). Swarms showed a segregate distribution as only swarms identified as “high” (i.e., at about 2 meters from the ground level), or “low” (at about 0.5 meters from the ground level) were observed.

Sixty-six “high” and sixty “low” swarms were collected (Supplementary Tables S3 and S4). Among the high swarms, characteristic of *A. zammitii*, 41 out of 66 were homospecific (i.e., including only *A. zammitii* individuals) and the others were heterospecific (i.e., including also a few individuals of *A. mariae*). Likewise, among the low swarms analyzed, characteristic of *A. mariae*, 40 out of 60 were homospecific (i.e., including only *A. mariae* individuals), and the others 20 were heterospecific, including also individuals of *A. zammitii* (Fig. 2, Supplementary Tables S3 and S4). As shown in Fig. 2, the high and low heterospecific swarms were composed mostly by individuals belonging to one species: the “high” heterospecific swarms were mainly formed by *A. zammitii* individuals while the “low” heterospecific swarms were mainly formed by *A. mariae* individuals ( $> 90\%$ ); males that entered in the “wrong” swarm were more numerous than conspecific females in both high and low swarms; few F1 hybrids were also found in both high and low swarms (Fig. 2, Supplementary Table S3). Heterospecific swarms were observed in all years of

collection (Fig. 2, Supplementary Table S3), and no significant differences were found among the frequencies of heterospecific mating swarms among years (for all  $\chi^2$  test  $P > 0.05$ ) (Supplementary Table S4).

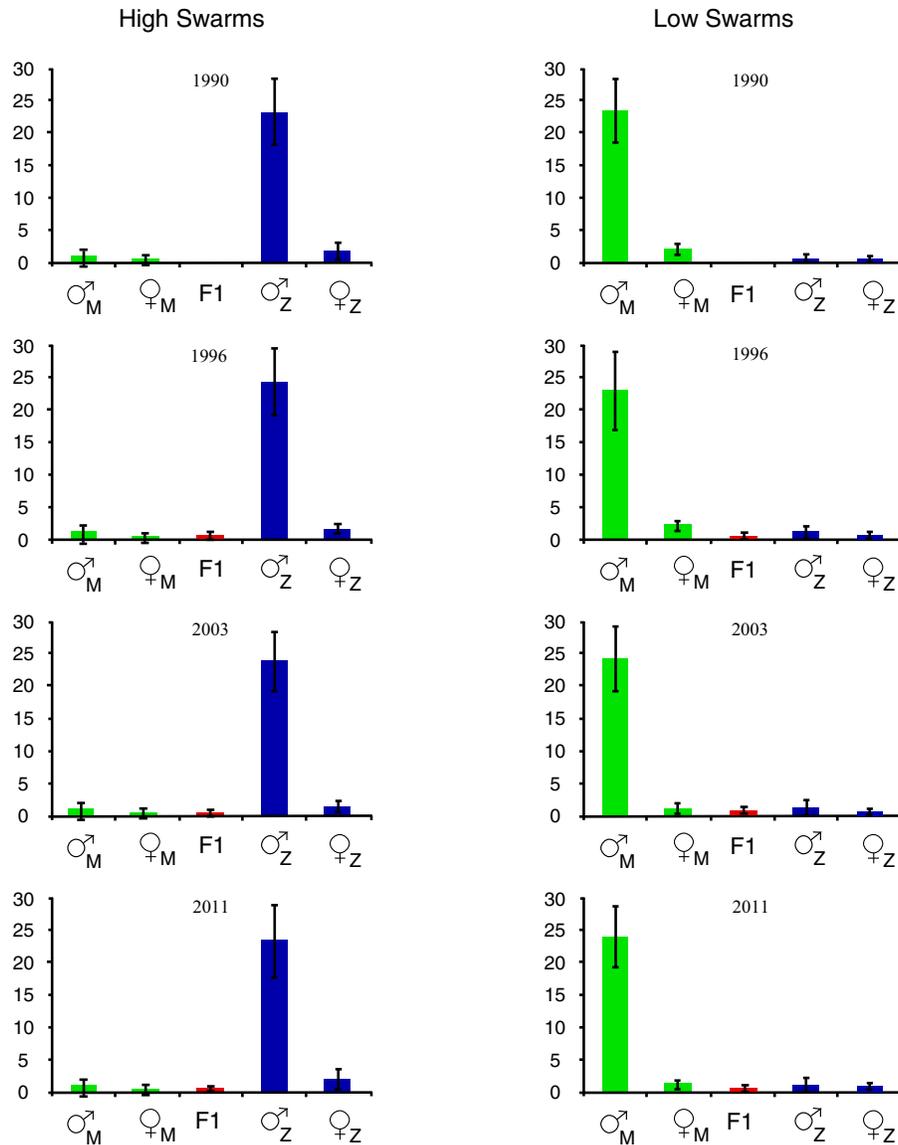
#### Hybridization survey

The power of the six allozymic diagnostic loci between *A. mariae* or *A. zammitii* to detect parental individuals and hybrids of specific classes was determined on simulated data (Vähä and Primmer 2006). NewHybrids showed high efficiency, accuracy and overall performance in assigning individuals to parental genotypic classes and F1 hybrid class: it assigned all simulated *A. mariae* or *A. zammitii* individuals to the correct parental genotypic class, and the efficiency, accuracy, and overall performance in assigning individuals to F1 hybrid class were 0.949, 0.904, and 0.858, respectively. Efficiency and accuracy dropped to 0.691 (overall performance 0.477) in assigning individuals to F2 genotypic class. The analysis of simulated data showed therefore that the markers used allow us to confidently detect parental individuals and F1 hybrids.

A total of 15,893 individuals were collected in the sympatric area across the 25 years following the first contact between the two species and genotyped. In the localities of the sympatric area were found mainly individuals that have only the alleles characteristic of *A. mariae* or *A. zammitii*, and that NewHybrids assigned to parental genotypic classes with a posterior probability ranging from 0.98 to 1.0; individuals that are heterozygous at all six loci for the diagnostic alleles (1.4–6.1%), that NewHybrids assigned to F1 hybrid class with posterior probability 0.99–1.00; individuals of hybrid ancestry that are heterozygous at 1–3 loci for the diagnostic alleles ( $< 5\%$ ), that were not assigned to hybrid classes or assigned to parental classes with 0.95 posterior probability threshold. None individual was assigned by NewHybrids to F2 class, as expected due to the sterility of F1 males (Coluzzi and Sabatini 1968).

F1 hybrid individuals were observed until 2011 with a percentage that ranged from 1.4 to 6.1% (Fig. 1). No differences were observed across years in the proportion of sites that contained F1 hybrids (intercept estimate =  $-90.457$  Std. Error =  $67.653$   $z$  value =  $-1.337$   $P = 0.181$ ).

The analyses of variation through time of departure from random mating, measured as deficit of heterozygotes  $F$ , are shown in Supplementary Table S5. The plots of  $F$  estimates across time for each sample and all data are shown in Supplementary Fig. S1. Assuming as null hypothesis  $a = 0$  (i.e., no  $t$ -dependence of  $F$ ), significant changes were observed in the sites Torre Gattarella and Pugnochiuso ( $a < 0$ ,  $P < 0.05$ ) and in the site of Baia dei Campi ( $a > 0$ ,  $P < 0.05$ ). When we used aggregate data no significant changes were observed showing a no  $t$  dependence of  $F$  (Supplementary Table S5).



**Figure 2.** Composition of the heterospecific mating swarms collected in the sympatric area between *Aedes zammitii* and *A. mariaae*. The histograms resume the heterospecific mating swarms (25 high and 20 low) collected from three sites (Baia dei Campi, Testa del Gargano, and Torre dei Campi) in four sampling years (1990, 1996, 2003, 2011) (Fig. 1, Supplementary Table S3). Red: F1 hybrids. Green: ♂<sub>M</sub> = *A. mariaae* males; ♀<sub>M</sub> = *A. mariaae* females. Blue: ♂<sub>Z</sub> = *A. zammitii* males; ♀<sub>Z</sub> = *A. zammitii* females.

## Discussion

It has been hypothesized that *A. mariaae* and *A. zammitii* originated during the Pliocene by fragmentation of an ancestral range with circum-Mediterranean distribution (Coluzzi et al. 1974). During the Plio-Pleistocene, drastic climatic, and geographic changes occurred along the Mediterranean coastline that led to repeated cycles of isolation and secondary contact among coastal populations (Carrión et al. 2003; Horn et al. 2006; Habel et al. 2009; Médail and Diadema 2009; Porretta et al. 2011; Porretta Mastrantonio et al. 2013). The possible occurrence of such cycles among populations of *A. mariaae* and *A. zammitii* has not been investi-

gated, and therefore it is unknown whether the two species came into contact at any time in their evolutionary history, or whether differences in the height of mating swarms originated through selection against hybridization or as a byproduct of allopatric divergence. Regardless, postmating isolation barriers developed (sterility of F1 hybrid males, Coluzzi and Sabatini 1968), and a hybridization rate of approximately 5% was found in 1986 when *A. mariaae* was translocated into the range of *A. zammitii*. Has natural selection against maladaptive hybridization lead to complete reproductive isolation between the two species? Our results clearly showed that *A. mariaae* and *A. zammitii* have not completed

reproductive isolation since their first contact in the artificial sympatric area.

At least four hypotheses can be carried out to explain these results. First, contact between the two species could be too recent, allowing insufficient time for the evolution of full reproductive isolation. However, populations of *Drosophila birchii* and *D. serrata* exposed to experimental sympatry required only nine generations to evolve full reproductive isolation (Higgie et al. 2000). A similar outcome was recently shown by Matute (2010). Using experimental populations of *Drosophila yakuba* and *D. santomea*, he found that reinforcement could promote the evolution of reproductive isolation within 5–10 generations. In the *A. mariae/A. zammitii* system, eight generations occur per year (Coluzzi and Bullini 1971; Bellini and Urbanelli, pers. obs.), and approximately 210 generations have occurred since their first contact; this number is comparable to or greater than that of other systems. The time required for the evolution of reproductive isolation depends on the strength of natural selection, which decreases with decreasing hybridization rate (Coyne and Orr 2004). From the above-mentioned study systems, natural selection is very strong in the *Drosophila birchii* and *D. serrata* populations, as all F1 hybrids were removed (Higgie et al. 2000). In the experimental populations of *D. yakuba* and *D. santomea* (Matute 2010), levels of hybridization below 0.07–0.12%, measured as the proportion of surviving F1 female hybrids each generation, were sufficient for reproductive isolation to evolve within 5–10 generations if gene flow among sympatric and allopatric populations was low. The hybridization rate found between *A. mariae* and *A. zammitii* in 1986 therefore may be sufficiently high for reproductive isolation between the species to be completed—considering that effects of intraspecific gene flow may be excluded for the introduced species—within the time that has past since their contact. Therefore, the hypothesis that contact is too recent seems unlikely.

Second, gene flow among sympatric and allopatric populations (i.e., populations that have never been exposed to the other species) is a critical factor in determining whether reproductive isolation will complete (Servedio and Noor 2003). Indeed, migration from allopatric parental populations can erode premating adaptations that evolve in sympatric zones (Coyne and Orr 2004). In our study system, migration of allopatric individuals could be suggested for *A. zammitii*, but not for *A. mariae* because it is surrounded by other *A. zammitii* populations and thus lacks a source of *A. mariae* migrants. At least for *A. mariae* we can exclude the possibility that gene flow could have played a major role.

Third, the absence of polymorphisms in mating traits could prevent increased mate discrimination. In the *A. mariae/A. zammitii* system, polymorphism in height of the mating swarm has been found (Bullini and Coluzzi 1980). Therefore, a baseline exists between these species upon which natural selection could act to complete reproductive isolation. Mating in mosquitoes is

a complex behavior characterized by the interplay of chemical, acoustic, and visual cues (Clements 1999). We analyzed height of the mating swarm, but variation in other mating traits that could be involved in reproductive isolation may exist (Clements 1999). If all heterospecific swarms found (25–41%) (Supplementary Table S4) would succeed in mating, a higher than observed estimate of F1 hybrids would result. This suggests that within-swarm form recognition may operate. Visual or auditory signals could be involved, as observed in several other mosquito species (Clements 1999; Gibson and Russell 2006; Cator et al. 2009, 2010, 2011; Warren et al. 2009; Pennetier et al. 2010).

A fourth argument to explain the absence of completion of reproductive isolation between these *Aedes* species may involve the costs of hybridization. On one hand, without an accurate estimate of the fitness cost of the sterility of the F1 males, we can not exclude that it was not enough to complete reproductive isolation. On the other hand, the cost of male sterility could be balanced by the advantage of F1 females, which would weaken the strength of reinforcing selection. Indeed, the F1 females could have greater fitness than pure females, as observed for example in the chorus frogs *Pseudacris feriarum* and *P. nigrita* (Lemmon and Lemmon 2010) and *Drosophila mojavensis* and *D. arizonae* (Bono and Markov 2009).

A final argument may involve the nature of the biological mechanism underlying prezygotic isolation. In a recent theoretical study, Bank et al. (2012) pointed out the importance of the scale of the effect of mutations that alter female mating preference, and suggested that the likelihood of reinforcing selection leading to reproductive isolation may depend on the mechanisms causing prezygotic isolation. A mutation that completely eliminates hybrid mating (large modifier) may lead to complete reproductive isolation between the hybridizing populations. This could occur, for example, when mate choice is based on pheromones in which single amino acid changes in a receptor could make an insect sensitive to one pheromone but completely insensitive to another. In contrast, mutations that result in quantitative changes (weak modifiers) in the responsiveness of females to particular stimuli (e.g., mutations in systems of mate choice based on acoustic cues) could make females insensitive to some signals but responsive to others, which may allow hybridization to occur. Because the strength of natural selection declines as hybrids become more rare (Coyne and Orr 2004), the evolution of weak modifiers may contrast the spread of other modifiers and hybridization rate may then not evolve to zero. These arguments appear to be supported by our results. The height of mating swarms, and other visual or auditory signals hypothesized to occur within swarms, are weak modifiers that limit but do not negate gene flow between the two species. The consequence is reduced selection strength, which may prevent the spread of another modifier (Bank et al. 2012).

In conclusion, among the several hypotheses here carried out to explain our results some of them could be excluded on the basis of the characteristics of our system (i.e., too recent contact between the two species, gene flow from allopatric populations, and lack of polymorphism in mating traits); some others could be instead quite plausible and interesting to further investigate (i.e., premating mechanism; costs of hybridization and fitness).

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#### DATA ARCHIVING

The doi for our data is 10.5061/dryad.39qf1.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

**Table S1.** Collection sites and sampling date of the populations of *Aedes mariaae* and *A. zammitii* analysed for allozymic markers before the translocation experiment.

**Table S2.** Alternative alleles found at discriminative allozymic loci between *Aedes mariaae* and *Aedes zammitii*.

**Table S3.** Composition of mating swarms collected in the sympatric area between *Aedes mariaae* and *A. zammitii*.

**Table S4.** Mating swarms sampled in the sympatric area between *Aedes mariaae* and *A. zammitii*.

**Table S5.** Estimates of the parameters  $a$ ,  $b$  and  $p$  using a maximum-likelihood approach for each site (Fig. 1) and for aggregate data.

**Figure S1.** Plots of  $F$  estimates across time for each sample (panels A–G) and aggregate data (panel H).