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Molecular and morphological data provide evidence for only one alien species of pearl oyster in the Mediterranean Sea

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Summary: Taxonomic identification of alien bivalve species in the Mediterranean Sea can be challenging because of high morphological variability and the occurrence of cryptic species complexes, as in the case of pearl oysters. While the presence of the Lessepsian species *Pinctada radiata* is well-established in the eastern Mediterranean Sea, the presence of *P. fucata* was recently suggested based on morphological data. In this study we performed an integrative assessment based on morphological and genetic data on pearl oysters collected across the Mediterranean Basin. Molecular species delimitation and phylogenetic analyses allowed a straightforward taxonomic assignment of all collected specimens to *P. radiata*. These specimens show the entire suite of morphological character states considered diagnostic of either *P. fucata* or *P. radiata* by previous studies. This finding clearly demonstrates that these morphological characters have no taxonomic value, and their variability observed in specimens from the Mediterranean Sea represents (part of) the intraspecific variability of *P. radiata*. While no evidence has been found for the presence of *P. fucata*, the earlier occurrences of *P. radiata* from the eastern and southern Mediterranean Sea are complemented with verified occurrence in the western and northern regions, demonstrating a further spreading of this non-native species throughout the Mediterranean Sea. This study clarifies the taxonomic identification and geographical distribution of pearl oysters in the Mediterranean Sea and substantiates the importance of molecular identification of alien bivalves characterized by extensive variation in shell characters.

Keywords: *Pinctada radiata*; *Pinctada fucata*; pearl oyster; allochthonous species; *cox1*; species delimitation; morphological analysis.

Evidencias morfológicas y moleculares proporcionan la presencia de una sola especie exótica de ostras perleras en el mar Mediterráneo

Resumen: La identificación taxonómica de especies de bivalvos exóticos en el mar Mediterráneo representa un desafío debido a la alta variabilidad morfológica y la aparición de complejos de especies crípticas, como en el caso de las ostras perleras. Si bien la presencia de la especie Lessepsiana *Pinctada radiata* se considera bien establecida en el Mediterráneo oriental, recientemente ha sido propuesta también la presencia de *P. fucata* sobre la base de datos morfológicos. En este estudio realizamos una evaluación integrada, basada en datos morfológicos y genéticos, de ostras perleras recolectadas en la cuenca mediterránea. La delimitación molecular de las especies y los análisis filogenéticos han permitido una asignación taxonómica clara y simple de todos los especímenes colectados a *P. radiata*. Estos especímenes muestran todo el conjunto de carácteres morfológicos considerados diagnósticos de *P. fucata* o *P. radiata* en estudios previos. Este hallazgo demuestra claramente que estos caracteres morfológicos no tienen valor taxonómico y la variabilidad observada en especímenes del mar Mediterráneo representa solo en parte la variabilidad intraespecífica de *P. radiata*. Si bien no se han encontrado evidencias de la presencia de *P. fucata*, las asignaciones anteriores a *P. radiata* en el este y sur del mar Mediterráneo se complementan con la presencia verificada en las regiones occidental y septentrional, lo que demuestra una mayor propagación de esta especie no nativa en todo el mar Mediterráneo. Este estudio ha permitido clarificar la identificación taxonómica y la distribución geográfica de

las ostras perleras en el mar Mediterráneo y corroborar la importancia de la identificación molecular de bivalvos exóticos caracterizados por una amplia variación en los caracteres de la concha.

Palabras clave: *Pinctada radiata*; *Pinctada fucata*; ostras perleras; especies exóticas; *cox1*; delimitación de especies; análisis morfológico.

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INTRODUCTION

In the last few decades, the number of alien species in the Mediterranean Sea has been constantly increasing (Zenetos and Galanidi 2020). However, the estimates rely heavily on correct identifications of alien species, a task that is often challenging, especially for taxa that show a high level of phenotypic plasticity and for cryptic species complexes (Faulwetter et al. 2017, Galià-Camps et al. 2020, Garzia et al. 2022). Lack of morphological diagnostic characters is frequent in marine bivalves, which account for more than 50 alien species established in the Mediterranean Sea (Zenetos et al. 2022). Incorrect identifications of alien species could misestimate the number of these taxa and provide a biased picture of their non-native distribution (Zenetos et al. 2017).

The species *Pinctada fucata* (A. Gould, 1850) and *Pinctada radiata* (Leach, 1814) are considered alien species in the Mediterranean Sea (Scuderi et al. 2019, Zenetos and Galanidi 2020) and belong to the species complex *P. fucata*/*imbricata*/*radiata* (Margaritidae Blainville, 1824), whose members share similar morphological characters that make their identification challenging. According to the phylogenetic analyses by Tëmkin (2010) based on three nuclear markers (28S, ITS2, and H3) and one mitochondrial (16S) marker, *P. fucata*/*imbricata*/*radiata* are distinct clades but show low phylogenetic divergence. These clades are interpreted as three valid species with quite distinct geographical distributions by Cunha et al. (2011) based on the mitochondrial marker *cox1* and the nuclear marker 18S: *P. imbricata* Röding, 1798 from the western Atlantic Ocean; *P. fucata* from the Indo-Pacific; and *P. radiata* from the eastern Indian Ocean and the Red Sea. This classification into three species is currently accepted by MolluscaBase (2023).

However, while molecular data have resolved the systematics of this species complex, the morphological identification of pearl oysters recorded in the Mediterranean Sea is still debated. First records of pearl oysters in the Mediterranean Sea were morphologically assigned to *Pinctada imbricata radiata* (Leach, 1814) (Doğan and Nerlović 2008, Mienis 2004, Zenetos et al. 2004) and later to *P. radiata* (e.g. Bellaaj-Zouari et al. 2012, Petović and Mačić 2018) following the taxonomic revision of the complex (Cunha et al. 2011). Barbieri et al. (2016) confirmed that all the sequenced specimens collected in the Mediterranean Sea belong to *P. radiata*. After this work, recent reports indicated the rapid spread of the alien *P. radiata* from the southern-central and eastern Mediterranean, where this species is considered well-established, towards the western Mediterranean (Ballesteros et al. 2020, Derbali et al. 2019, Gavrilović et al. 2017, Petović and Mačić 2018, Png-Gonzalez et al. 2021). Scuderi et al. (2019) and Cunningham Aparicio and Méndez (2021) claimed that another Lessepsian species, *P. fucata*, occurs throughout the Mediterranean Sea, in many cases in nearby localities with *P. radiata*. However, these studies are based on only morphological characters. According to Scuderi et al. (2019), *P. fucata* and *P. radiata* can be adequately discriminated by morphological characters. However, their diagnostic value has not been verified with molecular data. Despite the lack of molecular evidence, the most recent checklists have included both *P. fucata* and *P. radiata* as present in Italian waters (Renda et al. 2022) and in the Mediterranean Sea (Zenetos et al. 2022).

In this study, we performed an integrative assessment based on morphological and genetic data on pearl oysters collected across the central, southern and eastern Mediterranean Sea. Molecular species delimitation analyses based on the barcode marker *cox1* allowed a straightforward taxonomic assignment of collected specimens. Based on these results, we tested the diagnostic value of the morphological characters proposed by Scuderi et al. (2019). Finally, we provided an updated taxonomic classification and distribution of pearl oysters invading the Mediterranean Sea.

MATERIAL AND METHODS

Sampling areas

Pearl oysters were sampled between 2011-2021 in nine localities from the central, southern and eastern Mediterranean Sea. These also include localities where both *P. fucata* and *P. radiata* were previously recorded (Scuderi et al. 2019). Details of all the sampling local-

ities are listed in Table 1. All tissues of *Pinctada* specimens are stored in ethanol 95%. All dry shells are in the molluscan collection of Paolo Mariottini at the Roma Tre University, Rome, Italy.

Molecular analyses

A piece of adductor muscle was clipped and total DNA extraction was carried out using the high-salt protocol (Evans 1990). The standard barcoding fragment *cox1* was amplified using universal primers LCO1490 and HCO2198 (Folmer et al. 1994). The PCR conditions used were a 3 min denaturation step at 94°C; 35 cycles of 94°C/60 s, 48°C/60 s, 72°C/60 s; and a 10 min final extension at 72°C. The amplified products were sequenced by Macrogen Inc., Amsterdam, the Netherlands. The sequences are deposited in the GenBank database (https://www.ncbi.nlm.nih.gov/GenBank/) with accession numbers OR676328- OR676344.

We downloaded 263 *cox1* sequences of *Pinctada* spp. (Margaritidae) from GenBank (the GB dataset, updated to 31/12/2022) to build a dataset of 281 *cox1* sequences including the 17 newly generated sequences of *Pinctada* sp. and the outgroup *Pteria hirundo* (Linnaeus, 1758) (GB Accession Number AF120647). This dataset was aligned using the G-INS-i algorithm in the MAFFT v.7 server (Katoh et al. 2019) and trimmed to the standard *cox1* barcoding fragment (the 5′ portion of the gene) using Folmer's primers as a reference.

Molecular species identification was based on distance-based and phylogenetic tree-based approaches. A preliminary phylogenetic assessment was performed with a neighbour-joining (NJ) tree based on uncorrected *p*-distance calculated in MEGA X (Kumar et al. 2018) and the pairwise deletion option. The NJ tree allowed us to detect 41 misidentified sequences named as *P. margaritifera* but belonging to the freshwater *Margaritifera margaritifera* (Linnaeus, 1758) [Margaritiferidae J. Henderson, 1929 (1910)]. These misidentified sequences were excluded and a final dataset of 222 sequences was used for distance-based and tree-based species delimitation analyses.

Assemble species by automatic partitioning (ASAP) analysis were run using the ASAP web interface [https://bioinfo.mnhn.fr/abi/public/asap; (Puillandre et al. 2021)] with the Kimura-two parameter [K2P; (Kimura 1980)] substitution model and default parameters. ASAP delimitation was defined by evaluating both the partitions with the first and the second-best ASAP scores according to Puillandre et al. (2021).

As a tree-based method, we used the multirate Poisson tree processes (mPTP) model (Kapli et al. 2017) based on a maximum likelihood (ML) tree. ML analyses were performed in the W-IQ-TREE web server v.1.6.12 [http://iqtree.cibiv.univie.ac.at/; (Trifinopoulos et al. 2016)]. Node support was assessed with 1000 pseudoreplicates of ultrafast bootstrapping [uBS; (Minh et al. 2013)]. FigTree v.1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/) was used to visualize the tree. The previous ML tree was used as an input file (excluding the outgroup) for mPTP analysis using the web platform (https://mptp.h-its.org/#/tree).

Table 1. – Vouchers, GenBank accession number, sampling localities, coordinates and sampling dates of the pearl oyster specimens analysed in this study.

Voucher	Accession number	Sampling locality	Latitude	Longitude	Sampling date
RM3-PM-142a	OR676331	Malta: Blue Grotto	35° 49' 14.59'' N	144° 34' 32.37'' E	November 2014
RM3-PM-142b	OR676332	Malta: Blue Grotto	35° 49' 14.59'' N	144° 34' 32.37'' E	November 2014
RM3-PM-149a	OR676335	Greece: Rhodes, Lindos	36° 5' 47.31'' N	28° 5' 19.06'' E	August 2011
RM3-PM-149b	OR676337	Greece: Rhodes, Lindos	36° 5' 47.31'' N	28° 5' 19.06'' E	August 2011
RM3-PM-153a	OR676333	Greece: Astypalea Island	36° 32' 53.68'' N	26° 21' 23.08'' E	July 2012
RM3-PM-153b	OR676336	Greece: Astypalea Island	36° 32' 53.68'' N	26° 21' 23.08'' E	July 2012
RM3-PM-154a	OR676341	Italy: Taranto, Mar Piccolo di Taranto	40° 28' 52.03'' N	17° 14' 12.58'' E	Dicember 2020
RM3-PM-154b	OR676342	Italy: Taranto, Mar Piccolo di Taranto	40° 28' 52.03'' N	17° 14' 12.58'' E	Dicember 2020
RM3-PM-171a	OR676339	Italy: Briatico	38° 43' 41.48'' N	16° 1' 54.51'' E	November 2020
RM3-PM-171b	OR676340	Italy: Briatico	38° 43' 41.48'' N	16° 1' 54.51'' E	November 2020
RM3-PM-173	OR676334	Italy: Sassari, Olbia, Punta delle Saline	40° 54' 41.90'' N	9° 34' 32.09'' E	July 2021
RM3-PM-174a	OR676343	Italy: Sassari, Olbia, Lido Del Sole	40° 55' 8.69'' N	9° 33' 49.20'' E	January 2022
RM3-PM-174b	OR676344	Italy: Sassari, Olbia, Lido Del Sole	40° 55' 8.69'' N	9° 33' 49.20'' E	January 2022
RM3-PM-175a	OR676328	Cyprus: Polis	35° 2' 41.76'' N	32° 25' 3.81'' E	June 2019
RM3-PM-175b	OR676330	Cyprus: Polis	35° 2' 41.76'' N	32° 25' 3.81'' E	June 2019
RM3-PM-176a	OR676338	Italy: Messina, Faro Lake	38° 16' 8.30'' N	15° 38' 13.07'' E	July 2021
RM3-PM-176b	OR676329	Italy: Messina, Faro Lake	38° 16' 8.30'' N	15° 38' 13.07'' E	July 2021

Furthermore, we performed a phylogeographic analysis of pearl oysters' haplotype across the native and non-native (Mediterranean) distribution ranges, using phylogenetic network methods based on 57 *cox1* sequences assigned to the *Pinctada fucata*/*imbricata*/*radiata* species complex. We used POPART v1.7 (Leigh and Bryant 2015) to build phylogenetic networks based on the median-joining method (Bandelt et al. 1999) and the statistical parsimony method TCS (Clement et al. 2000). We visualized the geographic distribution of haplotypes by assigning them different colours according to the region of origin.

Finally, we mapped all the occurrences of specimens molecularly identified as *P. radiata* (Barbieri et al. 2016, Gavrilović et al. 2017, this study) along with Sardinian specimens morphologically identified as *P. radiata* [Stasolla et al. 2014, Grech and Caracciolo (2023) in Fortic et al. (2023)] and specimens identified either as *P. fucata* or *P. radiata* by Scuderi et al. (2019) and Cunningham Aparicio and Méndez (2021) based on the characters proposed by Scuderi et al. (2019). Occurrences of specimens analysed by Scuderi et al. (2019) are georeferenced according to the information on localities provided in Table 2 of their study.

Morphological analysis

To assess the diagnostic value of morphological characters proposed by Scuderi et al. (2019), we carried out a morphological assessment of the molecularly identified pearl oysters collected in the Mediterranean Sea. We analysed the following six morphological characters that are considered discriminant between *P. fucata* and *P. radiata* by Scuderi et al. (2019) (Table 2): (i) the general outline of the shell, (ii) the hinge structure (anterior tooth of the left valve), (iii) the shape of the ligament (hinge line), (iv) the extent of the ligament area, (v) the sculpture of the shell, and (vi) the prevalent colour of the valves. Characters' states were assigned based on Scuderi et al (2019: Figures 6-7, 10-11, 17-18, 20-21, 27-30 and 32-33 and Table 1). To better visualize character state distribution across the analysed specimens, we coded states into numbers, except for the "shell sculpture" and the "shell colour" because of the wide variability of these two characters, for which a short description of the state is provided. In "shell sculpture", the term "processes" used in Scuderi et al. (2019) refers to growths of the outer shell similar to imbricated lamellar structures. State "1" and "2" in Table 2 refer respectively to the diagnostic characters of *P. radiata* and *P. fucata sensu* Scuderi et al. (2019). We also added information relative to the specimen size and environmental characteristics of collection sites that are considered by Scuderi et al. (2019) (Table 2). According to Table 2, we selected two couples of voucher specimens to illustrate the morphological characteristics deemed as diagnostic of *P. radiata* (RM3-PM-149b and RM3-PM-174b) and *P. fucata* (RM3-PM-175a and RM3-PM-175b) *sensu* Scuderi et al. (2019).

RESULTS

Molecular analyses

All species delimitation analyses, whether distance-based or tree-based, support the three clades *P. fucata*/*imbricata*/*radiata* as distinct species (Fig. 1). In the ML tree, all 17 specimens collected in the Mediterranean clustered within the clade of *Pinctada radiata* (Fig. 2), which received high bootstrap support (uBS=98). The *P. radiata* clade is sister to the clades of *P. imbricata* and *P. fucata*, which also received high support (uBS=99-100).

The median-joining network showed three well distinct haplotype groups corresponding to sequences of (i) *P. imbricata* from the Atlantic Ocean, (ii) *P. fucata* from the Indo-Pacific Ocean, and (iii) *P. radiata* from the Mediterranean Sea and Persian Gulf (Fig. 2). These three haplogroups were separated by a high number (21-30) of mutational steps (nucleotide substitutions) and were recovered as separated sub-networks in the statistical parsimony analysis (result not shown).

Morphological analysis

Results of the screening of the six morphological characters proposed by Scuderi et al. (2019) in the 17 specimens analysed in this study are summarized in Table 2. The 17 specimens, molecularly identified as *P. radiata* (Fig. 1), show a mixture of character states that were considered as diagnostic of either *P. fucata* or *P. radiata* by Scuderi et al. (2019). Indeed, specimens of *P. radiata* show either an "oval" (Fig. 3G-H, J-K) or "rounded" (Fig. 3A-B, D-E) general outline, a "not duplicated" (Fig. 3L) or "duplicated" (Fig. 3C, F, I) hinge structure, a "straight" (Fig. S2c, S3c) or "curved" (Fig. 3C) ligament shape, and a "wide" (Fig. 3C, F) or "narrow" (Fig. 3I, L) ligament area. Furthermore, we observed intermediate states in the characters "hinge structure (A_TOO)" (Fig. S6, S13c, S14c) and "shape of the ligament (H_LIN)" (Fig. 3F, I, L). The variation of the sculpture and colours of the shells showed more than two alternative states described by Scuderi et al. (2019; Table 1) for *P. fucata* and *P. radiata* and included at least 7 and 12 states, respectively.

DISCUSSION

The low resolution of systematic relationships and taxonomic status of the *P. fucata*/*imbricata*/*radiata* species complex was previously due to extensive variation in shell characters among and within populations and wide geographical distribution. Phylogenetic studies based on molecular data have accurately identified three evolutionary and taxonomic units within this complex, recovered as reciprocally monophyletic clades (Cunha et al. 2011, Somrup et al. 2022, Tëmkin 2010) that are currently reported as distinct species in MolluscaBase (2023). Results from the present study are in close agreement with these findings. Molecular species delimitation and phylogenetic network analy-

Table 2. – Morphological characters that were considered discriminant between *P. fucata* and *P. radiata* by Scuderi et al. (2019): the general outline and shell (S_OUT), the hinge structure (anterior tooth of the left valve; A_TOO), the shape of the ligament (hinge line; H_LIN), and the extent of the ligament area (L_ARE). We also report the variability of the shell sculpture and the shell coloration. States 1 and 2 refer respectively to the diagnostic characters of *P. radiata* and *P. fucata sensu* Scuderi et al. (2019). Stars on the columns A_TOO and H_LIN correspond to intermediate characters for which the assignation of the state was challenging due to the presence of, respectively, "duplicated tooth barely visible" and "curved hinge line barely visible". Additional information on the height of the shell and on the environment of the sampling location are reported at the end of the table. Specimen vouchers in bold are present in Figure 3.

Species/specimens	S_OUT[a]	A_TOO[b]	H_LIN[c]	L_ARE[d]	Shell sculpture	Shell colour	Height of the shell	Environment
Pinctada radiata [Scuderi et al. (2019)]	1	1	1	1	Numerous rows of dense and pointed processes	Red-browndish with darker vertical strips	High up 75 mm	Hard substrate
Pinctada fucata [Scuderi et al. (2019)]	2	2	2	2	Low number of rows and sparse, blunt and wider processes	Greenish with paler vertical strips	high up to 45 mm	Sandy bottom near estuarine areas
RM3-PM-142a	1	2	1	2	Numerous rows of dense and pointed processes	Reddish with darker radial rows	41 mm	On a buoy rope
RM3-PM-142b	1	2	1	1	Numerous rows of dense and pointed processes	Reddish with darker radial rows	45 mm	On a buoy rope
RM3-PM-149a	2	1	1	2	Almost smooth; low number of rows of sparse processes	Light brownish with dark radial strips	46 mm	On rocky substrate
RM3-PM-149b	1	1	1	1	Numerous rows of dense and pointed processes	Reddish with white radial strips	45 mm	On rocky substrate
RM3-PM-153a	1	*	1	2	Numerous rows of dense and pointed processes	Yellowish with brownish radial strips	47 mm	On rocky substrate
RM3-PM-153b	1	*	1	2	Numerous rows of dense and pointed processes	Reddish with white radial strips	49 mm	On rocky substrate
RM3-PM-154a	1	1	1	2	Partially encrusted; dense and pointed processes visible only on the edge	Uniformly reddish	42 mm	On rocky substrate
RM3-PM-154b	1	*	1	2	Almost smooth; low number of rows of sparse processes	Uniformly reddish	44 mm	On rocky substrate
RM3-PM-171a	1	2	*	1	Uniformly smooth	Uniformly black	41 mm	On rocky substrate
RM3-PM-171b	1	*	1	1	Uniformly smooth	Uniformly black	43 mm	On rocky substrate
RM3-PM-173	1	1	2	1	Numerous rows of dense and pointed processes	Light green with dark green radial strips	58 mm	On rocky substrate
RM3-PM-174a	1	1	2	1	Numerous rows of dense and pointed processes	Green without radial strips	54 mm	Beached in front of a sandy coast
RM3-PM-174b	1	1	*	1	Low number of rows of sparse and wider processes	Light green with dark green strips	55 mm	Beached in front of a sandy coast
RM3-PM-175a	2	1	*	2	Numerous rows of dense and pointed processes	Dark brown reddish with white radial strips	43 mm	On rocky substrate
RM3-PM-175b	2	2	*	2	Low number of rows of sparse processes	Reddish with white radial strips	41 mm	On rocky substrate
RM3-PM-176a	1	2	1	2	Uniformly smooth	Encrusted	75 mm	On rocky substrate
RM3-PM-176b	1	*	1	2	Uniformly smooth	Encrusted	80 mm	On rocky substrate

[a] Rounded, 1 / Oval, 2
[b] Duplicated, 1 / Not duplicated, 2
[c] Curved near umbo, 1 / Straight, 2
[d] Narrow, 1 / Wide, 2

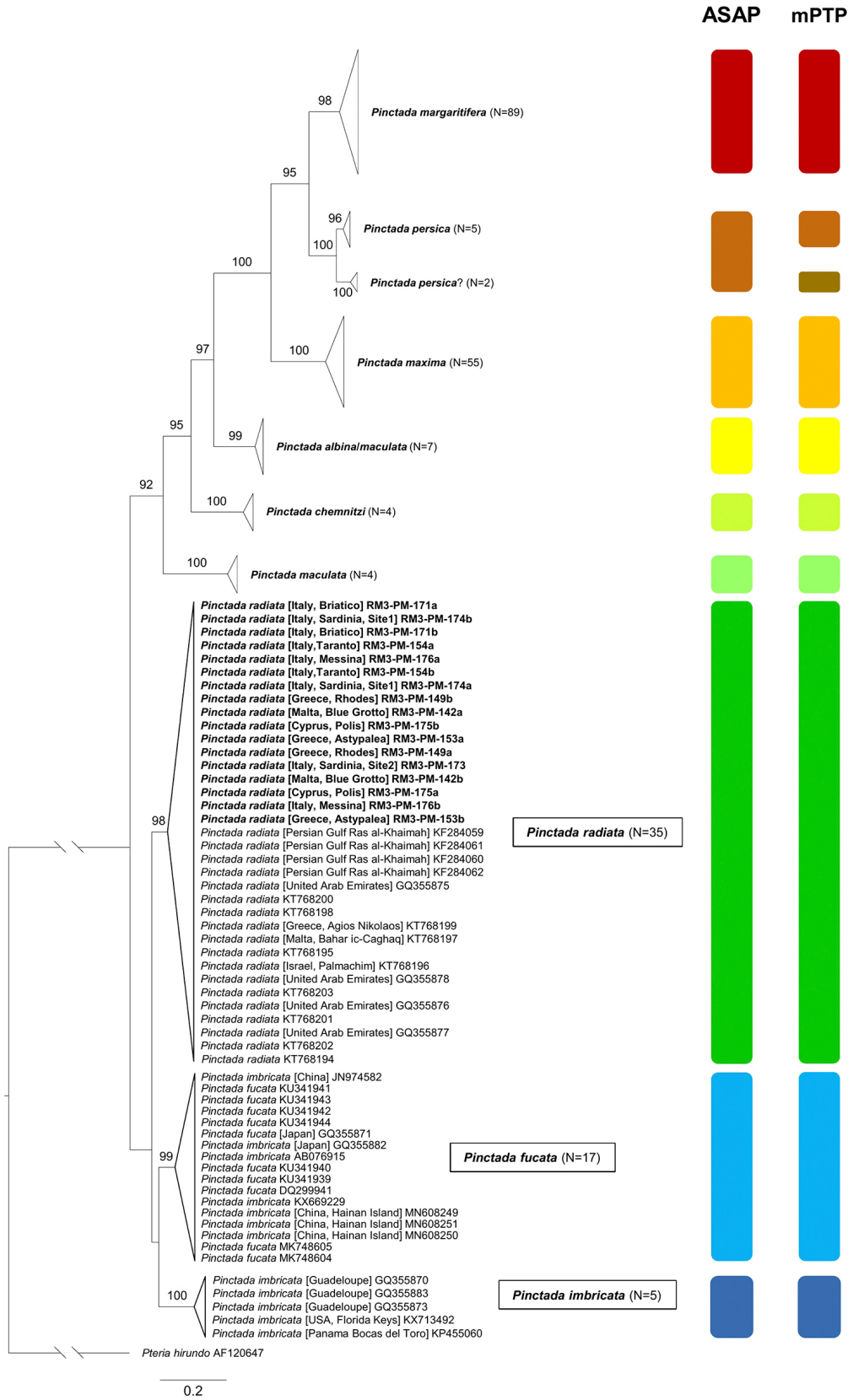

Fig. 1. – Maximum likelihood (ML) phylogenetic tree of Margaritidae based on COI sequences. The ML tree is rooted with *Pteria hirundo*; ultrafast bootstrap values are reported on the nodes (uBS >80%). Sequence names of *P. radiata* in bold are from this study. Taxonomic nomenclature of sequences reflects information from GenBank and is in line with previous phylogenetic works (Cunha et al. 2011, Somrup et al. 2022, Tëmkin 2010).

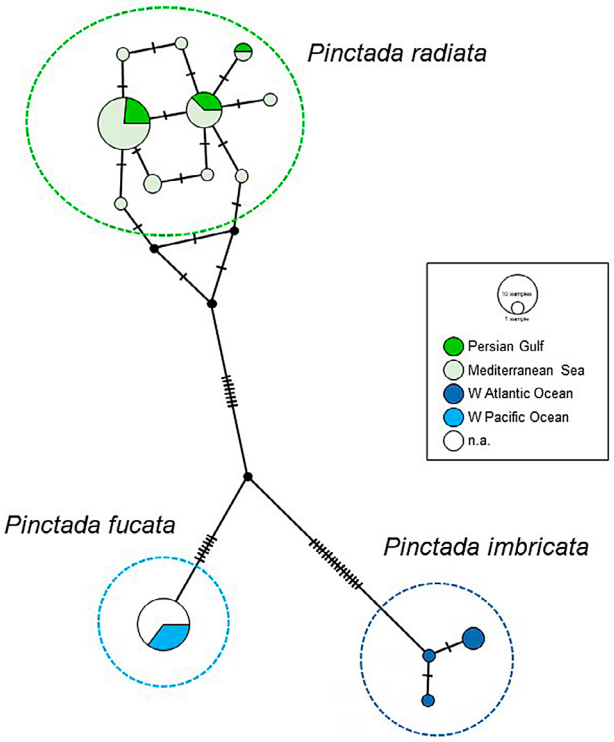

Fig. 2. – Median-joining network based on COI sequences of *P. fucata*/*imbricata* /*radiata* generated in this study and retrieved from Genbank. Haplotypes in the network are represented by circles with size proportional to their frequencies and coloured according to their geographic distribution; white colour represents haplotypes without geographic information (n.a.); small vertical bars represent nucleotide substitutions. Dashed lines represent haplogroups recovered as separated sub-networks in the TCS analysis (result not shown).

ses corroborate the taxonomic status of the species *P. fucata*, *P. imbricata* and *P. radiata* (Fig. 1 and Fig. 2) and provide further evidence of the utility of the *cox1* barcode marker in identifying specimens to these taxa.

All sequenced specimens collected in the Mediterranean Sea from 16 different localities, either in this study or in previous studies (Barbieri et al. 2016, Gavrilović et al. 2017), are unambiguously assigned to *P. radiata* (Figs 1 and 2). Compared with previously available data, this study contributes nine additional records of *P. radiata* from the southeastern Mediterranean Basin as well as northern regions and provides the first molecular validation of the presence of this species in Sardinia (Stasolla et al. 2014, Grech and Caracciolo 2023 in Fortic et al. 2023). Sardinian records indicate that this Lessepsian species is spreading further towards the northern and western sectors of the Mediterranean Sea. This concern is confirmed by recent molecular data, made available in GenBank at the time of preparation of our study, that indicate the presence of *P. radiata* also in the Adriatic Sea (Gavrilović et al. 2017). All these records verified by molecular data, including the recent data from the Adriatic Sea, are mapped in Figure 4, which represents the latest update on the distribution and spreading of *P. radiata* in the Mediterranean Sea.

Distinguishing specimens of these three closely related species based on morphological characters remains challenging. Scuderi et al. (2019) were the first to propose a set of six diagnostic morphological characters suitable to distinguish specimens of *P. fucata* and *P. radiata* collected from the Mediterranean Sea. However, our morphological assessment showed that molecularly identified specimens of *P. radiata* collected from several localities of the Mediterranean Sea encompass the entire suite of character states that Scuderi et al. (2019) considered diagnostic of either *P. fucata* or *P. radiata* (Table 2). This finding is quite surprising considering the much lower number of specimens assessed morphologically in our study (N=17) compared with the study by Scuderi and colleagues (N=1284) and clearly suggests that the variation in shell colour and sculpture of *P. radiata* is vastly larger than that observed by this study. Another character that proved challenging in the morphological assessment was the shape of the hinge line. Scuderi et al. (2019) described the hinge line either as straight or curved. We observed that in small specimens with an underdeveloped hinge region the hinge line seems straight or barely curved (see Supplementary Figs S2cd and S3cd), whereas in specimens with a more developed hinge area a well-defined curved hinge line occurs more frequently (see Supplementary Figs S5cd and S6cd). Therefore, while this character certainly has no taxonomic value, it seems that it may be related to the size variation of the hinge region.

The results of our combined and morphological assessments clearly demonstrated that the characters proposed by Scuderi et al. (2019) have no taxonomic value. The morphological variability observed in the pearl oyster specimens from the Mediterranean Sea clearly represents the intraspecific variability of *P. radiata* (Bellaaj-Zouari et al. 2012, Hmida et al. 2021). These findings are in agreement with previous morphometric investigations that documented an extensive variability of the shell morphology of *P. radiata* within populations in the native distribution area (Rajaei et al. 2014 2015), as well as within non-native populations in the Mediterranean Sea (Bellaaj-Zouari et al. 2012, Hmida et al. 2021). To date no diagnostic discrete morphological characters have been identified for populations of the closely related pearl oyster species *P. imbricata*, *P. fucata* and *P. radiata* (Huber 2010, Ranson 1961). In line with results from the present study, molecular data by Cunha et al. (2011) showed that morphological characters traditionally used to discriminate between *P. fucata* and *P. martensii* have no taxonomic value and represent the intraspecific variability of the *P. fucata*. Therefore, molecular validation remains essential for the identification of pearl oysters. In this respect, we have no evidence that *P. fucata* occurs in the Mediterranean Sea. Indeed, the Mediterranean records of *P. fucata* (Cunningham Aparicio and Méndez 2021, Scuderi et al. 2019) are based only on morphological characters that we have shown to encompass the morphological

variability of *P. radiata.* To date all the sequenced specimens of pearl oysters collected in the Mediterranean Sea are assigned to *P. radiata*, even from the same localities from where Scuderi et al. (2019) identified specimens as *P. fucata* based on morphological characters. It is therefore highly likely that samples identified as *P. fucata* based on morphological characters by Cunningham Aparicio and Méndez (2021) also belong to *P. radiata*, suggesting that the latter species has reached the westernmost region of the Mediterranean Sea.

CONCLUSION

This study clarifies the taxonomic identification and geographical distribution of pearl oysters in the Mediterranean Sea. Morphological characters pro-

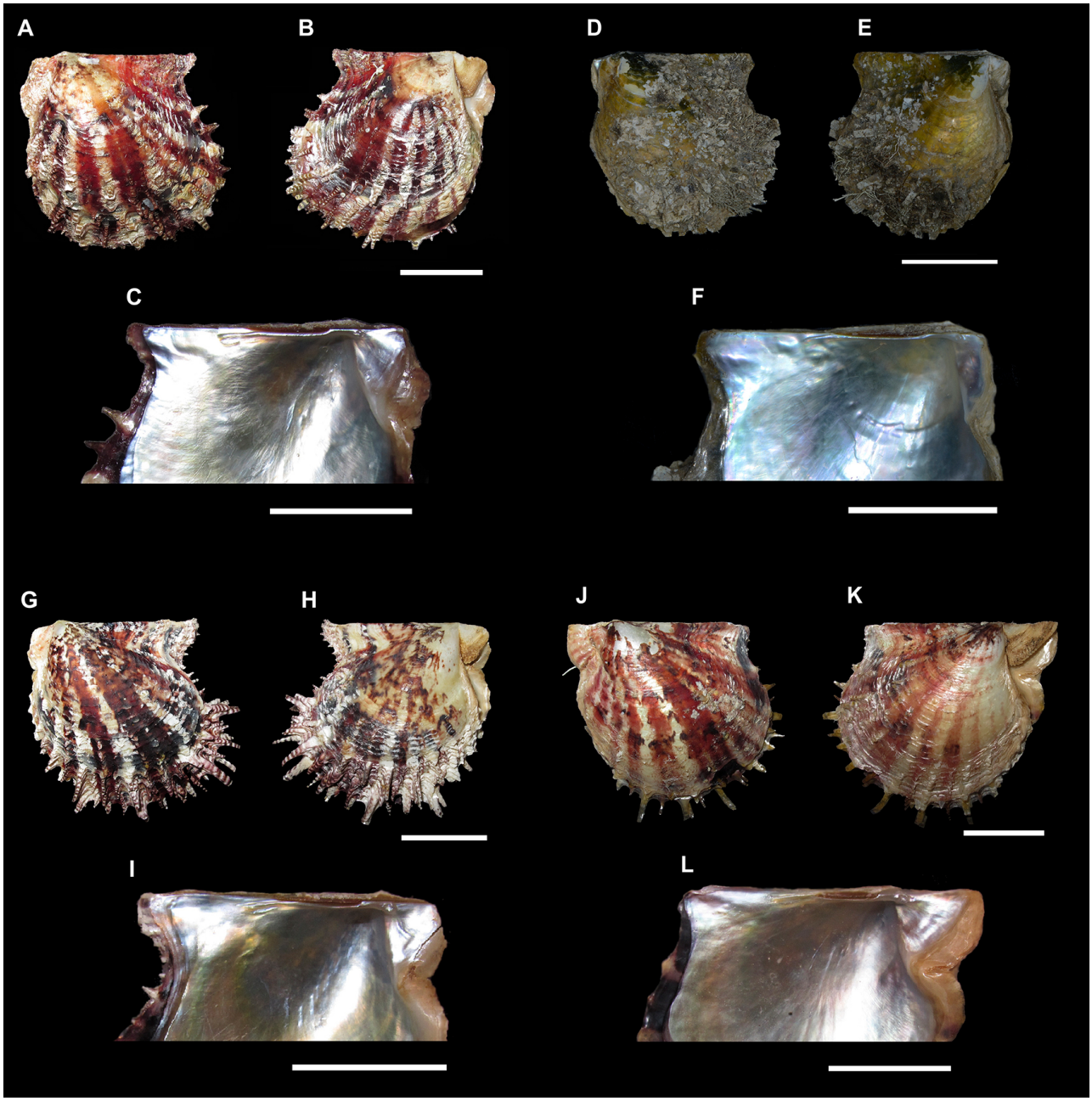

Fig. 3. – Variability of morphological characteristics in *P. radiata* specimens identified molecularly: the general outline and shell (S_OUT), the hinge structure (anterior tooth of the left valve; A_TOO), the shape of the ligament (hinge line; H_LIN), the extent of the ligament area (L_ARE), and shell sculpture and shell colour (see Table 2). (A-C) The voucher specimen RM3-PM-149b and (D-F) the voucher specimen RM3-PM-174b show morphological characteristics typical (i.e. diagnostic) of *P. radiata sensu* Scuderi et al. (2019). (G-I) The voucher specimen RM3-PM-175a and (J-L) the voucher specimen RM3-PM-175b show morphological characteristics typical of *P. fucata sensu* Scuderi et al. (2019).

posed by Scuderi et al. (2019) as diagnostic for either *P. fucata* or *P. radiata*, based on which the presence of *P. fucata* was newly reported in the Mediterranean, were not validated by the integrative morphological-molecular approach carried out in this study. The earlier occurrences of *P. radiata* from the eastern and southern Mediterranean Sea are complemented with verified records in the western and northern Mediterranean, demonstrating that this non-native species is spreading further throughout the Mediterranean Sea. The integrative approach used in this study, combining molecular and morphological data, proved fruitful for the taxonomic identification of alien bivalves characterized by extensive variation in shell characters. This approach demonstrates once again that, in many organism groups, relying on only morphological characters might determine wrong taxonomic identification and mislead our estimates on the number and distribution of alien species. For these organisms, we strongly suggest requesting the verification of any new records of alien species with molecular data and recommend including in checklists only molecularly verified occurrences.

SUPPLEMENTARY FILES

Supplementary material contains the Sardinian localities of *Pinctada radiata* and morphological plates with the inner and outer shell of all the new *Pinctada radiata* specimens analysed in this study.

DATA AVAILABILITY

All genetic data of new specimens analysed in this study are available on GenBank (http://www.ncbi.nlm.nih.gov/genbank/) with the accession numbers listed in the text (Table 1).

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AUTHOR CONTRIBUTION STATEMENT

Matteo Garzia: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original

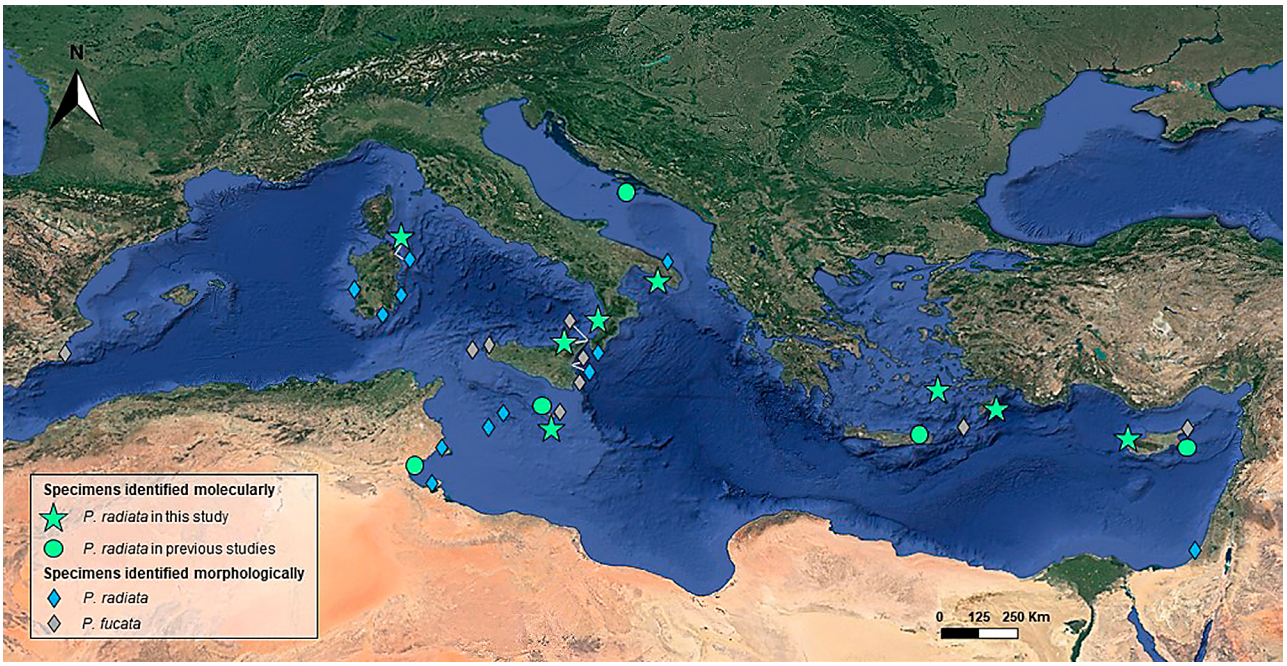

Fig. 4. – Map showing the distribution of specimens identified as *P. radiata* and *P. fucata* in the Mediterranean Sea based on molecular data and on the morphological characters proposed by Scuderi et al. (2019). Green symbols represent specimens identified molecularly as *P. radiata* in this study (green stars) or in previous studies (green circles; Barbieri et al. 2016; Gavrilović et al. 2021). Diamonds represent specimens identified morphologically as *P. radiata* (in blue) or *P. fucata* (in grey) by previous studies (Stasolla et al. 2014, Scuderi et al. 2019, Cunningham Aparicio and Mulero Méndez 2021; Grech and Caracciolo 2023 in Fortic et al. 2023). Specimens analysed by Scuderi et al. (2019) are georeferenced according to information provided in Table 2 of the same study. Map data: Google, 2023 CNES/Astrium, Maxar Technologies.

draft, Writing – review and editing. **Mauro Doneddu**: Sampling, Investigation, Writing – original draft, Writing – review and editing. **Salvatore Giacobbe**: Sampling, Investigation, Writing – review and editing. **Daniele Salvi**: Conceptualization, Methodology, Writing – original draft, Writing – review and editing. **Egidio Trainito**: Sampling, Investigation, Writing – review and editing. **Paolo Mariottini**: Sampling, Wet lab activities, Investigation, Writing – original draft, Writing – review and editing.

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