

EVIDENCE OF REINFORCEMENT OF PREMATING ISOLATION BETWEEN TWO SPECIES OF THE GENUS *OCHTHEBIUS* (COLEOPTERA: HYDRAENIDAE)

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The increase in premating reproductive isolation between recently diverged and potentially interbreeding taxa resulting from selection against hybridization (reinforcement) is one of the most contentious issues in evolutionary biology. After many years of debate, its plausibility under various conditions has been shown by theoretical studies and some cases have been documented. At present, interest is arising about the frequency and importance of reinforcement in nature. *Ochthebius quadricollis* and *Ochthebius* sp. A are two hydraenid beetles inhabiting marine rock pools in the Mediterranean basin. By molecular analysis of a contact zone between the two species along the Italian Tyrrhenian coast, full reproductive isolation between the two species was evidenced. However, the finding of introgressed specimens at some diagnostic loci suggested that gene flow occurred in the past but then ceased. In this article, by analyzing species composition of mating couples collected in sympatric localities, we show the existence of strong assortative mating between the two species in nature. In laboratory multiple-choice mating trials, sympatric populations showed greater assortative mating than allopatric populations. Reinforcement is suggested as the most parsimonious hypothesis to explain the evolution of discriminative mate recognition systems occurring among *O. quadricollis* and *Ochthebius* sp. A under sympatric, but not allopatric, populations.

KEY WORDS: Assortative mating, *Ochthebius*, reinforcement, speciation.

In the study of speciation the mechanisms that lead to the evolution of reproductive isolation between diverging populations are a central issue. One mechanism is via natural selection that can increase divergence in mate recognition between hybridizing populations by selecting against the production of hybrid or interspecific matings, and hence completing reproductive isolation (reviewed in Servedio and Noor 2003; Servedio 2004). This process, called reinforcement, has become an important feature of many models of allopatric speciation, but is yet one of the most debated issues in evolutionary biology (Coyne and Orr 2004 and references therein). The primary questions concerning speciation by reinforcement were whether it could occur and whether it could be documented. In the past decade, these questions found

an answer. The plausibility of reinforcement under various conditions has been shown by theoretical studies, and some cases have been documented of species that have diverged via reinforcement (Servedio 2004 and references therein; Hoskin et al. 2005; Kronforst et al. 2007). However it is still deeply uncertain how central reinforcement is in explaining the biological diversity we see today and at present the debate is particularly about its role in speciation (Servedio 2004). As emphasized by several authors, to assess the frequency of reinforcement in nature we must look for its signature in the most likely places (Servedio and Noor 2003 and references therein). The importance has long been highlighted by examining hybrid zones and areas of geographic overlap between pairs of species for evidence of reinforcement (Ritchie et al.

1989; Harrison 1990; Howard et al. 2003). To suggest completion of speciation by reinforcement there must be, or must have been, gene flow between diverging populations and selection against hybridization (Butlin 1989; Howard 1993). Genetic population analysis can help us to detect the occurrence of these two necessary items in the process of reinforcement (Countermand and Noor 2006; Mullen and Andr es 2007).

If reinforcement occurs, selection against hybrids or interspecific matings influences the components of the mate recognition system leading them to diverge in the area of contact. The outcome will be the decrease in the frequency of interspecific matings as a result of the increased assortative mating between the two diverging taxa. The pattern of increased premating isolation in sympatric versus allopatric populations has been traditionally considered to be evidence of reinforcement (Coyne and Orr 1989, 1997). However, it can be caused by processes other than reinforcement and the alternative hypotheses have to be ruled out to ascribe this pattern to reinforcement alone (Noor 1999; Coyne and Orr 2004; Lemmon et al. 2004).

Ochthebius quadricollis and *Ochthebius* sp. A are two morphologically indistinguishable species of hydraenid beetles inhabiting marine rock pools in the Mediterranean basin. Their ranges are largely allopatric, but sympatric areas have been detected along the Tyrrhenian coasts in Tuscany and mainly in Campania, Basilicata, and Calabria (Urbanelli et al. 1996; Urbanelli 2002). On the basis of an extensive allozymic analysis, no F1 hybrids or their offspring nor backcrosses were found in these sympatric areas, which shows that at present there is full reproductive isolation among the two taxa (Urbanelli 2002). The same study showed, however, the existence of introgressed genotypes exclusively in the sympatric areas, signature of past gene flow among the two species. Urbanelli (2002) hypothesized the occurrence of reinforcement to explain the pattern of past hybridization

observed between *O. quadricollis* and *Ochthebius* sp. A in the sympatric area along the Italian Tyrrhenian coasts, although other causes such as ecological character displacement could not be ruled out.

In this article, to distinguish among the hypotheses above, we measured premating isolation between *O. quadricollis* and *Ochthebius* sp. A in both allopatric and sympatric populations. During mating, females and males of both species get close until a couple is formed with the male (smaller) on the back of the female (bigger) (Fig. 1). Mating lasts for about 1 h during which time the female swims and walks on rocks with the male on her back (Beier 1956 and our personal observations). First we checked the species composition of couples collected from the field in sympatric areas to verify the occurrence of assortative mating among the two species in nature. Second, we tested the degree of assortative mating in sympatric versus allopatric samples through multiple-choice mating trials under laboratory conditions.

Materials and Methods

SAMPLING

To analyze the species composition of mating couples from natural populations in the sympatric area between *O. quadricollis* and *Ochthebius* sp. A, couples were collected in July 2005 directly from rock pools in the localities shown in Figure 2, separately frozen in liquid nitrogen, and brought to the laboratory for genetic identification (Urbanelli 2002).

Samples used in mating trials were collected in two sampling campaigns in July and September 2006 from Italian allopatric and sympatric sites (Fig. 2). The sympatric samples come from the main area of sympatry between *O. quadricollis* and *Ochthebius* sp. A found along the Tyrrhenian coasts of Campania, Basilicata, and Calabria (Urbanelli et al. 1996; Urbanelli 2002).

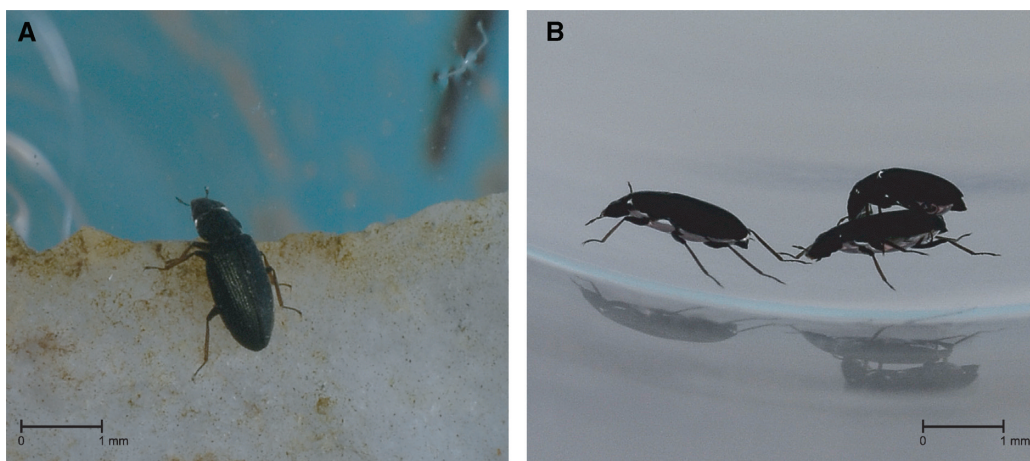


Figure 1. (A) Female of *Ochthebius quadricollis* (Coleoptera: Hydraenidae); (B) mating couple with male (smaller) on the back of the female (bigger). Courtesy: Paolo Ragazzini.

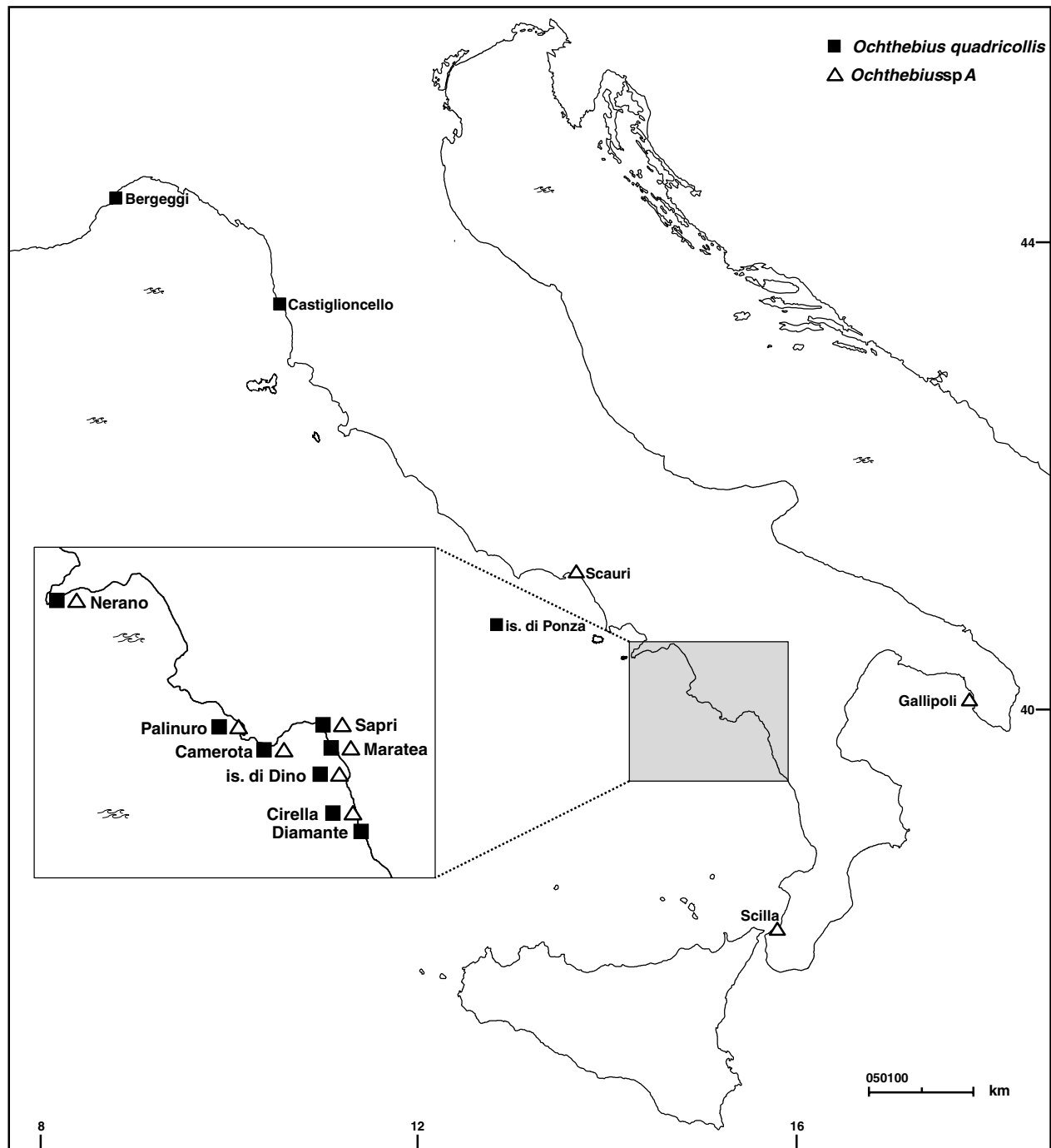


Figure 2. Collection sites of the samples of *Ochthebius quadricollis* and *Ochthebius sp. A* studied. In the square the sympatric area is shown. In all sites both species occur syntopically, but Diamante sample consisted of only specimens genetically identified as *O. quadricollis* and by introgressed specimens (Urbanelli 2002).

BREEDING EXPERIMENTS

Adult specimens were collected for breeding: females and males were manually sorted by body size (females are bigger than males, Beier 1956), kept in different plastic bottles filled with the water of the rock pool of origin, and brought to the laboratory.

Experiments consisted in multiple-choice mating without replacement (Coyne et al. 2005).

Within two days after the sampling, breeding experiments were performed in aquaria half filled with seawater and covered with a net to prevent the insects from flying out. Environmental conditions were as follows (Beier 1956): 26°C; 12-h dark : 12-h light. Small rocks covered with algae collected from the sampling sites were put in the aquaria as physical support and food source for the adults during the breeding experiments. All four sidewalls

of the aquaria were blinded with black paper so that light came in only from above. All in all the experiments were conducted under conditions very similar to those naturally occurring in rock pools (see Coyne et al. 2005). In nature, the number of beetles may vary according to the chemical and physical status of the rock pools in which they live, and high density may be reached (several hundred adult specimens per liter). We conducted mating trials at a density within the range we observed during the sampling of mating couples in the field. Males and females of both species, 400 specimens in all, were placed in a $50 \times 50 \times 30 \text{ cm}^3$ aquarium, and mating pairs were observed. The recorded crosses are summarized in Table 2. For allopatric crossings an aquarium was prepared as described above, hosting 100 females and 100 males of *O. quadricollis* and 100 females and 100 males of *Ochthebius* sp. A (Table 2).

The specimens tested for assortative mating in the sympatric area came from introgressed populations (i.e., containing genotypes with characteristic alleles of both species) (Urbanelli 2002). Because *O. quadricollis* and *Ochthebius* sp. A are morphologically indistinguishable species, we can distinguish them only by genetic analysis (see paragraph below). To put a similar number of specimens of the two species in the mating chamber, the sympatric trials were performed as follows:

- (1) Crossing males and females from Cirella, where the two species were found syntopically at about the same proportion (Urbanelli 2002). In this trial 200 females and 200 males were used.
- (2) Crossing specimens from Diamante and Maratea, where a high percentage of *O. quadricollis* and *Ochthebius* sp. A was, respectively, found (Urbanelli 2002) (100 females and 100 males from each locality).

The observed couples were removed from the aquaria at intervals of 2 h within the 12 h after the beginning of the trial. The trials were not lengthened to obtain the maximum number of mates because in a multiple-choice design the element of choice may diminish as specimens are removed from the aquaria (Casares et al. 1998). Couples collected were kept at -80° C until genetic analysis.

ELECTROPHORETIC ANALYSIS

Because the two species are morphologically indistinguishable, the specimens in each couple were identified by allozymic analysis. Standard horizontal starch gel electrophoresis was performed on single specimens following the protocols described by Urbanelli et al. (1996) and Urbanelli (2002). The following loci were assayed:

- (1) The locus Glucose-6-phosphate isomerase (*Gpi*, EC 5.3.1.9), where alleles Gpi^{100} , Gpi^{110} , Gpi^{112} , Gpi^{117} were found in *O. quadricollis*, and Gpi^{93} , Gpi^{98} , Gpi^{104} in *Ochthebius* sp. A.

- (2) The locus Phosphoglucosomutase (*Pgm*, EC 5.4.2.2) where alleles Pgm^{95} , Pgm^{100} , Pgm^{105} were found in *O. quadricollis*, and alleles Pgm^{108} , Pgm^{110} , Pgm^{114} in *Ochthebius* sp. A.

- (3) The locus Aspartate aminotransferase (*Aat-1*, EC 2.6.1.1) where the allele $Aat-1^{100}$ was found in *O. quadricollis*, and alleles $Aat-1^{80}$ and $Aat-1^{90}$ in *Ochthebius* sp. A.

- (4) The locus Aminopeptidase (*Pep-1*, EC 3.4.11.11) where the allele $Pep-1^{100}$ was found in *O. quadricollis*, and allele $Pep-1^{96}$ in *Ochthebius* sp. A (Urbanelli 2002).

STATISTICAL ANALYSIS

To assess the degree of sexual isolation among *O. quadricollis* and *Ochthebius* sp. A we used the I_{PSI} estimator proposed by Rolán-Alvarez and Caballero (2000) calculated as follows:

$$I_{PSI} = \frac{PSI_{aa} + PSI_{bb} - PSI_{ab} - PSI_{ba}}{PSI_{aa} + PSI_{bb} + PSI_{ab} + PSI_{ba}},$$

where PSI estimators are defined for every pair combination as the number of observed pair types divided by the number of expected pair types calculated from matings (Rolán-Alvarez and Caballero 2000). PSIs estimate the mate choice coefficient for each type of mating pair (homospecific combinations *aa* and *bb* and heterospecific *ab* and *ba*).

In a comparative study of the estimation properties of different statistics for measuring sexual isolation from mating frequencies, Pérez-Figuero et al. (2005) proposed the I_{PSI} as the safest estimator of sexual isolation for biological sample sizes. In fact, this estimator proved to be unbiased on average and showed very small variances across different scenarios of morph frequencies and mate propensity (the tendency for different phenotypes/genotypes to intrinsically mate more frequently than others). This is particularly important in our experiments because upon preparing the crosses among sympatric samples we are not able to evaluate the exact number of males and females of each species and in general we cannot evaluate the sexual maturation of the specimens used.

Like other estimators of sexual isolation, I_{PSI} can take values between -1 and 1 . Zero indicates random mating; 1 and -1 indicate that only homospecific or heterospecific matings were observed, respectively. We calculated the I_{PSI} estimator for each multiple-choice mating trial performed as described in JMATING software (Carvajal-Rodríguez and Rolán-Alvarez 2006). With the same software, we calculated the indices of asymmetry (IA_{PSI}) by taking the ratio of PSI values for the heterospecific combinations (Carvajal-Rodríguez and Rolán-Alvarez 2006). An IA_{PSI} of 1 indicates symmetric mating frequencies between the two mating combinations that were compared. Bootstrapping (100,000 resamplings) in JMATING was used to calculate mean bootstrap values, standard deviations, and two-tail probabilities for rejecting the null hypothesis that our estimates of I_{PSI} and IA_{PSI} are

different from 0 (0 = random mating) and different from 1 (1 = symmetry in mating), respectively. This method has been shown to be more appropriate than the parametric tests available as these show relatively high type-I errors (Pérez-Figuero et al. 2005).

The Mann–Whitney U -test was used to compare the sexual isolation indexes (I_{PSI}) among allopatric and sympatric samples to test the null hypothesis that the level of assortative mating does not differ among them.

Results

A total of 433 couples were collected in eight localities of the sympatric area of *O. quadricollis* and *Ochthebius* sp. A along the southern Tyrrhenian coast. By analyzing the species composition of mating couples we aimed to evaluate the sexual isolation between the two species in nature. I_{PSI} statistics of sexual isolation were used to verify the occurrence of random or assortative mating. Table 1 shows the number of homospecific and heterospecific couples collected for each locality. In all the samples a significant deviation from random mating was observed (for all I_{PSI} statistics, two-tail probability of rejecting the null hypothesis of random mating in the bootstrap resampling distribution with 100,000 replicates $P < 0.05$), which shows a strong skew toward species assortative matings.

Multiple-choice mating trials were conducted in the laboratory to verify the hypothesis of greater assortative mating in sympatric than allopatric samples. Results are shown in Table 2. No deviations from random mating were observed in allopatric crosses (for all I_{PSI} statistics, two-tail probability of rejecting the null hypothesis of random mating in the bootstrap resampling distribution with 100,000 replicates $P > 0.05$) regardless of the geographic origin of the samples. Conversely, in the trials with sym-

patric samples a significant departure from random mating was observed (for all I_{PSI} statistics, two-tail probability of rejecting the null hypothesis of random mating in the bootstrap resampling distribution with 100,000 replicates $P < 0.05$). No deviations from symmetry in the heterospecific combinations were observed in all (I_{PSI} , two-tail probability in the bootstrap resampling distribution with 100,000 replicates, $P > 0.05$), but one trial (Cirella-July) in which we observed an excess of *Ochthebius* sp. A females/*O. q.* males mating pairs (I_{PSI} , $P < 0.05$).

When comparing the sexual isolation observed in the allopatric and sympatric trials by Mann–Whitney U -test, highly significant differences were found ($Z = 2.717$, $P < 0.01$).

Discussion

In previous works conducted through genetic analysis using allozymic markers, *O. quadricollis* and *Ochthebius* sp. A have been shown to be separate species (Urbanelli et al. 1996; Urbanelli 2002). Eight allozymic loci were found diagnostic between them (i.e., each species has characteristic alleles) and no F1 hybrids or their offspring nor backcrosses were found in sympatric areas, showing that at present there is a full reproductive isolation among the two taxa. However, in some populations of the sympatric areas we recorded the sharing of characteristic alleles between the two species at some loci (i.e., alleles characteristic of each taxon were found in specimens of the other species) (Urbanelli 2002).

The presence of shared alleles only in sympatric areas (localized vs. widely or randomly distributed), despite the extensive sampling performed, indicates the occurrence of introgressive hybridization events, which rules out the potential alternative explanations for such a pattern, that is, stochastic sorting of ancestral polymorphism or the presence of isoelectrophoretic alleles (Klein

Table 1. Mating couples from natural populations in the sympatric area between *Ochthebius quadricollis* and *Ochthebius* sp. A; homo-specific (aa, *O. q./O. q.*; bb *O. sp. A/O sp. A*) and heterospecific (ab *O. q.* female/*O. sp. A* male; ba *O. sp. A* female/*O. q.* male) matings. N = total couples collected in each site; I_{PSI} statistic of sexual isolation (SD, Standard Deviation) and the two-tail probability of rejecting the null hypothesis being true ($I_{PSI} = 0$, random mating) in the bootstrap resampling distribution (100,000 replicates).

Samples	Homospecific pairs		Heterospecific pairs		N	I_{PSI}	SD	P
	aa	Bb	ab	ba				
Nerano	16	38	3	1	58	0.867	0.0650	<0.001
Palinuro	34	14	–	–	48	1	0.000	<0.001
Camerota	18	20	1	1	40	0.904	0.067	<0.001
Sapri	16	32	1	1	50	0.922	0.044	<0.001
Maratea	6	45	–	–	51	1	0.000	<0.001
Isola di Dino	30	26	1	3	60	0.874	0.610	<0.001
S. Nicola	36	30	–	–	66	1	0.000	<0.001
Cirella	32	28	–	–	60	1	0.000	<0.001
Total	188	233	6	6	433			

Table 2. Mating choice trials between *Ochthebius quadricollis* (O.q.) and *Ochthebius* sp. A. samples. *N* = total number of couples collected. Number of homospecific (aa, O.q/O.q; bb O. sp.A/O. sp. A) and heterospecific (ab O.q female/O. sp. A male/O. sp. A female/O. q. male) matings. I_{PSI} estimator of sexual isolation; $I_{A_{PSI}}$ ab/ba asymmetry index for heterospecific matings; *P* = two-tail probability of rejecting the null hypothesis in the bootstrap resampling distribution with 100,000 replicates

Samples	Comparison		Date of collection	Homospecific pairs			Heterospecific pairs			<i>N</i>	I_{PSI} (SD)	<i>P</i>	$I_{A_{PSI}}$ ab/ba (SD)	<i>P</i>
	<i>Ochthebius</i> q.	<i>Ochthebius</i> sp. A		aa	bb	ab	ba							
Allopatric	Bergeggi	× Scauri	July 2006	19	17	13	11	60	0.204 (0.129)	0.126	1.049 (0.152)	0.802		
			September 2006	26	18	22	12	78	0.143 (0.117)	0.230	1.112 (0.155)	0.310		
			July 2006	22	15	16	10	63	0.181 (0.129)	0.169	1.114 (0.187)	0.410		
	Castiglioneello	× Scilla	September 2006	26	18	19	12	75	0.180 (0.119)	0.134	1.105 (0.150)	0.350		
			July 2006	20	16	12	17	65	0.114 (0.127)	0.372	0.956 (0.085)	0.540		
			September 2006	22	19	16	13	70	0.175 (0.121)	0.157	1.048 (0.117)	0.711		
	Ponza	× Scauri	July 2006	19	23	17	15	74	0.136 (0.118)	0.260	1.023 (0.087)	0.714		
			September 2006	21	19	17	12	69	0.168 (0.121)	0.168	1.076 (0.138)	0.511		
			July 2006	35	29	4	6	74	0.736 (0.079)	<0.001	0.834 (0.538)	0.423		
Cirella	× Maratea	September 2006	22	30	1	5	58	0.812 (0.070)	<0.001	0.299 (0.344)	0.050			
		July 2006	28	12	2	8	50	0.631 (0.112)	<0.001	0.434 (0.278)	0.028			
		September 2006	19	16	2	5	42	0.689 (0.111)	<0.001	0.579 (0.439)	0.171			
Sympatric	Cirella	× Cirella	September 2006	19	16	2	5	42	0.689 (0.111)	<0.001	0.579 (0.439)	0.171		

et al. 1998). Therefore, the pattern observed in these two species in sympatric areas could be viewed as a signature of past gene flow between them which must have occurred when the two species first met and which was later interrupted. The occurrence of reinforcement was hypothesized although other causes such as ecological character displacement may not be precluded (Urbanelli 2002).

In this article, we aimed to distinguish among the hypotheses above, measuring premating isolation between *O. quadricollis* and *Ochthebius* sp. A in both allopatric and sympatric populations. First, we checked the species composition of couples collected from the field in sympatric areas. Of all the couples collected in the sympatric localities 97.3% were homospecific, which shows the existence of strong assortative mating between the two species.

Second, we assessed the possible occurrence of reproductive character displacement in sympatric, but not allopatric, areas by multiple-choice mating trials: under laboratory conditions, we tested the degree of assortative mating in sympatric versus allopatric samples. The presence of a significant departure from random mating toward homospecific matings was observed between samples of *O. quadricollis* and *Ochthebius* sp. A from the sympatric areas studied. Conversely, frequent heterospecific matings—not deviating from those expected under random mating—were observed between the two species in the allopatric populations.

Although greater premating isolation in sympatric than in allopatric populations is a signature that could be left by reinforcement, some other processes could account for this pattern (Noor 1999; Lemmon et al. 2004). Two of these hypotheses could be viewed as an alternative to reinforcement in assessing the evolution of reproductive isolation between *O. quadricollis* and *Ochthebius* sp. A.

First, when two similar species come into contact, competition for resources may drive population divergence (ecological character displacement, Brown and Wilson 1956; Losos 2000; Dayan and Simberloff 2005). This phenomenon results in greater adaptive divergence in populations that are in geographical contact with one another than in allopatric pairs of populations. As a consequence of this greater trait divergence, mating discrimination can be stronger between sympatric or parapatric than between allopatric pairs of populations. If ecological character displacement occurred between *O. quadricollis* and *Ochthebius* sp. A. in the areas of geographical overlap, the evolution of size and/or shape differences should be expected that would reduce resource-use overlap and hence interspecific competition (Dayan and Simberloff 2005, for a review). This pattern does not emerge from the morphological studies conducted on these species. Before Urbanelli et al. (1996) reviewed the taxonomy of the beetles of the genus *Ochthebius* through genetic analysis, taxonomists had described five species in Mediterranean and Macaronesian regions, on the basis of slight morphological differences. In particular, the

specimens recognized by genetic studies as belonging to *Ochthebius* sp. A and *O. quadricollis* from populations collected on the French, Corsican, and Italian Tyrrhenian coast had all been considered as a single subspecies named *Ochthebius q. quadricollis* (Ienistea 1977 and Urbanelli 1996 for a brief review). A subsequent revision conducted by Jäch (1993) on the basis of the copulatory male organ (aedeagus), put into evidence only slight morphological differences in the shine of the top surface and in the shape of the eadeagus. Overall, morphological studies do not support the existence of morphological differences in sympatric versus allopatric populations of both *O. quadricollis* and *Ochthebius* sp. A since: (1) no significant differences were found among sympatric versus allopatric populations in both *O. quadricollis* and *Ochthebius* sp. A; (2) the syntopic occurrence of the two species along the Tyrrhenian coast has been shown only by genetic analysis, as morphological studies failed to find significant differences among the two species as would be expected if ecological character displacement had occurred (Urbanelli et al. 1996; Urbanelli 2002; Losos 2000). None of these support the hypothesis that adaptive divergence resulting from other ecological factors has played a major role in the differences observed in mating choice between sympatric and allopatric populations.

Another hypothesis that could account for the increased mating discrimination in parapatric/sympatric populations with respect to allopatric populations is termed “differential fusion” (Templeton 1981). It suggests that only populations with strong mating discrimination persist in sympatry, whereas those populations lacking such discrimination fuse or lose their distinctness (see Noor 1999; Coyne and Orr 2004). This hypothesis was regarded as less likely than reinforcement to explain high sexual isolation in sympatry, at least in the majority of cases (Noor 1999; Coyne and Orr 1989, 2004 for a discussion). The key prediction of the differential fusion hypothesis is that some allopatric populations will exhibit levels of mating discrimination similar to those observed in sympatric populations. In consideration of the results of this study—all mating trials conducted between allopatric populations of *O. quadricollis* and *Ochthebius* sp. A exhibit lower discrimination than sympatric populations (Table 2)—this hypothesis seems unlikely, although we may not unequivocally reject it (Coyne and Orr 2004).

The current overlapping distributions of *O. quadricollis* and *Ochthebius* sp. A along the southern Tyrrhenian coast putatively reflect a secondary contact that followed divergence in geographic isolation (Urbanelli et al. 1996). Gene flow occurred in past as shown by introgression found in sympatric areas, a prerequisite for reinforcement, whereas at present there is no evidence of gene flow between the species (Urbanelli 2002). The geography of the overlap area is consistent with a model of symmetrical migration between two demes, which accounts for the evolution of reinforcement better than the one-way migration model between demes

(Servedio and Kirkpatrick 1997). The existence of a further signature typically ascribed to the occurrence of reinforcement was shown in this article: greater assortative mating in sympatric than in allopatric pairs of populations. In conclusion, considering the above discussed unlikelihood of the other causes that could lead to the observed mating pattern, we think that the hypothesis of reinforcement not only may not be rejected, but also seems, in this context, the most parsimonious one to explain the evolution of reproductive isolation between *O. quadricollis* and *Ochthebius* sp. A.

The beetles of the *Ochthebius* genus could be an interesting study system not only to find putative cases in which reinforcement has occurred, but also to investigate another outcome of reinforcement in speciation processes recently reported (Hoskin et al. 2005; Smadja and Butlin 2006). In the green-eyed tree-frog *Litoria genimaculata* reinforcement also drove premating isolation between intraspecific allopatric populations resulting in rapid allopatric speciation (Hoskin et al. 2005). Has the evolution of discriminative mate recognition systems in sympatric populations of *O. quadricollis* and *Ochthebius* sp. A influenced their intraspecific premating pattern as well?

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