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The bivalve mollusc *Mactra corallina*: genetic evidence of existing sibling species

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The rayed trough-shell Mactra corallina *Linnaeus 1758 is a surf clam that inhabits the Atlantic Ocean, Black Sea and Mediterranean Sea and represents a commercially important bivalve. This species is present with two different and well-defined sympatric morphotypes, which differ mainly for the colour of the shell (white in the* corallina *morph, and brown-banded in the* lignaria *morph). The aim of this work is to resolve the confused and contradictory systematics of the bivalves belonging to* M. corallina *putative species by analysing molecular and morphological features. Fifteen specimens of* M. corallina corallina *(white variant) and 19 specimens of* M. corallina lignaria *(brown variant) were collected in the North Adriatic Sea and analysed by four molecular markers (12S, 16S, 18S and COI genes, partial sequences). Genetic analyses clearly support the presence of two different species, which were previously ascribed to* M. corallina. *In addition, 35 specimens identified on a morphological basis as* M. c. corallina *and 28 specimens identified as* M. c. lignaria *collected in the same area were used for a morphometric analysis. A positive correlation was found between the maximum width of shell (W), antero-posterior length and between W and the height of specimens from umbo to ventral margin, thus adding to molecular data.*

Keywords: genetic diversity, molecular taxonomy, bivalves, *Mactra*

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INTRODUCTION

Surf clams (also known as duck clams or trough shells), belonging to the genus *Mactra* Linnaeus 1767, live in the surf zone of exposed beaches and are widely distributed along mud–sandy coasts of the Pacific Ocean, Atlantic Ocean, Black Sea and Mediterranean Sea (Conroy *et al.*, 1993). They represent commercially important bivalves in many countries and are extensively utilized as seafood, raw materials for manufacturing flavouring materials and live feed at various aquaculture farms (Hou *et al.*, 2006).

The rayed trough-shell *Mactra corallina* (=*M. stultorum*) Linnaeus 1758 inhabits sandy bottoms at depths between 5 and 30 m, and it is distributed along coasts of the Black Sea, Mediterranean Sea and the eastern Atlantic Ocean from Norway to Senegal. It is a medium sized marine bivalve with a very thin and delicate shell with concentric growth lines. This species is present with two different and well-defined morphotypes, which, although they live sympatrically, are generally classified as two different sub-species. These morphotypes are easily distinguishable by the colour of the shell: the white variant, named *M. corallina corallina* Linnaeus 1758, has a shell of a hyaline white with weak ivory radial bands, whereas *M. corallina lignaria* Monterosato 1878 shows brownish radiating bands (D'Angelo & Gargiulo, 1987; Fischer *et al.*, 1987).

The correct specific name for the rayed trough-shell *M. corallina* is a longstanding issue for zoologists and malacologists. As reported in the Mediterranean marine molluscs checklist (Chiarelli, 1999), three species belonging to the genus *Mactra* are present: *M. stultorum* (=*M. corallina*) Linnaeus 1758, *M. glauca* Von Born 1778 and *M. olorina* Philippi 1846. Within *M. corallina*, two taxa, *M. c. corallina* and *M. c. lignaria*, are recognized.

Nevertheless, based on analyses of partial region of 18S rDNA by PCR-SSCP, Livi *et al.* (2006) found preliminary genetic evidences that the traditional classification of *M. c. corallina* and *M. c. lignaria* as subspecies was in contrast with the high genetic distance observed between the two taxa. Besides, *M. c. corallina* formed a highly supported cluster with a further unknown genetic profile, giving evidence of a third taxon belonging to the *M. corallina* complex (Livi *et al.*, 2006).

In his handbook *Carta d'Identità delle Conchiglie del Mediterraneo* Parenzan (1976) describes five distinct phenotypes ascribable to the genus *Mactra*. But actually the most plausible hypothesis is that *M. corallina* is a complex formed by two or more species (Livi *et al.*, 2006).

The official Italian checklists of marine fauna (compiled in their latest version in 2006 and available at http://www.sibm.it/CHECKLIST/principalechecklistfauna.htm) refer to these clams as belonging to the single species *M. stultorum* whereas the FAO identification handbook of Mediterranean species

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(Fischer *et al.*, 1987) and Riedl (1991) indicate *M. corallina* as the valid name for this species and *M. stultorum* as a synonym.

We decided to adopt the FAO specific designation and thus we refer to the white variant as *M. c. corallina* and to the brown habitus as *M. c. lignaria* as described in D'Angelo & Gargiulo (1987).

This work represents a first attempt to resolve the confused and contradictory systematics of bivalves belonging to *M. corallina* putative species by analysing molecular and morphological characters of the two morphotypes observed. Analysed samples were collected along the north Adriatic coasts of Cesenatico (Italy). In the present study we analysed molecular data obtained by four DNA markers: a nuclear ribosomal DNA subunit (18S) and the mitochondrial genes cytochrome oxidase I (COI), small (12S) and large (16S) ribosomal subunits, in order to provide a stable and robust phylogenetic estimate of the target. In addition, a morphological analysis was carried out on the basis of five parameters of the shell.

MATERIALS AND METHODS

Sampling and DNA extraction

Samples were collected in the north Adriatic Sea in front of Cesenatico (Italy) during a single diving in September 2006 and stored at −80°C. To avoid the problem of collecting paralogous mtDNAs, as found in doubly uniparental inheritance (DUI) bivalve species (see Passamonti & Ghiselli, 2009, and references therein, for a review on the issue), foot muscle tissue was dissected from each individual using a sterile cutter and stored in 80% ethanol at 4°C for the following DNA extraction. DUI has not been searched for in *Mactra*, because of the lack of specimens with fully developed gonads, but even if it would be present, foot muscle is expected to mostly carry mtDNA of maternal origin (Garrido-Ramos *et al.*, 1998). Total genomic DNA was prepared from 25 mg of muscle tissue according to the DNeasy Tissue Kit (Quiagen) protocol.

DNA amplification, cloning and sequencing

Sequences from partial 12S, 16S, 18S and COI were obtained. PCR amplifications were carried out in a 50 µl volume, as follows: 5 µl reaction buffer, 150 nmol $MgCl_2$, 10 nmol each dNTP, 25 pmol each primer, 20 ng genomic DNA, 1.25 units of DNA polymerase (Invitrogen, Carlsbad, CA, USA), water up to 50 µl. Thermal cycling consisted of 35 cycles at 94°C for 60 seconds, the specific annealing temperature (48°C for 12S and 16S; 50°C for 18S and COI) for 60 seconds, and 72°C for 60 seconds. An initial denaturation step (94°C for 5 minutes) and a final extension holding (72°C for 7 minutes) were added to the first and last cycle, respectively. Primer pairs were SR-J14197 ÷ SR-N14745 for 12S (Simon *et al.*, 2006), 16SbrH(32) ÷ 16Sar(34) (5′–CGCCTGTTTAACAAAAACAT–3′) for 16S (modified from Palumbi *et al.*, 1996), 18SF ÷ 18SR for 18S (Livi *et al.*, 2006), and LCO1490 ÷ HCO2198 (Folmer *et al.*, 1994) for COI. Amplified DNAs were treated with Wizard® SV Gel and PCR Clean-Up System (Promega). For a single *Mactra corallina lignaria* individual it was necessary to clone the 18S rDNA gene fragment with Ultramax DH5α–Competent Cells (Invitrogen) following the manufacturer's instructions.

Purified amplifications were either cycle sequenced using the ABIPrism BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and run on an ABI310 Genetic Analyser (Applied Biosystems) or sent to Macrogen (Seoul, EE Korea) for sequencing. Polymorphisms were confirmed by sequencing both strands.

Sequence analysis

Haplotypes (GenBank Accession Numbers FJ830395–FJ830446; Appendix 1) were aligned using the MAFFT multiple sequence alignment tool (Katoh *et al.*, 2002) available online at http://align.bmr.kyushu-u.ac.jp/mafft/online/server. Q-INS-i (Katoh & Toh, 2008) and G-INS-i (Katoh *et al.*, 2005) algorithms were chosen for ribosomal- and protein-coding genes, respectively. Sequences of species belonging to different families of heterodont bivalves were downloaded from the NCBI databank and added to alignment as reference data. In order to compare orthologous characters, only female mtDNA sequences from GenBank were used for DUI species. Gaps were coded as presence/absence data following the simple indel coding method of Simmons & Ochoterena (2000) with the software GapCoder (Young & Healy, 2003).

The analysis of molecular variance (AMOVA) framework (Excoffier *et al.*, 1992) implemented in Arlequin v3.11 software (Excoffier *et al.*, 2005) was used to test the overall genetic heterogeneity of surf clam samples. In this statistical method, a hierarchical AMOVA was carried out on the partitioning of molecular variability at arbitrarily chosen levels (i.e. from the individual to the group of samples level). In the present analysis, groups were obtained by pooling bivalve samples in two groups corresponding to the two subspecies *Mactra corallina corallina* and *M. c. lignaria*. Kimura 2-parameters distances (K-2-P; Kimura, 1980) were computed with MEGA4 software (Tamura *et al.*, 2007) with pairwise deletion of gaps/missing data and with a uniform mutation rate. Φ_{ST} and F_{ST} fixation indices (mitochondrial and nuclear genome respectively) as implemented in Arlequin were calculated to assess the genetic divergence. Statistical significance was estimated by comparing the observed distribution with a null distribution generated by 1000 permutations, in which individuals were randomly re-distributed into samples.

A barcoding-like approach was used to analyse genetic distances computed as formerly described. Frequencies of intra- and inter- specific distances were separately plotted in histograms to provide a visual output of genetic differentiation between the two morphs.

Phylogenetic relationships were inferred through Bayesian analyses implemented in MrBayes 3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). All analyses employed a cold chain and three incrementally heated chains. Starting trees for each chain were randomly chosen and the default values of MrBayes were used for all settings (including prior distributions). Each metropolis coupled Markov Chain Monte Carlo (MCMC) was run for ten million generations, with trees sampled every 100 generations. Burn-in was visually determined for each gene fragment by plotting average standard deviation of split frequencies over generation seeking for apparent convergence. Chains had always converged to a stable average standard deviation of split frequencies values <0.01.

Posterior probabilities (PP) were used to assess clade support. Analyses were performed using the evolutionary

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models selected for each gene fragment by the Bayesian information criterion of Modeltest (Posada & Crandall, 1998). Selected models were K81uf + Γ (Kimura, 1981) for 12S and 16S, K80 + Γ (Kimura, 1980) for 18S, and TVM + Γ for COI. They were implemented into MrBayes with the more similar and more complex model available in the program. *Mytilus galloprovincialis* (female) was used as outgroup to root phylogenetic trees. Nodes with PP < 0.95 were collapsed with the exception of 12S gene fragment data (PP < 0.85). Trees were graphically edited by MrEnt v2.0 (Zuccon & Zuccon, 2006).

Morphological analysis

Five morphological variables were measured: (i) shell length (antero-posterior, L); (ii) height of specimens (ventro-dorsal, H); (iii) maximum width of shell (left–right, W); (iv) distance between the points of intersection of the adductor muscles impressions and the pallial line (AP); and (v) distances between the points of intersection of the adductor muscles impressions and the apex of the umbo (UA and UP). Parameters were measured to 0.01 cm with a caliper. On the basis of such measures, the ratios H/L, W/L and W/H were obtained. Plots were graphically edited by R (Ihaka & Gentleman, 1996). Morphological data were statistically treated with Pearson's coefficient (r) to assess correlation between different sizes; ratios were examined by analysis of F test and the Welch two samples t-test to assess mean differences. The F test is a statistic used to test the hypothesis that two parameters have the same variance against the alternative hypothesis that the variances are different. Degrees of freedom were calculated taking into account number of groups (i.e. $gl_1 = 2 - 1 = 1$) and number of specimens (i.e. $gl_2 = [35 - 1] + [28 - 1] = 61$). The critical values of F with $P = 0.975$ were calculated with the function *qf(p, gl₁, gl₂)* as implemented in R statistical computing software (Ihaka & Gentleman, 1996; R Development Core Team, 2009). Welch's t-test is an adaptation of the Student's t-test intended for use with two samples having possibly unequal variances. Values of t-test were calculated using the function *t.test(x1, x2)* implemented in R software.

RESULTS

Genetic data

Twenty individuals for each morphotype were collected. A total of 34 specimens, 15 ascribed to *Mactra corallina corallina* and 19 to *M. c. lignaria*, were amplified and sequenced for the 12S, 16S, 18S and COI genes (partial sequences). Fragments of 397, 513, 516 and 571 bp respectively were obtained. Variable sites (including maximum parsimony informative sites), haplotype frequencies, specimen numbers and GenBank IDs are given in Appendix 1.

Data obtained by aligning the 12S partial sequence appeared quite soon less powerful than other gene fragments probably because of sampling artefacts. Actually, technical problems occurred during amplification and sequencing of the 12S and only four individuals of each group gave suitable PCR amplicons and electropherograms. Twenty-six repeated null amplifications were observed (11 in *M. c. corallina* and 15 in *M. c. lignaria*), accounting for the presence of point mutations in the annealing site of either primer. Further analyses will be required to unravel this latest issue.

In any case, examining sequence alignments for all the analysed gene fragments, high genetic divergences were observed between specimens of the two different morphs here considered (i.e. var. *corallina* and var. *lignaria*). Diagnostic sites were 7 out of 397 for 12S, 8 out of 513 for 16S, 25 out of 516 for 18S, and 43 out of 571 for COI (Appendix 1).

No mutation was observed at the amino acid level for the COI gene. Most point-mutations occurred at the third position of the codon. Six out of 60, however, were found at 2nd position (343, 358, 370, 412, 475 and 478).

Levels of genetic variability within the same morphotype were remarkably low and some shared haplotypes were observed (Appendix 1). A weak polymorphism was observed in the 18S fragment within both morphotypes, in the proportion of one different haplotype out of eleven in *M. c. lignaria* (sample n. 14 C2; C/T transition in position 467) and one out of six in *M. c. corallina* (sample n. 32; C/A, A/G, C/A transversion/transition in position 198, 200 and 202 respectively). Incidentally, the *M. c. lignaria* observed single 18S variant was found in a cloned sequence (see Appendix 1).

The higher proportion of overall molecular variance was always found at 'between morphotypes' hierarchical level (from 77.78%, $P < 0.05$; to 99.23%, $P < 0.01$; Table 1). All fixation indices values were high and significant or even highly significant. With the only exception of the 12S fragment ($F_{ST} = 0.778$, $P = 0.025$), fixation indices values were higher than 0.90 and ranged from 0.902 (COI) to 0.992 (18S; Table 1).

Figure 1 shows histograms obtained by plotting intra- and inter- specific K-2-P distances for the four analysed gene fragments. Intra- and inter- morphotype distances are well separated and the gap between these distances ranges from about 0.005 (16S) to about 0.064 (COI), respectively.

The Bayesian analysis performed with different combinations of data yielded differently resolved but comparable

Table 1. Analysis of partition of molecular variance (AMOVA) and fixation indices values (F_{ST} for diploid data, Φ_{ST} for haploid data). *, $P = 0.05$; **, $P = 0.01$; ***, $P = 0.001$.

Locus	Source of variation	df	Sum of squares	Variance components	Percentage of variation	Fixation index	*P* value
12S	Among morphotypes	1	7.500	1.75000 Va	77.78	$\Phi_{ST} = 0.778$	*
	Within morphotypes	6	3.000	0.50000 Vb	22.22		
16S	Among morphotypes	1	22.750	3.01339 Va	92.34	$\Phi_{ST} = 0.923$	***
	Within morphotypes	13	3.250	0.25000 Vb	7.66		
18S	Among morphotypes	1	60.797	7.82208 Va	99.23	$F_{ST} = 0.992$	***
	Within morphotypes	15	0.909	0.06061 Vb	0.77		
COI	Among morphotypes	1	108.614	18.27869 Va	90.19	$\Phi_{ST} = 0.902$	**
	Within morphotypes	10	19.886	1.98857 Vb	9.81		

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Table 2. Analysis of F test with $P = 0.975$ calculated with the function *qf(p, gl₁, gl₂)* (degrees of freedom: $gl_1 = 1$ and $gl_2 = 61$) and of the Welch two samples *t*-test calculated using the function *t.test(x1, x2)* applied to H/L, W/L and W/H ratios.

Ratio	*Mactra corallina*	*Mactra lignaria*	F test	$P = 0.975$	*t* value	*P* value
H/L	0.82997 ± 0.007	0.82068 ± 0.009	0.0800	5.281162	1.5476	0.183
W/L	0.53866 ± 0.009	0.43579 ± 0.009	7.6597	5.281162	15.6507	<2.2e−16
W/H	0.64924 ± 0.011	0.53122 ± 0.011	7.0448	5.281162	14.9967	<2.2e−16

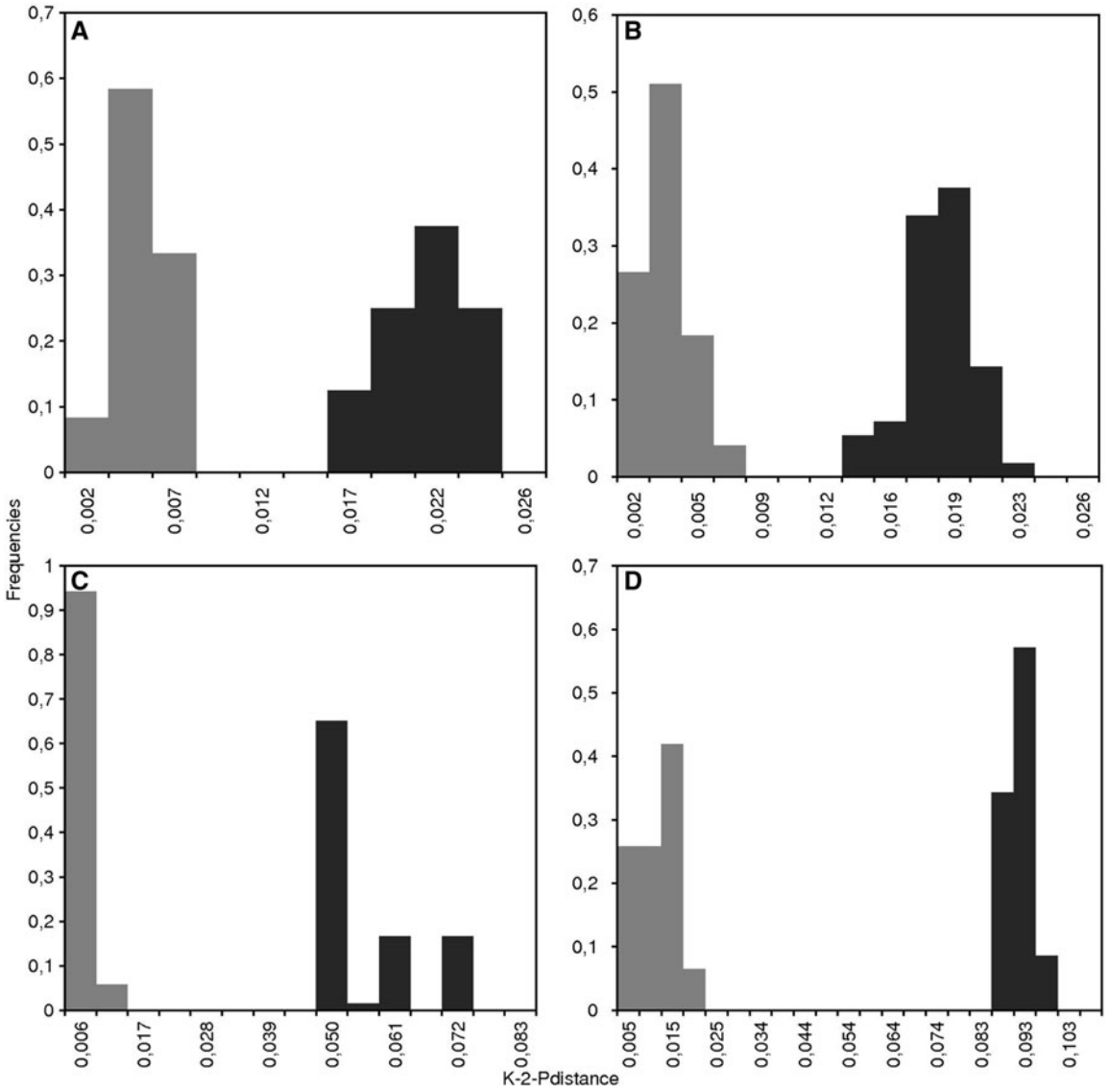

Fig. 1. Histogram illustrating K-2-P distances distribution among *Mactra corallina*/*M. lignaria* group, as resulting from the four characterized genes. K-2-P distance values are reported on x-axis, whereas their frequencies are reported on y-axis. A, 12S; B, 16S; C, 18S; D, COI; light grey: intra-specific distances; dark grey: inter-specific distances.

and well supported tree topologies (Figures 2–5). In all trees, the two morphotypes clustered separately from all other sequence data with $0.95 < PP < 1.00$. *Mactra c. corallina* was resolved as a monophyletic group for 12S ($PP = 0.88$), 18S ($PP = 1.00$) and COI ($PP = 1.00$). Similarly, *M. c. lignaria* was resolved as monophyletic for 16S ($PP = 0.96$), 18S ($PP = 1.00$) and COI ($PP = 1.00$). Both morphotypes were paraphyletic in other cases (i.e. 16S and 12S respectively). At a higher taxonomic level, the superfamily Mactroidea (= Mactracea) Lamarck 1809 (Mactridae Lamarck 1809 + Anatinellidae Gray, 1853 + Cardiliidae Fischer, 1887 + Mesodesmatidae Gray 1840) appear to be monophyletic in all obtained trees, with PP values ranging from 0.97 to 1.00, while the superfamily Veneroidea Rafinesque 1815 showed a complex situation that would require further investigations.

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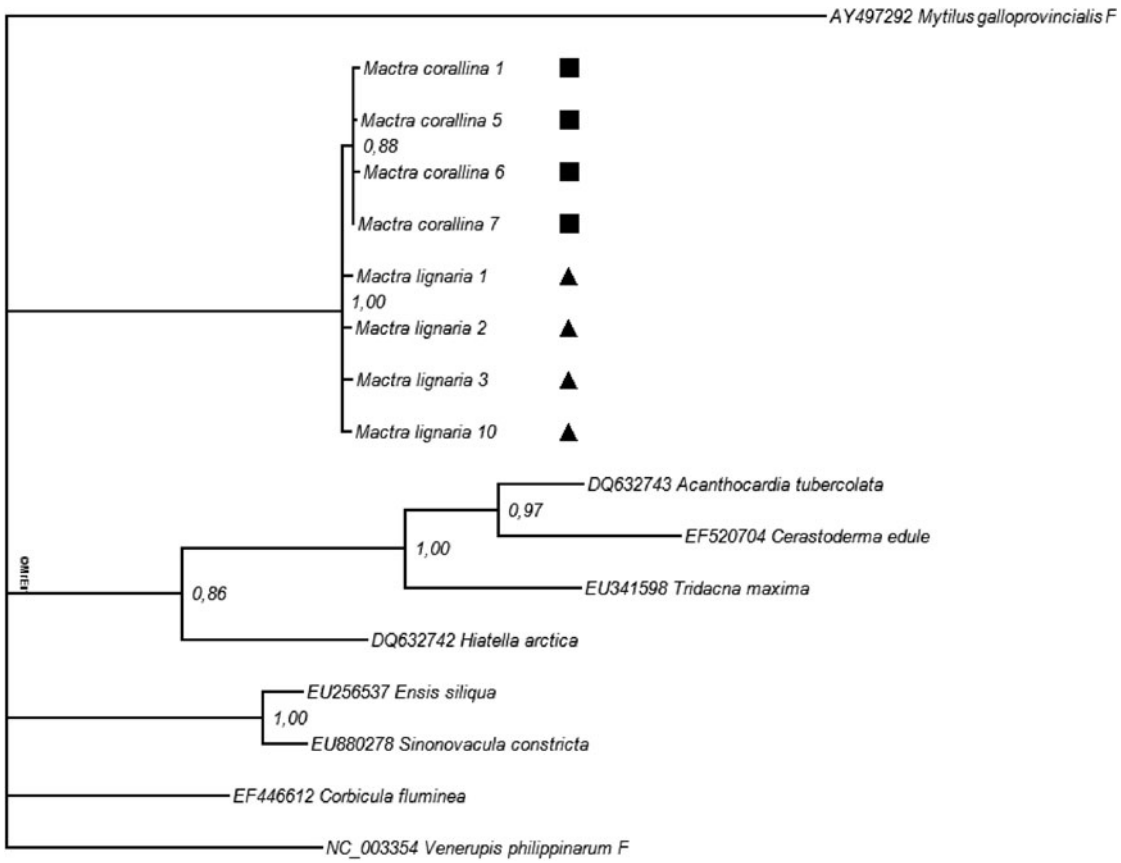

Fig. 2. Bayesian phylogeny of *Mactra corallina/M. lignaria* samples inferred by 12S sequence data. Individuals belonging to the *corallina* morphotype are marked with a square whereas individuals belonging to the *lignaria* morphotype are marked with a triangle. For correspondences to the GenBank accession number, see Appendix 1.

Morphological data

Morphological analyses showed that only three parameters (i.e. L, H and W) were statistically significant, while AP, UA and UP did not present any element of significance on discriminating the two morphotypes (data not shown). As a consequence, the last three parameters were not considered and here we will take into account ratios that only involve the former three parameters.

The analysis of Pearson's correlation reflects the degree to which two variables are related. The correlation between the considered sizes gives the following r values: in *M. c. corallina* $r_{H/L} = 0.915$, $r_{W/L} = 0.741$ and $r_{W/H} = 0.749$; in *M. c. lignaria* $r_{H/L} = 0.941$, $r_{W/L} = 0.781$ and $r_{W/H} = 0.777$.

Both in *M. c. corallina* and *M. c. lignaria*, all morphological features considered were positively correlated. In particular, high values of r were found for correlation between H and L. Morphometric ratios found are given in Figure 6.

The F test applied to W/L and W/H ratios showed statistically significant values, while for H/L the null hypothesis cannot be rejected (Table 2). Similarly, the *t*-test assessed a significant difference in W/H and W/L ratios. No significant difference was found in H/L ratio (Table 2).

DISCUSSION

The development of molecular tools for species identification scored an increased importance because of difficulties of discriminating them on the basis of morphological characters only. This is mostly true for organisms at early developmental stages and in cases of morphological stasis of adults or presence of sibling species (Øines & Heuch, 2005; Livi *et al.*, 2006).

Molecular assays presented in this paper brought to light a stable genetic divergence between *M. c. corallina* and *M. c. lignaria*. The clams analysed in this work were caught during a single dive in the very same area. The sympatric occurrence of the two morphotypes, coupled with the genetic divergence detected, is strong evidence of separate gene pools, thus supporting a reproductive isolation between the two morphs. Therefore, the taxon previously described as *M. corallina* should be rather considered as two different biological species, *M. corallina* and *M. lignaria*. A very similar experimental procedure, although based on allozyme analysis, was reported in Backeljau *et al.* (1994), who identify *Chamelea gallina* and *C. striatula*, previously considered as two subspecies of *C. gallina*, as two distinct and reproductively isolated biological species; actually, despite the probable overlap in breeding season between the two *Chamelea* morphotypes, they maintained a large genetic distance in sympatric conditions, giving evidence of two different biological species (Backeljau *et al.*, 1994).

For our *Mactra*, more genetic data obtained are consistent with two different species: the magnitude of genetic distances observed between *M. c. corallina* and *M. c. lignaria* were comparable to, if not greater than, distances detected among different genera belonging to the family Mactridae (K-2-P distance values, Figures 1 & 4B). The intra-specific pairwise

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K-2-P genetic distances were an order of magnitude lower than inter-specific comparisons (Figure 1). This divergence is also clearly shown by the high and statistically supported values of fixation indices, which were close to one and indicated the presence of a sharp dichotomy between genotypes, and the unbalanced partition of molecular variance with the majority of percentage detected at the higher hierarchical level, i.e. 'among morphotypes'. In the phylogenetic trees, albeit in two cases a soft paraphyly was observed (Figures 2 & 3) we observed a separation of *M. c. corallina* clusters from *M. c. lignaria* clusters, supported by robust node values.

Finally, the observed variability in the 18S gene well falls within the range of expected variability for this locus. This gene, generally highly conserved within species, shows variability higher in bivalves than in other taxa (Adamkewicz *et al.*, 1997; Passamaneck *et al.*, 2004). Moreover, the unique different haplotype found in *M. c. lignaria* was collected from a clone, which might have brought to light a rare variant (i.e. intra-individual variability among 18S repeats within the nuclear genome).

Preliminary morphological analyses seem also concordant with genetic data, although only one shell character (other than the colour) was significantly different; in fact, the main morphological character discriminating the two morphs seems to be the W value (maximum width of shell, i.e. the convexity) which differentiates morphometrical ratios in specimens with the same length or height. According to the data, the ratios W/L and W/H assume a clear (and classic) diagnostic value and allows us to take the following value to discriminate the two groups: in *M. c. corallina* $W/L > 0.50$ and $W/H > 0.60$, while in *M. c. lignaria* $W/L < 0.50$ and $W/H < 0.60$.

The effective reproductive isolation between *M. c. corallina* and *M. c. lignaria* (and/or sterility of hybrids) has still to be directly demonstrated, but obtained data are sound enough to support the species level for both morphs. Nevertheless, an additional sampling along the Adriatic coasts has already been planned to better describe the genetic landscape of *Mactra*, which seems to represent a complex of at least two (but probably more) different species (Livi *et al.*, 2006).

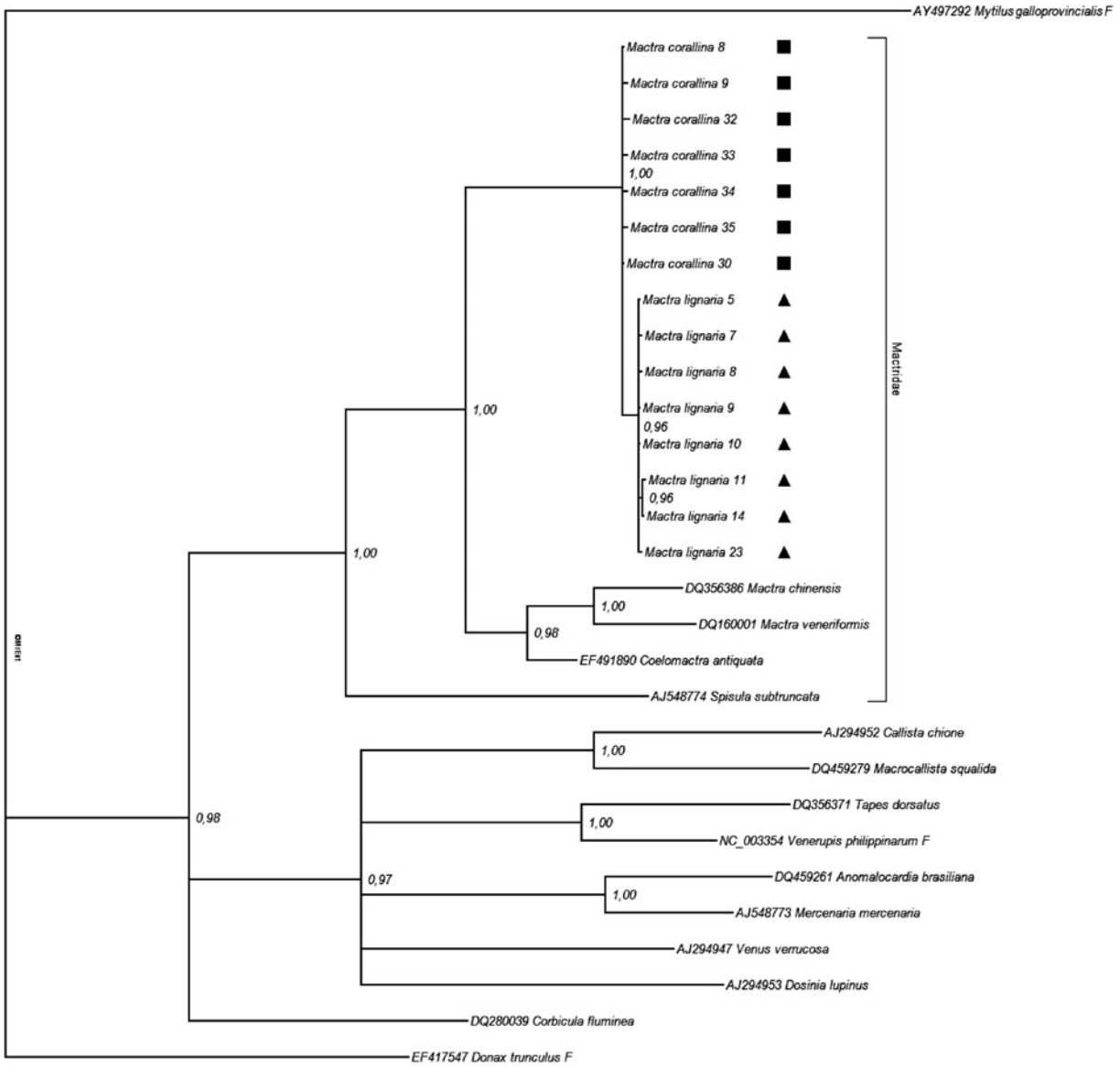

Fig. 3. Bayesian phylogeny of *Mactra corallina/M. lignaria* samples inferred by 16S sequence data. Taxon symbols as in Figure 2.

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Fig. 4. (A) Bayesian phylogeny of *Mactra corallina/M. lignaria* samples inferred by 18S sequence data. Taxon symbols as in Figure 2. Grey arrow heads point to Mesodesmatidae species; (B) histogram illustrating intergeneric K-2-P distances distribution among Mactridae: K-2-P distance values are reported on x-axis, whereas their frequencies are reported on y-axis; data from established genera of Mactridae are shown in white, whereas data from inter-specific comparisons among *Mactra corallina/M. lignaria* group are shown in dark grey, as in Figure 1C.

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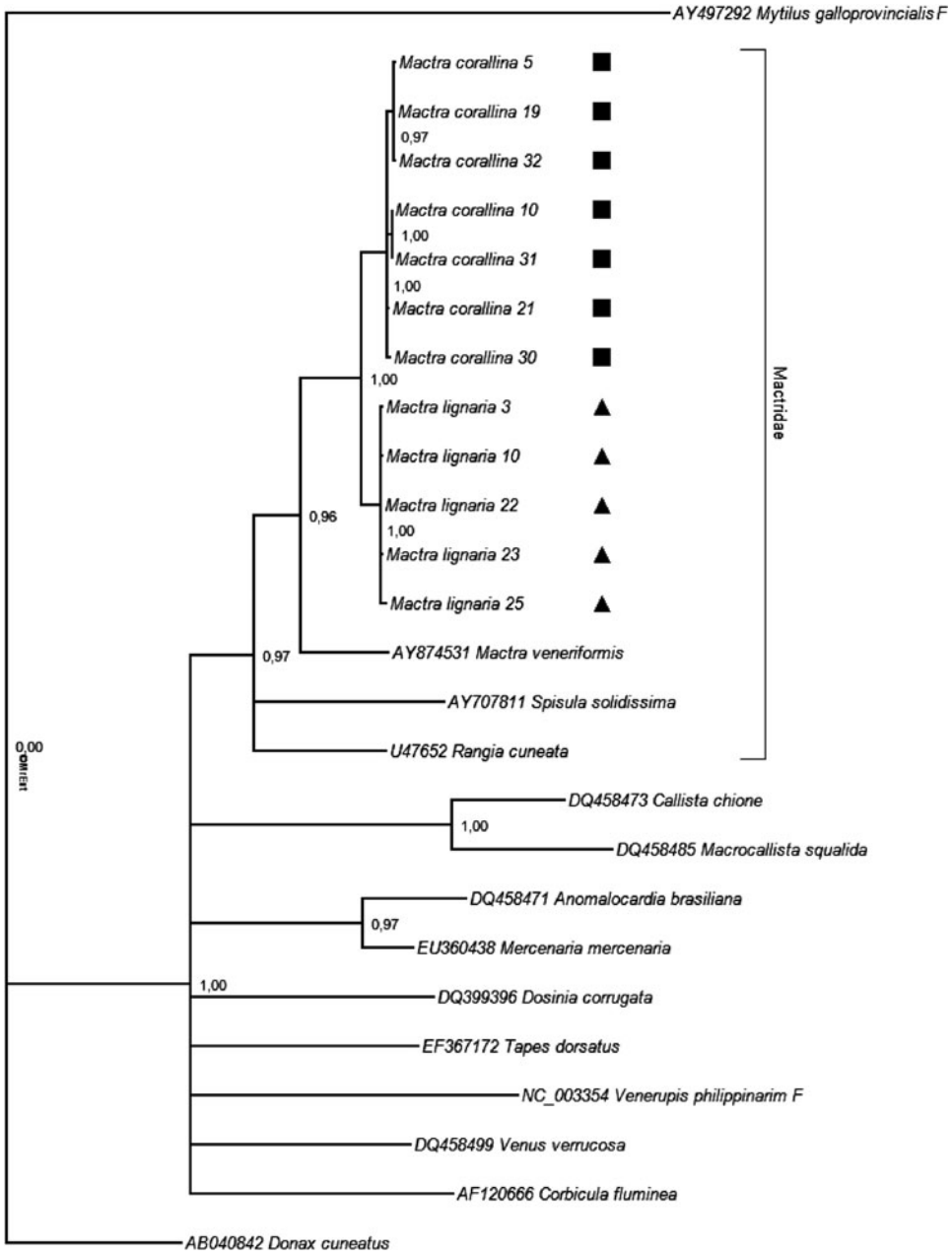

Fig. 5. Bayesian phylogeny of *Mactra corallina*/*M. lignaria* samples inferred by COI sequence data. Taxon symbols as in Figure 2.

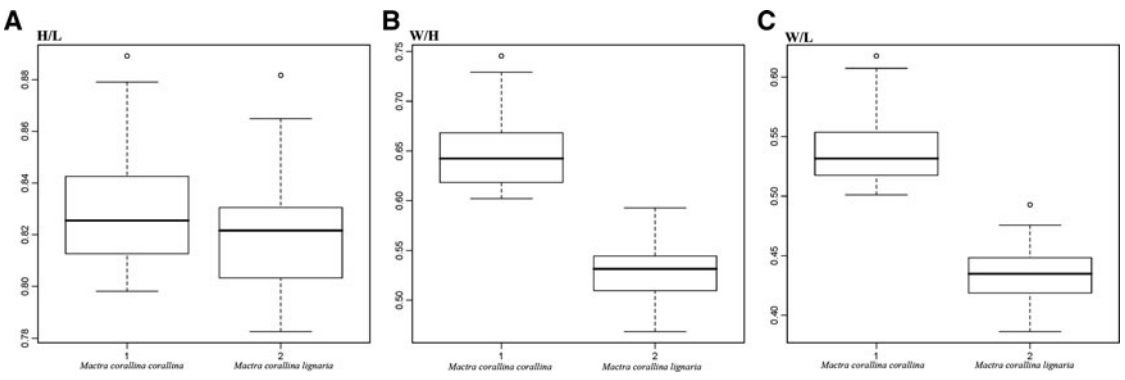

Fig. 6. Morphometric ratios in *Mactra corallina* and *M. lignaria*.

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Finally, the phylogenetic position of *Mactra* was addressed in this study. On the basis of 18S and 28S rRNA genes, it was previously found that the superfamily MACTROIDEA, traditionally classified near to the superfamily CARDIOIDEA (=CARDIACEA) Lamarck 1809 with an implicit sister-group relationship, showed grater affinity to UNGULINIDAE H. & A. Adams 1857 and the group of VENERIDAE Rafinesque 1815—CORBICULARIDAE Gray 1847—ARCTIDAE Newton 1891—CHAMIDAE Blainville 1825, but no connection with CARDIOIDEA (Taylor *et al.*, 2007). In our preliminary phylogenetic analysis, the genus *Mactra* was always monophyletic, although the 16S sequence of *Coelomactra antiquata* obtained from GenBank generates a polyphyly in the clade of *Mactra* (polyphyly supported by a significant PP nodal value of 0.98). Moreover, the superfamily MACTROIDEA clustered separately in all trees and was statistically well supported. Finally, in the 18S tree, individuals belonging to families MACTRIDAE and MESODESMATIDAE were intermingled (Figure 4A). This situation suggests further investigation focused on these species to assess the monophyly of the genus *Mactra* and to validate the family status of MESODESMATIDAE.

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Appendix 1. Alignment of the two variants of *Mactra corallina* analysed (*lig*: *lignaria, cor*: *corallina*), related frequencies (*f*), specimen numbers as in figures 2 to 5 and GenBank accession number. Only variable sites are reported.

Locus	Variable	Variable sites	*f*	Specimen number	GenBank accession number
12S		1224444566 6			
		9660124305 8			
		9024574506 9			
	lig	TCCATATTGA T	1	1	FJ830395
	lig	C.	2	2,10	FJ830396
	lig	C. C	1	3	FJ830397
	cor	C.TGAGACAG .	1	1	FJ830399
	cor	C.TGAGAC.G .	1	5	FJ830400
	cor	CTTGAGAC.G .	1	6	FJ830401
	cor	C.TGAGA..G .	1	7	FJ830402
16S		4455566 668			
		4895601867 891			
		8004562306 479			
	lig	CCTGGAAGAT TTT	4	5,7,9,10	FJ830403
	lig	. . . . . .C.	1	8	FJ830405
	lig	T. . . . . T.	1	11	FJ830408
	lig	. . . . . T.	1	14	FJ830409
	lig	. . . . . T.	1	23	FJ830410
	cor	.T.AA..AGC G ..	2	8,30	FJ830411
	cor	.TCAA..AGC G ..	4	9,33,34,35	FJ830412
	cor	.TCAA..AGC G.G	1	32	FJ830414
18S		111111 1111222222 233334			
		2223222366 7799000112 714586			
		0694679689 0158027464 311837			
	lig	CAAGACGTGC TTGCACGACA TCGTAC	10	10,11,13,14 C1,16,17,19,21,23,31	FJ830418
	lig	. . . . . .T	1	14 C2	FJ830422
	cor	ATTTCAACAG CCC...ATTG AAACC.	5	5,6,10,30,31	FJ830430
	cor	ATTTCAACAG CCCAGAATTG AAACC.	1	32	FJ830434
COI		111111111 1112222222 2333333333 3444444444 5555555555			
		1223466778 9122334467 7880122458 8224455788 9134456777 0013345667			
		5470506587 0703584724 7097629685 8173518047 9284762158 1791708140			
	lig	GCGGTCTATA GGATCGATAT CTTGTACCAT AGCTAATTTT TCCTCTCATT AGATTCCTCG	2	3,10	FJ830435
	lig	. . . .C. . . . . .C.	1	22	FJ830435
	lig	.........G	1	23	FJ830438
	lig	....C. . . . . .A. . . . .G. . . . . .T. . . . . .T.	1	25	FJ830439

Continued

Appendix 1. Continued

Locus	Variable	Variable sites	*f*	Specimen number	GenBank accession number
	cor	ATTA.TCGCG A.GC.AG.G. TC.TCG.TGA TA.CGGCCCC CTT.T.TTCC GAGCCT..TA	1	5	FJ830440
	cor	AT.A.TCGCG A.GC.AGCG. T..TCGTTGA TA.C.GCC.C CTT.T.TTCC GAGCCTTCTA	2	10,31	FJ830441
	cor	AT.A.TCGCG A.GC.AG.G. T..TCG.TGA TA.CGGCCCC CTT.T.TTCC GAGCCT..TA	1	19	FJ830442
	cor	AT.A.TCG.G A.GC.AGCG. T.CTCG.TGA TA.C.GCC.C CTTCT.TT.C GAGCCT.CTA	1	21	FJ830443
	cor	AT.A.TCGCG A.GC.AGCGC T..TCG.TGA TA.C.GCC.C CTT.T.TTCC GAGCCT.CTA	1	30	FJ830444
	cor	AT.A.TCGCG A.GCTAG.G. T..TCG.TGA TA..GGCCCC CTT.T.TTCC GAGCCT..TA	1	32	FJ830446