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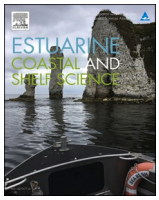

Genetic diversity of the *Ruditapes decussatus* and evidence of its hybridization with the alien *R. philippinarum* in the Western Mediterranean Sea

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ABSTRACT

The introduction of alien species in marine ecosystems is often driven by the increasing demand of fishery resources. This is the case of the Manila clam (*R. philippinarum*), imported in Europe from Japan since the 1970s, to meet the growing demand for clams that the native species, the grooved carpet shell clam (*Ruditapes decussatus*), could not satisfy. Alien species introduction could threaten the genetic diversity and integrity of the native clam, also causing hybridization (i.e., gene flow from one species into the gene pool of another). Since *R. philippinarum* recently spread in a few important Mediterranean coastal areas, a combined approach based on morphological characteristics, length differences of two nuclear species-specific markers (ITS2, 5SrDNA) and the sequence of the mitochondrial gene cytochrome *c* oxidase subunit I (COI), was used to investigate the presence of hybrids in six Mediterranean wetlands (Sardinia, Italy). Eight individuals morphologically identified as *R. decussatus* were hybrids, having sequences specific to both *R. decussatus* and *R. philippinarum* in their nuclear DNA (ITS2 and 5SrDNA). Most of these individuals were found to be post-first generation (F1) hybrids indicating that F1-hybrids may be fertile. Secondly, to study the genetic diversity of *R. decussatus* in the Sardinian wetlands as well as in its whole distribution area, >380 new COI sequences from the eastern Atlantic Ocean and Mediterranean Sea were analysed along with those available from public databases. Mitochondrial COI data revealed variable haplotype and nucleotide diversities in different areas, which were not dependent on sample sizes. The aquaculture breeding activities and clam transplantation between different countries, along with the long pelagic larval dispersal and the commercial import of other bivalve species might have promoted gene exchange between different sites and thus higher diversity levels in a few wild populations. Our research, evaluating the genetic makeup of wild and hatchery stocks and clarifying the degree of hybridization, can contribute to develop further recommendations for conserving the genetic integrity of *R. decussatus*.

1. Introduction

In recent decades, aquaculture-based production has risen to meet increasing food demand as a result of global population growth and decline in fishery resources (Ottinger et al., 2016). Among the factors responsible for the fast expansion of aquaculture, the introduction and the use of non-native species plays one of the most key roles (Lin et al., 2015).

A crucial concern linked to the aquaculture of non-indigenous species is the hybridization (i.e., gene flow from one species into the gene pool of another) between the introduced and the indigenous species. Hybridization, indeed, can cause the loss of genetic diversity and the unique characteristics of the native species, reducing its ability to adapt to environmental changes or anthropogenic threats. For these reasons, hybridization is an important concern for conservation and management efforts (Allendorf et al., 2001; Olenin et al., 2010).

Evidence of hybridization driven by anthropogenic activities in marine resources has been reported for many species of bivalves and in particular for the family Veneridae (Huvet et al., 2004; Crego-Prieto et al., 2015). Since the 1970's, one of the main fished shellfish species in Europe is *Ruditapes decussatus* (Linnaeus 1758), which inhabits muddy-sandy sediments of estuaries, lagoons, and shallow waters along

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the North-Eastern (NE) Atlantic and Mediterranean coasts (Chiesa et al., 2016; Cordero et al., 2017). However, its slow growth rate and high susceptibility to environmental diseases, pollution, degradation of the habitat, pathogens, and important levels of exploitation lead to the decrease of population sizes (Aranguren et al., 2014; Cruz et al., 2020).

To face the increasing commercial demand of clams, the Manila clam *Ruditapes philippinarum* (Adams and Reeve, 1850), native to the Pacific coast of Asia, was deliberately introduced in Europe (Moura et al., 2017 and references therein). *Ruditapes philippinarum* was first introduced in 1972–1974 in France to cope with production problems and later in the UK for experimental trials (Flassch and Leborgne, 1992). Subsequently, clams of French and British origin were transported to Italy, Spain, and Norway (Breber, 1985; Flassch and Leborgne, 1992; Hopkins, 2002; Chiesa et al., 2017). The expansion of the species does not seem to have stopped, as shown by the finding of self-recruiting populations along the Atlantic coasts of Europe (e.g., in southern UK) and in the Mediterranean Sea (e.g., with records from areas located far away from exploitation grounds, such as Turkey; Jensen et al., 2004). After the introduction of the alien species, the annual global capture production of *R. decussatus* seriously decreased, from 17300 tonnes (t) in 1992 to 760 t in 2020 (Fisheries Global Information System - FAO-FGIS, 2021), probably due to its low adaptation to climatic changes, increasing anthropogenic impacts and the competition with *R. philippinarum.* Indeed, the latter has higher fecundity, a longer larval phase, broader optimal salinity (15–50 practical salinity unit [PSU] versus 20–40 PSU of *R. decussatus*) and temperature range (6–30 °C vs 15–23 °C of the *R. decussatus*; Rato et al., 2022). The expansion of the alien species has been further boosted by the development of hatcheries for artificial production that allow it to be easily spread in the wild. Clams, indeed, are often farmed using hatchery-raised seeds at high density, in intertidal beach areas or in shallow oyster ponds, and thus they could be accidently transported into other areas by currents (Jones et al., 1993).

In Europe, *R. philippinarum* became naturalized immediately after its introduction and has shown typical invasive characteristics (i.e., quick reproduction, large numbers of offspring that can rapidly colonize new habitats, broad environmental tolerance, competition for food and space with the native clam species; Chiesa et al., 2017). Furthermore, hybrids between the two species have been recently found in northern Spain, in areas of intensive aquaculture, both in wild samples, and in the seeds used for population supplementation (Hurtado et al., 2011; Habtemariam et al., 2015).

Within this framework, the island of Sardinia in the Western Mediterranean Sea is a peculiar case because i) the collection of clams is still typically done by hand or rakes on natural beds and ii) no form of supplementation aquaculture is currently performed except for small ongoing scientific projects based on the use of local seeds (i.e., Muravera lagoons; R. Demurtas, personal communication, February 4, 2023). These two points are expected to slow down or even hamper the invasion of the *R. philippinarum* and subsequent hybridization, making Sardinia a peculiar case study.

R. philippinarum was introduced in Sardinia in 1980s, but its occurrence has been reported to be rare and localized until a few years ago (i.e., Santa Gilla lagoon; Cottiglia and Masala Tagliasacchi, 1988a, 1988b; Mura et al., 2012; Sanna et al., 2017). During recent years, the populations of *R. philippinarum* exploded in a few Sardinian lagoons, and we cannot rule out the possibility that unrecorded releasing of allochthonous seeds (also known as alien; refers to species introduced into new areas through human activities) may have occurred. For example, in Santa Gilla, which is considered one of the most important wetlands in Sardinia (Fois et al., 2021), *R. philippinarum* became the major contributor to clam's landings from 2015 to 2018, with yields of >300 t in 2017, 60 times higher than those of *R. decussatus* (Official data from Consorzio Ittico Santa Gilla; data available upon request). Despite this, to date, no study has assessed whether, and to what extent, hybridization between the two species may occur in Sardinian lagoons.

Due to the plasticity of the shell morphology and the presence of both species on the same beds, it is difficult to distinguish hybrids from their parental species only on the basis of morphological characteristics (e.g., shell characteristics, degree of siphon separation).

Considering the current decline of the yields and the potential impact of the alien species on the genetic integrity of the native clams, there is an urgent need to perform novel studies to evaluate the presence of hybrids between the two species and assess the genetic diversity of *R. decussatus*.

To detect hybridization between the two species, fragment length analysis of nuclear genes has proved to be successful (Hurtado et al., 2011; Habtemariam et al., 2015). Whereas at the population-level, several metrics have been proposed for assessing the mitochondrial genetic diversity, but the most commonly reported are two: the haplotype diversity Hd (i.e., the probability that two randomly chosen haplotypes are different; Nei, 1987), and the nucleotide diversity π (i.e., the average number of nucleotide differences per site between two randomly chosen DNA sequences; Nei and Li, 1979).

In this study, we aim to i) evaluate the occurrence of hybridization and extent of genetic introgression of the *R. philippinarum* genes into the autochthonous (also known as native; refers to species indigenous to a specific ecosystem or geographical area and its presence is the result of only local natural evolution) *R. decussatus* in six Sardinian sites. This has been done using a combined approach based on morphological identification, species-specific length differences of two nuclear markers (ITS2 e 5SrDNA), and the sequence of the mitochondrial gene COI. Additionally, we aim to ii) study the genetic diversity of *R. decussatus* at small (Sardinia) and large spatial scales (from the North-Eastern Atlantic to the Marmara Sea) by analysing the polymorphism of the COI region. This has been done by including in the analysis >380 newly produced sequences and the homologous ones mined from public databases.

2. Materials and methods

2.1. Hybrids' identification in Sardinian lagoons

Ruditapes decussatus is a bivalve mollusc that inhabits muddy-sandy sediments along the Mediterranean and eastern Atlantic shallow coastal areas. The congeneric Manila clam (*R. philippinarum*), native to the western Pacific Ocean, lives in low intertidal and shallow subtidal waters, often in estuaries and lagoons, thus occupying similar ecological niches of *R. decussatus*. *Ruditapes decussatus* and *R. philippinarum* share a great number of morphological and anatomical characteristics and sometimes specimens can show intermediate characteristics (i.e., shell features). All these reasons and the presence of both species on the same beds may hinder their correct morphological identification. In order to solve this problem, a genetic approach has been proposed to identify individuals and determine if the specimens showing intermediate morphology were interspecific hybrids. PCR amplification of two nuclear (ITS2 and 5SrDNA) and a mitochondrial gene (cytochrome *c* oxidase subunit I, COI) proved to be useful for discriminating the two species and detecting hybrids (Hurtado et al., 2011; Habtemariam et al., 2015).

2.1.1. Sample collection

In this study, 174 clams from six Sardinian sites were randomly collected in 2020 via Scuba diving. (Marceddì [MAR], Is Benas [ISB], Olbia [OLB], Orosei [ORO], Santa Gilla [SGL], Santa Giusta [SGI]; see Table 1 for sampling details).

Firstly, in the laboratory, each clam was cleaned and opened, and the siphon was observed as described in Mahè et al. (2022). If the siphon was entirely separated, the individual was identified as *R. decussatus*; if not, it was determined to be *R. philippinarum* (Hurtado et al., 2011).

2.1.2. DNA extraction and PCR amplification

Foot tissues were sampled to limit the presence of different mitochondrial lineages, which is a consequence of mitochondrial doubly

Table 1
Estimates of the genetic diversity for the COI gene in 35 populations of *R. decussatus*. Sampling sites and their respective code used in the map (Fig. 1), number of sequences (NS), number of haplotypes (H), haplotype diversity (Hd), and nucleotide diversity (π).

Marine ecoregion	Geographic Area	Sampling site	Code	NS	H	Hd	π
Atlantic Ocean	Portugal	Azores	AZZ	15	1	0	0
	Brittany, France	Golfe de Morbihan	GMO	32	6	0.343	0.00086
	Nouvelle-Aquitaine, France	Arcachon	ARC	14	2	0.143	0.00033
	Galicia, Spain	Lombos do Ulla	LUL	33	2	0.061	0.00014
	Galicia, Spain	Mugardos	MUG	32	3	0.28	0.00067
	Algarve, Portugal	Milfontes	MLF	17	1	0	0
	Algarve, Portugal	Rio Formosa	FOR	43	5	0.298	0.00084
Western Mediterranean	Murcia, Spain	Mar Menor	MME	33	4	0.28	0.00096
	Catalonia, Spain	Ebro delta	EBR	33	7	0.426	0.00138
	Languedoc-Roussillon, France	Thau	THA	34	4	0.406	0.00201
	Tuscany, Italy	Orbetello	ORB	20	4	0.363	0.0009
	Corsica, France	Aleria	COR	24	3	0.163	0.00039
	Sardinia, Italy	Alghero	ALG	33	2	0.117	0.00027
	Sardinia, Italy	Is Benas	ISB	30	3	0.131	0.00031
	Sardinia, Italy	Santa Giusta	SGI	49	4	0.12	0.00047
	Sardinia, Italy	Marceddì	MAR	43	5	0.26	0.00074
	Sardinia, Italy	Sant'Antioco	SAN	12	3	0.318	0.00077
	Sardinia, Italy	Santa Gilla	SGL	74	7	0.377	0.00135
	Sardinia, Italy	Feraxi	FEX	13	7	0.795	0.00243
	Sardinia, Italy	Muravera	MUR	29	2	0.069	0.00016
	Sardinia, Italy	Sa Praia	SPR	19	4	0.38	0.00187
	Sardinia, Italy	Tortolì	TOR	51	5	0.221	0.00143
	Sardinia, Italy	Orosei	ORO	29	5	0.318	0.00079
	Sardinia, Italy	San Teodoro	STE	29	4	0.313	0.00076
	Sardinia, Italy	Olbia	OLB	71	9	0.309	0.0009
	Sardinia, Italy	Poltu Qualtu	POQ	20	1	0	0
	Sardinia, Italy	Lu Canaloni	LUC	20	2	0.1	0.00023
Central Mediterranean	Tunisia	Sfax	SFX	31	3	0.239	0.00057
	Sicily, Italy	Messina	MES	19	2	0.105	0.00024
Adriatic Sea	Veneto, Italy	Goro	GOR	10	3	0.511	0.00129
	Veneto, Italy	Venice Lagoon	VEN	46	9	0.479	0.00136
	Albania	Albania	ALB	24	2	0.083	0.00019
Eastern Mediterranean	Macedonia, Greece	Halkidiki	GRE	33	6	0.767	0.013
	Aegean region, Turkey	Izmir	IZM	31	7	0.576	0.00549
Marmara Sea	Marmara region, Turkey	Marmara Sea	MAB	20	1	0	0

uniparental inheritance (DUI) (Passamonti et al., 2003). Tissues were stored in 90% ethanol at −20 °C until the analysis (Chiesa et al., 2017).

DNA was extracted using the PureLink® Genomic DNA Kit following the manufacturer's instructions.

In order to amplify the ITS2 and 5SrDNA genes, a combination of specific primers for ITS2 (ITS3: 5'-GCATCGATGAAGAACGCAGC-3' and ITS4: 5'-TCCTCCGCTTATTGATATGC-3') and 5SrDNA (5SD: 5'-CAACGTGATATGGTCGTAGAC-3'; 5SR: 5'-AACACCGGTTCTCGTCC-GATC-3'), were used (Hurtado et al., 2011). PCR amplification started with an initial denaturation at 95 °C for 2 min, followed by 35 cycles for ITS2 and 30 cycles for 5S, of 95 °C for 30 s, 48 °C for ITS2 and 44 °C for 5S for 30 s, 72 °C for 35 s for ITS2 and 45 s for 5S, followed by a final extension at 72 °C for 5 min (Hurtado et al., 2011).

Furthermore, a fragment of the COI gene was amplified using specific primers (COIdecF: 5'-ATCTTTTCTTTCTGAGCTGG-3' and COIdecR:5'-AGTATTAAAGTTTCGGTCCG-3') designed by the authors. In detail, the amplification of the COI gene was initially realized using the universal primers LCO1490/HCO2198 (Folmer et al., 1994), but it resulted in nonspecific bands (data not shown). Therefore, a new pair of primers was designed starting from the COI sequence of *R. decussatus* (GenBank Accession Number DQ184830; Mikkelsen et al., 2006) using the program Oligo Explorer (http://www.genelink.com/) and default parameters. COI PCR amplification used a touchdown approach with an initial denaturation at 94 °C for 3 min, following by 2 cycles at 94 °C for 40 s, annealing at 58 °C for 40 s, extension at 72 °C for 1 min and a final extension at 72 °C for 4 min. Annealing temperature was lowered 2 °C every two cycles until 52 °C was reached, followed by 30 cycles with the 52 °C annealing temperature (Table S1). During the annealing step, the temperature is lowered to enable the DNA primers to attach to the template DNA. The chosen annealing temperature relies directly on length and composition of the primers; it depends on the strand-melting temperature of the primers and the desired specificity.

All PCR products were examined by electrophoresis on a 2% agarose gel for ITS2 and 5SrDNA genes, and on a 1.5% agarose gel for COI gene.

2.1.3. Fragment size analysis of nuclear genes

For ITS2 and 5SrDNA, clams were identified by visualizing the amplified PCR fragments in the agarose gel. The nuclear products (ITS2 and 5SrDNA) were loaded along with the Thermo Scientific GeneRuler DNA Ladder Mix and allowed to run for 60–80 min at 100 V. The fragments were visualized under the UV light and photographed using the UVIDOC HD6 imaging system (UVITEC Cambridge). PCR fragments of ITS2 and 5SrDNA with lengths of 482 and 593 bp, respectively, are specific for *R. decussatus*; whereas the specific amplification fragments of *R. philippinarum* are 565 bp long and 526 bp for ITS2 and 5SrDNA, respectively (Hurtado et al., 2011). Individuals with both PCR products (482 bp/565 bp and/or 526 bp/593 bp for ITS2 and 5SrDNA, respectively) were classified as hybrids between the two species. When two fragments of different length for both markers were observed using gel electrophoresis, the individual was classified as a 'first-generation hybrid' (F1), having half of their nuclear genes from the maternal species and the other half from the paternal species. When a hybrid pattern for only one of the markers was observed, the offspring was regarded as a 'post-F1', that is a F1 hybrid that has reproduced with one of its parent species or with another F1 hybrid (Allendorf et al., 2001).

2.1.4. Sequencing of COI

COI PCR products were checked by running gel electrophoresis for 25 min at 100 V, quantified using NanoDrop (Thermo ScientificTM NanoDropTM OneC) and Sanger-sequenced at Macrogen Inc., The Netherlands. Raw chromatograms were visually checked and trimmed at both ends using MEGA v.10 (Kumar et al., 2018). Then, the NCBI

BLAST algorithm (Altschul et al., 1990) was used to identify the species represented by COI sequences. For species identification, the top hits (i.e., the best matches with the highest bit scores and percent identities) showing more than 98% identity were selected.

2.2. Genetic diversity analysis of R. decussatus

2.2.1. Sample collection

An additional 207 individuals, morphologically identified as *R. decussatus*, were randomly collected in 2008 by SCUBA diving at 12 sites located in the North-Eastern (NE) Atlantic Ocean (i.e., French Atlantic coast), the Western (W) Mediterranean (i.e., Sardinia, Spanish and French coasts), and the Central (C) Mediterranean (i.e., Adriatic Sea; Fig. 1; Cannas and Cau, unpublished data; Table S2).

2.3. DNA extraction, PCR amplification and sequencing of COI

DNA extractions, PCR amplifications and sequencing of COI have been performed as described above (Section 2.1, DNA extraction and PCR amplification).

2.3.1. Sequences' analyses

All the COI sequences (n = 381), obtained from 2008 and 2020 sampling activities, were aligned using the Clustal W multiple sequence alignment algorithm (Thompson et al., 1994) implemented in MEGA v10.

A more comprehensive dataset was built by supplementing our COI sequences with homologous ones mined from the GenBank and BOLD databases (see Table S3). The final alignment consisted of 1066 sequences from 35 sites, covering the NE Atlantic Ocean, the W, C, and Eastern (E) Mediterranean Sea and the Sea of Marmara (Table 1). Sites with only one sequence were not included in the analyses, as well as locations with published haplotypes but without specification of their frequencies by site.

In order to estimate the genetic diversity of populations, polymorphic sites (S), haplotypes (i.e., a specific combination of genetic variants), number of haplotypes (Ha), haplotype diversity (Hd) and nucleotide diversity (π) were obtained using DNAsp v. 6.12 (Rozas et al., 2017).

The relationships among haplotypes were investigated by the TCS method (haplotype network estimation also known as Statistical Parsimony, named after Templeton et al., 1992, and fully described in Clement et al., 2004), using the PopART v 1.7 software (Leigh and Bryant, 2015). In the network, circles represent the different haplotypes, and their size is proportional to the number of sequences that compose them. The circles are connected by segments, interspersed with dashes which indicate mutations (i.e., a mutation is a change of a base pair between two nucleic acid sequences).

In order to measure genetic differentiation between different populations, pairwise Fixation Indices (Φ_{st}) were calculated using the software Arlequin v3.5 (Excoffier and Lischer, 2010) using the ideal evolutionary model for our dataset identified in MEGA v10 (i.e., Tamura 3 parameter, TN93, for both the Sardinian and the whole dataset). In pairwise comparisons, the probability values were adjusted for multiple

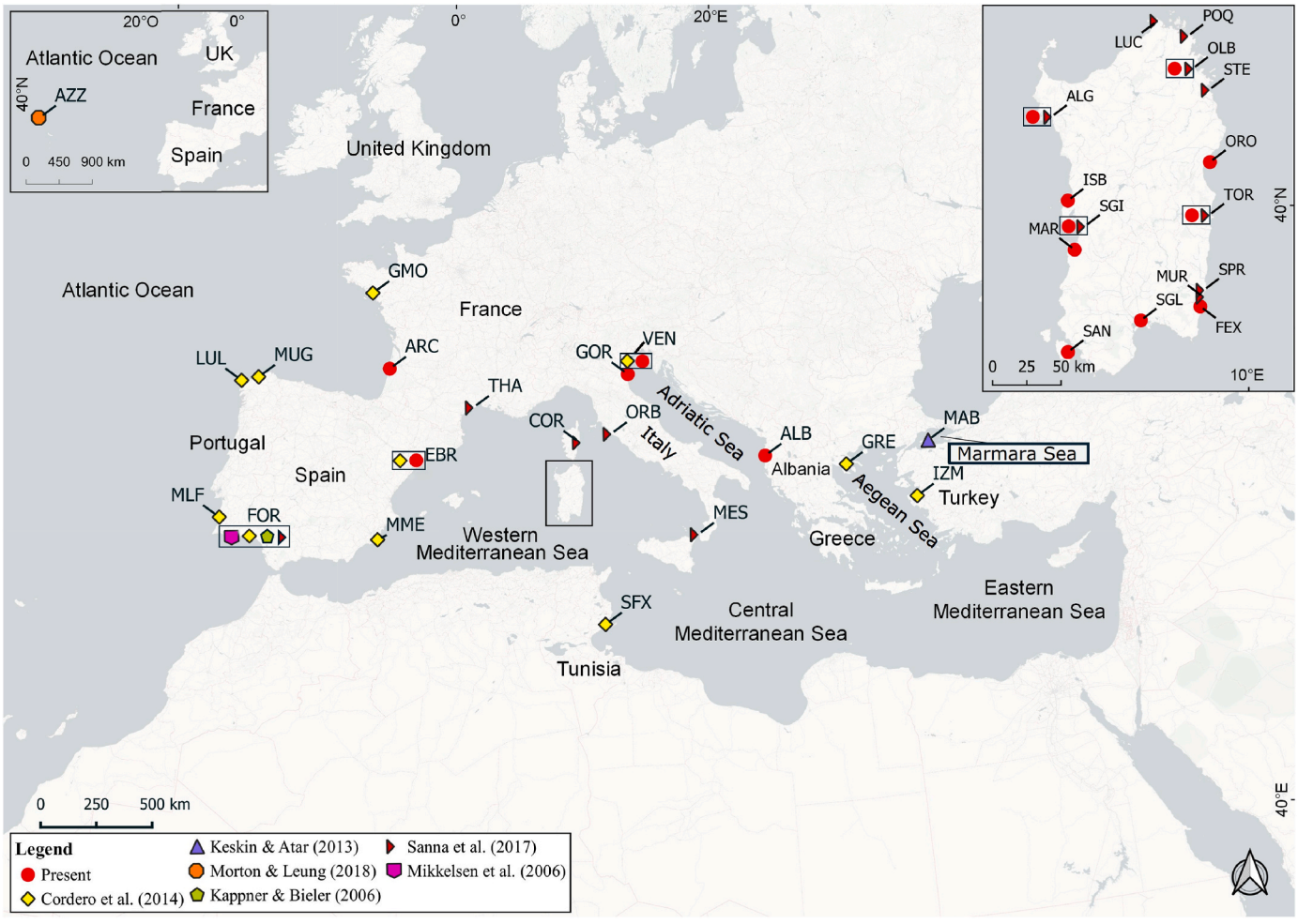

Fig. 1. Map of the sampling sites. The map shows the geographical origin of the *Ruditapes decussatus* sequences produced in the present study along with those from previous studies. All the localities are labelled in Table 1.

tests using the Bonferroni method (Dunn, 1961) as included in the software Myriads v1.1 (Carvajal-Rodríguez, 2018). The obtained Φ_{ST} matrix was also graphically represented using R Studio packages ("XML","corrplot","magrittr" and "dplyr").

Furthermore, the occurrence of population structuring was investigated with the Analysis of Molecular Variance (AMOVA) in Arlequin. The significance of fixation indices values was computed by a non-parametric permutation procedure with 10,000 iterations. Considering our sampling effort in Sardinia, the program SAMOVA 2.0 (Dupanloup et al., 2002) was also used to define groups of populations that are homogeneous in this area and maximally differentiated from each other.

Finally, the software Bayesian Analysis of Population Structure (BAPS) v.6.0 (Cheng et al., 2013) was employed to identify differentiated genetic groups (haplogroups or 'clusters'). BAPS was run using the method of "clustering for linked loci" and a codon model, with five independent runs, and setting the maximum number of clusters (K) to 12. The BAPS haplogroups were visualized as pie charts on a map using GENGIS 2.5.3 (Parks et al., 2013). The European coastline shapefile used to produce the maps was downloaded from the EEA website (http://www.eea.europa.eu/data-and-maps/data/eea-coastline-for-analysis-1/gis-data/europe-coastline-shapefile).

3. Results

3.1. Detection of hybrids

Siphons were fully separated in all of the 174 clams collected in 2020, thus all the samples used for hybrids' analysis were morphologically identified as *R. decussatus* (Table 2). We acknowledge the presence of 39 individuals (10 collected from ORO, 15 from OLB and 14 from SGL) which exhibited fully separated syphons, but dark colouration (see Fig. 2; Table S4).

Both PCR-based diagnostic genetic markers produced PCR products of the expected size. Classification based on the nuclear markers (ITS2 and 5SrDNA) assigned all samples to *R. decussatus* (482 bp of ITS2 and 593 bp of 5SrDNA; Table 2; Fig. 3A and B), except for 8 individuals (Table 2). One specimen from Marceddì (M8) was classified as an F1 hybrid showing signals of 482 bp and 565 bp for the ITS2 gene and of 593 bp and 526 bp for 5SrDNA. Three individuals from Marceddì (M1, M6, M7) and four from Is Benas (IB13, IB14, IB15, IB19) were post-F1 hybrid with only one band for the ITS2 (Fig. 3A) gene and two signals for 5SrDNA (Fig. 3B; Table 2). Details about the number of potential hybrids are reported in Table 2.

Furthermore, the COI sequences were compared to the reference sequences available in GenBank. The NCBI-BLAST algorithm results showed that all our sequences were identical to those deposited for *R. decussatus* (100% identical nucleotide matches and 0.0 E-values).

Table 2
Summary of the hybrids found after morphological and genetic assessment of *R. decussatus* individuals sampled in six Sardinian sites. RD: *Ruditapes decussatus;* RD*: *R. decussatus* with separate siphons, but dark coloured; Hyb: hybrid; IE: introgression event (as proposed by Habtemariam et al., 2015).

Number of cases	Sampling site	Species identification criteria			
		Morphology	Genetics		
			COI	ITS2	5S
127	SGI MAR ISB ORO OLB SGL	RD	RD	**RD**	**RD**
1	MAR	RD	RD	**Hyb (IE)**	**Hyb (IE)**
7	MAR	RD	RD	RD	**Hyb (IE)**
10	ORO	RD*	RD	RD	RD
15	OLB	RD*	RD	RD	RD
14	SGL	RD*	RD	RD	RD

3.2. Genetic diversity of R. decussatus

Each of the individuals sampled in 2008 and 2020 has been successfully amplified and sequenced. The alignment of the newly produced 381 COI sequences of *R. decussatus* consisted of 432 bp and included 27 haplotypes which were made available in GenBank under the following accession numbers: OR742137-OR742163 (Table S2). Most of the new sequences were from Sardinian individuals (n = 298) in 10 wetlands, six of which had never been investigated before. A total of 14 new haplotypes were discovered, seven of which were in Sardinian sites.

Combining our sequences with the public data, we obtained a final alignment 432bp long and a total of 45 haplotypes. Temporal replicates (i.e., sequences obtained from individuals sampled in the same location in different years) were merged, being not statistically significant different (see details in Table S5 and Fig. S1). Overall, 15 out of 35 sites included in the analyses were localized in Sardinia, and about 49% of sequences were of Sardinian origin.

In Sardinia, COI sequences showed variable values of both haplotype diversity (Hd; ranging from 0 to 0.795) and nucleotide diversity (π; ranging from 0 to 0.00243). Feraxi is the site showing the highest values (Hd = 0.795; π = 0.00243), whereas the lowest ones were found in Poltu Qualtu (Hd and π = 0) (Table 1). Similarly, in the whole dataset, haplotype diversity (Hd) and nucleotide diversity (π) showed different values, with the lowest ones (Hd and π = 0) found in the Azores, Milfontes, Poltu Qualtu and Marmara Sea) and the highest ones (Hd = 0.795; π = 0.013) found in Feraxi and Halkidiki, respectively (Table 1, Fig. S2).

The TCS network revealed a star-like shape with the occurrence of a central common haplotype (Hap_1; Fig. 4) which was shared among 852 individuals coming from all the sampling locations except for the Sea of Marmara. Overall, we found a high level of haplotype sharing among the populations dwelling in the Central and Western Mediterranean Sea and NE Atlantic Ocean. Most of the haplotypes diverged from Hap_1 by one mutation step, except for Hap_45 (composed by 20 individuals from the Sea of Marmara) which diverged for three mutations. The group of haplotypes from the Aegean Sea (Hap_35, Hap_38–44) diverged from Hap_1 by nine mutational steps. Among the Sardinian samples, Hap_35 was shared by 1 individual from Tortolì and others from the Aegean Sea (1 and 13 individuals from Turkey and Greece, respectively). In addition, there were 29 private haplotypes (i.e., haplotypes composed by sequences coming from the same area): eight in the NE Atlantic Ocean, seven each in Sardinia and the Aegean Sea, five in the Adriatic, and one each in the Western Mediterranean and the Sea of Marmara.

The amount of genetic differentiation among populations was measured using fixation indices. Pairwise Φ_{ST} values indicated a significant ($p < 0.001$) genetic divergence between the Sea of Marmara (MAB), Turkey (IZM), Greece (GRE) and other areas in all the comparisons. All the other pairwise Φ_{ST} values were not significant after Bonferroni correction with the only exceptions of FEX vs MLF and POQ, OLB vs THA, and VEN vs SGI (Fig. 5).

This genetic differentiation was further supported by the one-way AMOVA analyses which revealed the presence of 58% genetic variation among populations (Φ_{ST} = 0.57879, $p < 0.001$; Table 3). The component of genetic variation due to differences among groups was the highest (89.83%; Φ_{CT} = 0.89830; $p < 0.001$; Table 3) when the samples from Marmara Sea, Greece, Turkey, and other populations were considered four separated groups. Excluding eastern Mediterranean and the Marmara Sea, the percentage of total variation decreased to 1.82% or even to 1.11% when considering only the Sardinian samples. After the exclusion of the most eastern locations, we found a weak but significant genetic structure when the populations were divided in five groups (Φ_{CT} = 0.00715, $p < 0.05$; Table 3): the Atlantic locations, the Western Mediterranean, the central Mediterranean, the Adriatic and Sardinian populations.

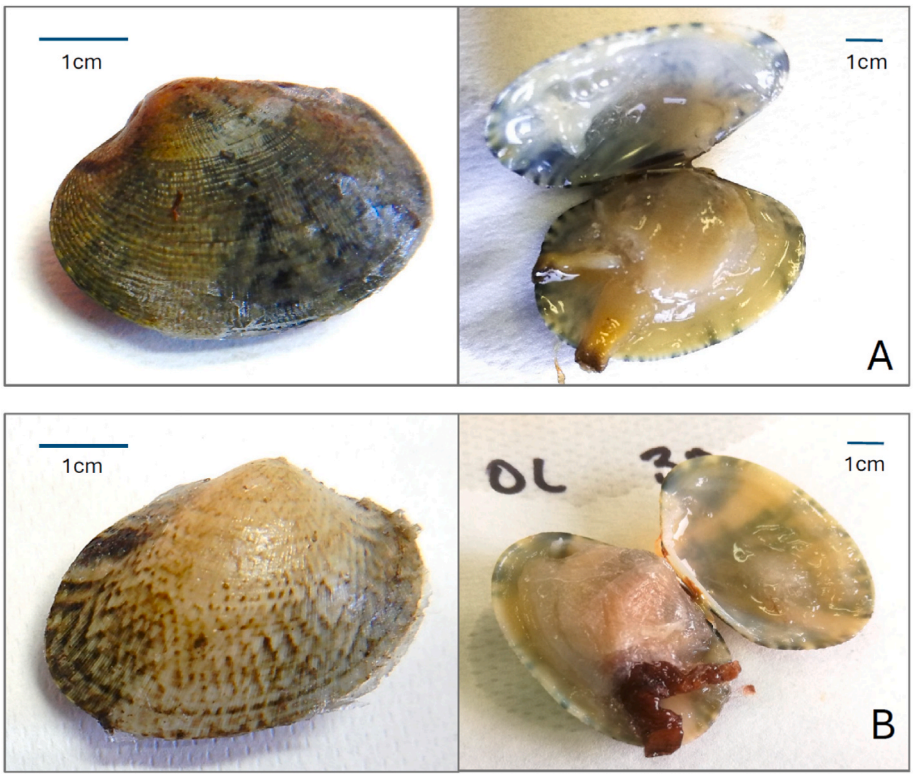

Fig. 2. Morphological features of shells and mollusc bodies of *R. decussatus*. (A) Individual of *R. decussatus* with typical separate whitish siphons. (B) Individual of *R. decussatus* with separate dark-coloured ones. The scale bar represents 1 cm.

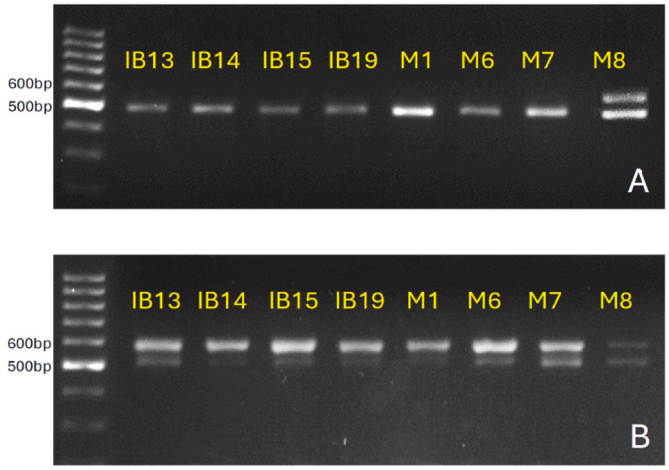

Fig. 3. Genetic assessments for nuclear genes of the sampled *R. decussatus* in this study. The analyses revealed the presence of 8 hybrids in Sardinian populations having sequences specific to both *R. decussatus* and *R. philippinarum*. (A) Amplified PCR products from ITS2 showing the single characteristic band of *R. decussatus* (482 bp) and the double bands of the hybrids for the M8 individual (482 bp and 565 bp). (B) Amplified PCR products from 5SrDNA showing the double bands of the hybrids for eight individuals (593 bp and 526 bp).

No significant structuring was observed dividing the Sardinian populations geographically in four groups (west/east/south/north: $\Phi_{CT} = 0.00108$, $p > 0.05$). However, testing different configurations with SAMOVA (from K = 2 to K = 14), significant differentiation was found for all structures except K = 2, with the highest significant Φ_{CT} for K = 3 and K = 14 (Table 3).

BAPS retrieved six as the most probable number of genetic clusters, with K6 (pink in Fig. 6) grouping individuals only from MAB. The Aegean populations were mostly assigned to K3 (orange in Fig. 6); outside this area, K3 was found only in one individual from TOR. Cluster K2 (violet in Fig. 6) was the most common and widespread, found everywhere except for MAB. The populations sampled in the Atlantic Ocean had high average population membership probabilities to K2 (>90%), and variable minor frequencies to K4 (yellow in Fig. 6). Individuals from the Central and Western Mediterranean sites were mostly assigned to K2, with the remaining membership probabilities distributed among K1 (green in Fig. 6), K4 and K5 (light blue in Fig. 6). K1 and K5 slightly differed in their distribution: K1 was found, with decreasing frequencies, from the Aegean and the Adriatic to the Mediterranean Spanish coasts, while K5 was more abundant in the western locations.

4. Discussion

4.1. Evidence of hybridization in Sardinian samples

The morphological identification of specimens followed by their molecular classification based on a suite of genetic markers (ITS2, 5S rDNA and COI) demonstrated, for the first time, the occurrence of hybridization between *R. decussatus* and *R. philippinarum* in a few Sardinian lagoons (Western Mediterranean Sea). Indeed, the morphological identity coincided with the 'genetic' one in most of the sampled clams, except for 4.5% individuals which were morphologically identified as *R. decussatus* but showed genetic signals specific for both species. Introgression events (based on ITS2 and 5SrDNA) were detected in specimens with pure phenotypes, where there was no occurrence of interspecific morphological characteristics. Our study confirmed the usefulness of both nuclear markers, ITS2 and 5SrDNA, to detect hybrids (Hurtado et al., 2011; Habtemariam et al., 2015), conversely to what has been recently reported by Markaide et al. (2021) which encountered numerous problems related to 5SrDNA such as the lack of amplification and extra bands. In particular, 5SrDNA is organized in tandemly-arranged repeat units that consist of a highly conserved 120bp

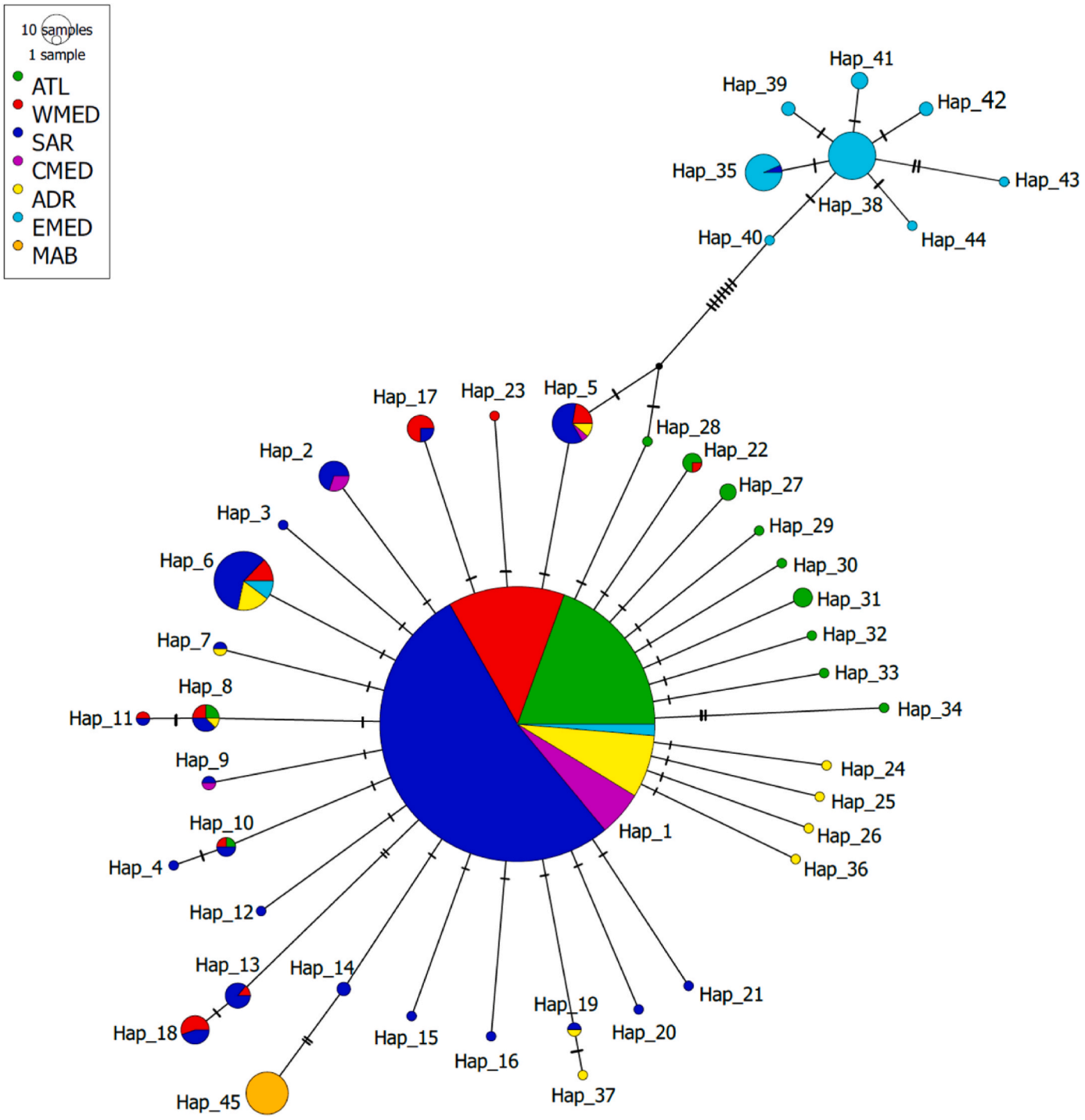

Fig. 4. TCS network analysis of *R. decussatus* populations. The analyses revealed the presence of two groups of haplotypes, with the biggest one including individuals from the NE Atlantic Ocean and the C Mediterranean Sea and the smaller one composed by specimens from the Aegean Sea. Each circle represents a unique haplotype with the circle size proportional to the frequency of each haplotype; circles are connected by segments, interspersed with dashes which indicate the number of mutations. The small black dot on the node represents the hypothetical missing/unsampled haplotype that is necessary for a fully connected network.

transcribing sequence and variable non-transcribed spacers (NTS), not necessarily identical within an individual. Variations in NTS, due to insertions-deletions, mini-repeats and pseudo genes have successfully been used as markers in evolutionary studies (Alves-Costa et al., 2006; He et al., 2012). The analysis of the 5SrDNA (both by fluorescence in situ hybridization and PCR) has proven to be effective to detect hybrids in different animal species (e.g., fish; Zhang et al., 2015) including clams (Hurtado et al., 2011; Habtemariam et al., 2015). Indeed, Hurtado et al. (2011) demonstrated the usefulness of the size-based analyses of 5SrDNA PCR product to detect hybridization between *R. decussatus* and *R. philippinarum* at a low cost and in a short time frame. The application of this method to the study of 328 specimens of carpet shell clams clearly demonstrated the existence of hybrids: some of the individuals morphologically identified as *R. decussatus* show signals specific for both species. The hybrid nature of those individuals was further highlighted by the chromosomal location of these sequences on the chromosomes of the hybrids. More recently Habtemariam et al. (2015) confirmed the utility of the size-based analyses of 5SrDNA PCR product to detect the

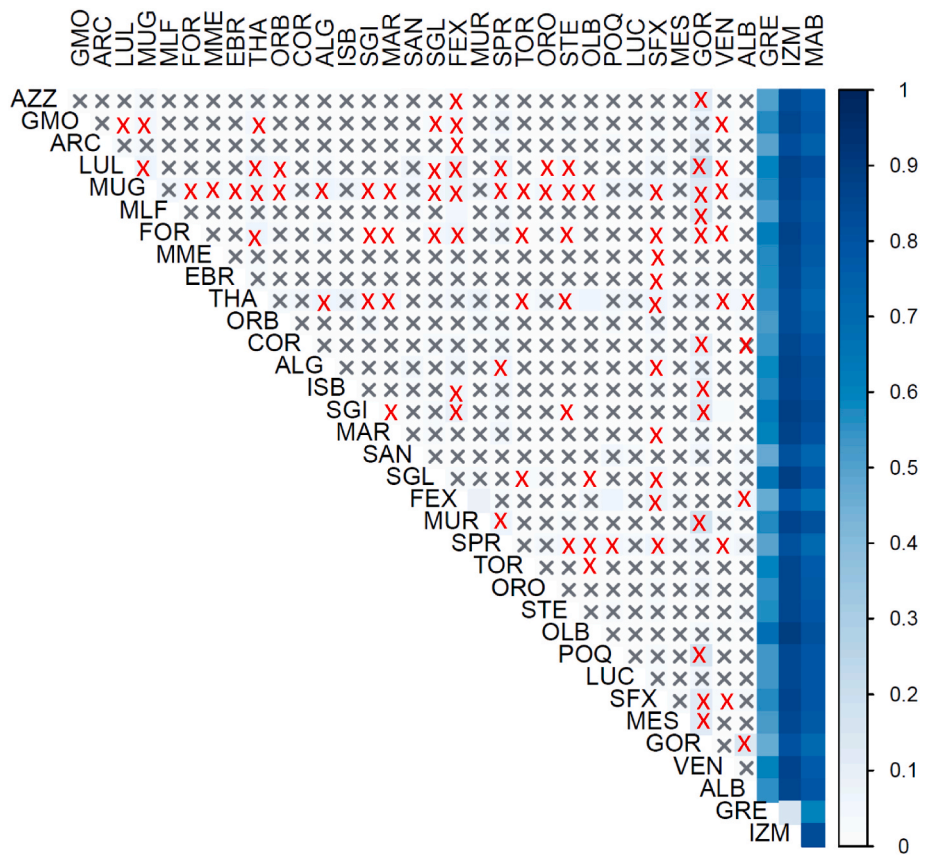

Fig. 5. Pairwise Fixation Indices (Φ_{ST}) estimated among populations of *R. decussatus*. The figure shows a significant ($p < 0.001$) genetic divergence between the Sea of Marmara (MAB), Turkey (IZM), Greece (GRE) and other areas in all the comparisons. The colour gradient in the crossing area of pairwise populations indicates the genetic distance: dark blue = higher differentiation; light blue = lower differentiation. Not significant values are indicated with "x". Red "x" indicates not significant values after correction for multiple testing (Bonferroni method). Codes are explained in Table 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

occurrence of hybridization between the two species in Asturian samples.

Among the hybrids found in our study, seven are post-F1 generation indicating that hybrids of the F1 generation could be fertile. The genetic approach proved to be a powerful tool to reliably identify hybrids, also considering that the morphological identification of post-F1 hybrids is particularly difficult since they are increasingly similar to the recurrent parent species in successive generations (Allendorf et al., 2001). In Sardinia, all hybrid individuals had mitochondrial sequences of *R. decussatus*, matrilineally transmitted, thus suggesting that the hybridization involved females *R. decussatus* and males *R. philippinarum*.

It is noteworthy to highlight that 22% of individuals, morphologically and genetically assigned to *R. decussatus,* showed separate siphons but dark colouration which is not a typical feature of the grooved carpet shell, where siphons are commonly whitish. It is well known that morphological differences within a species can be due to genetic mutations, environmental effects (i.e., phenotypic plasticity) or, more generally, a combination of both (Zieritz et al., 2010; Stamp and Hadfield, 2020). The phenotypic plasticity of the *Ruditapes* found in this study, i.e., the different colour of the siphons, could be influenced by several ecological factors, such as the type of sediment, tidal cycle, hydrodynamic force (e.g., currents or water turbulence), nutritional condition and salinity (Costa et al., 2008; Watanabe and Katayama, 2010; Caill-Milly et al., 2014). However, the lack of environmental data did not allow us to further investigate which factor could drive the different morphology found.

The presence of hybrids among species of the genus *Ruditapes* is not new to science, even if it has never been reported so far in the Mediterranean Sea. As outlined above, Hurtado et al. (2011) found hybrids between *R. decussatus* and *R. philippinarum* in North-western Spain. Kitada et al. (2013) also demonstrated the presence of hybrids between *R. philippinarum* and other *Ruditapes* species in Japan. Habtemariam et al. (2015) evidenced species misidentification and genetic introgression from the non-indigenous *R. philippinarum* into wild *R. decussatus* samples and in the aquaculture seeds used for population supplementation in two estuaries of the northern Iberian Peninsula. Conversely, hybrids were not found in the recent experiment performed by Markaide et al. (2021) in mixed cultures of *R. decussatus* and *R. philippinarum*, suggesting that hybridization may be infrequent, or the hybrid offspring may have extremely low survival rate. The existence of hybrids between two species belonging to the same genus was described for other marine bivalves in different parts of the world, as between the marine mussels *Mytilus edulis* and *Mytilus galloprovincialis* along the Atlantic coast of Europe (Bierne et al., 2003) and among *M. galloprovincialis* and *Mytilus trossulus* along the American Pacific coasts (Crego-Prieto et al., 2015). Hybridization has been also reported between *Pinctada fucata* and *Pinctada maculata* among wild pearl oysters collected for private pearl farming in Japan (Masaoka and Kobayashi, 2005) as well as between *Crassostrea gigas* and *Crassostrea angulata* along the European Atlantic and Asian Pacific coasts (Huvet et al., 2004).

The presence of hybridization had a key role in the evolution of plants and animals, but the growing presence of hybrids in nature can have negative impacts on native species with serious consequences on marine biodiversity and ecosystem functioning (Allendorf et al., 2001; Clavero and García-Berthou, 2005; Gilman and Behm, 2011). Further studies are needed in order to deepen our knowledge on the anthropogenic practices that can boost the presence of hybrids, their relative fitness and the evolutionary forces affecting their faith. Furthermore, the

Table 3
Analysis of molecular variance (AMOVA) of *R. decussatus* populations. The groups were defined according to geographical hypothesis for the whole dataset and the SAMOVA analyses for the Sardinian dataset. *** = pvalue <0.001; ** = pvalue <0.01; * = pvalue <0.05; ns = not significant.

Source of variation	Percentage of variation	Fixation Index	p-value
One group			
Among populations	57.88	$\Phi_{ST} = 0.57879$	***
Within populations	42.12		
Four groups (MAB/IZM/GRE/others)			
Among groups	89.83	$\Phi_{CT} = 0.89830$	***
Among populations	−0.02	$\Phi_{SC} = -0.00246$	***
Within populations	10.19	$\Phi_{ST} = 0.89805$	***
One group (excluding MAB + IZM + GRE)			
Among populations	1.82	$\Phi_{ST} = 0.01824$	***
Within populations	98.18		
Five groups (excluding MAB + IZM + GRE) (AZZ + GMO + ARC + LUL + MUG + MLF + FOR/MME + EBR + THA + ORB + COR/GOR + VEN + ALB/MES + SFX/MUR + LUC + POQ + STE + ORO + TOR + SPR + OLB + FEX + SGL + ALG + ISB + SGI + MAR + SAN)			
Among groups	0.72	$\Phi_{CT} = 0.00715$	*
Among populations	1.34	$\Phi_{SC} = 0.02051$	**
Within populations	97.95	$\Phi_{ST} = 0.01345$	***
One group (only Sardinia)			
Among populations	1.11	$\Phi_{ST} = 0.01111$	*
Within populations	98.89		
Four groups Sardinia (ALG + ISB + SGI/MAR + SAN + SGL + SPR + FEX/MUR + TOR + ORO + STE + OLB/POQ + LUC)			
Among groups	0.11	$\Phi_{CT} = 0.0108$	ns
Among populations	1.03	$\Phi_{SC} = -0.01030$	ns
Within populations	98.86	$\Phi_{ST} = 0.01137$	*
Three groups Sardinia (SPR/FEX/ALG + ISB + SGI + MAR + SAN + SGL + MUR + TOR + ORO + STE + OLB + POQ + LUC)			
Among groups	5.71	$\Phi_{CT} = 0.05713$	*
Among populations	0.32	$\Phi_{SC} = 0.00344$	ns
Within populations	93.96	$\Phi_{ST} = 0.06038$	*
Fourteen groups Sardinia (SPR/FEX/SGL/ALG/ISB/SGI/MAR/SAN/MUR/TOR/ORO/STE/OLB/POQ + LUC)			
Among groups	5.4	$\Phi_{CT} = 0.05397$	**
Among populations	−4.27	$\Phi_{SC} = -0.04513$	ns
Within populations	98.87	$\Phi_{ST} = 0.01128$	*

development of additional genomic tools could be helpful to investigate the existence and the type of genetic barriers between *R. decussatus* and *R. philippinarum*, as for example recently performed for divergent lineages of the Pacific cupped oyster, *C. gigas* and *C. angulata* (Gagnaire et al., 2018). Both species were recently co-introduced in Europe offering a unique opportunity to test how genetic differentiation is maintained under new environmental and demographic conditions.

4.2. Genetic diversity of R. decussatus

Studies performed on different aquatic organisms evidenced that the aquaculture of alien species may provoke the decline of the native ones and the alteration of their genetic diversity and integrity (see e.g., Cambray, 2003; Arismendi et al., 2009; Pilliod et al., 2010). In several disturbed sites of Europe, such as the Arcachon Bay (France) and the lagoon of Venice (Italy), the alien *R. philippinarum* has already supplanted the native *R. decussatus* by occupying almost entirely its ecological niche (Pranovi et al., 2006; Juanes et al., 2012; Bidegain and Juanes, 2013). Considering the increasing spread of *R. philippinarum* in different European coastal areas including Sardinia, here we aim to analyse the genetic diversity of *R. decussatus* both at small spatial scale (in Sardinia) and in its whole distributional range.

In our study, the analyses of the diversity indices evidenced variable haplotype and nucleotide diversities for all the investigated sites, without any correlation with the sample size (Fig. S2). Indeed, the highest values of haplotype and nucleotide diversity were found in Feraxi, Sa Praia, and Goro which were amongst the sites with the smallest sample size (Table 1). These metrics can be influenced by a variety of biological factors and methodological issues; in particular, the under-sampling (sampling of small numbers of individuals) can bias Hd and π estimates, both inflating or deflating them (Goodall-Copestake et al., 2012 and references therein). The high values recorded in our study can be explained by the small sample sizes used in the investigated sites, even if it has been described that samples of >5 specimens could be sufficient to discriminate extremes of high and low population-level diversity (Goodall-Copestake et al., 2012). However, in order to have a more accurate result, the use of >25 individuals is highly recommended (Goodall-Copestake et al., 2012).

An alternative explanation for the increased genetic diversity in specific sites is aquaculture restocking activities with seeds from diverse/distant populations. As far as we know, the use of allochthonous seeds to sustain *R. decussatus* production is not allowed in Sardinia, but we cannot exclude unauthorised or unrecorded releasing of them, or the effect of past experiments. In fact, a few decades ago, seeds from Greek and/or Turkish populations of *R. decussatus* were sporadically used for experimental restocking funded by the Autonomous Region of Sardinia (Sanna et al., 2017). Of course, other reasons could also be taken into consideration to explain such high levels of diversity like the "carrier role" of other bivalve species (e.g., *Mytilus galloprovincialis*) usually imported in Sardinia from other geographic areas, such as the Adriatic Sea or Greece, to sustain aquaculture productivity. Among the areas approved by the Sardinian authority for relaying live mussels imported from abroad, there are some of the most productive lagoons involved in these imports, where indeed the clam genetic diversity was high (i.e., SGL, OLB). In the congeneric *R. philippinarum*, restocking or even

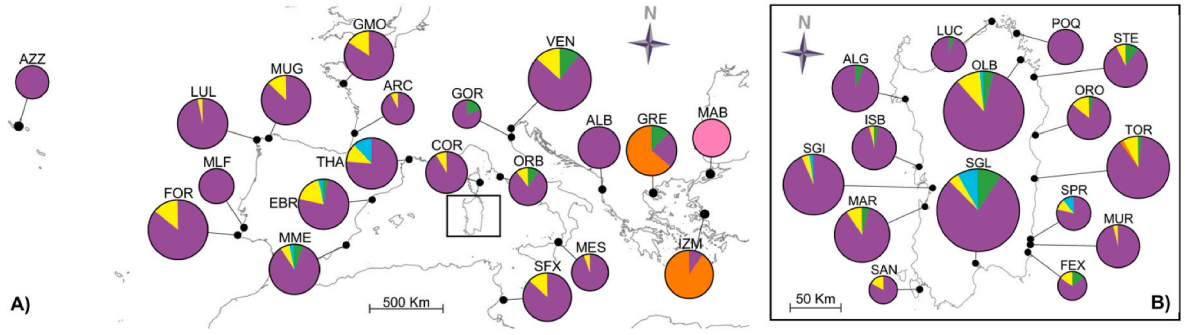

Fig. 6. Geographical distribution of six haplogroups of *R. decussatus* identified by BAPS analyses based on COI sequences 432 bp long. In (A) all of the sites are shown except for Sardinian lagoons which are depicted in the enlarged inset in (B). The analyses confirmed the striking differentiation of the eastern populations of the Aegean and Marmara seas from all the other ones. The pies define the frequencies of each haplogroup in each location. Haplogroups are identified by distinct colours: K1 = green, K2 = violet, K3 = orange, K4 = yellow, K5 = light blue, K6 = pink. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

transplants from distant areas, along with peculiar coastal flow and the long planktonic larval duration of the species, was recognized as a valid mechanism that promotes gene exchange among populations and thus favours the increase of genetic diversity (Cordero et al., 2017; Tan et al., 2020).

In the whole dataset, higher values of nucleotide and haplotype diversity were registered in the eastern Mediterranean (Hd $\geq$ 0.55: GRE, IZM) and the Adriatic Sea (Hd $\geq$ 0.45: VEN, GOR), while lower values (Hd $>$ 0.35) were found in the western Mediterranean (EBR, THA, ORB). When we add in the diversity comparison, sequences coming from Tunisia and northern Spain (not included in the previous analyses because of the lack of haplotype frequencies), we found again different values of haplotype and nucleotide diversity (Fig. S2; Gharbi et al., 2015; Habtemariam et al., 2015). The highest haplotype diversity values were recorded in Tunisia (i.e., Kerkennah Island) and in Spain (Cordero et al., 2014; Sanna et al., 2017; present study), where a higher genetic diversity was measured in the hatchery stock (Eo-H) in respect to the wild populations (Vill-W and Eo-W; Habtemariam et al., 2015). Captive breeding of shellfish species with high fecundity is known to produce a reduction of genetic variability in cultured stocks compared to their wild conspecifics, as a small number of parents are typically employed in the production of a large number of offspring (Frost et al., 2006). However, in this case, stock mixtures could have contributed to the increasing diversity in the hatchery stock (Habtemariam et al., 2015).

On the contrary, apart from POQ, the lowest values of haplotype diversity (Hd = 0) were found in a few sites (i.e., among the others, the most eastern site MAB), followed by moderate-low haplotype diversity values (0.1<Hd $<$ 0.3) recorded in the Sardinian western locations (ALG, ISB, SGI, MAR) and in most of the Atlantic sites (mean Hd = 0.16). According to previous studies involving *R. philippinarum* populations, low levels of haplotype and nucleotide diversities suggest that a population bottleneck or an establishment effect by a single, small population (e.g., from a hatchery restocking), has recently occurred (Wei et al., 2023). Overexploitation and habitat degradation might have eroded the allelic diversity of the wild clam populations in some areas. The star-shape haplotype network of COI with one geographically widespread haplotype is indeed commonly interpreted as a sign of a recent expansion following population bottlenecks (Xu et al., 2012).

The network analysis revealed the presence of at least two groups of haplotypes, with the central, biggest one including individuals from the NE Atlantic Ocean and the C Mediterranean Sea, and the smaller one composed of populations from the Aegean Sea. The AMOVA and BAPS analyses confirmed the striking differentiation of the eastern populations from the Aegean and Marmara seas. A genetic break in the Aegean Sea could be explained by the presence of land barriers that limited the water flow between the Aegean and the Mediterranean Sea during the sea level drops that occurred in the Pleistocene (Perissoratis and Conispoliatis, 2003). Furthermore, populations dwelling in the Sea of Marmara genetically diverged from all the others probably due to its peculiar geographic position, making clams from this area a semi-isolated population (Demir, 2003). Regarding the genetic differentiation at the mitochondrial level between Atlantic and Western Mediterranean basin, it was almost inexistent (Cordero et al., 2014; Sanna et al., 2017; present study). However, a clear genetic change in *R. decussatus* populations across the Atlantic-Western Mediterranean boundary was found using nuclear markers (Cordero et al., 2014). Despite a wealth of historical and oceanographic data, the Atlantic-Mediterranean transition remains controversial as there are discordant results regarding the biogeographical separation between the two areas.

At the local scale, in Sardinia, the differentiation among all sites was small but significant (overall Φ_{ST} = 0.01111, p $<$ 0.05; 14-group structure Φ_{CT} = 0.05397, p $<$ 0.01). In general, the western (ALG, ISB, SGI) and northern sites (POQ and LUC) were characterized by lower levels of diversity, that in turn peaked in FEX, SPR and SGL. Intermediate values were recorded in the eastern locations (TOR, ORO, STE, OLB; Table 1, Fig. S2). However, it is difficult to interpret these values, all being exploited sites for which pristine conditions are not known. Moreover, the origin of seeds and effect of local restocking was never documented. Within this context, it is hard to disentangle the effects of the natural connectivity among sites, due to the planktonic larval dispersion which might promote gene exchange, from the effects related to anthropogenic activities (overexploitation and restocking) that could increase/reduce the diversity of the affected populations. Despite these uncertainties, the data described here can represent a valid baseline reference, useful for the future genetic monitoring of wild populations in Sardinia, and the development of proper conservation actions and management plans for the species.

5. Conclusion

Our study outlined the presence of hybrids between *R. decussatus* and *R. philippinarum* in Sardinia, even if at a low extent. This confirms that Sardinian populations of *R. decussatus* are only weakly involved in negative competition with *R. philippinarum* due to its localised presence, but further attention should be given to investigate the occurrence of hybrids in Sardinia Island and the whole distribution area. In addition, we found variable haplotype and nucleotide diversities of the species without any clear relationship with sample size. Considering the lack of relevant competition with *R. philippinarum* and the formal absence of restocking programmes, the long pelagic larval dispersal and the commercial import of other organisms may play a key role in shaping the local species' genetic variation. However, we cannot exclude the possibility that unrecorded releasing of allochthonous seeds may have occurred in Sardinia to a greater extent than we suppose. Considering the growing concerns regarding the enhancement programmes, it becomes urgent to further study the current genetic makeup of wild populations as well as of the hatchery stocks used for supplementation aquaculture, to avoid disruption of local genetic diversity. The use of a single gene system, such as the mitochondrial DNA, may have some limitations since it is four times smaller than the nuclear genome and it is also maternally inherited, thereby representing historical processes in females only. Thus, it could be useful to further investigate the genetic diversity of *R. decussatus* using additional nuclear markers, such as microsatellites or single-nucleotide polymorphism, in a broader geographical context, including more specimens especially from the north Atlantic area.

Stakeholders, along with scientists and managers should collaborate in order to avoid the release of alien *R. philippinarum* into wild beds, detect the presence of potential hybrids and thus bolster the conservation of the native *R. decussatus*, which is one of the most important fishery resources along Atlantic and Mediterranean coasts.

Data availability statement

Data is contained within the article or supplementary material.

CRediT authorship contribution statement

Laura Carugati: Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Valentina Pinna:** Writing – review & editing, Formal analysis, Data curation. **Riccardo Demurtas:** Writing – review & editing, Data curation. **Angelo Cau:** Writing – review & editing, Resources. **Rita Cannas:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecss.2024.108903.

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