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Cytochrome c oxidase subunit I variability in *Ruditapes decussatus* (Veneridae) from the western Mediterranean

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Abstract
Ruditapes decussatus (Linnaeus, 1758) (Veneridae) is an Atlanto-Mediterranean bivalve whose populations have experienced reductions and, in some instances, hybridisation with allochthonous *R. philippinarum*. Acquisition of additional genetic knowledge concerning the present *R. decussatus* populations is essential to address adequate conservation plans for this species. For this purpose, we analysed a portion of the mtDNA cytochrome c oxidase subunit I (COI) region in populations from the western Mediterranean, where this species represents a harvested fishery resource. Our analyses revealed an overall lack of genetic structure within the western Mediterranean area, and the occurrence of mtDNA substructuring between the Aegean and Marmara seas and the remaining populations from the whole Mediterranean basin and the South European Atlantic coast. The results obtained for populations from Sardinia, where extensive restocking programmes have not been reported and where *R. philippinarum* is rare and localised, suggest that intensive harvesting and potential dispersal alone may have been able to shape the genetic variation identified in the local *R. decussatus* populations.

Keywords: Ruditapes decussatus, *western Mediterranean, Sardinia, COI sequencing, spatial genetic patterns*

Introduction

The grooved carpet shell, *Ruditapes decussatus* (Linnaeus, 1758) (Veneridae), is a bivalve mollusc that inhabits muddy-sandy sediments along the Mediterranean and Eastern Atlantic shallow coastal areas (Fischer-Piette & Métivier 1971). This species is gonochoric with external fertilisation and planktotrophic larval stages. Reproduction occurs during spring and summer seasons and hatchery experiments have shown that the planktonic larval duration (PLD) is approximately 10–20 days, depending on temperature (Pérez Camacho 1976; Borsa & Millet 1992).

Ruditapes decussatus represents a commercially important fishery resource; indeed, the global production of *R. decussatus* was 4002 t in 2014 (http://www.fao.org/fishery/species/3542/en). The high commercial demand for *R. decussatus* led to intensive exploitation in several areas and, as a consequence, natural *R. decussatus* populations have declined in many European and Mediterranean sites (http://www.fao.org/fishery/culturedspecies/Ruditapes_decussatus/en). This species is further threatened by the introduction of the Manila clam (*Ruditapes philippinarum*), native to the West Pacific coasts, into Europe in the early 1970s (see Breber 2002, and references therein). Competition between the two species led to dramatic reductions in *R. decussatus* populations in many geographic areas (see e.g. Pranovi et al. 2006; Juanes et al. 2012; Bidegain & Juanes 2013), and, in some instances, hybridisation was observed between the two species (Hurtado et al. 2011; Habtemariam et al. 2015).

Since acquiring population genetic data is of primary importance for informing species management strategies, several studies on the population genetics of *R. decussatus* have been published in

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recent decades (Borsa et al. 1994; Jordaens et al. 2000; Cordero et al. 2008, 2014; Gharbi et al. 2010, 2011; Pereira et al. 2011; Borrell et al. 2014; Habtemariam et al. 2015; Arias-Pérez et al. 2016). However, only three studies sampled a significant number of populations (Borsa et al. 1994; Cordero et al. 2014; Arias-Pérez et al. 2016), and these gave somewhat different results. Based on allozymes, the genetic variability of populations from the Mediterranean Sea and Southern Portugal was comparable to that of other bivalve species (Borsa et al. 1994). These authors also pointed out a very low population differentiation, as no heterogeneity was detected at the within-region scale. Cordero et al. (2014) investigated spatial genetic patterns in populations ranging from the Atlantic coast of France to Turkey using restriction fragment length polymorphism of introns (iRFLP) and partial sequences of the mitochondrial gene cytochrome c oxidase subunit I (COI). These authors found a general subdivision of populations into three groups: (1) Atlantic, (2) Mediterranean plus Tunisia, and (3) Adriatic and Aegean seas. Nuclear and mitochondrial markers showed levels of genetic variation not fully consistent with each other. Indeed, mtDNA showed a further phylogenetic break located at the transition from the western Mediterranean to the Adriatic and Aegean seas. Contrasting levels of genetic differentiation between mitochondrial and nuclear markers that were found across the Atlantic–Mediterranean transition were explained by the authors as due to the possible existence of endogenous genetic rather than physical barriers to larval migration.

Arias-Pérez et al. (2016) analysed Atlantic and Mediterranean populations along the Spanish coast using both iRFLP (the same as Cordero et al. 2014) and microsatellite markers, and confirmed the presence of a genetic break at the transition between Atlantic and western Mediterranean populations previously detected with introns (but not with mtDNA) by Cordero et al. (2014). In the present study, we investigated the level of mtDNA genetic variability in *R. decussatus* populations from under-sampled regions of the western Mediterranean, where the species is an important marine fishery resource (see e.g. Turolla 2008; Chessa et al. 2013). In this area, we mainly focused on populations from Sardinia. Although several experimental studies of restocking were carried out in the past century using allochthonous populations (University of Cagliari and University of Ferrara, Italy, funded by the Autonomous Region of Sardinia), no form of commercial breeding of *R. decussatus* is being performed at present. Indeed, collection is mostly done by hand or rakes on natural beds during low tides, and harvested clams are sold exclusively in the local markets (Chessa et al. 2013). Moreover, Sardinia is one of the few Mediterranean regions where the spread of *R. philippinarum* is still limited (Cottiglia & Masala Tagliasacchi 1988a,b; Cannas et al. 2009; Mura et al. 2012). To achieve this goal, we used partial sequences of COI, as this marker proved to be very useful at elucidating the historical processes that contribute to the spatial distribution of genetic diversity (see Remerie et al. 2009, and references therein) in different marine molluscan species (e.g. Gharbi et al. 2010; Nakano et al. 2010; Sá-Pinto et al. 2010, 2012; Bird et al. 2011; González-Wevar et al. 2011; Sanna et al. 2013; Cordero et al. 2014; Cossu et al. 2015).

To shed new light on the mtDNA patterns of *R. decussatus* populations, we supplemented our sequences from the western Mediterranean with those we obtained from Portugal (southern coast), France (Mediterranean coast), Italy (Ionian coast of Sicily) and Tunisia (Northern coast). Furthermore, the availability of many COI sequences from other Atlanto–Mediterranean sites (Brittany, Portugal, Spain, Tunisia, Adriatic, Aegean Sea and Marmara Sea) enabled us to compare our results with those previously reported for other populations.

Materials and methods

Sampling

We sampled 361 *Ruditapes decussatus* specimens from 13 locations in the western Mediterranean (Corsica island and southern coast of France, Sardinia and north-western coast of Italy, and the northern coast of Tunisia; 328 individuals), one location on the Ionian coast of Sicily (21 individuals) and one on the southern coast of Portugal (12 individuals). Overall, our sampling covered the following Mediterranean and Atlantic marine ecoregions (*sensu* Spalding et al. 2007): western Mediterranean (WM), Ionian Sea (IO), and the South European Atlantic Shelf (AT) (see Table I and Figure 1 for details).

DNA extraction and PCR

DNA was isolated from a portion of the abductor muscle using the Qiagen DNeasy tissue kit. Mitochondrial regions were amplified using specific primers for COI (L: 5′-gtaattattcggatagagtt-3′ and H: 5′-cgtcgggtcaaaaaa-3′) designed by the authors, as COI universal primers (Folmer et al. 1994) did not

Table I. Origin of the *Ruditapes decussatus* individuals used in this study. Sampling localities and sample sizes (N) of the specimens are indicated for each of the marine ecoregions proposed by Spalding et al. (2007).

Marine ecoregion	Geographic area	Locality	Code	N	Study
Western Mediterranean	Sardinia, Italy	Olbia	OL	44	Present
		Lu Canaloni	PA	22	
		Poltu Qualtu	PQ	24	
		Tortolì	TR	38	
		San Teodoro	ST	30	
		Alghero	CA	21	
		Muravera	MU	29	
		Sa Praia	SP	20	
		Santa Giusta	SG	20	
	Tuscany, Italy	Orbetello	LO	23	
	Corsica, France	Aleria	DN	25	
	Languedoc-Roussillon, France	Thau	TH	26	
	North-east Tunisia	Tunisis North-Lake	TL	6	
	Catalonia, Spain	Ebro delta	EBR	22	Cordero et al. (2014)
	Murcia, Spain	Mar Menor	MME	33	
Ionian Sea	Sicily, Italy	Messina	MG	21	Present
Adriatic Sea	Veneto, Italy	Venice Lagoon	VEN	32	Cordero et al. (2014)
Tunisian Plateau/Gulf of Sidra	Central-east Tunisia	Sfax	SFX	31	
Aegean Sea	Macedonia, Greece	Halkidiki	HAL	33	
	Aegean region,Turkey	Izmir	IZM	30	
Black Sea	Marmara region, Turkey	Marmara Sea	MAR	23	Keskin & Atar (2013), GenBank database*
South European Atlantic Shelf	Algarve, Portugal	Rio Formosa	RF	12	Present
			FOR	32	Cordero et al. (2014)
		Milfontes	MLF	17	
	Galicia, Spain	Mugardos	MUG	32	
		Lombos do Ulla	LUL	33	
	Brittany, France	Golfe du Morbihan	GMO	32	

Note: *See the main text for GenBank accession numbers.
Please note that, according to a personal communication from Prof. Emre Keskin, all Turkish sequences used in this study were from Marmara Sea (MAR).

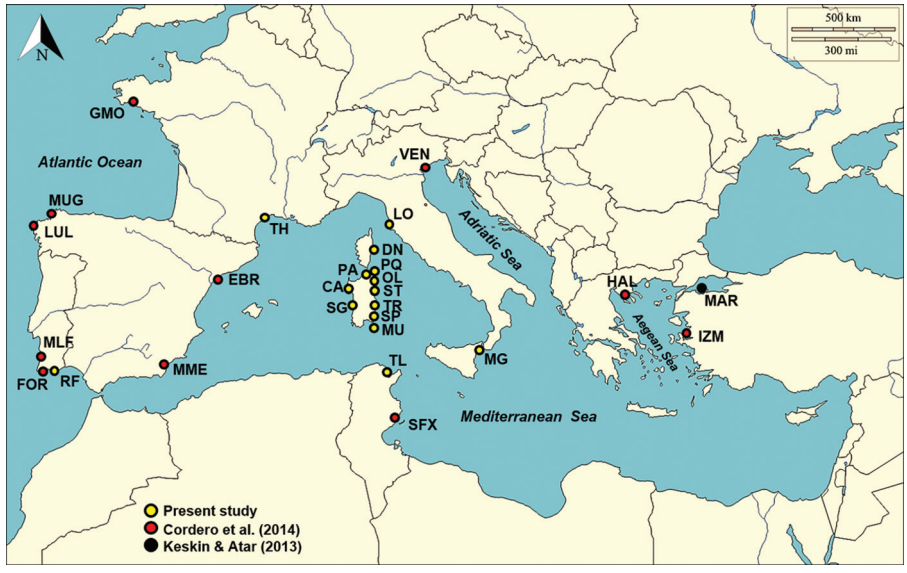

Figure 1. Sampling plan. The map indicates the localisation of the collecting sites. The localities are labelled as in Table I.

consistently provide satisfactory results in several samples. Each 25-μL polymerase chain reaction (PCR) mixture contained approximately 100 ng of total genomic DNA, 0.32 μM of each primer, 2.5 U of EuroTaq DNA Polymerase (Euroclone), 1× reaction buffer and 200 μM of each dNTP. The $MgCl_2$ concentration was set at 3 mM, and 25 μg of bovine serum albumin (BSA) (5 ng/mL) was added to the reaction mixture. PCR amplifications were performed according to the following steps: 1 cycle of 2 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 50°C and 1 min 30 s at 72°C, and a final post-treatment of 5 min at 72°C. After electrophoresis on 2% agarose gels, the PCR products were purified and sequenced using an external sequencing core service (Macrogen Inc., The Netherlands). Dual peaks of similar height, which could be interpreted as evidence of possible heteroplasmy, were not observed in any of the electropherograms. To eliminate the possibility of mitochondrial pseudogenes in the nucleus (Numts), sequences were translated to amino acids.

Statistical analysis

The sequences were aligned using the program Clustal W (Thompson et al. 1994), implemented in the BioEdit 7.1.3.0 software package (Hall 1999), and newly discovered haplotypes were deposited in GenBank (accession nos. KU557025–KU557044; see Supplementary Table S1 for details). Estimates of the number of polymorphic sites (S), number of haplotypes (H), haplotype diversity (h), and nucleotide diversity (π) were obtained using the software package DnaSP 5.10 (Librado & Rozas 2009).

The final data set, constructed using the sequences obtained in the present study, with 350 COI sequences available in the literature (Keskin & Atar 2013; Cordero et al. 2014) and those in the GenBank database deposited by Keskin in 2012 (GenBank accession nos. JQ623975, KC789414–KC789433), spanned the following marine ecoregions (*sensu* Spalding et al. 2007): the South European Atlantic Shelf (hereafter referred to as the AT ecoregion), western Mediterranean (hereafter referred to as the WM ecoregion), Ionian Sea (hereafter referred to as the IO ecoregion), Adriatic Sea (hereafter referred to as the AD ecoregion), Tunisian Plateau/Gulf of Sidra (hereafter referred to as the TU ecoregion), Aegean Sea (hereafter referred to as the AE ecoregion) and Black Sea (hereafter referred to as the BL ecoregion) (see Table I and Figure 1 for details). To analyse the same COI fragment obtained in this study, we deleted the first 84 bp (19% of the total length) from the sequences provided by Cordero et al. (2014), losing two out of the 29 haplotypes described by the authors (A14, B4). We also deleted the first 136 bp and the last 170 bp (47% of the total length) from the sequences provided by Keskin and Atar (2013), losing one of the two haplotypes they described. Overall, only five of 36 polymorphic sites (14% of the total number) described by Cordero et al. (2014) and Keskin and Atar (2013) were removed. Among the removed polymorphic sites, only two were informative, while three were single-nucleotide polymorphisms specific to single individuals. When we merged our samples with data from Keskin and Atar (2013) and Cordero et al. (2014), we obtained a data set of 711 sequences of 348 bp length. Genetic relationships among haplotypes were investigated by the median-joining network using the software package Network 4.5.0.1 (www.fluxus-engineering.com). Patterns of genetic differentiation at the population level were further assessed using analysis of molecular variance (AMOVA; Excoffier et al. 1992) as implemented in the software Arlequin 3.5.1.3 (Excoffier & Lischer 2010). AMOVA was carried out using Tamura and Nei's (1993) matrix of genetic distances with a gamma correction (alfa shape parameter = 0.8430) set according to the best-fitting model of sequence evolution selected by JModeltest (Posada 2008) using the Akaike information criterion (AIC) after a maximum likelihood-optimised search. The model was TPM2uf+G. First, we calculated pairwise Φ_{ST} values between sampling localities. The sequential Bonferroni method of correction was applied to P values to avoid errors due to multiple testing (Hochberg 1988). Next, we conducted a hierarchical AMOVA. Total genetic variation was partitioned taking into account alternative groupings of main marine ecoregions.

The presence of population genetic structure was determined by the Bayesian model-based clustering algorithm implemented in Baps 6.0 (Corander et al. 2013). Clustering among populations was performed using the codon linkage model available in the module for linked molecular data, which is appropriate for sequences. Each analysis was performed 10 times with a vector of K values ranging from 1 to 12, each with six replicates, where K is the number of assumed genetic clusters. The pairwise Φ_{ST} values between Bayesian groups were estimated using the software Arlequin 3.5.1.3.

Results

Within the whole data set of 711 sequences from seven marine ecoregions, we found 52 polymorphic sites (S), which resulted in 49 different haplotypes (see Table II and Supplementary Table S1 for details

Table II. Estimates of genetic diversity obtained for each *Ruditapes decussatus* population. Sample sizes (N), number of polymorphic sites (S), number of haplotypes (H), haplotype diversity (h), and nucleotide diversity (π) are shown. The localities are labelled as in Table I.

Marine ecoregion	Sample	N	S	H	h	π
Western Mediterranean	OL	44	10	11	0.614	0.00284
	PA	22	5	4	0.260	0.00131
	PQ	24	3	4	0.308	0.00153
	TR	38	12	6	0.334	0.00236
	ST	30	4	5	0.359	0.00111
	CA	21	2	3	0.186	0.00055
	MU	29	1	2	0.069	0.00020
	SP	20	6	5	0.442	0.00250
	SG	20	3	2	0.100	0.00086
	LO	23	6	7	0.522	0.00195
	DN	25	3	4	0.230	0.00069
	TH	26	10	7	0.523	0.00302
	TL	6	1	2	0.333	0.00096
	EBR*	22	2	3	0.255	0.00076
	MME*	33	5	4	0.280	0.00120
Ionian Sea	MG	21	5	4	0.271	0.00186
Adriatic Sea	VEN*	32	6	7	0.514	0.00185
Tunisian Plateau/Gulf of Sidra	SFX*	31	2	3	0.239	0.00070
Aegean Sea	HAL*	33	10	5	0.748	0.01306
	IZM*	30	13	7	0.591	0.00576
Black Sea	MAR§	23	0	1	0	0
South European Atlantic Shelf	RF	12	6	6	0.682	0.00287
	FOR*	32	3	4	0.236	0.00071
	MLF*	17	0	1	0	0
	MUG*	32	2	3	0.280	0.00143
	LUL*	33	0	1	0	0
	GMO*	32	5	6	0.343	0.00107
	Tot	711	52	49	0.449	0.00481

Note: *Samples whose sequences were from Cordero et al. (2014).

§Samples whose sequences were from Keskin and Atar (2013) and from the GenBank database.

Please note that according to a personal communication from Prof. Emre Keskin, all Turkish sequences used in this study were from Marmara Sea (MAR).

and GenBank accession numbers), 21 of which were newly described (A22–A42) and represented a 68% increase in the number of previously described haplotypes (31) for the species. In the WM region (Mediterranean coast of France, Corsica, Sardinia and north-west coast of Italy), 17 new haplotypes were found; two were found in IO (Sicily), and three were found in the Atlantic population of southern Portugal (AT).

Ten haplotypes, recorded by Cordero et al. (2014: A1, A2, A4, A5, A7, A8, A9, A16, A18 and B2), were also found in the present study. Overall, a total of 26 haplotypes were found among the new populations from the western Mediterranean. In particular, 23 haplotypes were discovered in populations from Sardinia. Consistent with Cordero et al. (2014), the haplotype A1 was the most common haplotype among the new western Mediterranean populations. A unique private haplotype was found in the Marmara Sea population (C1).

For the whole data set, the total mean haplotype and nucleotide diversity were $h = 0.449$ and $\pi = 0.00481$, respectively. The lowest values ($h = 0$ and $\pi = 0$) were found in the population from the Marmara Sea and in two populations from the Atlantic coasts of Portugal (MLF) and Spain (LUL), respectively. In our analysis, the highest levels of h were identified in one Greek population from the Aegean Sea (HAL) and in two Atlantic and western Mediterranean populations from Portugal (RF) and Sardinia (OL), respectively. The highest levels of π were discovered in the Greek (HAL) and Turkish (IZM) populations from the Aegean Sea (see Table II for details).

Overall, western Mediterranean populations (Mediterranean coasts of France, Corsica, Sardinia, the north-west coast of Italy and the northern coast of Tunisia) exhibited lower levels of genetic variation than those found within other ecoregions: a total mean haplotype and nucleotide diversity corresponding to $h = 0.356$ and $\pi = 0.00162$, respectively. Additionally, within the WM ecoregion, Sardinian populations (OL, PA, PQ, TR, ST, CA, MU, SP and SG) exhibited 25 polymorphic sites and a total mean haplotype and nucleotide diversity corresponding to $h = 0.335$ and $\pi = 0.00167$, respectively.

The median-joining network analysis revealed a star-shaped phylogeny with a central, most common haplotype (A1; Figure 2). Derived haplotypes diverged from the central haplotype by one or two point mutations. Overall, a high level of haplotype sharing among the WM populations was pointed out, without strong evidence of geographic structuring among regions (see inset in Figure 3). Individuals from the Aegean and Marmara Sea populations diverged from the central haplotype A1 by five and three point mutations, respectively. One individual from the Sardinian population of Tortolì was nested within the Aegean second most common exclusive haplotype, B2.

Pairwise Φ_{ST} values indicated significant genetic differentiation in 75 of 351 comparisons (21%). In particular, the populations of Marmara Sea (in the BL ecoregion), Greece (HAL) and Turkey (IZM) (in the AE ecoregion) significantly diverged from the other populations in almost 100% of the comparisons with the only exception being the population from HAL, which did not diverge either from the unique Tunisian population in the WM ecoregion (TL) or from the Atlantic population from Rio Formosa – (RF) in the AT ecoregion whose

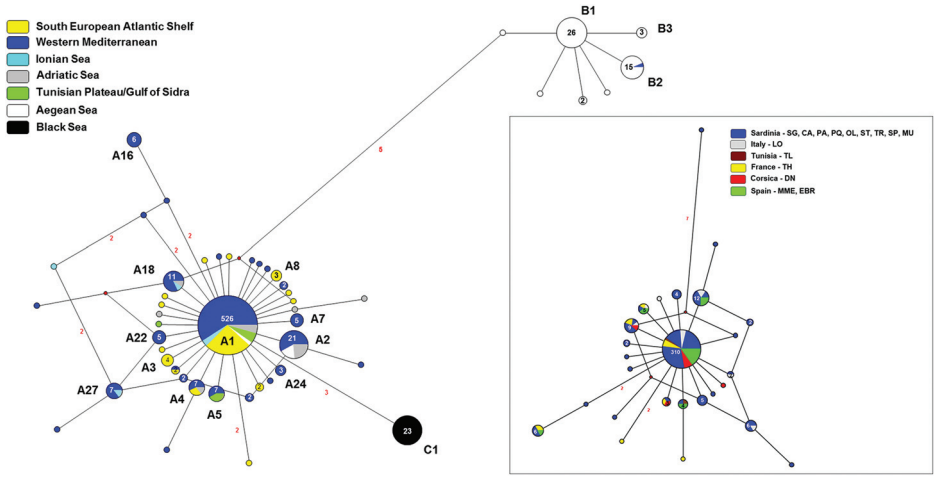

Figure 2. Median-joining network analysis. The haplotypes in the network are shown according to the proportion of individuals sampled in each of the marine ecoregions, as proposed by Spalding et al. (2007). The inset shows the haplotypes for western Mediterranean populations. The small white dots on the nodes correspond to median vectors representing the hypothetical missing/unsampled haplotypes that are necessary for a fully connected network estimated using the maximum parsimony method. The number of mutations between haplotypes that are greater than one is reported on the network branches. Haplotypes are represented by circles; those shared by at least two individuals are marked with a number indicating the individuals sharing the same haplotype. For each sequence shared by more than two individuals, capital letters followed by numbers indicate the name of the haplotype labelled as in Supplementary Table S1.

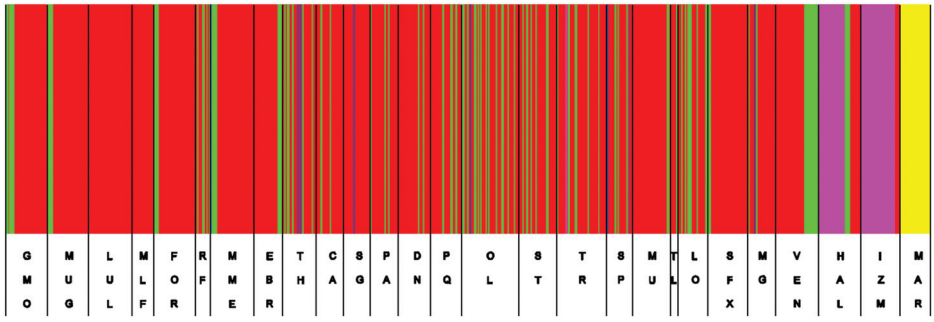

Figure 3. Bayesian model-based clustering analysis of genetic structure. Each individual is represented in the barplot by a thin vertical line; the shadow/colour gradient of segments is indicative of the individual membership of one of the four clusters retrieved. Black lines separate individuals from different sampling sites. The localities are labelled as in Table I.

individuals were collected during this study (Supplementary Table S2). The two Aegean populations from the AE ecoregion (HAL and IZM) were not divergent; furthermore, the Atlantic population from Milfontes (MLF), in the AT ecoregion, significantly diverged from the other Atlantic population, RF, sampled during this study. Finally, the unique population from the Adriatic Sea (VEN in the AD ecoregion) significantly diverged not only from Aegean (HAL and IZM in the AE ecoregion) and Marmara (in the BL ecoregion) seas populations, but also from the unique Tunisian population in the TU ecoregion (SFX).

AMOVA was carried out by defining the groups of marine ecoregions according to biogeographic criteria (see Table III, schemes A, B, C, and Supplementary Figure S1 for details); an overall significant fixation index value (Φ_{ST} = 0.592, $P < 0.001$) was obtained. The component of genetic variation due to differences among groups was high when the samples were grouped according to scheme A in Table III, in which the Aegean Sea (AE ecoregion) and the Marmara Sea (BL ecoregion) were considered two separate groups, whereas the other marine ecoregions (AT, WM, IO, AD and TU) were grouped together (Φ_{CT} = 0.853, $P < 0.001$). However, genetic divergence was maximised among groups (Φ_{CT} = 0.862, $P < 0.001$) when the two populations (HAL, IZM) from the Aegean Sea were considered separate groups (see Table III – scheme B for details). Slightly lower genetic divergence was also found (Φ_{CT} = 0.816, $P < 0.001$)

Table III. Results of the analysis of molecular variance (AMOVA). The groups were defined according to biogeographic criteria. df: degrees of freedom; SSD: sum of squared deviations; var. comp.: variance component; % var: percentage of variation. Populations are labelled as in Table I.

Source of variation	df	SSD	Var. comp.	% var	Fixation indices	*P*-value
A						
Group 1: Marmara Sea (BL).						
Group 2: Aegean Sea (AE).						
Group 3: South European Atlantic Shelf (AT), Western Mediterranean (WM), Ionian Sea (IO), Adriatic Sea (AD), Tunisian Plateau/Gulf of Sidra (TU).						
Among groups	2	355.896	2.280	85.35	Φ_{ct} 0.853	< 0.001
Among populations within groups	24	21.909	0.021	0.77	Φ_{sc} 0.053	< 0.001
Within populations	685	253.904	0.371	13.88	Φ_{st} 0.861	< 0.001
B						
Group 1: Marmara Sea (BL).						
Group 2: IZM.						
Group 3: HAL.						
Group 4: South European Atlantic Shelf (AT), Western Mediterranean (WM), Ionian Sea (IO), Adriatic Sea (AD), Tunisian Plateau/Gulf of Sidra (TU).						
Among groups	3	368.169	2.321	86.17	Φ_{ct} 0.862	< 0.001
Among populations within groups	23	9.636	0.002	0.07	Φ_{sc} 0.005	< 0.001
Within populations	685	253.904	0.371	13.76	Φ_{st} 0.862	< 0.001
C						
Group 1: Marmara Sea (BL).						
Group 2: IZM.						
Group 3: HAL.						
Group 4: Adriatic Sea (AD), Tunisian Plateau/Gulf of Sidra (TU).						
Group 5: South European Atlantic Shelf (AT), Western Mediterranean (WM), Ionian Sea (IO).						
Among groups	4	368.852	1.651	81.61	Φ_{ct} 0.816	< 0.001
Among populations within groups	22	8.953	0.001	0.07	Φ_{sc} 0.004	< 0.001
Within populations	685	253.904	0.371	18.32	Φ_{st} 0.817	< 0.001

when the populations from AD and TU ecoregions were considered a further separate fifth group (see Table III – scheme C for details). When alternative groupings of marine ecoregions were tested, AMOVA showed a strong decrease in the proportion of variance and significance (data not shown). The Bayesian analysis identified five distinct haplotype groups (R1, R2, R3, R4 and R5; see Figure 3 and Supplementary Figure S2). Overall, the most widespread group (R1), spanning all the marine ecoregions considered in this study except MAR (in BL ecoregion), did not display a clear geographic structuring. The second most common group (R2) was spread widely across the Atlantic (AT ecoregion) and the Mediterranean (WM, IO, AD, TU and AE ecoregions) and occurred in all populations, except MAR (in the BL ecoregion), IZM (from Turkey in the AE ecoregion), MLF, and LUL (from Portugal and Spain, respectively, in the AT ecoregion), and SG (from Sardinia in the WM ecoregion). Group R3 showed the lowest average frequency of distribution and was restricted to several western Mediterranean populations from Sardinia (OL, SP, SG), southern Mediterranean coasts of France (TH), and Spain (MME) and the Sicilian population (MG) in the Ionian Sea. Groups R4 and R5 were exclusive to the Marmara and Aegean seas, respectively, with the exception of one individual from the Sardinian population of Tortolì in the WM ecoregion (assigned to R5). All pairwise Φ_{ST} values calculated between Bayesian groups (Supplementary Table S3) indicated significant genetic differentiation in all the comparisons. High levels of differentiation were pointed out between all the groups with the exception of R2, which exhibited lower divergence with each of the other groups (R1, R3, R4 and R5). Pairwise Φ_{ST} values also indicated that the Bayesian groups R4 and R5 were the most divergent from the other groups.

Discussion

In this study, 21 new COI haplotypes (corresponding to 16 new polymorphic sites) were found for *Ruditapes decussatus*, increasing by 68% the number of haplotypes (31) previously reported by Keskin and Atar (2013) and Cordero et al. (2014).

The estimates of genetic diversity (haplotype and nucleotide diversities) recorded in the western Mediterranean are similar to those obtained in previous studies investigating exploited *R. decussatus* populations from the Atlantic Ocean, Spain,

Tunisia, Adriatic and Aegean Sea (e.g. Gharbi et al. 2010; Cordero et al. 2014).

A possible decline in population sizes due to overexploitation may have negatively affected the mtDNA genetic variability of *R. decussatus*. Indeed, according to the Food and Agriculture Organization of the United Nations (FAO) statistics (http://www.fao.org/fishery/species/3542/en), the species was intensively harvested throughout its distribution range until the early 1980s and global production seems to be declining (e.g. since 2007, production has dropped by more than half; http://www.fao.org/fishery/species/3542/en). Low levels of genetic variability have been reported in several overfished aquatic organisms (see e.g. Wilson & Clarke 1996; Roberts et al. 2001; Pérez-Ruzafa et al. 2006; García-Cisneros et al. 2016; and references therein). Furthermore, we need to consider the possible impact of the allochthonous *R. philippinarum*, which may affect the genetic variability of *R. decussatus* (see e.g. Juanes et al. 2012; Bidegain & Juanes 2013; and references therein) in at least two different ways. First, the complete replacement of *R. decussatus* by *R. philippinarum* in several areas (Pranovi et al. 2006; Juanes et al. 2012; Bidegain & Juanes 2013) may cause either loss of (local) genetic variability or changes in the connectivity patterns, as evidenced in other aquatic species (see e.g. Cambray 2003; Arismendi et al. 2009; Pilliod et al. 2010; and references therein). Moreover, when the two species share the same area, hybridisation may occur, as evidenced by the low rates of introgression of *R. philippinarum* genes in *R. decussatus* in northern Spain (Hurtado et al. 2011; Habtemariam et al. 2015). This second point represents a crucial issue in most geographic areas in which the Manila clam has been introduced, as hybridisation and genetic introgression are the most commonly reported genetic consequences of aquatic invasions (see e.g. Rhymer & Simberloff 1996; Echelle & Echelle 1997).

In this context, Sardinian populations, which show low levels of genetic variation as in other western Mediterranean sites, may be useful to understand the forces involved in decreasing the species' genetic variability. Indeed, most *R. decussatus* populations in Sardinian lagoons are not presently strongly affected by competition with *R. philippinarum*. In fact, introductions of *R. philippinarum* were realised during the 1980s, but these had only limited success in a few Sardinian sites (e.g. Sant'Antionco and Santa Gilla lagoons, southern Sardinia; see Cottiglia & Masala Tagliasacchi 1988a,b). At present, only one established population of Manila clam has been reported in north-east Sardinia, in the Gulf of Olbia where the introduction is likely the result of accidental release of adults due to careless management by fishery operators during legal stabulation processes (Cannas et al. 2009; Mura et al. 2012). Therefore, in Sardinia, overexploitation of the species, also enhanced by the common practice of illegal harvesting (Chessa et al. 2013), could be the main threat to the conservation of genetic variability in *R. decussatus*.

The high level of haplotype sharing found in the western Mediterranean may be explained by the long PLD of *R. decussatus*, which enhances the potential for dispersal in the marine realm (see Kelly & Palumbi 2010, and references therein). However, the recovery of exhausted natural beds of *R. decussatus* in different European localities, often achieved by the release of seeds collected in distant geographic areas (see e.g. Passamonti et al. 1997; Turolla 2008; Pereira et al. 2011; Arias-Pérez et al. 2016), may have also contributed to the genetic homogenisation of *R. decussatus* across its distribution range.

Even taking into consideration the possibility that this practice may occur in Sardinia, not being recorded or formally authorised by the administration, we should consider this irrelevant to the genetic variability, at least for most of the Sardinian populations. Indeed, despite the growing demand for *R. decussatus* in both the local markets and the tourist trade (Saba 2011), to our knowledge only in a few instances, e.g. in the Tortolì lagoon, have seeds from allochthonous populations of *R. decussatus* (mainly from Greece and Turkey) been released for restocking (projects funded by the Autonomous Region of Sardinia and carried out by the University of Cagliari and University of Ferrara, Italy). Therefore, it is not surprising that an individual from the Sardinian population of Tortolì (western Mediterranean) shared a haplotype (B2) found only in the Aegean Sea. Nonetheless, considering the likely limited extent of these experiments, the occurrence of many haplotypes among the Sardinian populations diffused throughout the western Mediterranean is suggestive of efficient dispersal rather than anthropic factors. Interestingly, despite a background of high gene flow, 20% of individuals of *R. decussatus* from Olbia (north-eastern Sardinia) show private haplotypes (i.e. haplotypes not shared with other populations). This occurrence could indicate the persistence of "wild" genetic variability not yet exposed to the effect of genetic homogenisation.

The acquisition of COI sequences for 13 new sites from the western Mediterranean allowed us to strengthen the knowledge of the genetic structuring of *R. decussatus* recorded by Cordero et al. (2014) at the mtDNA level. Consistent with that study, our results revealed the occurrence of at least two main groups of haplotypes, one of which includes populations spanning an area from the Atlantic to the

Adriatic Sea, the other being almost restricted to the Aegean Sea.

Conversely, albeit different molecular markers were used and the sampling areas do not completely overlap, our results are different from those previously obtained by Borsa et al. (1994) and Arias-Pérez et al. (2016). Indeed, Borsa et al. (1994) found low levels of genetic divergence without substructuring across the Atlanto–Mediterranean range (five Mediterranean populations and one from southern Portugal). Arias-Pérez et al. (2016) detected a further genetic transition between the Atlantic and western Mediterranean, investigating eight Atlantic and two Mediterranean populations from the Spanish coasts.

Our study also determined the occurrence of genetic substructuring between the Marmara Sea and the other Mediterranean and Atlantic populations. Although the limited extent of sampling in this region does not allow us to draw a definitive conclusion, based on our knowledge, we hypothesise that the peculiar genetic traits of MAR may be related to the geographic position of this population, as the Marmara Sea represents the edge of *R. decussatus* distribution in the Mediterranean (Demir 2003). In this context, MAR may represent a peripheral isolate (sensu Frey 1993, i.e. relatively small isolated or semi-isolated populations distributed around the periphery of a large central portion of a species' range). In particular, the *R. decussatus* sample from the Marmara Sea was surprisingly highly divergent from the geographically close sample of the Aegean Sea. Several studies observed the occurrence of a genetic break among the Aegean Sea and the other Mediterranean basins that was explained by hydrographical isolation (Nikula & Vaïnölä 2003; Domingues et al. 2005; Peijnenburg et al. 2006; Zulliger et al. 2009; Sanna et al. 2013). Indeed, this finding likely reflects the enclosed nature of the Aegean Sea, which represents a well-defined phylogeographic region (Georgiou et al. 2015). The geographical complexity of this area, which mostly encompasses the North Aegean Islands, the Cyclades, the Dodecanese and Crete, among others, and the complex hydrodynamic regime influenced by the cold northern winds and upwelling of cold water along the Anatolian coast (Shemesh et al. 2009), may account for the observed genetic structuring. The upwelling favours larval retention in coastal species, thus promoting genetic divergence, as shown in the red rock lobster *Panulirus interruptus* (Iacchei et al. 2013).

On the other hand, few Aegean haplotypes (12 from the Greek population of Halkidiki and three from the Turkish population of Izmir) are nested within the two most widespread Atlanto–Mediterranean haplotypes (A1, A2). Their occurrence may represent the footprint of a possible ancient Mediterranean panmictic population, whose present genetic structure was shaped by sea level fluctuations during the Pleistocene glaciations (see Cordero et al. 2014). However, the present data do not allow us to rule out the influence of human activities: e.g. restocking programmes, or passive transport of seeds by ships, the latter being a common finding in many marine mollusc invasions that can circumvent geographic barriers (see e.g. Carlton & Hodder 1995; Johnson & Carlton 1996; Apte et al. 2000; Gollasch 2007). Estimates of the pairwise Φ_{ST} values obtained between populations and between Bayesian groups, respectively, showed an overall genetic divergence between western and eastern Mediterranean basins as a consequence of the significant differentiation between samples from Aegean and Marmara seas and all the other Mediterranean samples. Furthermore, AMOVA pointed out a significant, if slight, genetic divergence between the area including the Adriatic Sea (AD) and Tunisian Plateau/Gulf of Sidra (TU) and the other westernmost and easternmost areas. Such results suggest that the occurrence of the genetic break between the western and eastern Mediterranean is likely located south-eastwards from the Siculo–Tunisian Strait (STS). This finding is consistent with the model proposed by Bianchi and Morri (2000), who set the position of the boundary between the western and eastern Mediterranean east of the eastern coast of Sicily (see also Bianchi 2007 for more details). This finding partially contrasts with the results obtained by Gharbi et al. (2010, 2011). Based on COI and the internal transcribed spacer region ITS1, Gharbi and colleagues (2010) found no significant differentiation among eastern and western Tunisian populations on either side of the STS; conversely, based on allozymes, they set the genetic break through the STS (Gharbi et al. 2011). The discrepancy between our results and those of the latter study may be due to the different molecular markers used. Nevertheless, it is remarkable that the augmented sampling plan allowed us to pinpoint a boundary between the western and eastern Mediterranean, as has been seen in other species with high dispersal capabilities (e.g. Varney et al. 2009; Xiao et al. 2010; Borrero-Pérez et al. 2011; Dailianis et al. 2011; Lazoski et al. 2011; Mokhtar-Jamaï et al. 2011; Sanna et al. 2013).

In conclusion, the present study extended the knowledge of the genetic patterns of *R. decussatus* populations in the western Mediterranean, particularly in a specific geographic region (Sardinia) where

populations of *R. decussatus* are presently wild and only weakly involved in negative competition with *R. philippinarum* due to its rare and very localised occurrence. This unique condition allows us to hypothesise that, in the absence of relevant competition with *R. philippinarum* and systematic restocking programmes, unplanned human harvesting and potential dispersal may play an important role in shaping the local species' genetic variation.

However, we cannot rule out the possibility that unauthorised and unrecorded releasing of allochthonous seeds may have occurred in Sardinia to a greater extent than we suppose. For this reason, future studies on a larger number of Sardinian individuals and localities will help to shed further light on the *R. decussatus* genetic scenario of this Mediterranean island.

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Supplemental material

Supplemental data for this article can be accessed here.

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