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Population genetic structure of *Paramuricea clavata* (Risso, 1826) in the eastern Adriatic as revealed by microsatellite markers

Graduation Thesis

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This Thesis,
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BASIC DOCUMENTATION CARD

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The red gorgonian *Paramuricea clavata* is a long-lived, slow-growing coral that contributes significantly to the biomass and structural complexity of the Mediterranean hard-bottom communities. In this research, the population genetic structure of *P. clavata* in the eastern Adriatic was assessed for the first time. Thirteen locations in four geographical regions along the Adriatic Sea were genotyped using seven microsatellites. Gene diversity, population structure, dispersal patterns and connectivity among populations were analysed using a hierarchical sampling scheme, from local to regional scale. Results showed strong genetic differentiation which was previously never recorded in the population genetic studies of benthic species in the Adriatic. Discussed results should be valuable for creating or improving management plans for *Paramuricea clavata* and other endangered species with similar life-history traits in the Adriatic Sea.

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Key words: population genetics, red gorgonian, conservation biology, genetic clustering, isolation by distance

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Genetička struktura populacija vrste *Paramuricea clavata* (Risso, 1826) na istočnom Jadranu određena pomoću mikrosatelita

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Crvena gorgonija, *Paramuricea clavata* je dugoživući, spororastući sesilni koralj koji čini vrlo bitan dio kompleksnog koraligenskog staništa u cijelom Sredozemnom moru. Ovim istraživanjem, po prvi puta je određena genetska raznolikost ove vrste u Jadranu. Trinaest lokacija iz četiri geografske regije duž istočne jadranske obale je genotipizirano pomoću sedam mikrosatelitskih markera. Genetska raznolikost, struktura populacije, uzorci disperzije te povezanost među populacijama su analizirani na lokalnoj i regionalnoj razini. Također, po prvi puta je pokazana jaka genetska diferencijacija kod bentoskih vrsta u Jadranu. Rezultati ovog istraživanja mogu poslužiti kao vrlo vrijedni podaci za uspostavu i unaprijeđenja menadžment planova za vrstu *Paramuricea clavata* te druge Jadranske ugrožene vrste sa sličnim životnim ciklusom i karakteristikama.

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1 INTRODUCTION

1.1 Unknown but threatened marine diversity

There are less than 200.000 catalogued marine species living in the ocean and around 2 million more waiting to be discovered (Mora *et al*. 2011). The biggest knowledge gap is the deep-sea diversity where data is extremely scarce (Webb *et al.* 2010). Unfortunately, with the present rate of discoveries of new species and their extinctions, many of them will disappear before being even discovered and evolutionary relationships among them will not be possible to describe and understand (Hendry *et al.* 2010).

Furthermore, it is also demanding to determine the conservation status of already known species in the ocean. Since little is known of many species' distribution or range, a lot of effort is needed to determine whether populations are stable or changing, and if they are threatened or endangered. A research on conservation priorities of tropical coral reefs revealed that up to 53.6% of some taxons have highly restricted ranges, rendering them vulnerable to extinction (Roberts *et al.* 2002). These data show a lot of reasons for serious concern about world's marine biodiversity.

Mediterranean Sea is one of the world's biodiversity hotspots (Myers *et al*. 2000). With 0.82% of the world's ocean area and 0.3% of its volume, it comprises 4 to 18% of the world's marine species. The main reasons for this high diversity are Mediterranean's tormented geological history which caused numerous environmental changes and led to high speciation due to species' adjustment to cold, temperate and sub-tropical conditions co-occurring in the sea (Bianchi & Morri 2000). Additionally, the rate of endemic species in the Mediterranean is remarkably high, with the average of more than 25% (Lejeusne *et al*. 2009).

Although population genetics is becoming an increasingly frequent topic of research in the world, such studies about the Adriatic species are very scarce. Moreover, they are being conducted only on charismatic species like marine mammals (Gaspari *et al*. 2007) or commercially exploited species like fish (Rossi *et al*. 2006; Tinti *et al*. 2002), cephalopods (Garoia *et al*. 2004) and mussels

(Štambuk *et al.* 2013). Other invertebrates, even endangered ones, are being completely cast aside. Studies based on global scale, even the ones on extremely vagile spacies (Gaspari *et al*. 2007; Rossi *et al*. 2006) show distinction of Adriatic populations from the NW Mediterranean populations. Research by Garoia *et al*. (2004) and Štambuk *et al*. (2013) thus showed both very low genetic differentiation among populations suggesting the occurrence of single genetically homogeneous populations along the whole Adriatic.

According to these studies, it is of great significance to reveal the spatial genetic structure of the species in natural populations. Defining the spatial distribution of genotypes, it might be possible to understand historical events and selection pressures on investigated species (Vekemans & Hardy 2004).

1.2 Spatial population genetic structure: studying dispersal in sessile organisms

Spatial population genetic studies are based on tracing genetic variation (allele frequencies) between populations along a geographical area. They allow insight into various parameters characterizing the biology of populations such as dispersal (Broquet & Petit 2009). In sessile species, such as marine invertebrates as well as plants, genetic dispersal occurs through propagule. In the marine realm, this propagule (i.e. larvae) is carried away by sea currents and plays an important role in connecting spatially disjunct populations (Gaines *et al.* 2003). Since this environment is a three-dimensional and immense space compared to the size of larvae, revealing their route to settlement can be a challenging task (Hellberg *et al.* 2002) and population genetics approaches are useful tool for overcoming this concern (Féral 2002).

Marine populations are usually much less connected then it would be expected based on dispersal capacity of species' propagules (Marshall *et al*. 2010). Connectivity among populations can be affected by various factors, of which the most obvious ones are physical barriers to larval dispersal (Pineda *et al.* 2007). Place of settlement is determined by characteristics of the oceanographic flow field and two biological variables: time larvae spend in the water column before they become developmentally and physiologically capable of settling (precompetency period), and the time over which larvae settle (competency period) (Jackson & Strathmann 1981). Furthermore, natural selection also seems to play an important role in population connectivity which may be reduced by ”phenotype–environment mismatches” i.e. reduction of fitness when an organism that is specialized to one environment finds itself in an alternative environment (DeWitt *et al.* 1998). Consequently, oceanographic barriers and biological characteristics of marine invertebrates can affect gene flows among populations and thereby shape the spatial genetic structure of the species (Baums *et al.* 2006; Ledoux *et al.* 2010).

Until recently, connectivity among marine populations was usually explained by Wright’s (1943, 1946) island model, stating all populations are the same size, equally distant one from another, and linked by the equal gene flow, with a

proportion of migrants every generation (Palumbi 2003). Since these conditions are not common in natural environments and there are various other aspects affecting the gene flow, this model is not realistic for populations in nature.

On the other hand, the stepping-stone model assumes that populations that are geographically closer exchange larger amounts of genetic material i.e. genetic differentiation increases with geographical distance. (Kimura & Weiss 1946, Malécot 1950) (Figure 1).

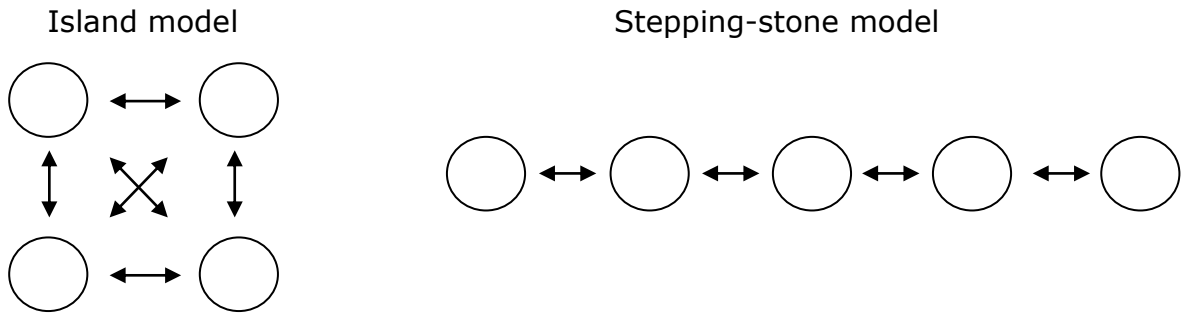

Figure 1 Two common models of population structure: Sewell Wright's island model and Stepping-stone model (Palumbi 2003).

1.3 Implications for conservation of marine biodiversity

Besides species and ecosystems diversity, genetic diversity is the third level of biodiversity that needs particular conservation efforts (McNeely *et al.* 1990) but it is often being neglected. Unfortunately, short-term economic and political interests overcome scientific opinions when creating management and conservation plans (Lande 1988). Additionally, one of the main contributiors for huge development in discovering, describing, and organizing biodiversity is the recent improvement of technology and methods. Conservation genetics is an extremely effective tool for identifying the environmental drivers of biodiversity and revealing biodiversity hotspots or areas of endemism. (Hendry *et al*. 2010).

Before the work of Reusch *et al.* (2005) there was no evidence of genetic diversity enhancing ecosystem functionality. Populations of a species are becoming rapidly depleted and consequently the genetic variation is reduced (Frankham 2005a). Depleted ecosystems may not contain the species and genetic diversity necessary to enable them to recover or survive major environmental changes and stresses, such as climate changes, diseases or new predators (Garrabou *et al.* 2009). Furthermore, the decline of one species can lead to the increase or decline of others and thus pose a threat to the stability of the ecosystem. Loss of genetic diversity typically impacts over the long-term, associated with environmental change, while inbreeding depression has an immediate impact on the whole ecosystem (Frankham 2005b).

Since climate changes are not something we can stop, it is necessary to gain insights into species' life history and diversity in order to prevent, or at least minimise, future loss of biodiversity. Additionally, habitat degradation due to human population growth is not helping in species preservation (Lotze *et al.* 2006). A clear understanding of a species' life history and diversity is essential for creating effective and scientifically-based management plans. If both, demography and population genetics are not taken into concern, protecting the habitat may have no effect on ensuring population sustainability (Lande 1988). Habitat restoration and preservation of healthy populations will be possible only by determining the effectiveness of marine protected areas according to dispersal, genetic traits and other species' characteristics (Gaines *et al.* 2003).

Furthermore, population genetic studies can show us how current and past biodiversity patterns have been influenced by past evolutionary processes. Consecutively, these studies can help predict what might happen to biodiversity patterns in the face of future changes in geography and climate (Hendry *et al*. 2010).

An evolutionary perspective is required to evaluate the biological importance of the data. In addition, these data will allow an understanding of past evolutionary events in endangered species, whether they are nonselective, such as bottlenecks or gene flow, or selective, such as detrimental or adaptive mutations (Hedrick 2001).

1.4 *Paramuricea clavata* as a studied species

Paramuricea clavata (Figure 2) is an anthozoan that thrives on steep, vertical or overhanging rocky walls but also on horizontal bottoms if the light level is low enough. It is widespread along the western part of the Mediterranean Sea and in the Adriatic Sea, and sporadically in the Aegean Sea and Atlantic Ocean (Carpine & Grashoff 1975). Populations show patchy distribution and can be found from 5 to 200 meters in depth (Mokhtar-Jamaï *et al.* 2011). As an ″ecosystem engineer″, it creates assemblages and it presents a very important part of structurally complex coralligenous habitats (Ballesteros 2006). In addition, it increases the biomass and diversity of the community by providing habitats for other small species.

Figure 2 A colony of *Paramuricea clavata* in the National park Kornati (photo: Hrvoje Čižmek).

Paramuricea clavata is a long-lived species with a low population dynamics. Colony survival rates reach from 63% up to 100% (mean of ~91%) and biggest colonies can be from 60 to 100 years old (Linares *et al.* 2007). Also, its growth rates are very low, from 1.8 to 2.7 cm per year (Coma *et al.* 1998), as well as mean recruitment rates which are around 3% (Linares *et al.* 2007). Compared to other marine invertebrate species, it obtains sexual maturity quite late, between 7 and 13 years of age (Coma *et al.* 1995).

In contrast to tropical gorgonian species where asexual reproduction is common (Bell 1982), *Paramuricea clavata* reproduces only sexually (Coma *et al.* 1995). It is a gonochoric species but there is no sexual dimorphism between male and female colonies. Furthermore, hermaphroditism is very rare. *P. clavata* is iteroparous and reproduces annually. Oogenesis lasts from 13 to 18 months and spawning takes place during June and July (correlated to the sea temperature and the lunar cycle). Since development of new oocytes starts before the ones of the previous year are released, there can be two generations of oocytes found in one polyp. Fertilization occurs internally or on the surface of the mother colony. Following the spawning, first planulas appear after only 48 hours, and may settle down on the substratum in a relatively short time, after spending just a few minutes in the water column (Coma *et al.* 1995).

Health of *Paramuricea clavata* populations is mostly affected by irresponsable SCUBA diving activities, utilization of destructive fishing gear and mass mortality events related to global warming. In areas with a high level of recreational diving, the estimations of the mortality rates of the red gorgonian populations are increasing from 2.7 to 7.4 % per year (Coma *et al.* 2004). During the 2003 heat wave, *P. clavata* was one of the most affected species in the north-western Mediterranean, reaching up to an average of 80% of the colonies impacted (Garrabou *et al.* 2009). Depending on the frequency of extended warm periods or other disturbance events, modelling of near-certain quasi-extinction results in 36 to 84 years (Linares *et al.* 2010). Therefore, it is necessary to gain as much data as possible on the species in order to enhance its conservation. In the eastern Adriatic Sea, the assessment of the *P. clavata* population structure and its conservation status has been initiated recently (Kipson *et al*. 2011).

Previous study on the spatial genetic structure of *Paramuricea clavata* showed strong genetic differentiation at both local and global scale, and a heterozygote deficiency at the sample level. Furthermore, a significant pattern of isolation by distance was observed (Mokhtar-Jamaï *et al.* 2011) at different spatial scale. Likewise, similar studies conducted on other gorgonian species (e.g. *Corallium rubrum*), show strong population genetic structure and limited larval dispersal (Ledoux *et al.* 2010a, Ledoux *et al.* 2010b, Constantini *et al.* 2007). In their study, Mokhtar-Jamaï *et al.* (2011) focused on the north-western Mediterranean populations (Spain, France and west Italy), whereas only one population was sampled at the Croatian coast. Regarding this population, the analysis of STRUCTURE shows that it represents its own cluster with a very low level of admixture with other clusters. This suggests the occurrence of a particular genetic pool in this region. This result combined, with the lack of knowledge regarding the genetic diversity in the Adriatic, call for the development of studies in this particular region of the Mediterranean Basin.

2 THE AIM OF THE RESEARCH

The major goal of this study was to define the spatial distribution of genetic diversity of *Paramuricea clavata* in the eastern part of the Adriatic Sea. To achieve this aim, using a hierarchical sampling scheme (from local to regional scale), two main aspects were analysed:

i. gene diversity and population structure
ii. dispersal patterns and connectivity among populations

Obtained results should inform adequate management and conservation of the red gorgonian *Paramuricea clavata* and other endangered species with similar life-history traits in the Adriatic Sea. Data should be taken into consideration for creating scientifically-based No Take Areas and Marine Protected Areas.

3 MATERIALS AND METHODS

3.1 Sampling design

Small tissue fragments of *Paramuricea clavata* colonies were collected at 13 sites (Table 1) following a hierarchical sampling scheme (Figure 4) while SCUBA diving. Locations are distributed in four geographic regions: Senj Archipelago, Kornati Archipelago, Rogoznica Area and Pakleni Islands (Figure 3). Samples were taken at depths from 30 to 50 meters in years 2009 and 2011. At each location, one apical fragment (size: 2 - 3 cm) was taken from around 35 colonies (from 23 to 55) bigger than 20 centimetres in height. Finally, 456 *Paramuricea clavata* tissue fragments were conserved in 95% ethanol and stored at -20°C prior to DNA extraction.

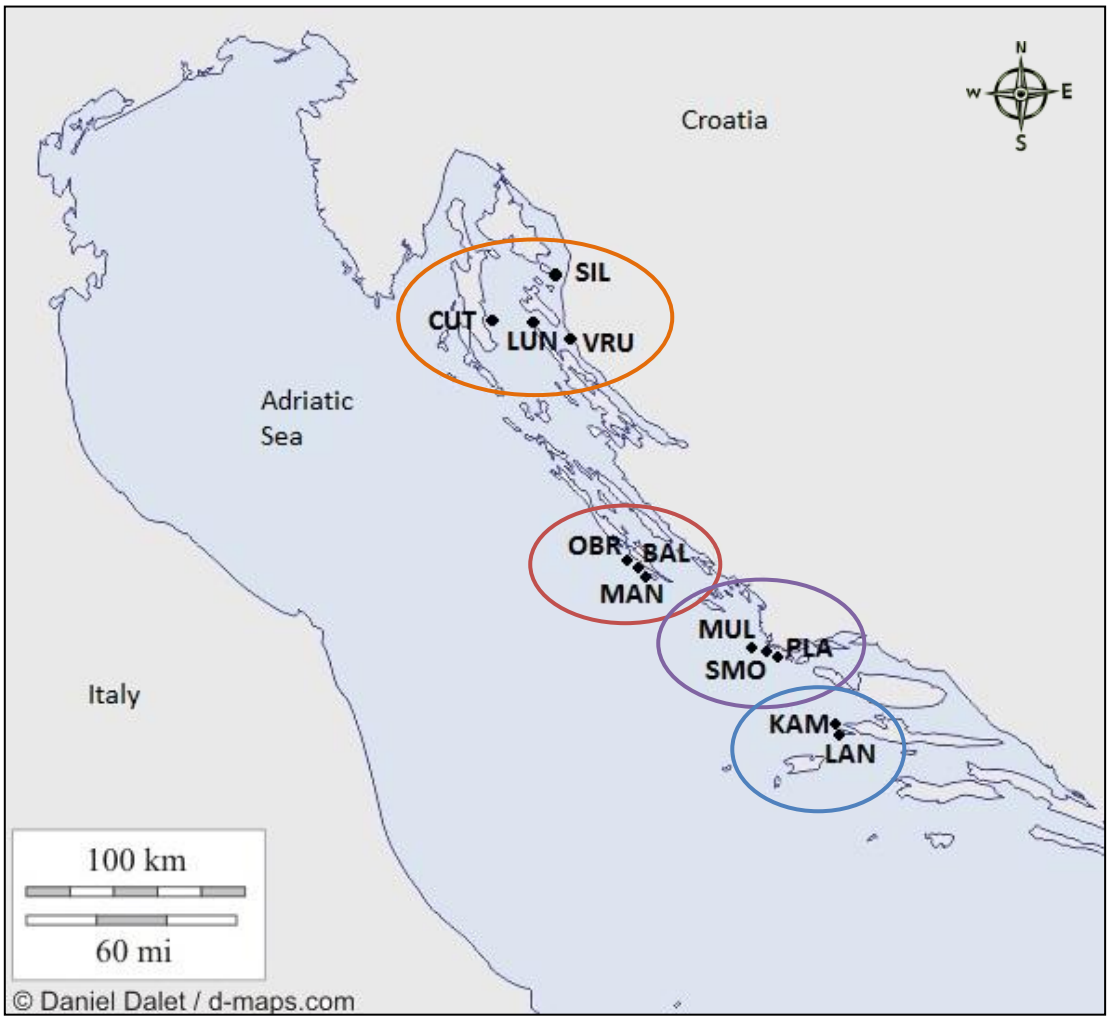

Figure 3 Map of 13 *Paramuricea clavata* sample sites. BAL and BLU are the same location but sampling took place at different depths.

Table 1 Details on sample locations of *Paramuricea clavata.*

Geographic region	Sample location	Protection status	Population label	Coordinates		Depth (m)	No. of samples
				Latitude	Longitude		
Senj Archipelago	Ćutin Mali	unprotected	CUT	44°43'29''N	14°29'36''E	30-40	32
Senj Archipelago	Lun	unprotected	LUN	44°42'15''N	14°44'02''E	30-40	23
Senj Archipelago	Rt Silo	unprotected	SIL	44°53'12''N	14°50'13''E	30-35	30
Senj Archipelago	Vrutak	unprotected	VRU	44°39'11''N	14°55'36''E	38-50	48
Rogoznica area	Smokvica	unprotected	SMO	43°30'38"N	15°56'32"E	30-40	35
Rogoznica area	Planka	National Ecological Network	PLA	43°29'36''N	15°58'09''E	30-40	35
Rogoznica area	Mulo	unprotected	MUL	43°30'48''N	15°55'07''E	30-40	35
Kornati Archipelago	Balun	Marine Protected Area	BAL	43°48'14''N	15°15'18''E	30-37	31
Kornati Archipelago	Balun	Marine Protected Area	BLU	43°48'14''N	15°15'18''E	35-40	39
Kornati Archipelago	Mali Obručan	Marine Protected Area	OBR	43°50'11''N	15°13'12''E	35-40	31
Kornati Archipelago	Mana	Marine Protected Area	MAN	43°48'01''N	15°15'59''E	35-40	29
Pakleni Islands	Kampanel	Protected landscape	KAM	43°10'44''N	16°18'34''E	30-40	55
Pakleni Islands	Lanterna	Protected landscape	LAN	43°10'05''N	16°18'38''E	30-40	33

Total genomic DNA was extracted from all 456 *P. clavata* individuals using a salting out procedure adjusted from Miller *et al.* (1988) (see Appendix 1 for details).

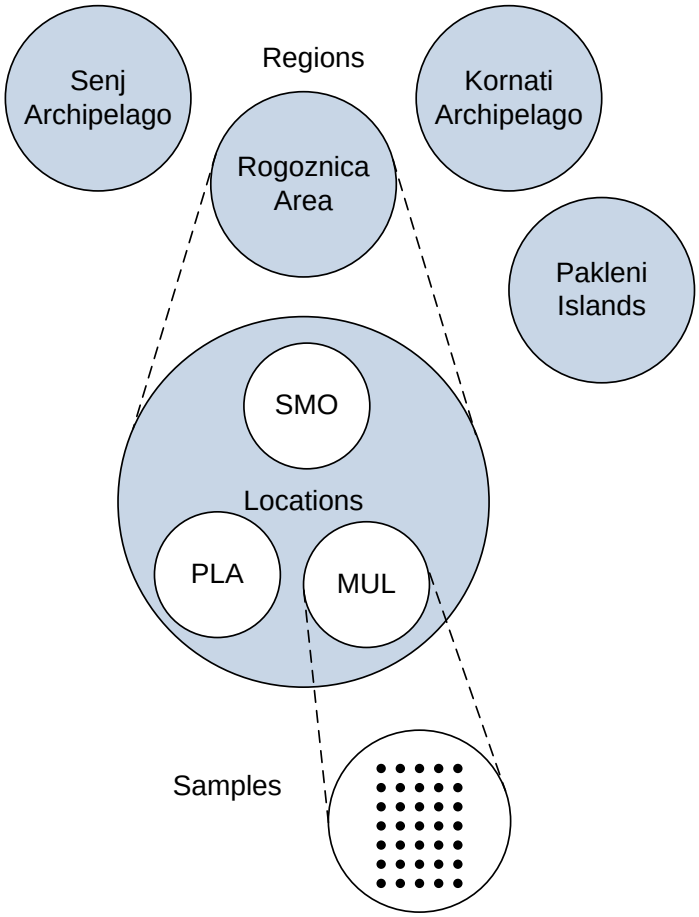

Figure 4 Hierarchical sampling scheme for one of the four regions sampled across the Eastern Adriatic. Figure modified after Cornell *et al.* (2007).

3.2 Microsatellite analysis

All individuals were genotyped using seven microsatellite loci: Pcla 09, Pcla 10, Pcla 12, Pcla 14, Pcla 17, Pcla 81 (Molecular Ecology Resources Primer Development Consortium *et al.* 2010) and Pcla-a (Agell *et al.* 2009) (Table 2). Markers Pcla 81 and Pcla-a were used for the first time while the rest were already utilized in the study of Mokhtar-Jamaï *et al*. (2011).

All the loci were amplified in 10 µL of PCR solution which contained:

20 ng/µL of genomic DNA,
1x PCR buffer,
2.5 mM $MgCl_2$,
0.5 µM of each primer,
0.125 mM dNTPs,
0,25 U of GoTaq DNA polymerase (Promega).

The forward primer was labelled with fluorescent dyes (Table 2).

The PCR program was:

94°C for 3 min;
30 cycles of 94°C for 1 min,
annealing temperature (60°C for all loci except for Pcla-a 59°C) for 1 min,
72°C for 1 min;
final extension at 72°C for 20 min.

PCR products were analysed on an ABI 3130 Genetic Analyser with GeneScan 600 LIZ internal size standard (Applied Biosystems) at the Plateforme Genome Transcriptome de Bordeaux (France; http://www4.bordeaux-aquitaine.inra.fr /pgtb). STRand version 2.2.30 was used for scoring alleles (UC Davis Veterinary Genetics Laboratory) (Figure 5).

Table 2 Primer sequences, PCR conditions and genetic characteristics of seven *Paramuricea clavata* microsatellites.

Locus name	Primer sequence (5'-3')	Repeat motif	Number of cycles	T_a (°C)	Allele size range (bp)	Genbank accession number
Pcla 09	F: 6-FAM_CCTGCTTTTCATGATTTTGC R: CTGTCTAATGTGGCACTGAATG	$(CA)_{21}$	30	60	91-126	GU386261
Pcla 10	F: 6-FAM_TCGCGTGACTGCTAACTCTC R: TGTATTGCAGCTGGGATGTG	$(GT)_{23}$	30	60	195-237	GU386262
Pcla 12	F: VIC_GAAATCGACAATAACACGTTCG R: TTATCACCTCCGCCAGTTTG	$(AC)_{15}$	30	60	100-149	GU386263
Pcla 14	F: NED_CTGGGAGTGGGTATCTGAGC R: ACAAGACTTTTCGGAGTGAGG	$(CA)_{26}$	30	60	165-215	GU386264
Pcla17	F: PET_TCGACGAACTTTGCAGATTG R: CAAATTTCCAAGAAAAGGAAGG	$(AC)_{14}$	30	60	170-191	GU386265
Pcla 81	F: NED_CCAACAACCGCAAGATAGGT R: TTGCTGCCATATCGTTCATC	$(ACA)_{10}$	30	60	175-201	GU386259
Pcla-a	F: VIC_TGGCAAACATAATTCCACCA R: CAAATGATGCTATTGAAGCCAGT	$(GTT)_9$	30	59	158-208	FJ560968

Forward primers are 5' fluorescently labelled with VIC, NED, PET or 6-FAM (Applied Biosystems); T_a annealing temperature; bp = base pair.

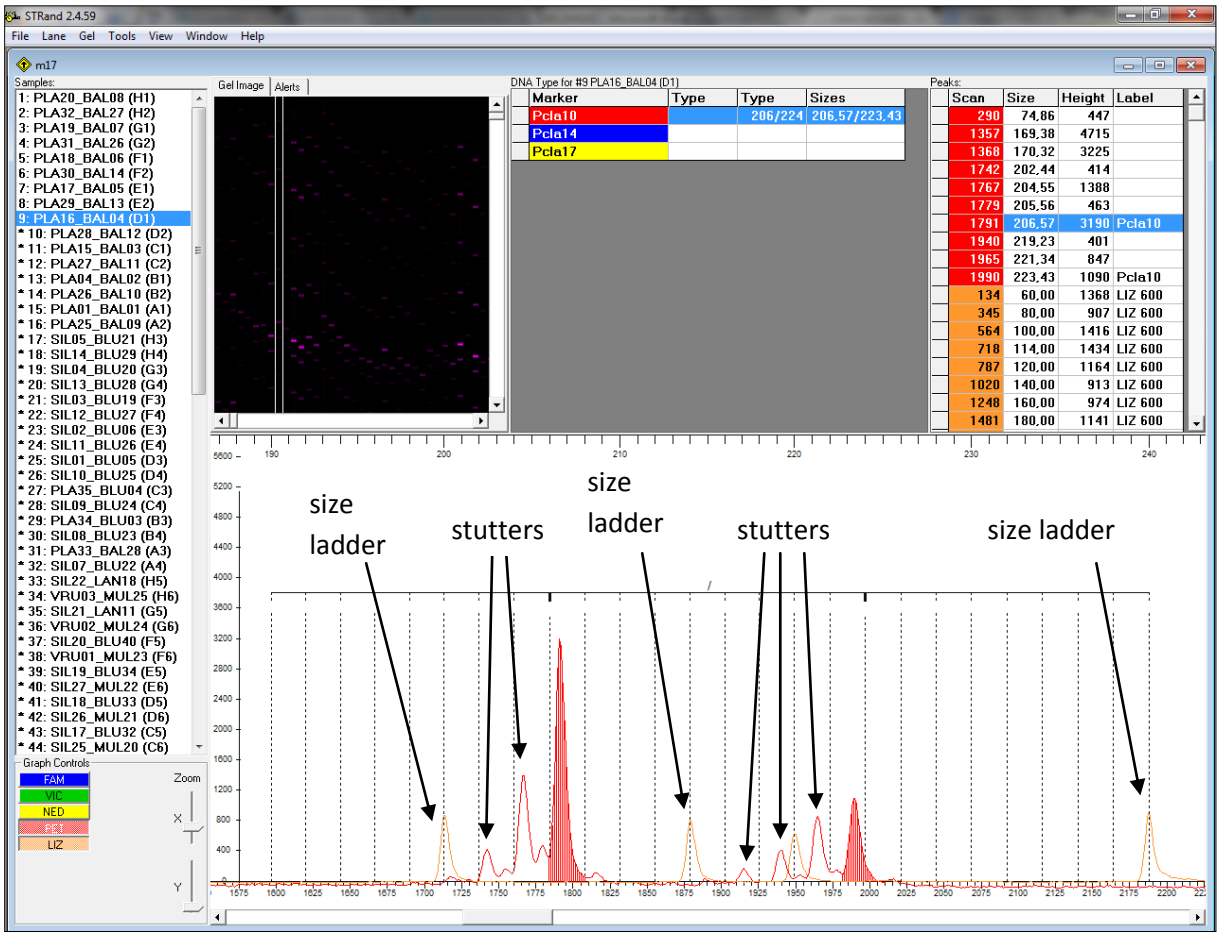

Figure 5 STRand version 2.2.30 showing electropherogram of individual PLA16 which is heterozygote for Pcla 10 with alleles 206 and 224 bp peaks. Orange (LIZ) peaks correspond to the size ladder.

3.3 Microsatellite characteristics and Hardy - Weinberg equilibrium

PCR amplification can create DNA fragments that are several repeats shorter than the actual allele. These fragments may be seen as stutter bands (Figure 5) and can be misleading while analysing samples (Ewen *et al.* 2000). Also, as a result of deviations from Hardy-Weinberg proportions, large allele dropouts are possible to occur. Preferential amplification of shorter alleles (Wattier *et al.* 1998) can result in an apparent deficiency in heterozygotes. Consequently, MICRO-CHECKER version 2.2.3 (Van Oosterhout *et al.* 2004) was used to test for scoring errors due to stutters and large allele dropout.

In addition, GIMLET version 1.3.3 was used for identification of identical genotypes that could result from mishandling during sampling processes or PCR preparation (Valière 2002). There is a possibility that same colony might have been sampled twice or that DNA of the same specimen has been replicated twice. Frequencies of null alleles were estimated for each locus and population following the Expectation Maximization (EM) algorithm (Dempster *et al.* 1977) using FreeNA software (Chapuis & Estoup 2007).

Total number of alleles per locus, observed heterozygosity (Ho) and gene diversity i.e. expected heterozygosity (He) (Nei 1973) per locus and *f* estimator of F_{IS} (Weir & Cockerham 1984) were computed with FSTAT version 2.9.3 (Goudet 2001). Observed heterozygosity (Ho) and gene diversity (He) (Nei 1973) per population were calculated using GENETIX version 4.05 (Belkhir *et al.* 2004).

Score test for heterozygote deficiency was conducted for each sample with GENEPOP version 4.1.4 (Rousset 2008) in order to test departure from panmixia. Significance of the result was computed by Markov Chain (MC) algorithm (Guo & Thompson 1992; Raymond & Rousset 1995) using default parameters. Null hypothesis of linkage equilibrium was tested for each pair of loci in each sample using 1000 permutations with GENETIX.

3.4 Pairwise genetic differentiation and Isolation by distance

F_{ST} for all populations and all population pairs was computed using Weir & Cockerham's estimator of F_{ST} (θ) in GENEPOP version 4.1.4 (Rousset 2008). Also, pairwise genotyping differentiation between samples was tested with the G test based on default parameters for the Markov chain algorithm (Guo & Thompson 1992).

Isolation by distance (IBD - populations are connected by continuous migration but the rate of gene flow is greatest between neighbouring populations) pattern was analysed through the correlation of genetic and geographic distances between populations (Rousset 1997). Geographic distances in meters were measured among locations using GOOGLE EARTH version 7.0 (http://earth.google.com) considering shortest path between each population but avoiding geographical barriers like islands and islets. Also, surface currents were taken into account when determining this route. In GENEPOP, linear regression was made on the genetic distance computed as $F_{ST}/(1-F_{ST})$ over logarithm of geographic distances, as recommended for two-dimensional model (Rousset 1997). The significance of the correlation between two distance matrices was tested by the Mantel test (1967) using 10000 permutations.

AMOVA tests i.e. analyses of molecular variance (Weir & Cockerham 1984, Excoffier *et. al.* 1992) were computed using ARLEQUIN version 3.5.1.3 (Excoffier *et. al.* 2005) in order to quantify genetic variation among groups (F_{CT}), among populations within groups (F_{SC}) and within populations (F_{ST}). Significance tests were conducted using 1000 permutations. Groups tested with AMOVA were determined by STRUCTURE version 2.2 (Pritchard *et al.* 2000; see below).

3.5 Clustering analyses

The whole dataset was submitted under the Bayesian analysis setup in STRUCTURE version 2.2 (Pritchard *et al.* 2000) in order to evaluate the number of genetic clusters (K) from the individuals' genotypes dataset without any information on their geographical locations or assumptions on any kind of population boundaries (Guillot *et al.* 2005). In computation, it regards admixture, correlated allele frequencies between clusters and uses the recessive allele option to deal with null alleles (Falush *et al.* 2003, 2007). Ten independent runs were preformed for each K (varying from 1 to 10) with a burn-in period of 200 000 followed by 500 000 iterations. These values were sufficient to reach stability in parameter estimations. A 'true' K value was then determined based on the logarithm of the likelihood of observing the data - *lnP(D)* as a function of genetic cluster - K (Waples & Gaggiotti 2006). The value capturing the major structure in the data is the K value (Pritchard *et al.* 2007).

CLUMPP version 1.1 (Jakobsson & Rosenberg 2007) was used to average the assignment scores over the 10 runs. Lastly, the graphical scheme of results was made using DISTRUCT version 1.1 (Rosenberg 2004).

For multiple tests, significance levels were corrected using a false discovery rate (FDR) correction (Benjamini & Hochberg 1995).

4 RESULTS

4.1 Microsatellite characteristics and Hardy - Weinberg equilibrium

According to MICRO-CHECKER no evidence of scoring errors due to stuttering or large allele dropout was found in the whole data set. In all 13 populations, all loci were polymorphic ($P < 0.01$).

Total number of alleles varied from 5 for Pcla 17 to 29 for Pcla 12, with a mean of 17 alleles per locus. Mean estimated null allele frequencies by locus varied from 0.008 for Pcla 81 to 0.076 for Pcla-a, with a mean value of 0.024 per locus (Appendix 2). No global significant linkage disequilibrium (LD) among loci was observed over all samples (all $P > 0.05$ after FDR correction). Observed heterozygosity ranged between 0.462 foPcla-a and 0.799 for Pcla 81 (mean of 0.692). Gene diversity varied from 0.484 for Pcla 17 to 0.813 for Pcla 81 (mean of 0.715). Mean He observed in MPAs is a bit higher (0.73) then in non protected areas (0.673) but not significantly. Weir & Cockerham's estimator of F_{IS} ranged from -0.107 for Pcla 17 to 0.187 for Pcla-a, with a mean value of 0.035 (Table 3).

Departure from panmixia varied between loci and samples. Heterozygote deficiency was found for two microsatellites, Pcla 10 and Pcla-a and in two populations, LAN and VRU, both with significance of $P < 0.001$ (Appendix 2).
Mean estimated null allele frequencies for populations varied between 0.007 for LUN and 0.057 for VRU with a mean value of 0.024 (Appendix 3). LD among pairs of loci was observed only in LAN population for one pair of loci and in OBR population for two pairs of loci. Observed heterozygosity ranged between 0.616 for VRU and 0.786 for KAM (mean of 0.692). Gene diversity varied from 0.610 for SIL to 0.788 for KAM (mean of 0.704). Multilocus values of Weir & Cockerham's estimator of F_{IS} ranged from -0.042 for SIL to 0.109 for VRU, with a mean value of 0.032 per population. All values were significant at the 0.05 level except VRU population (Table 4).

Table 3 Genetic characteristics of seven *Paramuricea clavata* microsatellites used in this research.

Locus name	Na	Ho	He	*f*	r
Pcla 09	20	0.796	0.785	**-0.015**	0.006
Pcla 10	21	0.726	0.784	**0.075**	0.025
Pcla 12	29	0.770	0.790	**0.019**	0.019
Pcla 14	17	0.753	0.790	**0.049**	0.024
Pcla17	5	0.537	0.484	-0.107	0.014
Pcla 81	19	0.799	0.813	**0.025**	0.008
Pcla-a	11	0.462	0.560	**0.187**	0.076
mean	17	0.692	0.715	**0.035**	0.024

Na - number of alleles, Ho - observed heterozygosity, He - gene diversity i.e. expected heterozygosity (Nei 1973), *f* - Weir & Cockerham (1984) estimator of F_{IS}, r - null allele frequency. Presented values correspond to the mean value over all samples. Values in bold are significant at the 0.05 level after FDR correction.

Table 4 Measures and estimations of genetic diversity for 13 populations of *Paramuricea clavata* based on seven microsatellite loci.

Population	Ho	He	f	r
BAL	0.678 (0.202)	0.679 (0.194)	**0.017**	0.017
BLU	0.690 (0.140)	0.724 (0.165)	**0.061**	0.016
CUT	0.627 (0.239)	0.640 (0.190)	**0.035**	0.021
KAM	0.786 (0.125)	0.788 (0.144)	**0.013**	0.021
LAN	0.707 (0.194)	0.771 (0.132)	**0.100**	0.050
LUN	0.645 (0.076)	0.626 (0.126)	**-0.008**	0.007
MAN	0.732 (0.124)	0.717 (0.134)	**-0.003**	0.009
MUL	0.713 (0.189)	0.748 (0.108)	**0.062**	0.034
OBR	0.727 (0.170)	0.731 (0.165)	**0.023**	0.014
PLA	0.694 (0.185)	0.702 (0.169)	**0.025**	0.029
SIL	0.646 (0.266)	0.610 (0.244)	**-0.042**	0.023
SMO	0.733 (0.129)	0.732 (0.115)	**0.014**	0.020
VRU	0.616 (0.224)	0.683 (0.127)	0.109	0.057
mean	0.692 (0.174)	0.704 (0.155)	**0.032**	0.024

Ho - observed heterozygosity, He - gene diversity (Nei 1973), f - Weir & Cockerham (1984) estimator of F_{IS}, r - null allele frequency. Presented values correspond to the mean value over all samples. Standard deviations are in brackets. Values in bold are significant at the 0.05 level. Shaded rows are populations in MPAs.

4.2 Pairwise genetic differentiation and Isolation by distance

Pairwise F_{ST} values among all pairs of populations were scattered from 0.0008 between BAL and BLU to 0.178 between BAL and SIL, with a mean value of 0.097 (Appendix 4). After FDR correction, all pairwise comparisons showed highly significant genotypic differentiation observed among all populations.

Presence of the Isolation by distance model of gene flow was confirmed by high significance ($P < 0.001$) of the correlation between $F_{ST}/(1-F_{ST})$ and logarithm of geographic distances (Figure 6).

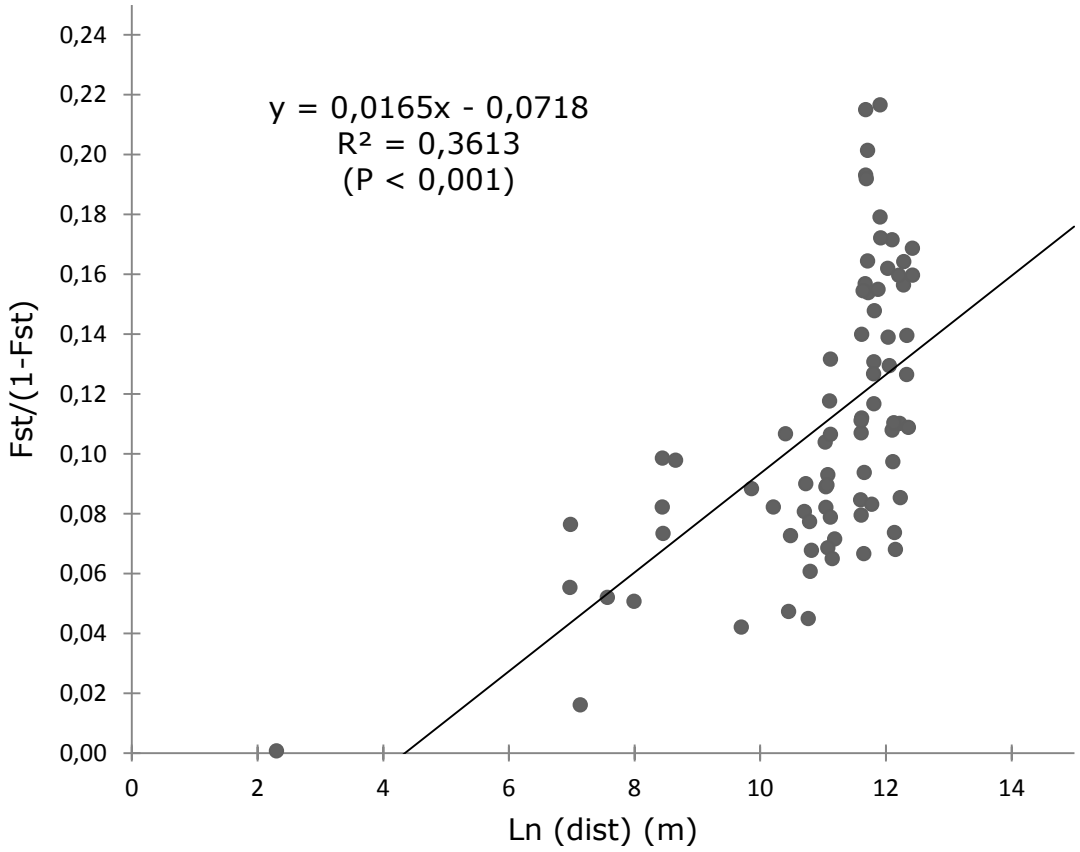

Figure 6 Linear regression of the genetic distance computed as $F_{ST}/(1-F_{ST})$ over logarithm of geographic distances (m).

The AMOVA showed genetic variation among four clusters of 7.58%, among populations within clusters of 5.39% and within populations 87.03%. Also, high significance of results was observed (Table 5; all $p < 0.001$).

Table 5 AMOVA test - results of the analysis of molecular variance within populations of *Paramuricea clavata*, among its populations within clusters and among clusters. Grouping was based on four clusters defined by STRUCTURE (K=4).

source of variation	d. f.	variation	P value
among clusters	3	7.58 %	< 0.001
among populations within clusters	9	5.39 %	< 0.001
within populations	443	87.03 %	< 0.001

4.3 Clustering analyses

Using STRUCTURE, analyses made for K spanning from 2 to 4 presented new clusters. For K=2, apparent distinction is shown between Senj archipelago and the rest of the regions. For K=3, Kornati area has separated from Pakleni islands and Rogoznica area with OBR population showing a high level of admixture. For K=4, Pakleni islands and Rogoznica area have clear distinction into two separate clusters (Figure 7, Figure 8).

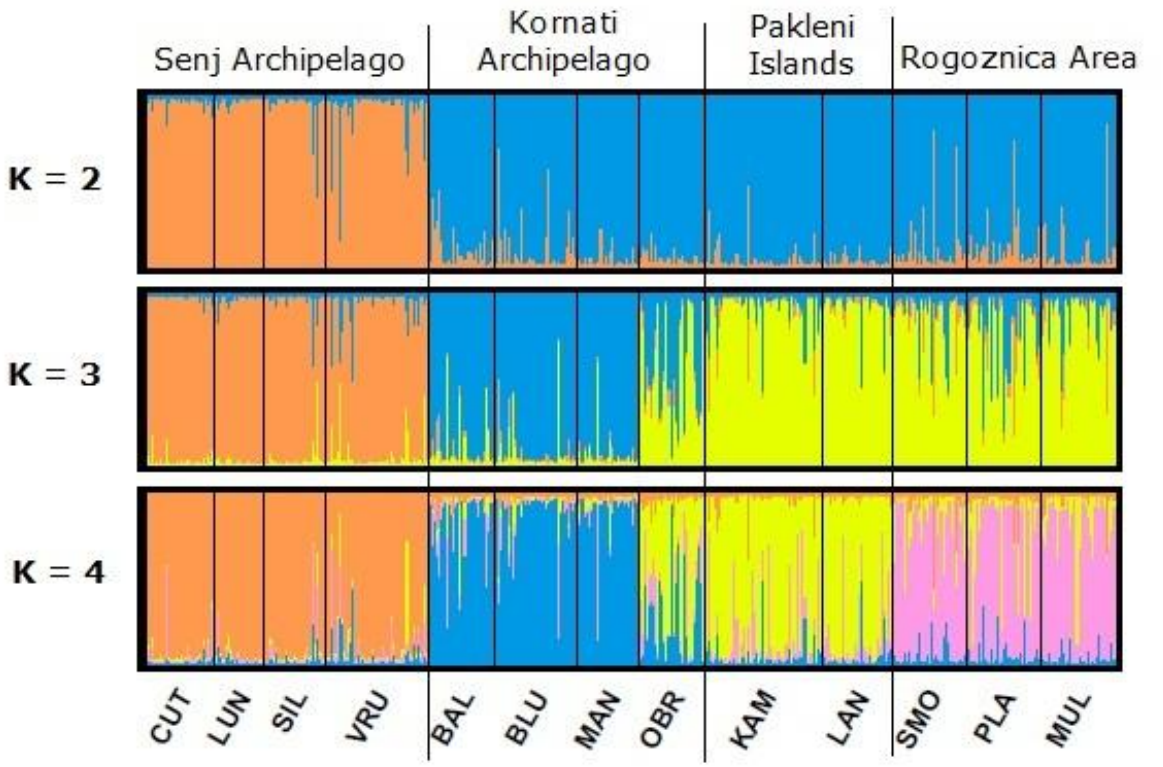

Figure 7 *Paramuricea clavata* population structure computed by STRUCTURE analysis. Every vertical line represents one colony in the population. Each colour shows individual's ratio in one cluster. Colonies were grouped into two, three and four clusters.

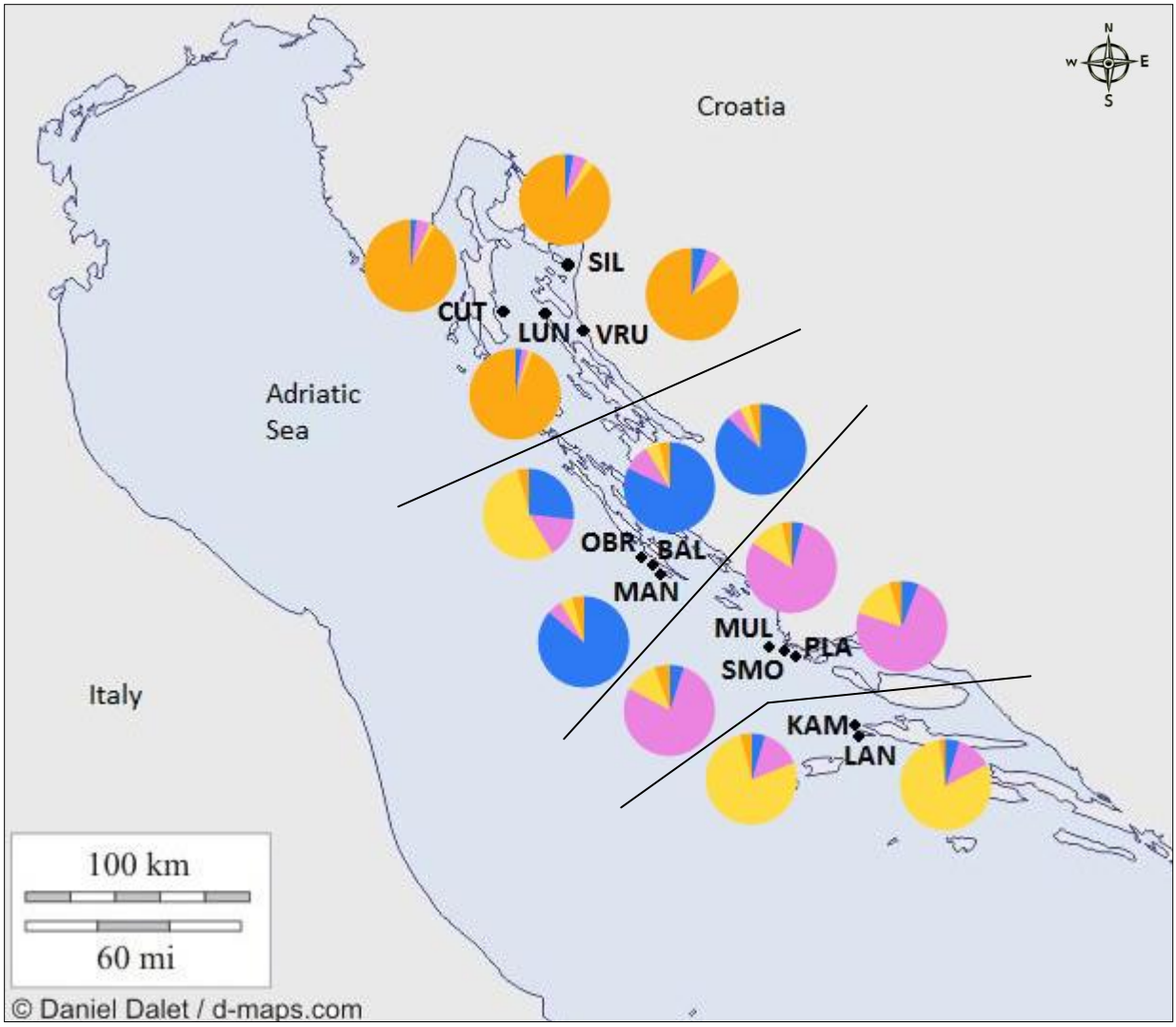

Figure 8 Map of 13 *Paramuricea clavata* sample sites. BAL and BLU are the same location but sampling took place at different depths. Lines divide geographical areas selected for hierarchical sampling scheme. Circles correspond to the level of assignment of each population in one of the four clusters defined by STRUCTURE.

5 DISCUSSION

5.1 Gene diversity and population structure

This study was the first one to characterize the genetic diversity of one engineering cnidarian species in the Adriatic Sea. Microsatelites Pcla 81 and Pcla-a were not used before in the research of *Paramuricea clavata* even if they were already in the gene bank. Therefore, they were efficiently developed at the population research for the first time and now complement previously used ones.

Departure from Hardy-Weinberg equilibrium was shown as well as in other marine sessile animals like sponges (Duran *et al.* 2004), ascidians (Dupont *et al*. 2009; Pérez-Portela & Turon 2008) and corals (Mokhtar-Jamaï *et al.* 2011; Ledoux *et al.* 2010a,b; Polato *et al.* 2010). High genetic diversity was comparable to the West-Mediterranean populations of *Paramuricea clavata* (mean He by samples: 0.71 vs. 0.74) while heterozygote deficiency and congruently null allele frequencies where much lower than previously described (Mokhtar-Jamaï *et al.* 2011). Still, it is expected for this species to show heterozygote deficiency at some rate due to its reproductive characteristics. Mating between related individuals is very likely because females brood the eggs on their surface and depend on low sperm dispersal (Coma & Lasker 1997a,b) and also larvae usually settle near the mother colony (Coma *et al.* 1995; Linares *et al.* 2008).

At the maximum of Pleistocenic glaciation, the water level in the Mediterranean Sea was more than 100 meters lower then today. Consequently, only the deepest parts of the Adriatic Sea, the Middle Adriatic Pit and the South Adriatic Pit (Appendix 5a) with surrounding areas remained submerged. A possible explanation for high genetic diversity and lower heterozygote deficiency might be a post-glacial re-colonisation of the Adriatic. Beginning with individuals that remained afterwards isolated in the deepest parts of the Adriatic and gradual colonisation of individuals from deeper Eastern Mediterranean (Ruggero *et al.* 2002). Previous STRUCTURE analysis from the study of Mokhtar-Jamaï *et al*. (2011) showed Adriatic population genetically most similar to the Aegean population which confirms gene flow from the east. A mixture of ancient pre-glaciation genes from the Adriatic and genes from the Eastern Mediterranean create a valuable source of genetic material.

5.2 Dispersal patterns and connectivity among populations

Since the eastern Adriatic coast is well-indented and there is a lack of knowledge on hydrodynamic processes of the sea, it is hard to predict the route of propagules to their settlement. Global surface currents flow from the Strait of Otranto in the south to Gulf of Piran in the north along the eastern coast and back to Otranto along the Italian coast (Appendix 5b, Poulain 2001). Moreover, some studies at local scale showed similarities between surface and deep currents (Vilibić *et al.* 2009) but only a few data exist on this topic in the Adriatic. The actual hydrodynamics occurring in the Adriatic are far more complex then described in the literature to date.

Positive and significant correlation between $F_{ST}/(1-F_{ST})$ and logarithm of geographic distances ($R^2 = 0{,}3613$; $P < 0{,}001$) suggests a strong pattern of Isolation by distance but with genetic discontinuities. Previous studies on gorgonian species confirmed the occurrence of IBD model of gene flow at Mediterranean scale, but not always on local scales (Mokhtar-Jamaï *et al.* 2011; Ledoux *et al.* 2010b). The capacity to detect IBD pattern depends on the range of distance values and the variance of estimators that are lower at shorter distances (Rousset 1997).

First round of clustering showed clear distinction of Senj Archipelago from all other populations meaning geographical distance is also an important segment of barriers to gene flow of *Paramuricea clavata*. The reason for this outcome may be a low dispersal of larvae i.e. its short swimming phase (Linares *et al.* 2008) and largest geographical distance from all other populations. AMOVA results confirmed strong cluster genetic differentiation showing higher differentiation among clusters then among populations within clusters (7.58% vs. 5.39%) which have not been recorded yet in octocoralian population genetics studies in the Mediterranean (6.0% vs. 7.2% in Mokhtar-Jamaï *et al.* 2011, 2.7% vs. 6.7% in Ledoux *et al.* 2010b). These high rates of differentiation suggest some heterogeneity between populations within the same group (Planes & Fauvelot 2002). Moreover, this pattern in AMOVA results suggests historical long-term isolation between different regions (Nesbø *et al*. 1998).

Possible explanation to Obručan population (OBR, Kornati Archipelago) belonging to the cluster with populations from the Pakleni Islands might be the strong currents at the southern sides of islands in the past when the contact zone between two clusters was around Kornati Archipelago. Unfortunately, there is no evidence of previous genetic diversity of *Paramuricea clavata* in the Adriatic Sea. Other explanation might be the impact of the sampling scheme. In some cases, different sampling schemes can make STRUCTURE detect different number of clusters (Aurelle & Ledoux 2013).

When it comes to the local scale, populations sampled at the same location but at different depths (30 and 40 meters) - BAL and BLU, did not show any barriers in gene flow as it was the case with some populations in the NW Mediterranean due to thermocline (Mokhtar-Jamaï *et al.* 2011). This outcome was expected since spring-summer thermocline in the middle Adriatic is formed down to a depth of 50 meters (Artegiani *et al.* 1996).

No significant differences in gene diversity were observed between populations inside Marine Protected Areas and the ones living in non-protected areas. Marine protected areas included in this study have been declared in 1963 and 1980, so even considering the low dynamics of *Paramuricea* clavata, some differences in gene diversity were expected in the Kornati National Park and protected landscape Pakleni Islands, in respect to the other non-protecetd investigated regions (Linares *et al*. 2011). Therefore, this data should be considered as a warning and stimulation to create scientifically-based management plans and a revision of MPA policies for other endangered species and habitats as well.

6 CONCLUSION

6.1 Implications for the conservation of *Paramuricea clavata*

The results of this study provide new information that could improve the management of *Paramuricea clavata* together with similar slow-growing sessile animals and the coralligenous habitat. Because of species' slow dynamics and delicate habitat structure, their recovery from anthropogenic and environmental disturbances is measured in decades (Garrabou *et al*. 2001). Additionally, the presence of strong genetic differentiation suggests these disturbances are usually recovered due to self recruitment, and much more rarely due to the inflow of new genetic material. Therefore, it is necessary to start systematic morphological and genetic monitoring of *P. clavata* and similar vulnerable species (Schwartz *et al.* 2007). Another core measure in conservation of this species is a severe control of diving activities on *P. clavata* sites (Linares *et al.* 2011) since high diving activities seriously damage populations and increase their mortality rates (Coma *et al*. 2004). Regarding extinction predictions due to global warming and other environmental effects (Linares & Doak 2010), it must not be permitted that any other disturbances, especially anthropogenic ones, affect the Adriatic populations.

It is necessary to develop a marine reserve network which would be formed based on the Paramuricea clavata dispersal abilities assuring connectivity of populations with a special focus on populations showcasing high genetic diversity (Palumbi 2004). The network of genetic connections among populations is the web that maintains the potential for evolution. Rather than a focus on longisolated populations, efforts should be directed at maintaining networks that capture the adaptive diversity within species (Crandall *et al.* 2000). Moreover, concerning the size of the Adriatic and high genetic differentiation of *Paramuricea clavata* populations, it is an extremely important area for their conservation.

Additionally, the transplantation of colonies in suitable habitats could be a useful solution due to restricted dispersal of larvae (Linares *et al*. 2008). Small populations of endangered species which have lost genetic variation might benefit from the introduction of individuals from related populations or even

subspecies for genetic restoration and recovery to normal levels of genetic variation (Hedrick 2001).

Genetic diversity is essential for further evolution in response to environmental changes and finally, survival of the species (Frankham 2005a). Therefore, genetic diversity definitely is one of the three levels of biodiversity that needs huge attention in conservation (McNeely *et al*. 1990).

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8 APPENDICES

Appendix 1

DNA extraction protocol: salting out procedure, adjusted from Miller *et al*. (1988)

Appendix 2

Hardy Weinberg test when H1= heterozygote deficit [Score (U) test], computed in GENEPOP v.4.1.4 (Rousset 2008)

Appendix 3

Table of estimated null allele frequencies, computed with FreeNA software using the EM algorithm (Dempster *et al*. 1977)

Appendix 4

Genetic and geographic distance matrices used for IBD analyses, computed in GENEPOP version 4.1.4 (Rousset 2008)

Appendix 5a

Adriatic Sea coastline and topography, included from Artegiani *et al*. (1996)

Appendix 5b

Map of surface mean flow in the Adriatic basin during summer, included from Poulain (2001)

Appendix 1

DNA extraction protocol: salting out procedure, adjusted from Miller *et al*. (1988)

1. For each colony/individual dissect around 10 polyps in a Petri dish with a clean scalpel
2. Incubation few minutes at 55°C to dry the remaining alcohol
3. Add 600 µL of TNES buffer
4. Add 20 µL of proteinase K (20mg/mL) + vortex + centrifuge
5. Incubation at 55°C for 4 to 5 hours
6. Put the digestion product in a new tube (without any remaining tissue)
7. Add 166,7 µL of NaCl (6M) (during this step the proteins will be precipitated)
8. Centrifugation for 15 min at 10000 g or 10,0 rcf or 12,2 rpm
9. Put the supernatant in a new tube
10. Add 780 µL of cold (-20°C) absolute alcohol (ethanol 100%)
11. Add 78 µL of Sodium Acetate (3M, pH=7)
12. Centrifugation for 15 min at 13000 g or 13,0 rcf or 13,9 rpm (DNA precipitation)
13. Remove the alcohol by pipetting
14. Add 780 µL of cold 70% ethanol
15. Centrifugation for 15 min at 13000 g
16. Remove the alcohol
17. Let the tubes dry over night
18. If needed, incubation for few minutes at 55°C to remove the remaining alcohol
19. Add 100 µL of milliQ sterilized H_2O

Appendix 2

Hardy Weinberg test when H1= heterozygote deficit [Score (U) test], computed in GENEPOP v.4.1.4 (Rousset 2008)

Markov chain parameters for all tests:

Dememorization: 10000
Batches: 20
Iterations per batch: 5000

Results by population (test multi-locus):

Population	P-val	S.E.	switches (ave.)
BAL31	0.1114	0.0145	13415.57
BLU40	0.0723	0.0120	16078.57
CUT33	0.1362	0.0145	19176.43
KAM55	0.0241	0.0092	19562.29
LAN35	**0.0006**	**0.0006**	**17665.43**
LUN24	0.5061	0.0120	20515.71
MAN30	0.3389	0.0306	15281.00
MUL35	0.0081	0.0020	15076.14
OBR31	0.1339	0.0143	18384.57
PLA35	0.0291	0.0055	19177.43
SIL30	0.2406	0.0180	17696.43
SMO36	0.1012	0.0146	14405.29
VRU48	**0.0000**	**0.0000**	**12861.57**

Results by locus (test multi-population):

Locus	P-val	S.E.	switches (ave.)
Pcla09	0.7398	0.0237	7438.69
Pcla10	**0.0002**	**0.0002**	**9332.46**
Pcla12	0.0109	0.0056	3614.69
Pcla14	0.0078	0.0029	9688.23
Pcla17	0.8911	0.0036	59898.23
Pcla81	0.2332	0.0159	9234.15
Pclaa	**0.0000**	**0.0000**	**18876.23**

Appendix 3

Table of estimated null allele frequencies

FreeNA: Estimating null allele frequency using the EM algorithm (Dempster *et al*. 1977)

Population:	BAL31	BLU40	CUT33	KAM55	LAN35	LUN24	MAN30	MUL35	OBR31	PLA35	SIL30	SMO36	VRU48
Locus:													
Pcla09	0,0001	0,0000	0,0000	0,0199	0,0503	0,0000	0,0028	0,0000	0,0000	0,0000	0,0000	0,0000	0,0000
Pcla10	0,0695	0,0216	0,0014	0,0162	0,0000	0,0005	0,0279	0,0000	0,0090	0,0252	0,0000	0,0852	0,0654
Pcla12	0,0000	0,0000	0,0000	0,0000	0,0572	0,0000	0,0075	0,0284	0,0000	0,0587	0,0581	0,0073	0,0286
Pcla14	0,0130	0,0544	0,0000	0,0274	0,0000	0,0459	0,0000	0,0713	0,0679	0,0326	0,0000	0,0000	0,0000
Pcla17	0,0376	0,0005	0,0001	0,0000	0,0000	0,0000	0,0000	0,1398	0,0046	0,0000	0,0000	0,0000	0,0000
Pcla81	0,0000	0,0352	0,0000	0,0000	0,0084	0,0000	0,0000	0,0000	0,0148	0,0000	0,0000	0,0000	0,0474
Pclaa	0,0000	0,0000	0,1456	0,0827	0,2357	0,0000	0,0280	0,0000	0,0000	0,0870	0,1014	0,0491	0,2563

Appendix 4

Genetic and geographic distance matrices used for IBD analyses computed in GENEPOP v.4.1.4 (Rousset 2008)

Fst/(1-Fst) estimates:

	BAL	BLU	CUT	KAM	LAN	LUN	MAN	MUL	OBR	PLA	SIL	SMO	VRU
BAL													
BLU	0,000801												
CUT	0,201441	0,088417											
KAM	0,164496	0,156476	0,171574										
LAN	0,107052	0,164271	0,060774	0,154542									
LUN	0,079577	0,076468	0,067809	0,097922	0,117718								
MAN	0,12655	0,055412	0,162027	0,07891	0,073448	0,106756							
MUL	0,13998	0,153908	0,103981	0,131695	0,071607	0,172205	0,107984						
OBR	0,112019	0,084681	0,098626	0,106568	0,216642	0,159714	0,045017	0,065042					
PLA	0,139618	0,11118	0,082283	0,097426	0,179182	0,155014	0,077423	0,050775	0,072719				
SIL	0,016175	0,191983	0,156895	0,08078	0,047379	0,085416	0,139032	0,110211	0,108893	0,147869			
SMO	0,215048	0,089054	0,066703	0,090073	0,15975	0,093084	0,089633	0,130803	0,1268	0,11043	0,068103		
VRU	0,193178	0,082158	0,093824	0,12952	0,168743	0,06866	0,052086	0,116784	0,042168	0,083203	0,082285	0,073752	

Ln (distance):

	BAL	BLU	CUT	KAM	LAN	LUN	MAN	MUL	OBR	PLA	SIL	SMO	VRU
BAL													
BLU	2,30259												
CUT	11,71	9,86246											
KAM	11,7099	12,2818	12,0995										
LAN	11,6082	12,2861	10,7962	11,6397									
LUN	11,6081	6,98193	10,8162	8,65434	11,1055								
MAN	12,3314	6,97261	12,0303	11,1184	8,45468	10,4038							
MUL	11,6159	11,7196	11,0354	11,1196	11,1861	11,9189	12,1014						
OBR	11,6158	11,5997	8,44484	11,1195	11,9087	12,2062	10,7657	11,1476					

PLA	12,3348	11,6074	8,44269	12,1122	11,9086	11,8765	10,7869	7,99294	10,4834			
SIL	7,13728	11,6897	11,672	10,7038	10,4529	12,2309	12,0367	12,2213	12,3594	11,8178		
SMO	11,6797	11,0467	11,6495	10,7265	12,4249	11,0776	11,0633	11,8101	11,806	12,1283	12,1532	
VRU	11,6796	11,0465	11,6569	12,0564	12,4237	11,0775	7,5689	11,81	9,70021	11,7755	10,2103	12,1378

distances (m):

	BAL	BLU	CUT	KAM	LAN	LUN	MAN	MUL	OBR	PLA	SIL	SMO	VRU
BAL													
BLU	10												
CUT	121782	121772											
KAM	109997	109987	226704										
LAN	110844	110834	227482	1258									
LUN	118147	118137	19196	215740	216656								
MAN	1077	1067	122959	109067	109906	119340							
MUL	62736	62726	179786	48834	49820	167756	62031						
OBR	4651	4641	117241	114635	115480	113519	5735	67401					
PLA	67483	67473	182088	44526	45547	172200	66535	4697	72118				
SIL	148548	148538	34644	248916	248617	32985	150072	200025	143846	205023			
SMO	64706	64696	180123	47369	48381	168832	63789	1937	69400	2960	203059		
VRU	134601	134591	35717	233149	134053	16321	135651	185035	130021	189705	27182	186803	

Mantel test, 10000 permutations

(statistic: Spearman Rank correlation coefficient)

Test of isolation by distance (One tailed Pvalue):

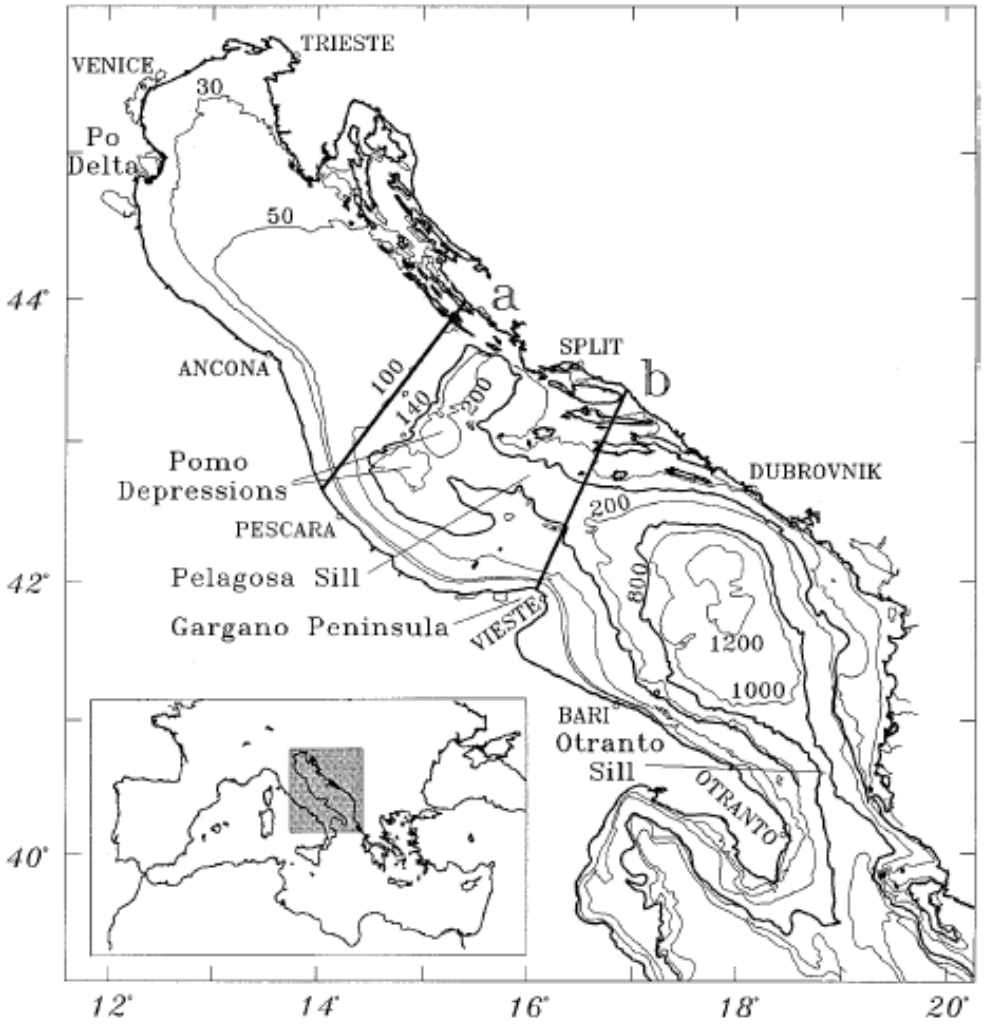
Pr (correlation > observed correlation) =0.0002 under null hypothesis

Other one tailed Pvalue:

Pr (correlation < observed correlation) =0.9998 under null hypothesis

Appendix 5

a Adriatic Sea coastline and topography, included from Artegiani *et al*. (1996)



b Seasonal maps of surface mean flow in the Adriatic basin, included from Poulain (2001)