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Pattern of genetic isolation in the crab *Pachygrapsus marmoratus* within the Tuscan Archipelago (Mediterranean Sea)

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ABSTRACT: The genetic connectivity level of the benthic crab *Pachygrapsus marmoratus* was assessed within the Tuscan Archipelago, an area between the Ligurian and Tyrrhenian Seas, Italy. The archipelago comprises 7 islands lying inside the largest marine protected area of the Mediterranean Sea. We genotyped a total of approximately 230 individuals from 8 populations (4 protected and 4 unprotected) at 8 microsatellites. Overall, our results showed a clear partitioning of genetic variation within this area; all populations were separated from one another, except the 2 southernmost islands. This genetic subdivision could be due to a 'sweepstakes reproductive effect' (i.e. only a small proportion of the available gene pool successfully contributes to the replenishment of each population). Furthermore, the lack of a pattern of isolation by distance, coupled with a low percentage of individuals assigned to their own populations, indicates that retention of larvae near the parent population is unlikely to be the main cause of the recorded genetic structure. However, we also found evidence of past demographic events in all populations, which may have played a pivotal role in shaping the recorded pattern of intraspecific differentiation. Finally, we did not detect any difference in the level of genetic variation between populations that were protected (i.e. experiencing less human pressure) and those that were unprotected. This suggests that, from a genetic point of view, the effects of protection have not yet been demonstrated.

KEY WORDS: Genetic structure · Microsatellites · Intertidal crabs · Mediterranean islands

INTRODUCTION

Understanding the level of connectivity of marine populations is fundamental in basic and applied ecological studies and for the management and protection of marine areas (Jones et al. 2007, Selkoe & Toonen 2011). Furthermore, in recent years, the growing concern about climate change has prompted scientists to look more deeply into its consequences for dispersal in the sea. It is already known that an increase in sea temperature shortens the duration of larval periods, with direct and predictable consequences for population connectivity, community structure and biodiversity distribution (for reviews see O'Connor et al. 2007 and Hoegh-Guldberg & Bruno 2010).

The reproduction of the majority of coastal marine organisms involves planktonic dispersal phases, such as gametes, eggs, larvae or spores (generically called propagules; Fairweather 1991), whose durations are extremely variable, from a few hours to years. These propagules are often released in the open sea and carried by currents to a suitable habitat where they develop into adults (Levin 2006). Although mortality due to recruitment failure is high, larval dispersion represents an important mechanism for the dispersal

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of marine species, especially for those whose adults are either sedentary or exhibit limited mobility during the maturity phase (DiBacco et al. 2006).

For years, a central ecological paradigm was the idea that the marine realm largely lacks geographic barriers to dispersion (see Ekman 1953). Following this broadly accepted view, the extent of marine species dispersion was simply related to the intensity of the currents and to the duration of their dispersal phases; generally, the more time propagules spend in the water column the further they tend to be dispersed. Recently, based on new evidence from phylogeographic and population genetic studies, a paradigm shift has started to occur (see Hauser & Carvalho 2008) and the existence of geographic barriers to species connectivity has become apparent in the sea. Another ecological paradigm under revision is the passive nature of larval dispersal in marine invertebrates, an assumption that pervaded marine biology until recently (Stobutzki 2001); however, it has now been challenged, based on studies of larval behaviour and adaptations in controlling their dispersion (for a review see Queiroga & Blanton 2005, Levin 2006).

As a consequence, dispersion in the marine realm is now recognised as a complex process, being affected by a number of biotic and abiotic factors and showing temporal variation (Shanks 1995, Carvalho & Hauser 1998, Shanks et al. 2003). In particular, the dispersal and successive recruitment of marine species and, ultimately, their dynamics and population genetic structure are strongly affected by the interplay among superficial and deep currents and winds (Palmer et al. 1996, Shanks et al. 2003). Moreover, the active role of adults (i.e. by controlling when and from where to spawn, Skov et al. 2005) and larvae (able to perform the so called ontogenetic and return migrations, Shanks 1995, Queiroga & Blanton 2005) also contribute to the complexity of such processes. Thus, it is not surprising that many population genetic studies suggest a higher complexity than expected in populations of marine organisms (see Hauser & Carvalho 2008). Despite the potential for dispersal, particularly at the larval stage, these organisms can achieve genetic differentiation (sometimes to a large degree), even at a local scale (see Hauser & Carvalho 2008). In particular, the ideas discussed above apply to those marine species inhabiting fragmented habitats, such as islands (Taylor & Hellberg 2003, Fauvelot & Borsa 2011), estuaries (Ikeda et al. 2003, Maltagliati et al. 2003), mangroves (Silva et al. 2010), coral reefs (Planes et al. 1998) and rocky shores (Riginos & Nachman 2001).

The crab *Pachygrapusus marmoratus* (Grapsidae: Brachyura) is an example of an intertidal species inhabiting rocky shores for which a higher than expected complexity in its genetic structure has been recorded (Fratini et al. 2008, 2011, Silva et al. 2009). *P. marmoratus* has several characteristics that suggest this species is likely to have a good dispersal ability, even at macro- and meso-geographic scales: a wide geographic range, high population density on rocky shores, high resilience to anthropogenic impact and a pelagic larval stage with a long duration (i.e. 1 mo in the water column). Fratini et al. (2011) confirmed this expectation by analysing genetic variation at mitochondrial haplotypes among distantly related Mediterranean and Atlantic populations. Conversely, other genetic studies that investigated polymorphisms at microsatellite loci at local scales recorded a genetic differentiation among populations separated by tens or a few hundreds of kilometres (Fratini et al. 2008, 2011 along the Tuscan coast, with a separation among Ligurian and Tyrrhenian populations; Silva et al. 2009 along the Portuguese coast, with a genetic patchiness among populations unrelated to a geographic gradient). Overall, these studies seem to indicate that dispersion of *P. marmoratus* on a meso- and macro-geographic scale is affected by oceanographic processes, while its fine-scale dispersal and recruitment processes are mainly affected by local hydrological conditions. Consequently, population genetic structures within specific local areas are not predictable *a priori*, based on pelagic larval duration and geographic distances.

Under these premises, this study aimed to assess connectivity among populations of *Pachygrapsus marmoratus* within a group of 7 islands belonging to the Tuscan Archipelago (Italy). The archipelago covers an area of about 750 km^2 and the distance between the northernmost and the southernmost islands is 150 km, and so within the order of the mean dispersal distance reported for fish and invertebrate species (Palumbi 2003). In this study we analysed the polymorphisms at 8 microsatellite loci in a total of approximately 230 individuals, collected from a population from each island and from a population located on the Tuscan coast adjacent to the archipelago. Two different scenarios and different explanations per scenario are possible. First, the *P. marmoratus* populations of the archipelago belong to a unique metapopulation with a high level of connectivity; this could be explained by the ability of larvae to disperse over the maximum distance between pairs of islands and/or by the presence of stepping-stone populations. Second, a genetic separation is arising within

this area, resulting in the presence of more or less isolated self-sustaining populations or groups of populations. This scenario could be promoted by subtle geographic barriers, such as those between the Ligurian and Tyrrhenian Seas, which separate populations along the Tuscan coast (Fratini et al. 2008, 2011). Alternatively, local retention could be at the basis of the recruitment process of each insular population; in this case, we hypothesise that the recorded population genetic structure will follow a pattern of isolation by distance.

Finally, since the Tuscan Archipelago belongs to a marine and terrestrial national park, we hypothesise that the crab populations from protected sites should experience a far weaker anthropogenic pressure with respect to those living in unprotected areas and, as a consequence, should have a higher level of genetic variation. During springtime and summertime, i.e. the reproductive and recruitment period of *Pachygrapsus marmoratus*, sandy and rocky shores of Tuscany are intensively visited by tourists that exert a direct (e.g. chasing of the crabs) and indirect (e.g. human trampling) disturbance on intertidal populations of crabs and molluscs (see Rossi et al. 2007).

Overall, the results of the present study should provide useful information on *Pachygrapsus marmoratus* larval dispersal processes for the future management and conservation of marine resources of the Tuscan Archipelago.

MATERIALS AND METHODS

Study species

Pachygrapsus marmoratus (Fabricius, 1787), known as the 'marbled crab', is an intertidal crab. It is widespread throughout the entire Mediterranean basin, in the Black Sea and along the European and east African Atlantic coasts, including the Canary and Azores Islands (Zariquiey Alvarez 1968, Ingle 1980). Recently, the distribution range of *P. marmoratus* has expanded northward, probably as a consequence of the warming of sea surface waters (Dauvin 2009). *P. marmoratus* occupies the upper and middle levels of rocky shores, dwelling in crevices of breakwaters and on pier pilings. As this species is resilient to anthropogenic disturbance, it is also found in ports and marinas (Fratini et al. 2008). *P. marmoratus* is an opportunistic feeder, able to shift its diet from algal browsing to mollusc predation, thus influencing the abundance and structure of a number of intertidal populations (Cannicci et al 2007). Therefore, the importance of the role of this species in ecosystem dynamics of Italian rocky shores is undisputed.

Pachygrapsus marmoratus adults are relatively sedentary, being faithful to a specific intertidal area (Cannicci et al. 1999); thus, dispersion is exclusively guaranteed by a larval planktonic stage lasting approximately 1 mo (Cuesta & Rodriguez 2000). This species breeds from late spring to late summer (Álvarez 1968, Ingle 1980) and megalopae settlement has a peak at the end of the summer. Settlement seems to follow a semi-lunar cycle in correspondence with the spring tides, at least on Atlantic coasts where tidal excursions are substantial in comparison to the Mediterranean basin (Flores et al. 2002).

Study area and collection sites

The Tuscan Archipelago (Fig. 1) is a chain of islands in the western Mediterranean Sea, west of Tuscany, Italy. It includes 7 islands, Gorgona (GOR), Capraia (CAP), Elba (EL, the largest island of the group), Pianosa (PIAN), Montecristo (MO), Giglio (GI) and Giannutri (GN). All of these islands are part of the marine and terrestrial Tuscan Archipelago

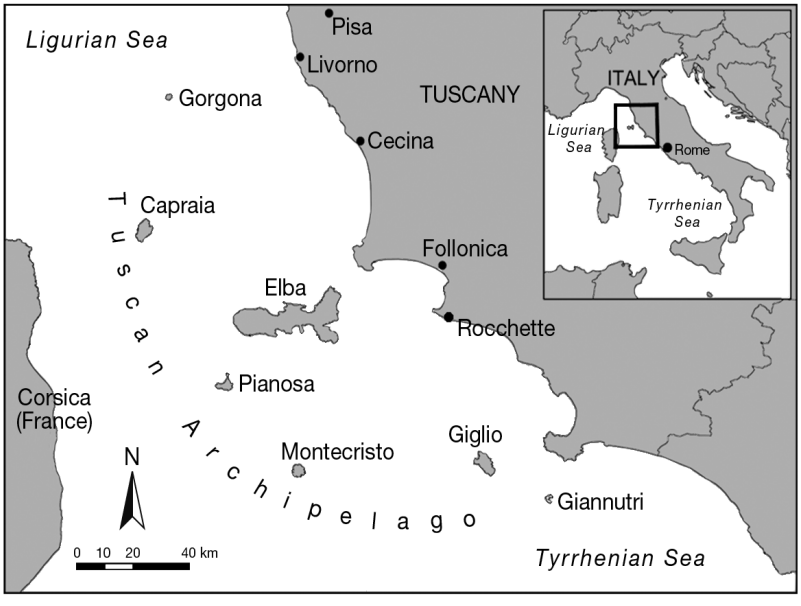

Fig. 1. Map of the study area, showing the locations of the islands of the Tuscan Archipelago

National Park, the largest European marine park, and are managed according to different regimes of protection. A strict protection regime (fishing, diving and anchoring not allowed without a special permit) is in force on GOR, PIAN and MO and on parts of GN and CAP. Conversely, EL and GI are intensively exploited by tourism.

The islands of the Tuscan Archipelago lie within 2 distinct basins, between the Ligurian Sea and Tyrrhenian Sea. The islands washed by the Ligurian Sea (GOR, CAP and the northern coast of EL) are under the influence of the cold West Corsican Current, a branch of the Atlantic Current (Millot 1987, Artale et al. 1994, Astraldi et al. 1994), while the southern part of the archipelago is crossed by the Tyrrhenian Current, which flows along the coasts of Latium and Tuscany, bringing water northward through the Corsican Channel from the Tyrrhenian Sea (Millot 1987, Artale et al. 1994, Astraldi et al. 1994).

Pachygrapsus marmoratus populations were collected at all of the 7 islands of the Tuscan Archipelago (Fig. 1 and Table 1, see 'Results') and from a site located on the Tuscan coast (Rocchette, R, Fig. 1 and Table 1). A chela or leg was removed from 27 to 31 crabs per population (Table 1) and afterwards each animal was released back into its natural habitat.

Laboratory analyses

Total genomic DNA was extracted from muscle tissue using the Puregene Kit (Gentra Systems). DNA was then re-suspended in sterile distilled water and stored at 4°C for routine use, or at −20°C for long-term storage.

A total of 232 individuals were screened for polymorphism at 8 microsatellite loci specifically isolated for *Pachygrapsus marmoratus.* Seven of the loci (pm-101, -79, -109, -108, -187, -183, -99) are described in Fratini et al. (2006) and were amplified using PCR conditions as reported in Fratini et al. (2006, 2008, 2011). The locus pm-84, however, is a newly described locus, isolated using the Fratini et al. (2006) procedure. Its PCR conditions are: 1 to 2 µl of DNA, 2 µl of Buffer 10X (Invitrogen), 2 mM of $MgCl_2$, 0.4 ml of each 10 µM primer, 200 µM of each dNTP and 0.4 U of Taq (Invitrogen) in 15 µl of final volume, with 35 cycles of 30 s for denaturation at 94°C, 45 s for annealing at 58°C and 180 s extension at 72°C, preceded by 5 min of initial denaturation at 94°C and followed by a 30 min final extension at 72°C.

For detection of polymorphisms, the forward primer for each locus was 5′-labelled and then labelled amplicons from the 8 loci were divided into 2 sets (pm-101 NED + pm-79 HEX + pm-84 FAM + pm-109 FAM; and pm-108 HEX + pm-187 NED + pm-183 FAM + pm-99 FAM). For each set, 1 to 5 µl of each PCR product was combined with water in a final volume of 10 µl for successive dimensional analysis. Sizing was performed in an ABI Prism 310 Genetic Analyzer (Applied Biosystems) with reference to the internal size standard ROX400 using Genotyper ver. 3.7 and GeneScan ver. 3.7 (Applied Biosystems).

Statistical analyses of microsatellite data

The number of alleles and the allelic richness for each locus and population were calculated using FSTAT ver. 2.9.3 (Goudet 1995). Linkage equilibrium among loci and Hardy-Weinberg equilibrium (HWE) were assessed for each population using Genepop ver. 3.4 (Raymond & Rousset 1995, http://genepop.curtin.edu.au). Since all populations showed heterozygote deficiencies, the data set was checked for genotyping errors (i.e. null alleles) by means of equation 2 from Brookfield (1996), as implemented in Microchecker ver. 2.2.3 (van Oosterhout et al. 2004). We then used the software FreeNA (www.montpellier.inra.fr/URLB) to compute a genotype dataset corrected for null alleles, following the INA method described in Chapuis & Estoup (2007). This new dataset was used in all further analyses (except the Bayesian clustering method) and for recalculating the measures of population variability.

To assess whether the samples were random or comprised related family groups, a pairwise comparison was made within each population using the software ML-Relate (Kalinowski et al. 2006), which estimated the percentage of unrelated individuals of each population pair-by-pair. The unrelated pairs were identified by log-likelihood ratios taking into account the incidence of null alleles (Wagner et al. 2006).

Genetic differentiation was estimated by the exact test of population differentiation (Raymond & Rousset 1995), implemented in Genepop. This test verifies the existence of differences in allele frequencies at each locus and for each population. Single locus p-values were calculated using a Markov chain with 1000 batches and 1000 iterations per batch, combined over loci using the Fisher Exact test.

The existence of population structure was also assessed by 1-level AMOVA (Excoffier et al. 1992), using Arlequin ver. 3.5 (Excoffier & Lischer 2010). The significance of F_{ST} values was computed by per-

mutation tests from 10000 random permutations. Additional 2-level AMOVAs were performed, grouping Tyrrhenian populations versus Ligurian populations, and on the basis of the results of pairwise F_{ST} comparisons.

As F_{ST} is based on sample heterozygosity and can therefore underestimate differentiation in cases where within-population heterozygosity is high or varies among populations (Jost 2008), we also calculated Jost's *D*, which accounts for allelic richness (Jost 2008) as an additional measure of population differentiation, using SMOGD ver. 1.2.5 (Crawford 2010).

Correlations between genetic (F_{ST} values) and geographic distances (calculated as effective linear distance by sea) were evaluated by the Mantel Test, as implemented in Arlequin, with 20000 random permutations.

We also applied the Bayesian clustering method proposed by Pritchard et al. (2000), using the software STRUCTURE 2.0, to infer the number of genetically distinct groups and to evaluate the relative contribution of each inferred population to the genotypes of each individual and to the original groups. STRUCTURE requires us to choose the ancestral model of population structure used; we chose the admixture model, which is indicated to be appropriate for populations that have recently experienced, or are currently experiencing, gene flow at sufficient rates that individuals may have recent ancestors from more than 1 population (Pritchard et al. 2000). With this model the probability of the number of populations (*k*) for the pooled data was estimated by fixing prior values of *k* (in our case $k = 1$ to 8) and comparing the ln likelihood of the data. If the hypothetical single 'population' is admixed and includes more than 1 subpopulation, the likelihood of the data will increase with k (Pritchard et al. 2000). We ran this model, selecting the option that the allele frequencies in the different populations are correlated; this means that the populations diverged from a single ancestral population at some point in the past and the differences in their allele frequencies are the result of drift that has occurred since their divergence. The method is implemented by a Markov chain Monte Carlo algorithm that allows the estimation of the posterior distribution of the allele frequencies in the inferred populations (Evanno et al. 2005). Data were obtained with runs of 1000000 iterations (conducted 3 times for each k value) and a burn-in of 20000 iterations.

Population structure was also investigated using the assignment and exclusion tests implemented in GeneClass software ver. 2.0 (Piry et al. 2004). This method consists of calculating the most probable origin of each individual by comparing the likelihood of the multi-locus genotype of a given individual in a set of pre-determined populations. The Bayesian method, proposed by Rannala & Mountain (1997) and suggested as the most accurate method by Cornuet et al. (1999), was chosen, together with a threshold of 0.05. Using GeneClass, we also calculated the probability that each individual may be a resident. For migrant detection, we calculated the probability *L* as the ratio of the likelihood computed from the population where the individual was sampled over the highest likelihood value among all population samples, including the population where the individual was sampled (Paetkau et al. 2004). The frequencies-based method described by Paetkau et al. (1995) was used and a threshold of 0.01 was set. For both the tests, the rejection probability was obtained by simulating 10000 individuals from allelic frequencies based on the simulation algorithm of Paetkau et al. (2004).

We then applied the BOTTLENECK test (Cornuet & Luikart 1997) to test for evidence of genetic bottlenecks in *Pachygrapsus marmoratus* populations, using the heterozygosity excess method of Luikart et al. (1998), as implemented in the BOTTLENECK software ver. 1.2.02 (Piry et al. 1999). These analyses of deviations from mutation-drift equilibrium were conducted using the 2-phase mutation model (TPM) of evolution that is best suited for microsatellites (Di Rienzo et al. 1994, Luikart & Cornuet 1998) and the step-wise mutation model (SMM). For the TPM, we set 70 multi-step mutations, with a variance among multi-steps of 12%, as recommended for microsatellites (Piry et al. 1999) and obtained statistical significance based on 1000 replications. For both the mutational models, the heterozygosity of each locus expected under mutation-drift equilibrium, given the observed number of alleles (He), was determined using 10000 simulations and then compared against observed heterozygosity (Ho). In a population at mutation-drift equilibrium (i.e. the effective size of which has remained constant in the past) there is approximately an equal probability that a locus shows a heterozygosity excess or a heterozygosity deficit. We then recorded the number of loci for which Ho was greater than He and determined whether the overall set of deviations was statistically significant using the Wilcoxon sign-rank test (Luikart et al. 1998). The more powerful standardised difference test recommended by Cornuet & Luikart (1997) cannot be used with data from

fewer than 20 polymorphic loci. Bottlenecked populations are also expected to exhibit a characteristic 'mode shift' in the frequency distribution of alleles away from the L-shaped distribution expected under mutation-drift equilibrium (Luikart et al. 1998). Consequently, BOTTLENECK was also used to generate a qualitative descriptor of whether the observed allele frequencies at each locus deviate from such a distribution.

For testing bottleneck events, we also applied the M-ratios test using the software Msvar (Garza & Williamson 2001). One locus (pm-99) was omitted from this analysis, because it did not conform to the stepwise mutation model. To determine the critical M-ratio value (below which population declines are inferred), we used the M-crit program developed by Garza & Williamson (2001). Three parameters are needed for this program: effective population size, percentage of mutations greater than 1 step and average size of a non 1-step mutation. Effective population size was estimated for each population using Arlequin 3.5 (Excoffier & Lischer 2010). We then used 0.12 for the proportion of non 1-step mutations and 2.8 for the average size of non 1-step mutations, as these were mean values found in a literature survey (Garza & Williamson 2001).

Finally, we applied 1-way Permutational ANOVAs (PERMANOVA), as implemented in PRIMER ver. 6.1 (Clarke & Gorley 2006), to compare genetic diversity indices (observed heterozygosity, allelic richness, number of private alleles) and the percentage of unrelated pairs of each population between populations from protected and unprotected sites (Table 1, see 'Results'). The factor 'protection status' was considered fixed and orthogonal and the percentage of unrelated individuals was arcsine transformed. Our hypothesis was that populations from protected sites would show higher genetic variation indices and a lower percentage of related individuals than populations from unprotected sites.

RESULTS

No significant linkage disequilibrium was recorded across all populations. All loci were polymorphic, with a number of alleles ranging from 5 (locus pm-84) to 43 (locus pm-109). Each population showed relatively high levels of molecular variation, with allelic richness varying from 10.75 to 11.95 (Table 1). The number of private alleles for each population was also relatively high, ranging from 3 to 7 (Table 1). In Table 1 the percentage of unrelated individuals per population is also shown; this value is approximately 90% for all populations, and thus below the prediction of randomly mating groups.

Based on the original dataset, all populations strongly deviated from HWE, with an excess of homozygotes, mainly due to locus pm-101, pm-108 and pm-99. For these loci, the software Micro-Checker suggested the presence of null alleles and thus we corrected the original matrix for null alleles. Using the revised dataset, all populations, except GN, were in HWE when considering all loci together (Table 1). However, when considering each specific locus, populations CAP, GI, GN and PIAN deviated from HWE at locus pm-108.

Overall, our samples showed a strong degree of genetic differentiation among populations. Based on the outcome of the Fisher exact test, we rejected the hypothesis of genetic homogeneity in distribution of allele frequencies for the entire set of microsatellites ($\chi^2 = \infty$, df = 16, $p < 0.001$), and locus by locus, with the exception of locus pm-109 (data not shown).

Table 1. *Pachygrapsus marmoratus* populations analysed. For each population, population abbreviation (Abb.), the basin within the Mediterranean Sea, GPS coordinates, protection status (P = protected area; UP = unprotected area), number of individuals analysed (N), allelic richness (Na), number of private alleles (Np), percentage of unrelated individuals (% un-r), observed heterozygosity (Ho) and expected unbiased heterozygosity (He), after correction for null alleles, and the p-value of departure from the Hardy-Weinberg equilibrium (p, significant values in bold) are reported

Population	Abb.	Basin	GPS coordinates		Protection status	N	Na	Np	% un-r	Ho	He	p
Gorgona Island	GOR	Ligurian	43°25′43″N	9°54′25″E	P	27	10.75	5	91.17	0.76	0.73	0.23
Capraia Island	CAP	Ligurian	43°01′31″N	9°50′06″E	UP	28	10.84	3	89.15	0.75	0.72	0.20
Elba Island, Lacona	EL	Tyrrhenian	42°45′34″N	10°18′02″E	UP	31	11.67	6	90.75	0.79	0.79	0.72
Pianosa Island	PIAN	Tyrrhenian	42°34′28″N	10°03′55″E	P	30	11.19	6	88.97	0.72	0.69	0.14
Montecristo Island	MO	Tyrrhenian	42°21′02″N	10°18′40″E	P	30	11.88	4	91.49	0.77	0.73	0.27
Giglio Island	GI	Tyrrhenian	42°22′31″N	10°52′57″E	UP	28	11.95	7	91.80	0.75	0.74	0.08
Giannutri Island	GN	Tyrrhenian	42°15′30″N	11°06′09″E	P	28	11.11	3	89.95	0.76	0.74	**0.03**
Rocchette	R	Tyrrhenian	42°46′33″N	10°47′38″E	UP	30	11.37	4	91.49	0.77	0.76	0.43

Table 2. Pairwise comparisons of genetic differentiation estimated from the χ^2 of the Fisher exact test (below the diagonal), the F_{ST} values (above the diagonal) and the Jost's *D* values (in parentheses). Significant χ^2 and F_{ST} differentiation values between populations indicated in **bold** ($p < 0.05$). Tests of significance for *D* values were not possible. Abbreviations as in Table 1

	GOR	CAP	EL	PIAN	MO	GI	GN	R
GOR	*	**0.013** (0.026)	**0.014** (0.019)	**0.025** (0.010)	**0.04** (0.006)	**0.012** (0.011)	**0.008** (0.003)	**0.01** (0.003)
CAP	25.28	*	**0.016** (0.015)	**0.012** (0.004)	**0.024** (0.015)	**0.026** (0.034)	**0.023** (0.009)	**0.02** (0.016)
EL	**49.53**	**33.49**	*	**0.007** (0.022)	**0.01** (0.067)	**0.012** (0.057)	**0.034** (0.060)	**0.023** (0.038)
PIAN	23.47	**33.13**	**50.36**	*	**0.009** (0.025)	**0.025** (0.017)	**0.012** (0.021)	**0.028** (0.029)
MO	23.49	**36.68**	∞	25.98	*	**0.015** (0.025)	**0.015** (0.004)	**0.015** (0.006)
GI	**40.9**	**29.43**	∞	∞	**32.11**	*	0.003 (0.004)	**0.012** (0.009)
GN	∞	**30.29**	∞	**42.76**	18.62	19.77	*	**0.02** (0.024)
R	25.55	**35.36**	∞	∞	∞	**32.71**	**35.5**	*

Genetic population differentiation for each population pair was also significant for most pairs, except GN vs. GI, CAP vs. GOR, GI vs. MO, GOR vs. PIAN, MO vs. PIAN and GOR vs. R (Table 2).

The AMOVA test also confirmed the hypothesis of partitioning of genetic variation among populations (F_{ST} = 0.02, df = 463, $p < 0.001$). All the pairwise F_{ST} values were significant, except for the comparison GI vs. GN (Table 2). Based on biogeographic information and on the significant pairwise population comparisons, we performed 3 additional 2-level AMOVAs. First, we grouped the 2 Ligurian populations (CAP and GOR) and the 6 Tyrrhenian populations (EL, PIAN, MO, R, GI and GN) and obtained an F_{CT} value of −0.004 and a corresponding p-value of 0.93. Second, we created 3 groups, splitting the Tyrrhenian group into 2 groups, corresponding to northern (EL, PIAN, MO and R) and southern (GI and GN) populations; in this case the F_{CT} value was 0.001 with a p-value of 0.43. Third, we grouped the 2 southern Tyrrhenian populations (GI and GN) against all the others, obtaining an F_{CT} value of 0.007 and a p-value of 0.03. Thus, only the third hypothesis seemed to explain the genetic separation among groups, even when a low value of F_{CT} was recorded.

The average Jost's *D* value (0.035 ± 0.001) was higher than the F_{ST}, and the pairwise *D* values ranged from 0.003 to 0.067 (Table 2). As with the F_{ST} pairwise values, the highest *D* values were found for the pairs CAP vs. GI, GI vs. EL, EL vs. MO and EL vs. R, while the lowest values were found for the pairs GI vs. GN, GN vs. GOR, GN vs. MO and GOR vs. R (Table 2).

Using the Mantel test, we found no relationship between pairwise genetic differentiation and geographic distance ($r = -0.11$, $p = 0.66$), indicating that the hypothesis of isolation by distance does not explain our data and other factors have to be considered in order to explain the recorded population differentiation.

Genetic differentiation among populations was also revealed based on genotypic assignment; the Bayesian cluster analysis recorded a consistent increase in the $\ln L$ values starting from $k = 1$ and estimated the maximum hierarchical level of structure at $k = 8$ (mean $\ln L = -6536.9$). Furthermore, only 28% of individuals were assigned by GeneClass (with a quality index equal to 17.2%) to the populations from which they were sampled. GeneClass also revealed that only 5 individuals were potential first generation migrants.

Table 3. Results of the heterozygosity excess test, allele frequency analysis and M-ratios tests. For the heterozygosity excess bottleneck detection method, we used the TPM and the SMM models. Exp Hexc indicates the expected number of loci with heterozygosity excess, Ratio represents the number of microsatellite loci exhibiting heterozygosity deficiency vs. excess and p indicates the probability values determined using Wilcoxon tests for heterozygote excess (significant values in **bold**). M-ratio is the mean value of M calculated across 7 loci and Mc is the critical M-value, below which population declines are inferred. Other abbreviations as in Table 1

Population	Exp Hexc	Ratio	p_{TPM}	p_{SSM}	Mode shift	M-ratio	Mc
GOR	4.78	5:3	0.195	**0.004**	None	0.62	0.76
CAP	4.77	7:1	**0.012**	**0.008**	None	0.66	0.76
EL	4.78	5:3	0.195	**0.019**	None	0.65	0.75
PIAN	4.56	5:3	0.195	0.990	None	0.63	0.76
MO	4.81	6:2	**0.039**	**0.008**	None	0.79	0.76
GI	4.76	8:0	**0.004**	**0.012**	None	0.49	0.76
GN	4.77	6:2	**0.019**	**0.012**	None	0.68	0.76
R	4.75	7:1	**0.012**	**0.008**	None	0.81	0.76

The heterozygosity excess approach of Luikart et al. (1998), under the TPM and SMM models, provided evidence for a recent population decline for all populations, except PIAN, while the distribution of allele frequencies was clearly L-shaped for all populations (Table 3). Furthermore, only the M-ratio values for MO and R were higher than the critical value for individual populations calculated by M-crit and the critical value of 0.68 proposed by Garza & Williamson (2001).

Finally, no differences existed in allelic richness ($F = 0.44$, df = 1, p = 0.53), number of private alleles ($F = 0.2$, df = 1, p = 0.67), observed heterozygosity ($F = 1.25$, df = 1, p = 0.32) and percentage of unrelated individuals ($F = 0.34$, df = 1, p = 0.58) between protected and unprotected sites.

DISCUSSION

In this paper we used a combination of statistical approaches to investigate the genetic differentiation of *Pachygrapsus marmoratus* within the Tuscan Archipelago, the largest marine park of the Mediterranean Sea, and a series of genetic variability descriptors and statistical tests to depict the main historical demographic events affecting the study populations.

We found clear evidence, based upon *F*-statistics, Jost's *D* values, a Fisher exact test and a Bayesian cluster analysis, of population genetic structure within the study area. The pairwise F_{ST} comparisons indicated that all populations were separated from each other, with the exception of GI and GN (the southernmost islands of the archipelago). These are the 2 islands that are the closest in proximity to each other within the archipelago, lying only 20 km apart. Based on the pairwise χ^2 values of the Fisher exact test, the pairs CAP-GOR, GI-MO, GOR-PIAN, MO-PIAN and GOR-R were also not genetically separated. Moreover, the Bayesian cluster analysis recorded the presence of 8 distinct groups.

This genetic partition is higher than we expected, considering the size of the study area (the archipelago spans a distance of approximately 150 km) and the biology of the species (*Pachygrapsus marmoratus* disperses by a larval phase in the water column with a duration of 1 mo). Furthermore, the recorded genetic structure cannot be explained by the sea circulation of this Mediterranean region, known to create a clear biogeographic separation between the Ligurian and Tyrrhenian Seas. Previous results obtained for the Tuscan coast (Fratini et al. 2008, 2011) also reported a genetic separation at a similar geographic scale, but an order of magnitude lower than that recorded in this study. These Tuscan coast populations were split into 2 groups, the first including all of the Ligurian populations, and the second grouping the Tyrrhenian populations, i.e. in line with the biogeographic break present between these 2 seas. Moreover, the coastal populations of *P. marmoratus* also had a notably lower number of private alleles (1 to 2 per population, Fratini et al. 2008, 2011) than insular populations (3 to 7 per population). This difference in level of genetic separation recorded for these 2 areas seems to indicate the influence of local hydrological conditions for local-scale *P. marmoratus* dispersion, as already hypothesised by Fratini et al. (2011).

We explained the genetic population structure recorded in this study area as due to a 'sweepstakes effect', the genetic drift among larval cohorts from the same or different populations that results from the random reproductive success of different small subsets of adults over time and space (Hedgecock 1994, Taylor & Hellberg 2003, Hedrick 2005). This effect is known to be relevant in marine species with high fecundity and high early mortality. In these species the effective population size can be significantly smaller than the total population size, with relatively few individuals per population reproducing at each reproduction cycle, and the variance in reproductive success among individuals can be high due to stochastic factors (see Hedgecock & Sly 1990, Hedgecock et al. 2007). The small size of the study islands should enhance this effect, making larval recruitment even harder. Comparison of genetic variation within temporal cohorts from the same populations may provide further support for this hypothesis; however, our study was limited to spatial comparisons among populations sampled only once.

Recent studies have demonstrated that self-recruitment as a dispersal mechanism of marine populations is more widespread than previously thought (for examples on crustaceans see Queiroga & Balton 2005, Marta-Almeida et al. 2006). This mechanism is beneficial to recruitment success since it guarantees conditions suitable for survival of new recruits (Strathman 2002, Taylor & Hellberg 2003). Vertical migration employed by larvae appears to be the predominant behavioural mechanism to avoid long distance dispersal (Queiroga & Balton 2005, Marta-Almeida et al. 2006) and DiBacco et al. (2001) reported that *Pachygrapsus* species employed this mechanism. However, the lack of a pattern of isolation by distance among populations of *P. marmoratus* within the Tuscan archipelago, as well as the low

number (less than 30%) of individuals assigned to their own populations, indicate that retention of larvae near the parent population may be less important in maintaining genetic structure with respect to other environmental and behavioural mechanisms.

We tested for the genetic signature of population decline using 3 different approaches (heterozygosity excess, shifts in allele frequencies and low ratios of allelic number to allelic size range) and, as expected, we found different results. These 3 tests, in fact, provide evidence of bottleneck events at different time scales; the M-ratio is known to have a long recovery time, while heterozygosity excess and allele frequency distributions tend to recover relatively quickly (Garza & Williamson 2001). For this reason, populations incurring recent decline will have evidence of bottleneck associated with all the detection methods. This seems to be the case for populations from GOR, EL, CAP, GI and GN. Conversely, the PIAN population, showing high M-ratio values, but significant heterozygosity excess, presumably experienced a historical decline. The MO and R populations showed evidence of decline using only the heterozygosity excess method; this could be due to the different sensitivities of bottleneck detection methods. Since the magnitude of the decrease of M is positively correlated with the severity and duration of the reduction in size, the population from GI seems to be the population that had experienced the highest reduction. Furthermore, these results indicate that our populations experienced a population size reduction in the recent or historical past and we may attribute this to habitat exploitation and fragmentation. The recorded genetic separation among populations may also be ascribed to past demographic events, able to mask the homogenising effects of actual gene flow.

The islands of the Tuscan Archipelago are part of a recently (1996) established national park. We expected that populations from protected sites would show higher levels of genetic variation, as an expression of more 'healthy conditions' (see Lacy 1997, Frankham 2005 for a review, Bouzat 2010), than populations from unprotected sites. Protected areas prevent the intensive human disturbance commonly exerted by large numbers of tourists visiting and trampling on Tuscan rocky shores during springtime and summer, i.e. during the reproductive and recruitment phase of our study crabs (see Rossi et al. 2007). However, we did not record any difference in genetic indices between protected and unprotected populations, indicating that, from a genetic point of view, the effects of protection have not yet been demonstrated.

Besides having the potential to provide protection of a habitat, a marine national park may be able to enhance adjacent populations of marine organisms through a 'spill-over' effect and promote larval recruitment to adjoining exploited areas (Carr & Reed 1993, Shanks et al. 2003). The role of the marine reserve as a centre of dispersal for propagules for a given species is strictly dependent on the dispersal abilities of the species, and the role is only realised when a species is able to disperse outside the borders of the protected area. Based on the results of our study, the Tuscan Archipelago National Park does not seem able to carry out this role, at least not for *Pachygrapsus marmoratus*, as the study populations within this area do not substantially exchange among themselves. However, it is possible that the national park functions as a centre of dispersal, considering the complex nature of population genetic structure and connectivity level, and their dependence on an interaction of abiotic and biotic factors, which is unique for any given species. Indeed, an enlargement of the protected zones within the archipelago may enhance the effectiveness of this marine park.

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