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Does GenBank provide a reliable DNA barcode reference to identify small alien oysters invading the Mediterranean Sea?

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The Mediterranean Sea is currently under siege by a conspicuous alien pressure, and, within some families (e.g. the Ostreidae), the number of native species seems to be remarkably outnumbered by that of the alien ones. We wanted to test the reliability of the molecular data currently available on the small alien oysters recently invading the Mediterranean Sea. Samples from Greece and Turkey, encompassing the known species-specific morphological variation, were sequenced for the markers with the widest taxonomic coverage in the group of small oysters (i.e. the 16S rDNA and the COI). The sequences obtained have been compared with those available in GenBank, and a possible identification at the species level has been finally tested in a DNA-barcoding fashion. The present results clearly demonstrated that our samples belong to a single, morphologically highly variable species. Their 16S sequences were closely related to a sequence registered under the name Dendostrea folium, *with a genetic distance which does not warrant conspecificity. Additionally, a remarkable number of sequences retrieved from the GenBank (of both genes) did not form a monophyletic group according to the published classification of the vouchers, suggesting—at least in part—an origin from specimens not properly identified. Both genes seem promising for use as DNA-barcode, although the COI will probably prove more effective. Therefore, we urge the availability of a baseline of oyster pedigreed DNA barcode sequences in the public databases, to allow the use of such genetic data to reliably monitor bio-invasions in the Mediterranean Sea.*

Keywords: DNA-barcoding, Mollusca, oysters, alien species, Mediterranean

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INTRODUCTION

The autochthonous Mediterranean marine fauna is mostly of Atlantic affinities (Sabelli & Taviani, 2014), having originated with the re-establishment of the Atlanto-Mediterranean connection (5.33 million years ago (Ma)), after the Messinian Salinity Crisis (from 5.971 to 5.33 Ma: Manzi *et al.*, 2013) had probably nearly exterminated the stenoecious marine biota (Taviani, 2002; Bianchi *et al.*, 2012). Local alpha diversity, however, is currently under siege by the continuous arrival of alien species (Sabelli & Taviani, 2014). Allochthonous elements mostly enter the Mediterranean via the Suez Canal, although a conspicuous contribution to the alien component currently comes from human activities (aquaculture, leisure boating, commercial maritime traffic). The early morphological detection of easily-identified shallow water species attracted a number of scientists, becoming one of the main local research fields, especially in the Eastern Mediterranean (Galil & Goren, 2014 and references therein).

Mollusca currently account for the highest number of known species introduced in the Mediterranean (~200) and, according to literature data, within some families the number of native species is currently outnumbered by that of the alien ones (Zenetos *et al.*, 2010, 2012; Sabelli & Taviani, 2014). Oysters and relatives, for instance, are represented in the Mediterranean by species belonging to three families: (1) the family Ostreidae Rafinesque, 1815, with two accepted native species, *Ostrea edulis* Linnaeus, 1758 and *Ostrea stentina* Payraudeau, 1826, and ~10 alien species reported by several authors during the last century; (2) the family Gryphaeidae Vialov, 1936, with one accepted native species and two Erythraean aliens; and (3) the family Plicatulidae Gray, 1854, with one Erythraean species only (Table 1 and main Mediterranean references therein).

However, given the severely misleading morphological patterns in these bivalve families (see discussion in, e.g. Lam & Morton, 2003, 2004; Huber, 2010), the need for a genetic confirmation is evident before assessing their presence and distribution in the basin. Nevertheless, studies confirming or elucidating the distribution in the Mediterranean of alien oysters are mostly lacking, with the sole exception of the occurrence in the northern Adriatic Sea of both sibling taxa *Crassostrea gigas* (Thunberg, 1793) and *Crassostrea angulata* (Lamarck, 1819) (Fabioux *et al.*, 2002). This has resulted often in chaos in the literature, with authors reporting or deleting species from lists of aliens according to subjective views only, and often without any discussion of the rationale.

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Table 1. Native and alien nominal species of oysters and relatives recorded from the Mediterranean Sea, with plausible/suspected vectors (V, for alien species only) and main references. AQ, aquaculture; SH, shipping; L, lessepsian entry.

Status	Taxa	V	Main literature references/reviews for the Mediterranean Sea
	Family Ostreidae Rafinesque, 1815		
Alien	*Alectryonella plicatula* (Gmelin, 1791)	L	Mienis *et al.* (2012b)
Alien	*Crassostrea angulata* (Lamarck, 1819)*	AQ	Monterosato (1915), Dantan & Heldt (1932), Mazzarelli (1936), Matta (1969), Blundo *et al.* (1972), Mizzan (1999), Fabioux *et al.* (2002)
Alien	*Crassostrea gigas* (Thunberg, 1793)*	AQ	Blundo *et al.* (1972), Cossignani *et al.* (1992), Mizzan (1999), Giannuzzi-Savelli *et al.* (2001), Fabioux *et al.* (2002), Zenetos *et al.* (2004, 2010), Albayrak (2011), Antit *et al.* (2011), Çinar *et al.* (2011), Crocetta (2011, 2012), Pećarevic *et al.* (2013)
Alien	*Crassostrea virginica* (Gmelin, 1791)	AQ	Monterosato (1915), Cossignani *et al.* (1992), Çevik *et al.* (2001), Giannuzzi-Savelli *et al.* (2001), Albayrak (2011), Cossignani & Ardovini (2011)
Alien	*Dendostrea folium* (Linnaeus, 1758) = *D. frons sensu* Mediterranean *auct. fide* Huber (2010)	L	Çeviker (1999, 2001), Zenetos *et al.* (2004, 2009, 2010, 2011), Delongueville & Scaillet (2006), Katsanevakis *et al.* (2009), Huber (2010), Çinar *et al.* (2011), Zenetos *et al.* (2013), Crocetta *et al.* (2013b)
Alien	*Dendostrea sandvichensis* (G. B. Sowerby II 1871) = *Ostrea crenulifera* G. B. Sowerby II, 1871	L	Sharon *et al.* (2005), Galil (2007), Zenetos *et al.* (2010), Mienis *et al.* (2012a)
Alien	*Nanostrea fluctigera* (Jousseaume in Lamy, 1925) = *Nanostrea exigua* Harry, 1985	L	Lubinevsky & Mienis (2005), Galil (2007), Mienis (2008), Zenetos *et al.* (2010)
Native	*Ostrea edulis* Linnaeus, 1758		
Native	*Ostrea stentina* Payraudeau, 1826		
Alien	*Planostrea pestigris* (Hanley, 1846)	L	Mienis (2004), Galil (2007)
Alien	*Saccostrea cucullata* (Born, 1778)	SH	Zenetos *et al.* (2004, 2010), Albayrak (2011), Çinar *et al.* (2011)
Alien	*Saccostrea glomerata* (Gould, 1850) = *Ostrea commercialis* Iredale & Roughley, 1933	AQ	Cesari & Pellizzato (1985), Cesari (1994), Mizzan (1999), Zenetos *et al.* (2004, 2010), Albayrak (2011)
	Family Gryphaeidae Vialov, 1936		
Alien	*Hyotissa hyotis* (Linnaeus, 1758)	L	Mienis (2004), Galil (2007)
Native	*Neopycnodonte cochlear* (Poli, 1795)		
Alien	*Parahyotissa inermis* (G.B. Sowerby II, 1871) = *Ostrea imbricata* Lamarck, 1819	L	Mienis (2004), Galil (2007)
	Family Plicatulidae Gray, 1854		
Alien	*Plicatula plicata* (Linnaeus, 1767)	L	Mienis (2004), Galil (2007)

*, the taxonomic status of *Crassostrea gigas* and *C. angulata* is still controversial, with some authors considering them as different species (e.g. Lapègue *et al.*, 2004; Liu *et al.*, 2011) and others regarding them as conspecific (e.g. López-Flores *et al.*, 2004; Reece *et al.*, 2008). They are here conservatively treated as different species.

Among small oysters (genera *Alectryonella* and *Dendostrea*), four nominal species have been cited from the eastern Mediterranean Sea (Table 1). *Dendostrea sandvichensis* (G. B. Sowerby II, 1871) and *Alectryonella plicatula* (Gmelin, 1791) have been recorded from Israel only on the basis of the following morphological features: *D. sandvichensis* (recorded as *A. crenulifera*) is diagnosed by a cup-shaped lower valve and a flat upper valve, a yellowish-green coloured interior and the exterior of the upper valve of a dirty white colour, often with some weak reddish lines, whilst *A. plicatula* has a shallow lower valve and a flat upper valve, a yellowish-green internal colour and the upper valve is purple–red externally (Mienis *et al.*, 2012b). *Dendostrea folium* (Linnaeus, 1758) and *Dendostrea frons* (Linnaeus, 1758) were recorded from Lebanon, Cyprus and Greece without any morphological description (Table 1). Both species are well known to largely overlap in shell morphology, being very variable and showing different ecomorphs, with or without a submedian ridge and a highly variable number of plications, and it is difficult to separate those records from the specimens of *D. sandvichensis* recorded from Israel. Huber (2010) suggested the exclusion of *D. frons* (a Caribbean native species: Huber, 2010) from the Mediterranean basin on the basis of biogeographical considerations, but his suggestion was not generally accepted, and *D. frons* has still been reported for some eastern Mediterranean localities (e.g. Zenetos *et al.*, 2013) and in general reviews (Nunes *et al.*, 2014). However, diagnostic characters differentiating *Dendrostrea* nominal species are weak, since general outline and shape of valves are known to reflect the nature of the substrate, and the internal and external colour may vary even in the same specimen (e.g. the specimen in Figure 2F, G starts with a red external colouration which then becomes white).

Therefore, a combined morphological–molecular approach may probably help in re-assessing the alpha taxonomy of this group and identifying effective diagnostic characters. To test the reliability of currently available molecular data, samples of small oysters showing extremely different morphological characters and encompassing the known morphological variation (cup-shaped or flat lower valve; flat or rounded upper valve; yellowish-green, whitish or with malachite-green patches internal colour; dark or light reddish, whitish or white with weak reddish lines external colour; presence/absence of regular/irregular rounded ribs; presence/absence of plications at the margins; and massive/scarce presence of chomata: see Figures 2 and 3) were collected at four Mediterranean localities (two in Greece, Astypalia Island and Rhodes Island, and two in Turkey, Kekova and Olympos) spanning most of their range in the Mediterranean. They were then sequenced for the two mitochondrial barcode markers (Liu *et al*., 2011) with the widest taxonomic coverage in oysters: the 16S rDNA and the COI (GenBank: accessed on 31 January 2014). The nuclear ITS2 rDNA is a further potential candidate barcode marker for bivalves (Salvi *et al*., 2010; Salvi & Mariottini, 2012), but the coverage of the sequence in GenBank was too low in the small oysters to allow a reliable comparison, and it was thus discarded. The sequences obtained have been compared with those available in GenBank, and a possible identification at the species level in a DNA-barcoding fashion has been finally tested.

MATERIALS AND METHODS

Samples were collected at two Greek ((1) Astypalia (36°35′13″N 026°24′08″E) and (2) Rhodes (36°02′51″N 027°58′93″E)) and two Turkish sites ((3) Kekova (36°10′19″N 029°50′46″E) and (4) Olympos (36°23′40″N 030°28′44″E)), at a depth of 0.1–3 m (Figure 1). At all sites, the oysters were present in high densities of up to 80–100 individuals per square metre. Specimens were collected by hand while diving and fixed in 100% ethanol upon collection. Then, an *a priori* morphological identification was attempted according to the most recent guides and papers dealing with the involved species (e.g. Sharabati, 1984; Oliver, 1992; Bosch *et al*., 1995; Sharon *et al*., 2005; Zenetos *et al*., 2009; Huber, 2010; Mienis *et al*., 2012a, b), but this failed due to the presence of an ambiguous combination of characters typical of distinct species. A piece of tissue was dissected from the foot for DNA extraction, and the remaining tissues were stored, preserved in 100% ethanol with the relevant voucher shell, at the Department of Biology and Biotechnologies, 'La Sapienza' University (Figures 2 and 3: voucher ID, BAU1412–BAU1419). Total genomic DNA was extracted using a standard proteinase K phenol/chloroform method with ethanol precipitation, as reported in Oliverio & Mariottini (2001). A fragment of the mitochondrial 16S rDNA was amplified by PCR from eight specimens using the universal primers 16Sar-L and 16Sbr-H (Palumbi *et al*., 2001). The DNA-barcode fragment of the mitochondrial cytochrome oxidase I (COI) was amplified by PCR from the same specimens using the universal primers LCO1490 and HCO2198 (Folmer *et al*., 1994). All amplicons were sequenced by Macrogen Inc. (Seoul, South Korea), using the same PCR primers. Forward and reverse sequences were assembled and edited, and the resulting consensus sequences of each specimen were readily aligned by hand. Accession numbers for the sequences are KJ946438–KJ946453.

A total of 650 (16S) and 923 (COI) ostreid sequences were retrieved from the GenBank, corresponding to specimens identified at species (43 taxa for 16S; 32 taxa for COI) or genus level (24 entities for 16S and 72 entities for COI, determined as 'sp.'). Of these, 166 (16S) and 145 (COI) sequences were excluded from the database being either too short or too divergent to be reliably included in the analysis. Since our focus was on the lower limit of the interspecific ranges, deleting highly divergent sequences would not bias the analysis. In a first phylogenetic analysis subsets of 92 (16S) and 74 (COI) ostreid sequences, representing all included nominal genera, plus the 16S and COI sequences generated in this study for the small-oysters ($N = 8$) and three gryphaeid sequences to serve as outgroup (alignments: 16S, 515 bp; COI, 620 bp), were used. Phylogenetic relationships among the sequences were inferred by neighbour-joining (NJ) and maximum likelihood (ML) using the best model of nucleotide substitution, by the software MEGA 5.0 (Tamura *et al*., 2011), bootstrapped over 1000 replicates.

According to the results of these first analyses the retained sequences (484 16S and 782 COI) were pooled into distinct matrices, corresponding to the major clades recovered, each aligned by Clustal_X 1.83 (Thompson *et al*., 1997), and the final complete alignments were trimmed to the minimum number of corresponding nucleotides (all alignments available from the authors on request). For each gene the three datasets were: a *Saccostrea*-dataset including all *Saccostrea* spp. sequences (192 16S sequences, 501 bp; 91 COI sequences, 548 bp); a *Crassostrea*-datset including all *Crassostrea* spp. sequences (158 16S sequence, 499 bp; 582 COI sequences, 548 bp); and an Ostreine-dataset including all remaining sequences (134 16S sequences, 497 bp; 109 COI sequences, 548 bp) ascribed to the genera *Ostrea*, *Ostreola* and *Lopha*, and also the so-called small oysters (genera *Dendostrea, Alectryonella* and allies, including our new sequences). Pooling the sequences in distinct matrices, allowed an easier

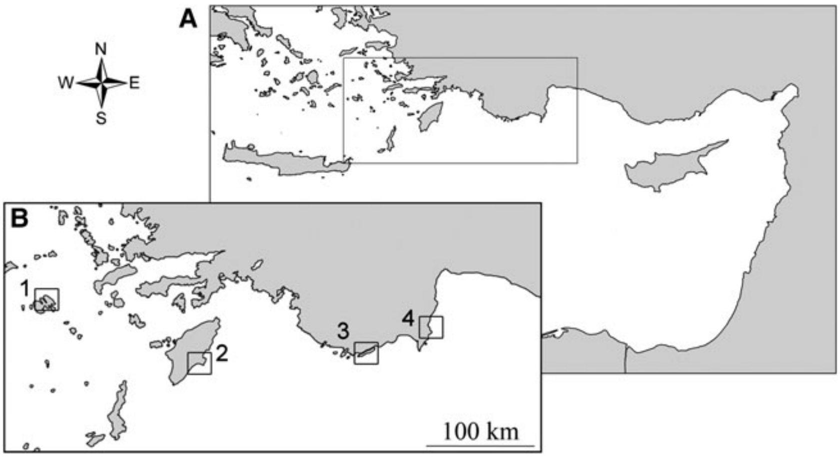

Fig. 1. (A) Location of the sampling area in the eastern Mediterranean Sea; (B) map of the sampling sites: 1, Astypalia; 2, Rhodes; 3, Kekova; 4, Olympos (see Material and Methods for details).

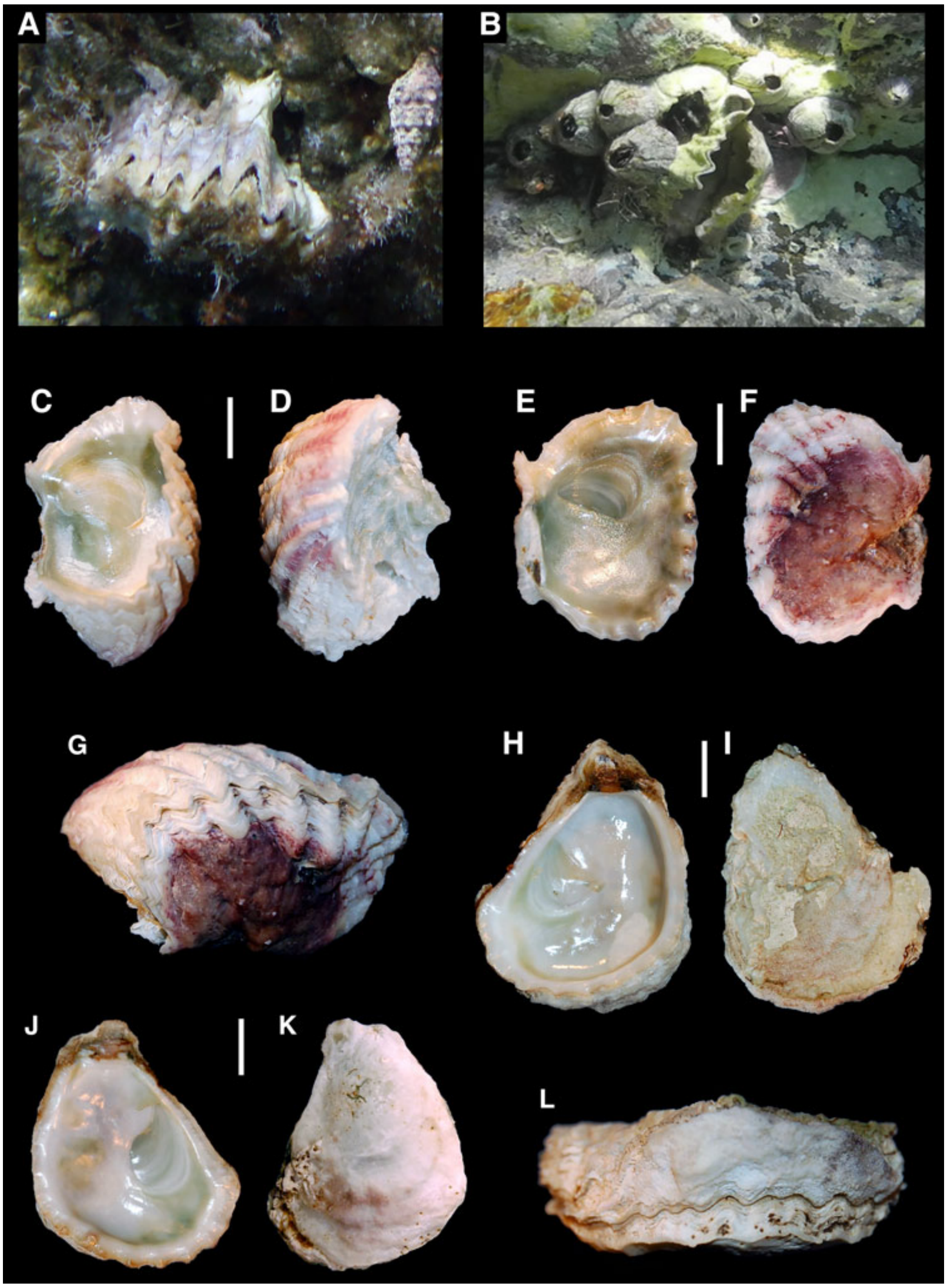

Fig. 2. Small oysters from the Mediterranean Sea: (A) Rhodes (Greece), 50 cm depth, *in situ* photograph; (B) Astypalia (Greece), 30 cm depth, *in situ* photograph; (C–G) BAU1412, Rhodes (Greece); (H–L) BAU1413, Rhodes (Greece). Scale bar: 1 cm.

alignment of the 16S sequences, and, by limiting the need for gaps, allowed retention of longer alignments without having to discard ambiguously aligned partitions. A cumulative matrix including all 484 16S sequences, aligned for 506 bp, required the exclusion of 40 positions of ambiguous alignment.

Analysis of the nucleotide sequences were performed by using MEGA version 5 (Kumar *et al.*, 2004; Tamura *et al.*, 2011). The observed '*p*' and the Kimura's 2-parameter (K2p) (Nei & Kumar, 2000) pairwise genetic distances were estimated within each dataset.

Updated nomenclature and taxonomy follow the World Register of Marine Species (accessed on 31 January 2014).

RESULTS

Phylogenetic patterns in the Ostreidae

The ML trees recovered by MEGA on the two subsets of 95 (16S) and 74 (COI) ostreid sequences (with three gryphaeids as outgroup), using a GTR (General Time Reversible; Tavaré 1986) model of evolution, and 1000 bootstrap replicates, are reported in Figure 4 (the topology of the respective NJ trees were almost identical, and only the bootstrap supports at the node are reported). For both datasets the sequences were split into three major clades: a *Saccostrea*-clade, a *Crassostrea*-clade, and an Ostreine-clade. The sequences of

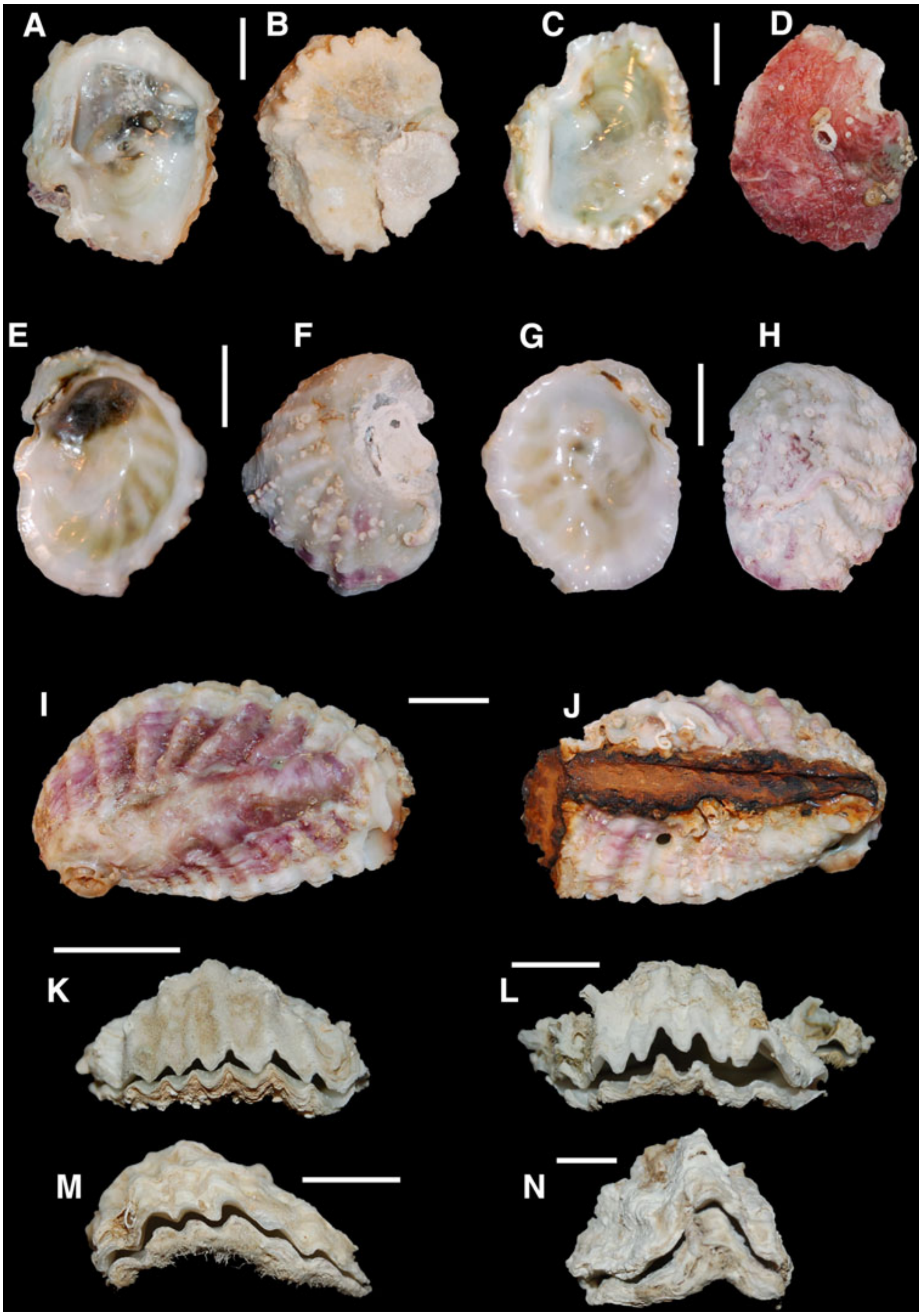

Fig. 3. Small oysters from the Mediterranean Sea: (A–D) BAU1414, Astypalia (Greece); (E–H) BAU1415, Astypalia (Greece); (I, J) Astypalia (Greece), −2 m depth, empty shell from the sampled population; (K) BAU1416, Kekova (Turkey); (L) BAU1417, Kekova (Turkey); (M) BAU1418, Olympos (Turkey); (N) BAU1419, Olympos (Turkey). Scale bar: 1 cm.

the small-oysters (*Dendostrea*-related, including GenBank sequences and our alien Mediterranean samples) were interspersed within the ostreine-clade. Accordingly, ML and NJ analyses (1000 bootstrap) were performed using the Ostreine-dataset with one *Crassostrea* and one *Saccostrea* as outgroups (16S: 134 + 2 sequences, alignment 555 bp; COI: 109 sequences, alignment 548 bp) producing the trees in Figure 5. The K2p genetic distances were calculated within each matrix, and the frequency of pairwise distance values among the sequences were plotted in the histograms of Figure 6.

The visual inspection of the NJ trees (Supplementary Figures S1–S6) derived from each distinct dataset, allowed to identify the clades corresponding to the most probable putative species. According to these patterns, the following maximum intraspecific and minimum interspecific K2p genetic distances were scored in each dataset:

CRASSOSTREA-DATASET

16S. With the exception of a highly diverging sequence from a single specimen ascribed to *Crassostrea rhizophorae*

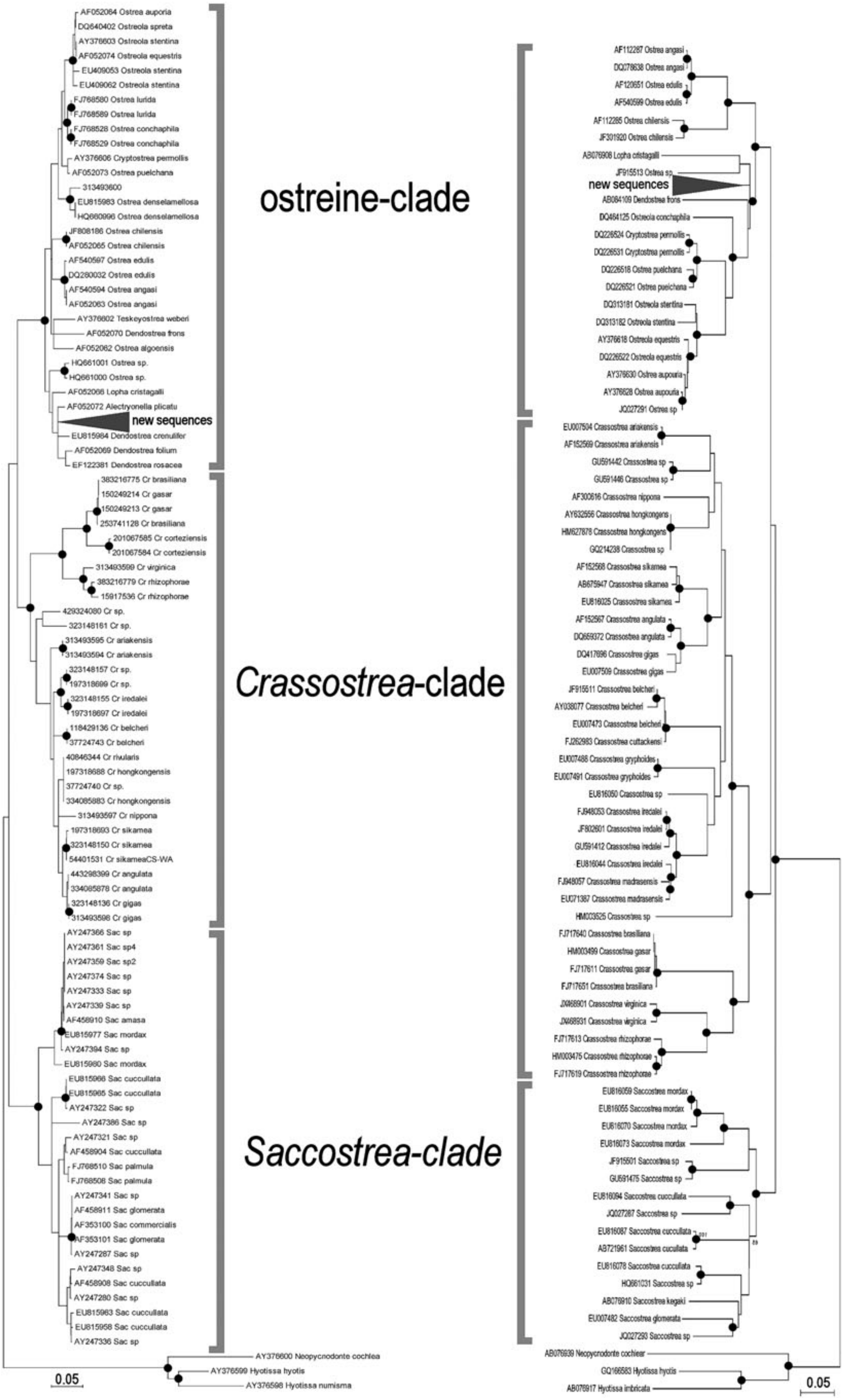

Fig. 4. Maximum likelihood (K2p evolutionary model) phylogenetic trees of selected sequences, encompassing all ostreid genera represented in the GenBank (16S left: log likelihood −3004.1772: COI right: log likelihood −9163.8453. The topologies obtained after NJ and BI analyses recovered the same major clades. Closed circles indicate a bootstrap support (1000 NJ and ML bootstrap replicates) >90% and BI posterior support >95%. Cr, *Crassostrea*; Sac, *Saccostrea*.

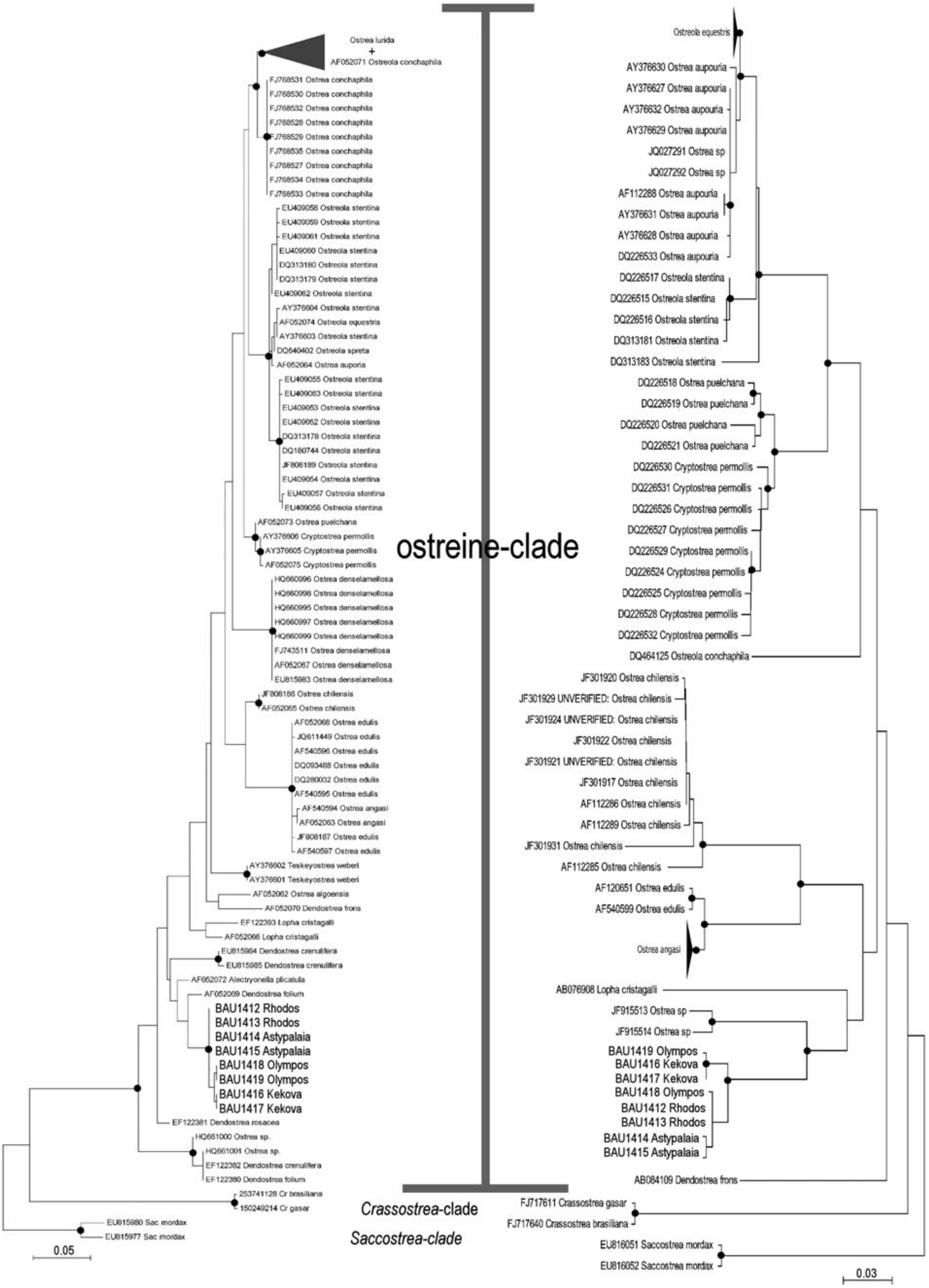

Fig. 5. Maximum likelihood (K2p evolutionary model) phylogenetic trees of the ostreine dataset (16S left: log likelihood −2968.3177: COI right: log likelihood −3634.5067). The topologies obtained after NJ and BI analyses recovered the same major clades. Closed circles indicate a bootstrap support (1000 NJ and ML bootstrap replicates) >90% and BI posterior support >95%. Cr, *Crassostrea*; Sac, *Saccostrea*.

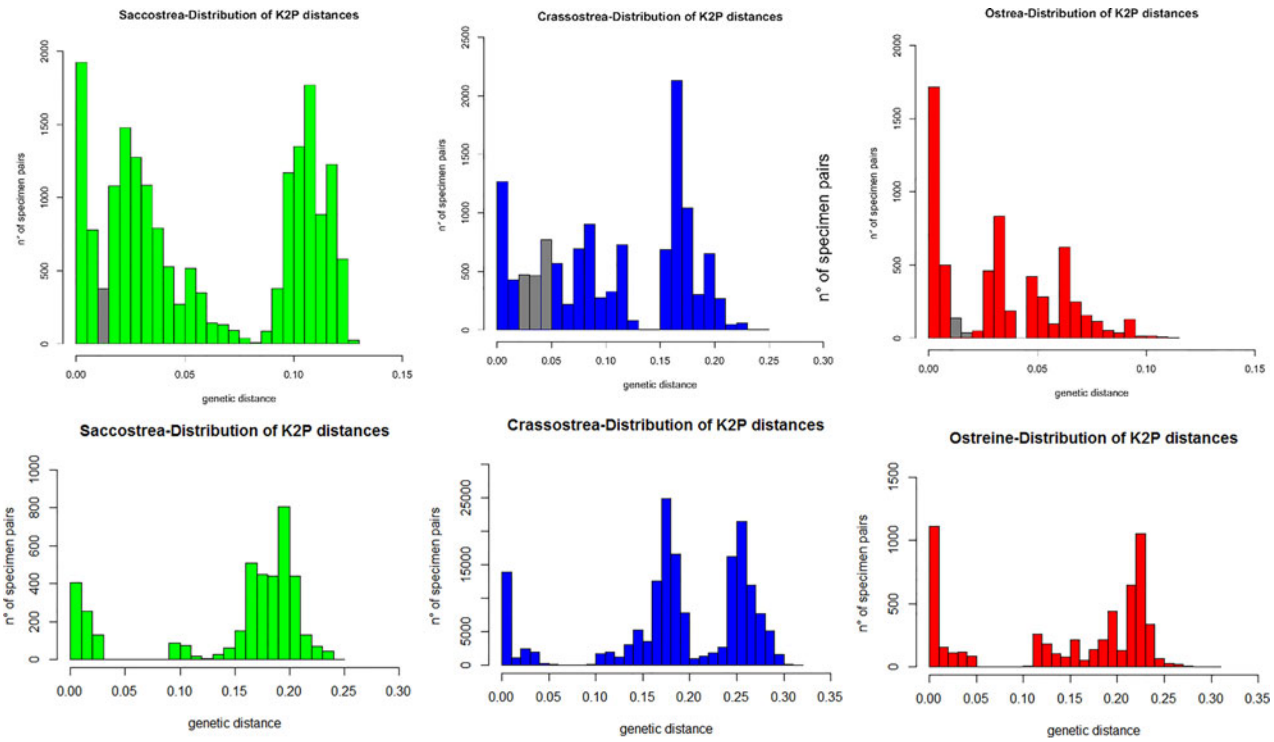

Fig. 6. Histogram representing the frequency of pairwise K2p distance values among 16S (upper row) and COI (lower row) sequences. The grey bars indicate overlaps of presumed intraspecific and interspecific pairwise distances in each dataset.

(AJ312938: with K2p distance 2% from all other *C. rhizophorae*) all intraspecific distances were <1%. The smallest interspecific distances were 1.2%.

COI. Eight sequences annotated as *Crassostrea belcheri* (GU591442–GU591447; JF915478, JF9154789) formed a separate clade in the NJ tree, with a minimum K2p divergence of 13.0% from other species, and a K2p divergence of 19.3% from other *C. belcheri*. The sequence FJ262985 annotated as *C. gryphoides* was likely a *C. belcheri* (1.1% K2p divergence from *C. belcheri*, 17.7%, from other *C. gryphoides*). The specimen FJ262983 ascribed to *C. cuttackensis* was likely a *C. belcheri* (0.55% K2p divergence with other *C. belcheri*). Very low distance values were obtained between sequences nominally ascribed to distinct species in complexes of doubtful taxonomic status (*C. gigas*/*C. angulata*, *C. madrasensis*/*C. iredalei* and *C. brasiliana*/*C. gasar*), which probably do not include more than one species each. Once accounting for these cases, all intraspecific K2p distances were <4.0% and all interspecific distances were >9.5%.

SACCOSTREA-DATASET

16S. The higher distance values (5%) were observed between specimens of the *Saccostrea cucullata*/*S. palmula* complex, where possibly more than one species were involved. The remaining sequences displayed values of distance <2% for presumed intraspecific comparisons, and >3% for interspecific ones.

COI. The sequences annotated as *S. cucullata* clustered in three lineages with divergence as high as 19.54%. Sequences identified as *S. mordax* formed two divergent clades with a minimum genetic distance of 12.05%. These values were similar to the average interspecific divergence between *Saccostrea* species (16.9%), thus suggesting that both *S. cucullata* and *S. mordax* may represent species complexes. The sequences AY038076 assigned to S. *cucullata* was likely from a specimen *S. mordax* complex. Several sequences identified as *Saccostrea* sp. have been assigned either to known species (N = 3) or to unidentified lineages (N = 24) which may subtend up to three undescribed species. Once accounting for possible species complexes and undescribed lineages, all the intraspecific distances were <3.37% and all interspecific distances were >12.24%.

OSTREINE-DATASET

16S. Within the ostreine-dataset, the presumed intraspecific distances ranged from 0 to a maximum of 2% (in comparisons among the *O. stentina*/*spreta* complex). Among the presumed interspecific comparisons, the lowest values were shown by pairwise distances of *O. conchaphila vs O. lurida* (1.6–2%; Polson *et al.* (2009) recently confirmed *O. conchaphila* as a different species from *O. lurida*), whilst all other comparisons yielded values >3%. Remarkably, the sequences from specimens of *Cryptostrea permollis* (AY376605–6, AF052075) resulted virtually identical to those of *Ostrea puelchana* (AF052073: 0.2–0.7%).

COI. Distance values up to 7.29% were recorded between sequences ascribed to *Ostrea chilensis*. On the other hand, species pairs such as *Ostrea edulis*/*Ostrea angasi*, *Ostreola equestris*/*Ostrea aupouria*, and *Cryptostrea permollis*/*Ostrea puelchana* showed allegedly interspecific distances lower than 2.81%.

DISCUSSION

A clear threshold between intraspecific and interspecific pairwise comparisons of 16S and COI ostreid sequences did not emerge after the analysis of datasets derived from the GenBank and including our new sequences, although many of the overlaps could be discussed in the framework of taxonomic revisions of the complexes involved (e.g. *Crassostrea rhizophorae*, *Saccostrea cucullata/palmula*, *Ostrea stentina/spreta* and *O. conchapila/lurida*). However, values higher than 3% for the 16S and of 4% for the COI, seemed to consistently indicate interspecific comparisons, while values lower than 1% (16S) and 10% (COI) were scored in intraspecific comparisons. The sequences from our Mediterranean samples formed highly supported monophyletic groups with both datasets, and pairwise distances ranged from 0 to 0.7% (16S) and from 0 to 0.8% (COI). The COI sequences in the GenBank did not provide any potential conspecific candidate. The closest sequences were JF915513–JF915514, labelled '*Ostrea* sp.' from Sungai Menghulu (Malaysia), 13–14% divergent from the Mediterranean ones, thus clearly not conspecific. Furthermore, they were not identified at the species level and the phylogenetic pattern clearly indicated that they do not belong in *Ostrea* but rather are in the *Dendostrea* clade. All 16S Mediterranean sequences resulted very close to the single sequence of a specimen from Aitutaki (Cook Islands) (AF052069) registered under the name *Dendostrea folium* by Jozefowicz & Ó Foighil (1998). The locality of this sample is within the range of this taxon (Pacific Ocean: see discussions in Huber, 2010). The distance values obtained with the Mediterranean samples (3–3.7%) support a close relationship, yet are not conclusive for testing conspecificity. Furthermore, sequences ascribed to the genus *Dendostrea* did not form a monophyletic group, nor did sequences ascribed to either *D. folium* (Linnaeus, 1758) or *D. crenulifera* (G.B. Sowerby II, 1871) (Figure 5).

Historically, taxonomy of both native and alien Mollusca of the Mediterranean has been almost entirely based on shell characters (e.g. Keller, 1883; Pallary, 1912; Haas, 1948; Barash & Danin, 1986), that are still commonly used in the early detection and discovery of alien species. Morphological variation of molluscan shells as an adaptive response to environmental pressures, however, has been widely documented (e.g.: Pascoal *et al.*, 2012; Solas *et al.*, 2013). Although the best known taxonomic problems concern the class Gastropoda, oysters (family Ostreidae and allied) represent typical examples of shell morphological plasticity due to environmental pressures, since valve morphology may strongly reflect the nature of the substrate and/or the tidal regime (Gunter 1950; Seilacher *et al.*, 1985). Morphological characters are often not reliable for differentiating closely related species, and nomenclatural confusion may derive by misidentifications (Korringa, 1952; Boudry *et al.*, 2003; Huber, 2010 and references therein). A combined use of paralleled molecular and morphological markers has become central, in the last decade, to solve issues of oyster identifications and taxonomic relationships (e.g. Lam & Morton 2003, 2004; López-Flores *et al.*, 2004, 2010; Wang *et al.*, 2004, 2008; Reece *et al.*, 2008; Liu *et al.*, 2011), further prompting for a wider use of genetic analyses also on alien species in the Mediterranean Sea to delineate dispersal pathways and clarify doubtful identifications (e.g. Albano *et al.*, 2009; Crocetta *et al.*, 2013a; Valdés *et al.*, 2013).

Our GenBank search, however, showed that for oysters the taxonomic coverage of the classical barcode gene (COI) was slightly lower than for 16S. Both markers (COI and 16S) revealed some potential utility for oyster DNA-taxonomy, albeit with some limitation since some non-homogeneous variation seems to exist across the different clades in the family. Nevertheless, the analysis of the pairwise distances in a phylogenetic context allowed the drawing of some conclusion and raised several concerns. The possibility of using a DNA-barcoding approach for oysters mostly relies on the availability of a baseline of pedigreed sequences in the GenBank (i.e. sequences from figured specimens properly identified by specialists, better if from type localities of the nominal taxa); this is still lacking, at least for the species here investigated. Furthermore, a major problem with the use of DNA sequences in identifying small oysters is that several COI and 16S sequences in the GenBank originated from specimens not properly identified. With the exception of *Dendostrea frons*, with a sequence largely diverging from the other small oysters, the remaining sequences ascribed to the genus *Dendostrea* show some close relationships, but do not form a holophyletic group, nor do sequences ascribed to either *D. folium* or *D. crenulifera*. Given the importance of oysters from many points of view, and the frequent translocation of specimens resulting in alien populations of oysters in many seas, it is certainly urgent to make available a baseline of pedigreed sequences for the species of Ostreidae. It would probably be desirable that the targeted barcode gene is the classical COI, but other candidates (e.g. the nuclear ITS2, see Salvi & Mariottini, 2012) may prove to be useful complements.

The Mediterranean specimens of small oysters collected at four localities in the Aegean Sea were morphologically so divergent that they could be *a priori* ascribed to different nominal species, all potential invaders in the semi-enclosed basin and, in fact, all recorded from the Mediterranean Sea by different authors. Actually, our specimens clearly belong to a single, morphologically highly variable species, included in an Indo-Pacific clade. This highlights the need for testing genetically the specimens used to record distinct species of small oysters as alien species in the Mediterranean (Çeviker, 1999, 2001; Sharon *et al.*, 2005; Zenetos *et al.*, 2009, 2011; Mienis *et al.*, 2012a, b; Crocetta *et al.*, 2013b). Unfortunately, with the sole exception of records published in Zenetos *et al.* (2011: *partim* from Astypalaia, from a population hereby investigated), most of the specimens were recorded as empty shells, or their soft parts were not properly preserved, therefore making impossible a molecular check.

Marine alien species feature among the qualitative descriptors of good environmental status in the European Union's Marine Strategy Framework Directive. In the case of framework builder species, such as oysters (Kružić, 2014), when they become invasive with high density in the invaded areas, their bearing on autochthonous communities may be remarkable. Therefore, alien species' inventories play important roles in informing regional policy and management decisions, as well as in identifying resource allocation priorities. The scientific community is called upon to pay particular attention to their accuracy and veracity (Ojaveer *et al.*, 2014), and genetic data are often the only tool to make them reliable.

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Supplementary materials and methods

The supplementary material for this article can be found at http://www.journals.cambridge.org/MBI

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