Mediterranean Marine Science

Vol 23, No 4 (2022)

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doi: [10.12681/mms.29218](https://doi.org/10.12681/mms.29218)

To cite this article:

GARZIA, M., FURFARO, G., RENDA, W., ROSATI, A.-M., MARIOTTINI, P., & GIACOBBE, S. (2022). Mediterranean spreading of the bicolor purse oyster, Isognomon bicolor, and the chicken trigger, Malleus sp., vs. the Lessepsian prejudice. *Mediterranean Marine Science*, *23*(4), 777–788. https://doi.org/10.12681/mms.29218

Research Article

Mediterranean Marine Science
Indexed in WoS (Web of Science, ISI Thomson) and SCOPUS
The journal is available on line at http://www.medit-mar-sc.net
www.hcmr.gr
DOI: http://doi.org/10.12681/mms.29218

Mediterranean spreading of the bicolor purse oyster, *Isognomon bicolor*, and the chicken trigger, *Malleus* sp., vs. the Lessepsian prejudice

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Contributing Editor: Argyro ZENETOS

Received: 13 January 2022; Accepted: 01 June 2022; Published online: 02 September 2022

Abstract

The introduction rate of alien species in the Mediterranean Sea is rapidly growing, and their taxonomical identification is increasingly challenging. This uncertain identification often leads to an incorrect estimation of the number of alien species, their route of introduction, and their potential negative effects. This is particularly true for some bivalves, which are characterized by high variation in their shells, resulting in uncertain morphological identification. This is the case for two alien bivalves, i.e., an Isognomonidae and a Malleidae species, both characterized by confused historical colonization records in the Mediterranean Sea, misidentifications, and controversial and changing nomenclatures that have insofar negatively affected our knowledge on their geographical distributions. In this respect, molecular approaches provide a strategy that is especially useful when traditional taxonomy fails, and DNA barcoding is a powerful and well-known tool to obtain reliable identifications through efficient molecular markers. In this work, we used the 16S rRNA marker to assess the preliminary identification of *Isognomon* sp. and *Malleus* sp. specimens from different localities in the Southern Mediterranean Sea. Bayesian inference (BI) and maximum likelihood (ML) methods were applied to test the monophyly of the phylogenetic linages and to clarify their taxonomic positions, allowing a complete overview of the colonization and spreading of these two alien bivalves in the Mediterranean Sea. In particular, the *Isognomon* sp. specimens were identified as the Atlantic *I. bicolor*, highlighting that previously suggested invasive migration patterns, (i.e., the Lessepsian migration), must be reconsidered with stronger critical attention in light of currently occurring global changes.

Keywords: alien species; DNA barcoding; molecular systematic; *Isognomon*; *Malleus*.

Introduction

The first record of the non-indigenous genus *Isognomon* [Lightfoot], 1786 in the Mediterranean Sea is due to Mienis (2004), who reported on an unspecified number of *Isognomon ephippium* (Linnaeus, 1758) specimens collected in Israel. Later, Zenetos *et al*. (2005) and Galil (2007) questioned the occurrence of this species, as it was recorded only once on an oil platform from the Red Sea. Still in Israel, a single initially undetermined "Hammer or Tree oyster" shell, was found in 2015 and, based on the iconography of Oliver (1992: 67, plt 10, figs. 4a-f) and Huber (2010: 176, t*ext-figure*), was identified by Mienis *et al*. (2016) as *Isognomon legumen* (Gmelin, 1791). Afterwards, the settlement of this latter species along the Israelian coast was confirmed by Marchini *et al*. (2020) and reported from Turkey by Ovalis & Zenetos in Stamouli *et al*. (2017) and from Greece by Angelidis in Lipej *et al*. (2017). The latter group mentioned the previous misidentification of *I. legumen* with the co-occurrent *Malleus regula* (Forsskål in Niebuhr, 1775) (see Angelidis in Lipej, 2017) and traced the establishment of this alien bivalve in the Aegean Sea to 2016. However, specimens reported as *M. regula* from Lybia were subsequently re-identified as *I. legumen* by Crocetta (2018), who suggested that this species might have occurred in the Mediterranean Sea before 1996. Concurrently, its rapid spread westward in the Mediterranean basin was confirmed by reports from the Ionian coast of Sicily (Scuderi & Viola, 2019) and the Strait of Messina (Giacobbe & Renda in Dragičević *et al*., 2019), whilst Angelidis & Polyzoulis (2018) reported *I. legumen* and *I. australica* (Reeve, 1858) (first record from the Mediterranean Sea) as sympatric in the South Aegean. The occurrence of *I. australicus* in the Eastern Mediterranean was confirmed by Manousis (2021). In this regard, we noted that juveniles *I. legumen* reported from Greece by Micali *et al*. (2017)*,* and other specimens reported as "*Malleus regula*" by Angelidis in Lipej *et al*. (2017) but re-identified as *I. legumen* by Crocetta *et al*. (2017), appear very similar to the *I. australica* specimens outlined by Angelidis & Polyzoulis (2018). However, according to Zenetos *et al*. (2018) "the contemporary presence in Astypalaia of two taxa belonging to *Isognomon* Lightfoot, 1786, one of which apparently absent from the Red Sea, seems unlikely", so that the same researchers preferred to ascribe both records to the Red Sea taxon *I. legumen* "to avoid the inflation of alien molluscan records and pending molecular confirmations". Partially in agreement with this latter opinion, Albano *et al*. (2021) prudentially reported "*I.* aff. *australica* (*sensu* Angelidis and Polyzoulis, 2018)" from Cyprus and Kriti. The current literature thus demonstrates how our knowledge on the genus *Isognomon* in the Mediterranean Sea is complicated by a series of misidentifications, conflicting opinions and interpretations. This is unsurprising when we consider that the genus *Isognomon* is characterized by marked habitat-dependent variability in shell shapes (Fischer-Piette 1976; Coan *et al*., 2000; Wilk & Bieler, 2009) and convergent interspecific morphologies (Benthotage *et al*., 2020). These features make shell-based discrimination in the species unreliable, as shown by Wilk & Bieler (2009) for *I. alatus* (Gmelin, 1791) vs. the sympatric *I. bicolor* (C.B. Adams, 1845) and the allopatric *I. ephippium* in the Florida Keys. Morphological similarities and shell shape variability may also explain the presumptive record from the Canary Islands of the exclusively Indo-Pacific *I. isognomum* (Linnaeus, 1758) (Gömez Rodriguez & Perez Sanchez, 1998), which should instead be assigned to the West African *I. dunkeri* (P. Fischer 1881), according to Huber (2015), or to the West Atlantic *I. bicolor*, according to Holmes *et al*. (2015). The entire systematics of the genus *Isognomon*, however, is controversial and requires an update supported by molecular data. For example, according to Huber (2015) the phylogenetic data of Tëmkin (2010) indicates that the two most common Indo-Pacific Isognomonidae, i.e., *Isognomon (I.) ephippium* (Linnaeus 1758) and *I. (I.) isognomum* (Linnaeus 1758), may be derived from two sibling species. It follows that a resolution of valid names, with the related species distributions and habitat preferences, requires thorough review, necessarily supported by detailed illustrations of the genetically analysed specimens. Similar considerations may concern the above-mentioned *M. regula*, whose putative spread across the Mediterranean remains uncertain, also due to the frequent erroneous attribution of *Isognomon* sp. specimens (Crocetta *et al*., 2017; Crocetta, 2018). *Malleus regula*, although a precocious Lessepsian migrant [the first Mediterranean record from Syria and Lebanon by Moazzo (1931)] has been reported as established in the Levantine Basin only within the last decade (Marchini *et al*., 2020) and in the Ionian Sea even more recently (Prato & Rubino in Kousteni *et al*., 2019; Bini & Pinasco, 2021), with an apparent disjunct distribution which could indicate unequal investigation efforts. In this paper, the first molecular identification of the above-mentioned presumptive species is provided through DNA barcoding and a phylogenetic approach using Bayesian inference (BI) and maximum likelihood (ML) methods. We use these data to clarify the taxonomic position of two controversial taxa, providing insight on the patterns and processes of their introduction and spread in the Mediterranean Sea.

Materials and Methods

Monitoring and sampling

Monitoring of Non-Indigenous Species, NIS, starting in the framework of the PRA 2008/2009 program "Settlement dynamics and colonization of allochthonous assemblages in the Capo Peloro Lagoon" (Strait of Messina, central Mediterranean), was gradually extended to the adjacent Tyrrhenian and Ionian basins and integrated with further investigation in North Ionian, Aegean, and Levantine Sea (PhD school contribution 2016; PON 2014-2020, grant AIM 1848751-2). Two sampling methods have been employed, i.e., by hand collection during snorkelling and SCUBA recognition and scraping of 30x30 cm hard bottom surface. The scraped samples, retained in a fine net (< 100 µm), were fixed in ethanol 70% in the field or, whenever possible, rapidly brought in the laboratory and immediately frozen. Subsequently, an accurate sorting was carried out under a stereomicroscope, and the selected specimens definitively fixed in 70% or 95% ethanol, depending on their destination for analysis. Shell characters are investigated to propose a morphological identification comparing the shells of the new Mediterranean specimens with shells of previous works (Huber, 2010; Mienis *et al*., 2016; Micali *et al*., 2017; Ovalis & Zenetos in Stamouli *et al*., 2017; Angelidis & Polyzoulis, 2018; Crocetta, 2018; Giacobbe & Renda in Dragičević *et al*., 2019; Prato & Rubino in Kousteni *et al*., 2019; Scuderi & Viola, 2019; Marchini *et al*., 2020; Albano *et al*., 2021; Bini & Pinasco, 2021; Manousis *et al*., 2021), and pictures of shell specimens of *I. bicolor* (AMNH:307896) from Florida Keys (USA) and *M. regula* (AMNH:319335 and AMNH:319339) from Darwin Harbour, Australia stored at the American Museum of Natural History (AMNH).

Molecular analysis

A piece of adductor muscle was clipped from each sample and total DNA was extracted using a standard high-salt protocol (Sambrook, 1989). Amplification of the 16S fragment gene was performed by PCR using universal primers 16Sar-L and 16Sbr-H (Palumbi *et al.,* 1991). The PCR conditions used were: 3 min denaturation step at 94°C; 35 cycles of 94°C/60 s, 51°C/60 s, 72°C/60 s; 10 min final extension at 72°C. Amplicons were sequenced by Genewiz GmbH (Germany) (https://www.genewiz.com). Sequences are deposited in GenBank database (https://www.ncbi.nlm.nih.gov/GenBank/) with accession numbers OK104096-OK104098 and OK166813. The Basic Local Alignment Search Tool (BLASTN) search (Altschul *et al.,* 1990, Johnson *et al*., 2008) was used in a preliminary identification of the sequences, and also to exclude contaminations. In addition, two 16S datasets, one for each family (Isognomonindae and Malleidae), were built including the newly produced sequences and all the GenBank sequences belonging to each of the families. Datasets were aligned with MAFFT v.7 online service (Katoh *et al*., 2019) using the E-INS-i iterative refinement algorithm. Polymorphic sites of the three dataset alignments were calculated using DnaSP 6 (Rozas *et al.*, 2017). Uncorrected *p*-distances and Kimura 2-paramer (K2p) distances were calculated using the program Mega X (Kumar *et al*., 2018). A third dataset, used for downstream phylogenetic analysis, was built by joining together the two previous datasets, and included the addition of *Atrina rigida* ([Lightfoot], 1786) (HQ329397) as an outgroup. The GTR+G model was selected as the best evolutionary model by JModelTest 0.1 (Posada, 2008) according to the Bayesian information criterion (BIC). Phylogenetic analyses were performed using Bayesian inference (BI) and maximum likelihood (ML) methods. BI was carried out using MrBayes 3.2.7 (Ronquist *et al*., 2012) running two Markov chains of 5 000 000 generations each, sampled every 1000 generations. Consensus trees were calculated on trees sampled after a burn-in of 25%. The ML tree was inferred with W-IQ-TREE web platform (Trifinopoulos *et al.,* 2016) using an ultrafast bootstrap (uBS) analysis with 1000 generations. The obtained phylogenetic trees were visualised and curated using FigTree v.1.4.4 (available at http:// http://tree.bio.ed.ac.uk/software/figtree/).

Results

Sampling localities and ecological notes

Several *Isognomon* sp. specimens have been collected in the South Tyrrhenian Sea, from the north-eastern coast of Sicily, in Cape Milazzo (38.230303°N, 15.249114°E) in June 2021, as well as from the north-western Calabrian coasts, namely Briatico (38.727822°N, 16.033433°E) in November 2020 and San Lucido (39.305881°N, 16.045556°E) in February 2021 (Fig. 1). Other specimens were collected in the Strait of Messina (38.259819°N, 15.628871°E) in September 2020, from the same Sicilian population reported by Giacobbe & Renda in Dragičević

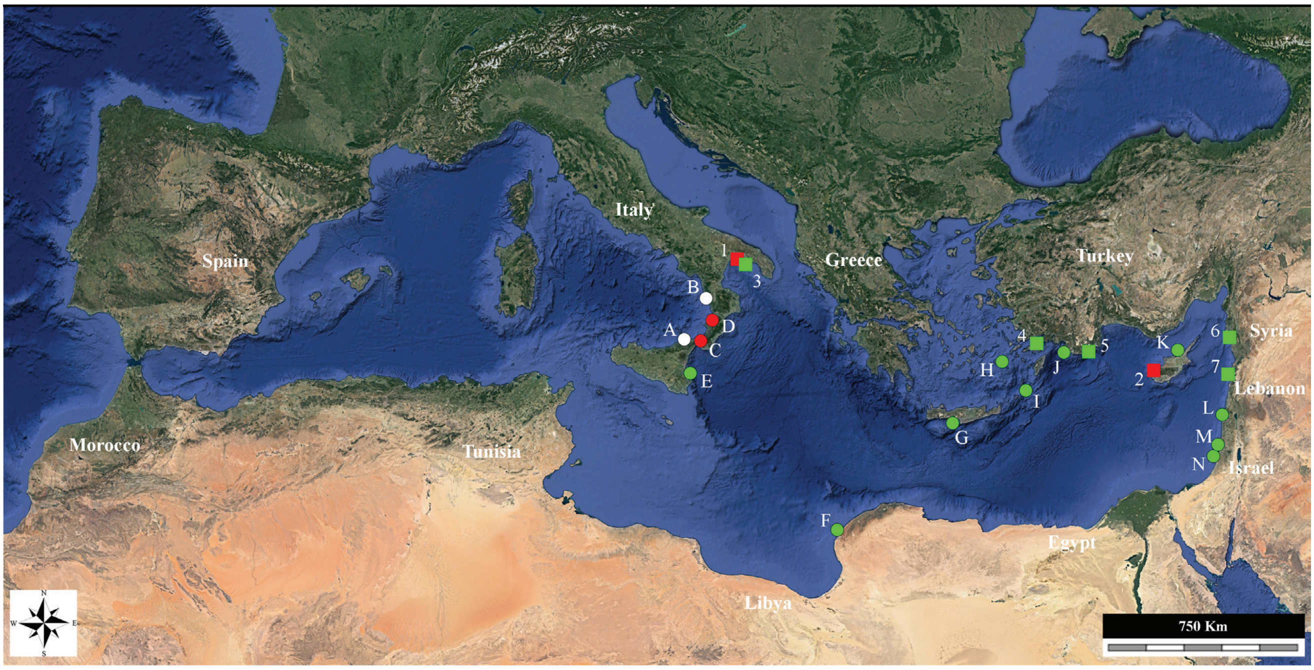

Fig. 1: Mediterranean distribution of Isognomonidae (circles) and Malleidae (squares) records. White circles indicate first reports in this work - A: Milazzo, Sicily, Italy; B: San Lucido, Calabria, Italy. Red circles indicate sequenced specimens in this work - C: Messina, Sicily, Italy; D: Briatico, Calabria, Italy. Green circles indicate reports of bibliography - E: Catania, Sicily, Italy (Scuderi & Viola, 2019); F: Bengasi, Lybia, identified as *Malleus regula* (Giannuzzi-Savelli *et al*., 2001); G: Plakias, Creta, Greece, identified as *I. aff australica* (Albano *et al.,* 2021); H: Astypalaia, Greece, identified as *Malleus regula* (Angelidis in Lipej *et al*., 2017) and as *I. australica* (Angelidis & Polyzoulis, 2018); I: Karpathos, Greece (Micali *et al.*, 2017); J: Dalyan, Iztuzu, Turkey (Ovalis & Zenetos in Stamouli *et al*., 2017); K: north of Cyprus, Turkey, identified as *I. aff australica* (Albano *et al*., 2021); L: Shiqmona, Israel (Mienis *et al*., 2016); M: Tel Aviv, Israel (Marchini *et al*., 2020); N: Ashqelon, Israel identified as *I. ephippium* (Mienis, 2004). Red squares indicate sequenced specimens in this work - 1: Mar Piccolo di Taranto, Apulia, Italy; 2: Polis, Cyprus. Green squares indicate reports in bibliography. 3: Mar Piccolo di Taranto, Apulia, Italy (Stamouli *et al*., 2017); 4: Simi Island, Greece (Giannuzzi-Savelli *et al*., 2001); 5: Kas, Turkey (Giannuzzi-Savelli *et al*., 2001); 6: Syria (Moazzo, 1931); 7: Lebanon (Moazzo, 1931).

et al. (2019) as the first report from the Italian coast (Fig. 1). *Isognomon* sp. specimens, (maximum sizes ranging from 4.7 mm to 13.9 mm; Fig. 2), were found deeply wedged between the roughness of vegetated hard substrates, mostly within red algae calcareous concretions (Fig. 3A). Only one specimen (maximum size 22 mm) was found adhering to a *Pinna rudis* Linnaeus, 1758 shell surface, partially masked by crustose poriferans (Fig. 3B).

A juvenile *Malleus* sp. (maximum size 12 mm) was collected at Polis, Cyprus (35.043453°N, 32.416217°E) Levantine Basin, in June 2019, on a rocky bottom substrate at 2 m depth, as an epibiont on *Chama pacifica* Broderip, 1835 and was analysed using molecular methods (Table 1). Additional specimens were sampled in July 2020, in "Mar Piccolo di Taranto" (40.481283° N, 17.268383° E), a sheltered area of the Ionian Apulian coast (Italy) (Fig. 1). The *Malleus* sp. specimens were found as epibionts on rocks (Fig. 4) and anthropic structures at 3 m depth, or as dead shells (maximum sizes ranging from 12 mm to 46 mm; Fig. 5).

Molecular analyses

It was impossible to unequivocally assign morphological identifications for any new Mediterranean specimens. Therefore, the Mediterranean samples, *Isognomon* sp. specimens from Briatico (OK104096) and from Messina (OK104097) and *Malleus* sp. specimens from Cyprus (OK104098) and from Taranto (OK166813) were analysed using the 16S mitochondrial barcode marker. BLASTN results of OK104096 and OK104097 nucleotide sequences showed 100% of similarity with the GenBank sequence identified as *I. bicolor* (HQ329406; voucher AMNH:307896). BLASTN results of OK104098 and OK166813 sequences showed less than 90% of similarity with any sequences stored in GenBank as *Malleus* spp. (Table 1). Therefore, it was only possible to assign an identification at genus level.

In addition, we built three different 16S sequence datasets. The 16S Isognomonidae sequences alignment consisted of 503 positions and a total number of 124 polymorphic sites. The 16S Malleidae sequences alignment

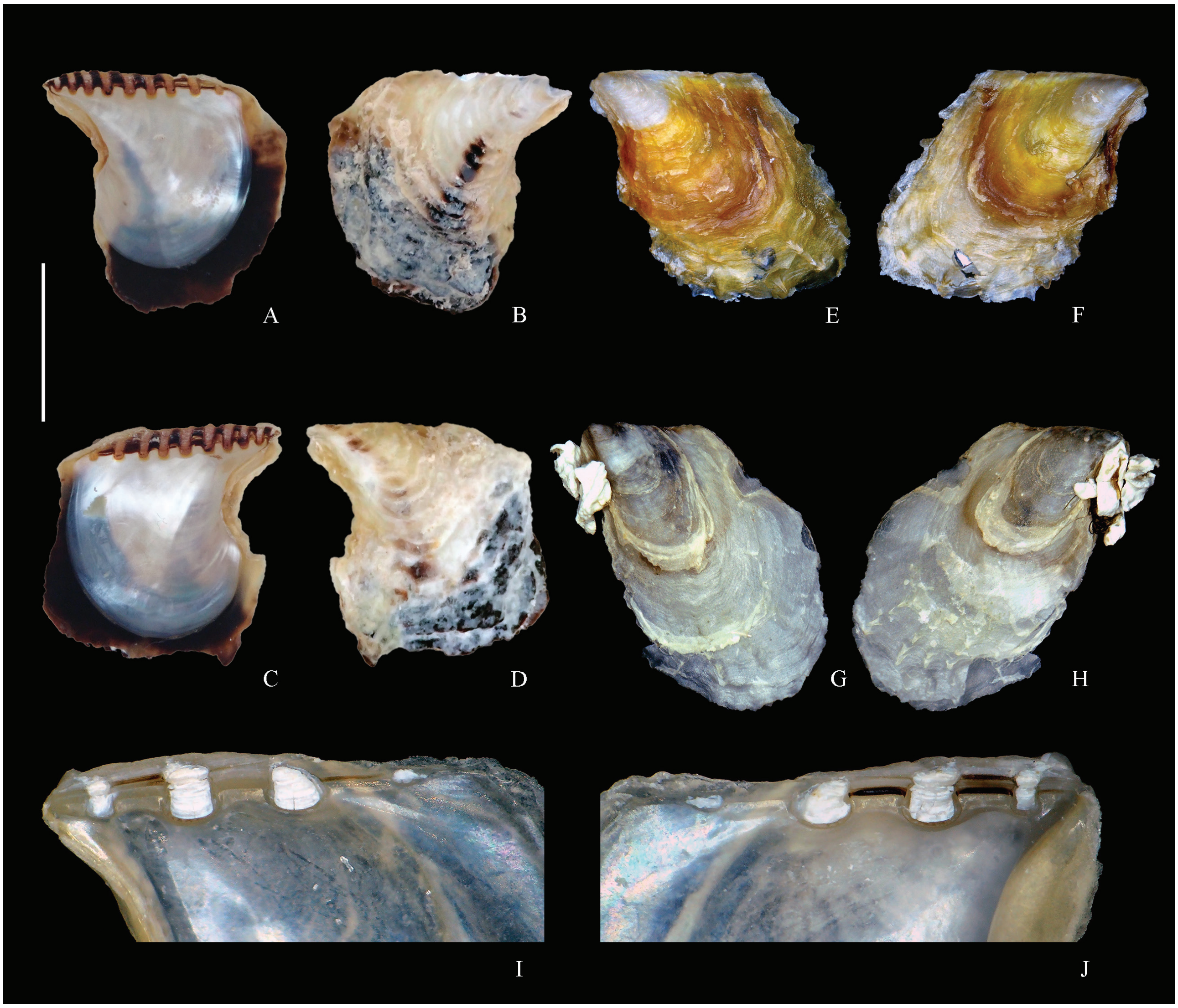

Fig. 2: *Isognomon bicolor* specimens. **A-D**, internal and external view of left and right shells of AMNH:307896 specimen (HQ329406), 11 mm (Florida Keys, USA). **E-F**, external view of the sequenced specimen OK104096, 10.5 mm (Briatico, Calabria, Italy). **G-H**, external view of the sequenced specimen OK104097, 13 mm (Messina, Sicily, Italy). **I-J**, details of the hinge of the specimens from Messina. Scale bar 10 mm.

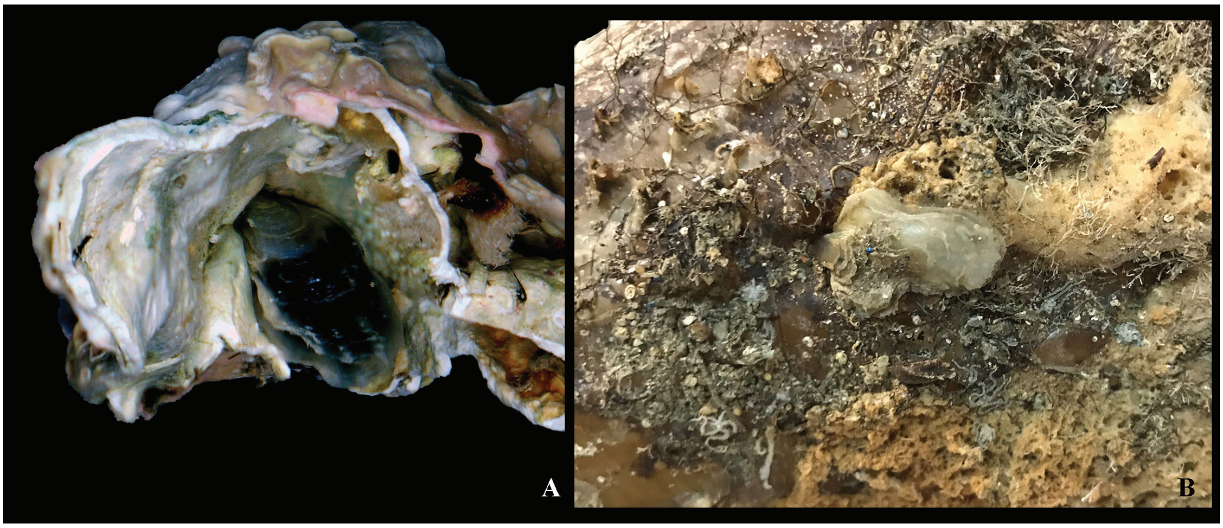

Fig. 3: *Isognomon bicolor* specimens *in situ.* **A**, *I. bicolor* specimenfound deeply wedged between the roughness of vegetated hard substrates, mostly within red algae calcareous concretions. **B**, *I. bicolor* specimen found adhering to a *Pinna rudis* Linnaeus, 1758 shell surface, partially masked by crustose poriferans (partially removed).

Table 1. Dataset based on 16S sequences of Isognomonidae and Malleidae species analysed. *: indicates the specimens sequenced in this work.

Species	Sample localities	Vouchers	Accession numbers 16S	References
Isognomonidae Woodring, 1925				
Isognomon alatus	USA: Florida Keys	AMNH:305129	HQ329405	Tëmkin, 2010
Isognomon alatus	Puerto Rico: Phophorescent Bay	--------------------	JN133622	Unpublished Kappner *et al.*
Isognomon alatus	USA: Florida Keys	BivAToL-30	KC429251	Sharma *et al.,* 2013
Isognomon bicolor	USA: Florida Keys	AMNH:307896	HQ329406	Tëmkin, 2010
*Isognomon bicolor**	Italy: Briatico	--------------------	OK104096	This study
*Isognomon bicolor**	Italy: Messina	--------------------	OK104097	This study
Isognomon cf. *ephippium*	Thailand: Kungkrabaen Bay	AMNH:319253	HQ329407	Tëmkin, 2010
Isognomon ephippium	--------------------	MIEE2015-6IE1	KY081325	Liu *et al.,* 2018
Isognomon radiatus	USA: Florida Keys	AMNH:305142	HQ329408	Tëmkin, 2010
Isognomon recognitus	Mexico: Baja California Sur, Laguna San Ignacio	--------------------	KT317424	Raith *et al*., 2015
Isognomon recognitus	Mexico: Sonora, Guaymas	--------------------	KT317425	Raith *et al*., 2015
Isognomon recognitus	Mexico: Sonora, Guaymas	--------------------	KT317426	Raith *et al*., 2015
Isognomon recognitus	Mexico: Sonora, Puerto Penasco	--------------------	KT317427	Raith *et al*., 2015
Isognomon sp. A2	Clipperton Island	CASIZ 104281	HQ329409	Tëmkin, 2010
Malleidae Lamarck, 1818				
Malleus albus	--------------------	BivAToL-79	KC429252	Sharma *et al*., 2013
Malleus cf. *albus*	Australia: Houtman Abrolhos	AMNH:319298	HQ329410	Tëmkin, 2010
Malleus candeanus	USA: Florida Keys	AMNH:FK-685	HQ329411	Tëmkin, 2010
Malleus malleus	Australia: Bohol Is.	MNHN:42755	HQ329412	Tëmkin, 2010
Malleus regula	Australia: Darwin Harbor	AMNH:319339	HQ329414	Tëmkin, 2010
Malleus regula	Australia: Darwin Harbor	AMNH:319335	HQ329413	Tëmkin, 2010
Malleus sp.1*	Cyprus	RM3-PM-151	OK104098	This study
Malleus sp.1*	Italy: Taranto	RM3-PM-147	OK166813	This study
Pinnidae Leach, 1819				
Atrina rigida	--------------------	AMNH:305138	HQ329397	Tëmkin, 2010

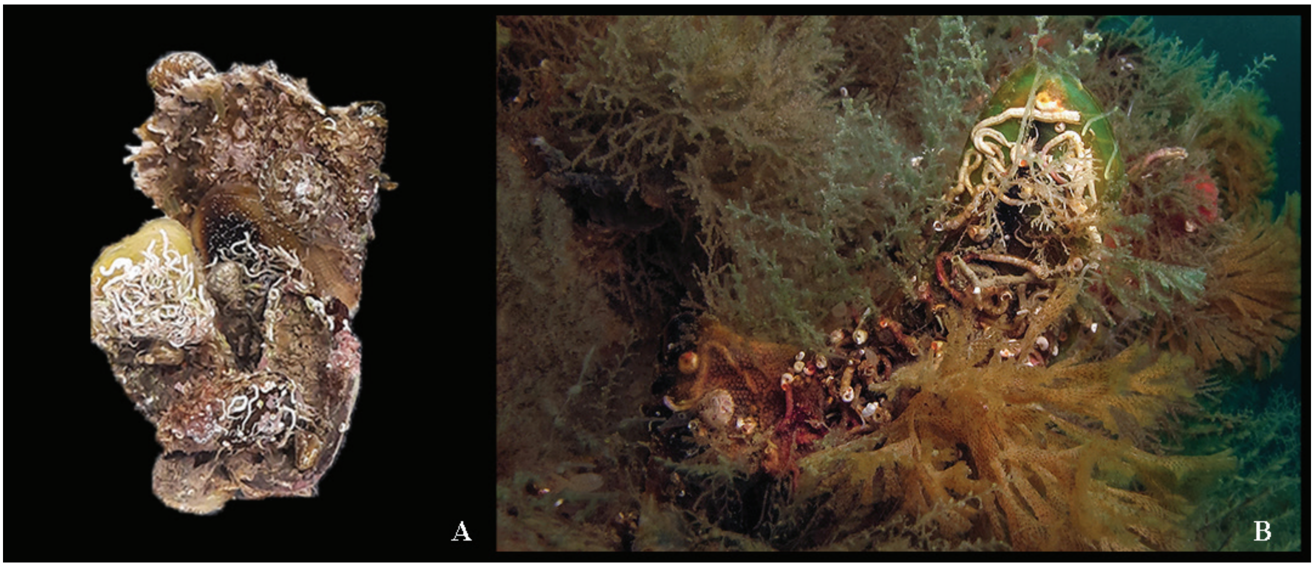

Fig. 4: *Malleus* sp. specimens *in situ*, inside hard (A) and soft (B) bioconcretions.

consisted of 438 positions and a total number of 85 polymorphic sites. The joined dataset (Isognomonidae+Malleidae+outgroup) consisted of 431 positions and a total number of 177 polymorphic sites. The list of species and relative GenBank accession numbers are reported in Table 1. Genetic distances (*p*-distance and K2p) were calculated considering any representatives of Isognomonidae and Malleidae families (Table 2). Results of the distance analyses showed no intraspecific divergence between each couple of new sequences (OK104096/OK104097 and OK104098/OK166813). Moreover, the interspecific genetic distance (*p*-distance/K2p) in Isognomonidae family ranged from 4.6%/4.8% to 23.1%/29%, while in Malleidae the genetic distances ranged from 5.1%/5.3% to 15.9%/18.1%. The phylogenetic results corroborate our preliminary identification attempts (Fig. 6). Bayesian posterior probabilities (BPP) and ultrafast bootstrap (uBS) values, based respectively on BI and ML analyses, are reported at each node of the tree. Each of the new sequenced specimens were placed in two distinct highly supported clades: BPP=1; uBS ≥98%.

Discussion

Taxonomic investigation of Mediterranean **Isognomon** ***sp. and*** **Malleus** ***sp. specimens***

The molecular data generated here provides compelling evidence that our Mediterranean *Isognomon* sp.

Table 2. 16S interspecific genetic distance values (*p*-distance: lower; K2p: upper) between representatives of Isognomonidae (a) and Malleidae (b) families. *: indicates the specimens sequenced in this work (*I. bicolor**: OK104096 and OK104097; *Malleus* sp.1*: OK166813 and OK104098).

a. Isognomonidae	*I. ala*	*I. bic*	*I. bic**	*I. bic**	*I. eph*	*I. rad*	*I. rec*	*I.* sp.
Isognomon alatus	-	17.7%	17.7%	23.9%	21.6%	23.9%	23.9%	29.0%
Isognomon bicolor	18.4%	-	1.9%	22.6%	22.2%	23.8%	23.8%	28.3%
*Isognomon bicolor**	18.4%	0.0%	-	22.0%	21.3%	23.8%	23.8%	27.6%
*Isognomon bicolor**	18.4%	0.0%	0.0%	-	0.0%	5.0%	5.0%	24.9%
Isognomon ephippium	16.0%	18.4%	18.4%	18.4%	-	4.8%	4.8%	23.8%
Isognomon radiatus	21.1%	22.1%	22.1%	22.1%	22.1%	-	0.0%	25.8%
Isognomon recognitus	17.7%	4.6%	4.6%	4.6%	19.2%	23.1%	-	25.8%
Isognomon sp.	21.8%	20.0%	20.0%	20.0%	22.9%	12.3%	20.7%	-

b. Malleidae	*M. alb*	*M. can*	*M.* cf. *alb*	*M. mal*	*M. reg*	*M.* sp.1*	*M.* sp.1*
Malleus albus	-	18.1%	4.8%	6.3%	6.0%	13.2%	13.2%
Malleus candeanus	15.9%	-	16.0%	15.7%	17.2%	14.7%	14.7%
Malleus cf. *albus*	4.8%	14.5%	-	5.3%	5.8%	11.5%	11.5%
Malleus malleus	6.1%	14.0%	5.1%	-	3.5%	11.8%	11.8%
Malleus regula	6.0%	15.2%	5.5%	3.8%	-	11.5%	11.5%
Malleus sp.1*	12.3%	13.3%	10.6%	10.7%	10.4%	-	0.0%
Malleus sp.1*	12.3%	13.3%	10.6%	10.7%	10.4%	0.0%	-

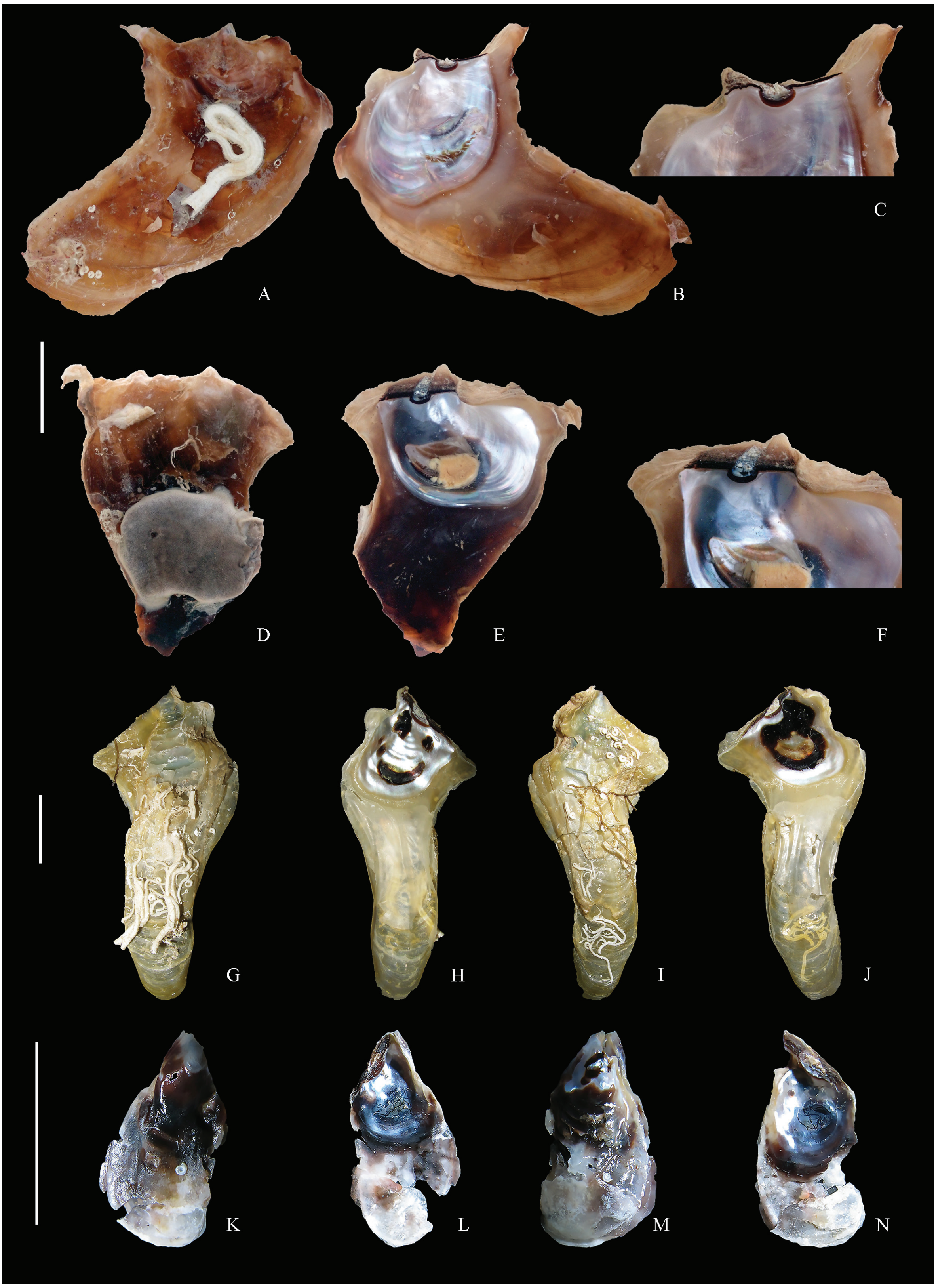

Fig. 5: *Malleus* spp. specimens. **A-C** *M. regula***,** AMNH:319335 specimens (HQ329413), 32 mm (Darwin Harbour, Australia). **D-F** *M. regula*, AMNH**:**319339 specimens (HQ329414), 32 mm (Darwin Harbour, Australia). **G-J** external and internal view of the sequenced *Malleus* sp. specimen OK166813, epibiont on rocks, 3 m depth, 46 mm [Mar Piccolo di Taranto, Apulia (Italy)]. **K-N** external and internal view of the sequenced *Malleus* sp. specimen OK104098 (juvenile), on rocks, 2 m depth, 12 mm (Polis, Cyprus). Scale bars 10 mm.

specimens represent *Isognomon bicolor*. It is well known that many reference sequences in GenBank are not reliable for barcoding studies and this causes uncertainty in the molecular identification of some molluscan taxa (see Briski *et al.,* 2016). However, our 16S sequences (OK104096 and OK104097) showed a 100% match-rate with a reference sequence from museum specimen (AMNH:307896) sampled in the Florida Keys (present-

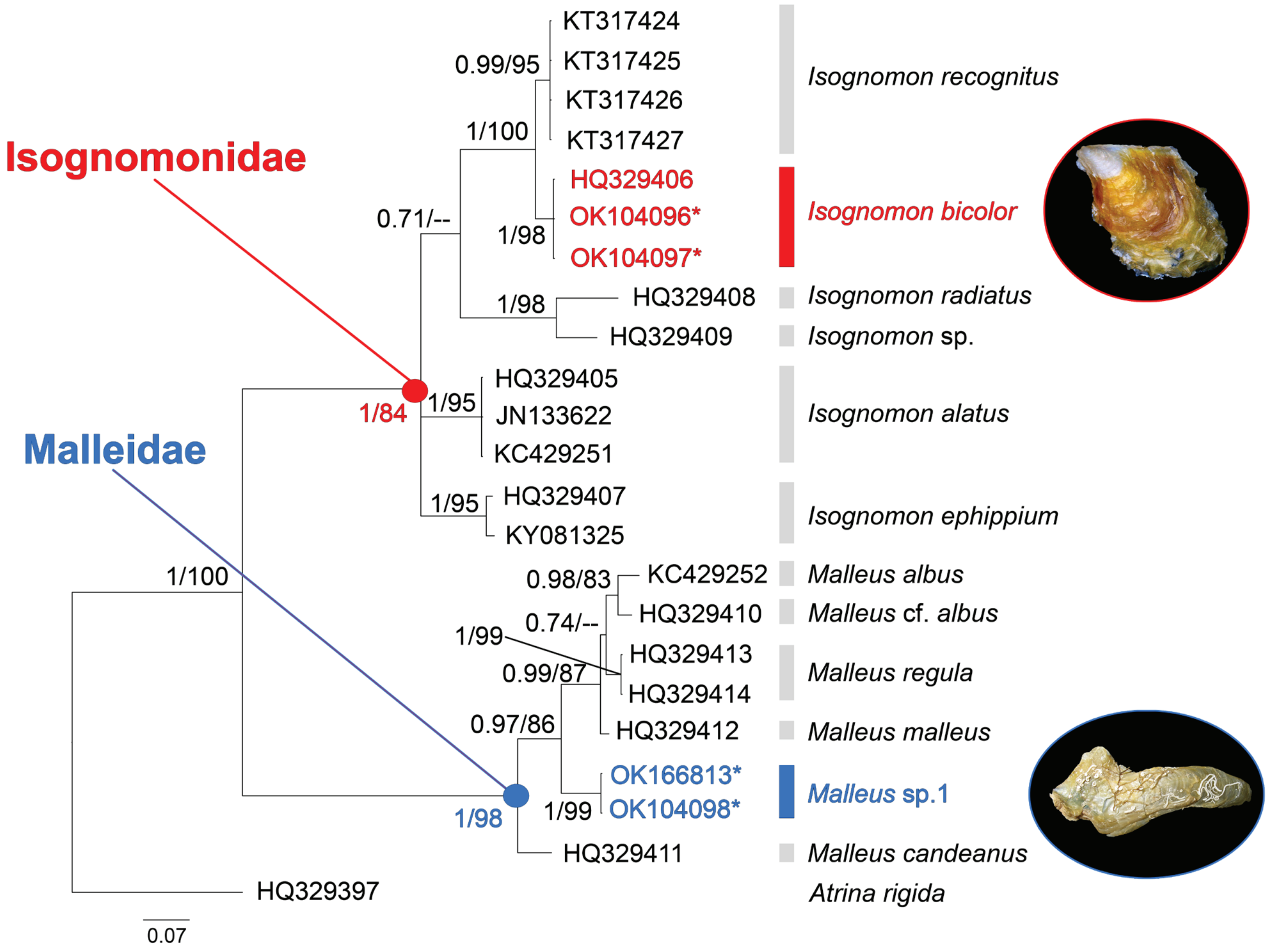

Fig. 6: Bayesian phylogenetic tree based on the joined 16S dataset. Bayesian posterior probability (left) and ultrafast bootstrap (right) values are indicated at each node. *: indicates the specimens sequenced in this work. Sequences of AMNH museum specimens of *Isognomon bicolor* (Fig. 2) and *Malleus regula* (Fig. 5) used for morphological comparisons are respectively HQ329406, and HQ329413/HQ329414.

ed in Fig. 2A-D). This sampling locality is north of the Caribbean region where the type locality of *I. bicolor* is found (Jamaican coasts) (Adams, 1845). In addition, this determination agrees with the redescription of *I. bicolor* by Domaneschi & Martins (2002), although this similarity cannot be considered by itself definitive proof, due to the well-known morphological variability characterizing *Isognomon* species (Wilk & Bieler, 2009). In this regard, the small number of ligamental resilifer teeth observed in the specimens that were analysed using molecular tools (Fig. 2I-J) can be explained by the high variability in this structure within the *Isognomon* genus, which in fact does not constitute a diagnostic character (Domaneschi & Martins, 2002). The 16S sequences of *Malleus* sp.1 (OK104098 and OK166813) did not match any sequences present in GenBank, leaving such specimens undetermined at the species level. In particular, *Malleus* sp.1 sequences are 10.4% distant from *M. regula* sequences of museum specimens (AMNH:319335 and AMNH:319339 from Australia; Fig. 5A-F). Moreover, *Malleus* sp.1 and *Malleus regula* sequences formed two well-separated and highly supported clades in the Bayesian phylogenetic tree (Fig. 6).

Molecular identification of previously misidentified alien *Isognomon* sp. And *Malleus* sp. Specimens from the Mediterranean Sea (Tyrrhenian, Ionian, and Aegean basins) have been provided here for the first time using the 16S rRNA marker. Furthermore, phylogenetic results based on BI and ML analyses confirmed that the analysed Mediterranean specimens belong to Isognomonidae and Malleidae families, representing two distinct well-supported clades.

Atlantic introduction

The molecular identification of *I. bicolor* brings into question all previous morphological identifications of Isognomonidae in the Mediterranean, whose shell morphologies always agree with the variability range of *I. bicolor*. It is remarkable that observations on the *I. bicolor* invasiveness and habitat preferences in the west Atlantic also agree with reports from the Mediterranean. As invasive species, for example, the “bicolor purse oyster”, originating from the native Caribbean region (Benthotage *et al*., 2020), has spread southward, reaching the north-eastern Brazilian coasts in the 1970s (Loebmann *et al.,* 2010; Dias *et al*., 2013; De Oliveira Gomes & Carvalho Da Silva, 2013), and then rafting to the Uruguayan coast (Breves *et al.,* 2014), representing one of the most important introductions to the benthic realm (Ferreira *et al.,* 2009). As for the Mediterranean reports, in Brazil this species was initially misidentified, i.e., with the alleged endemic *I. alatus* (Gmelin, 1791) (Domaneschi & Martins, 2002). In south Brazil, moderate wave exposure and availability of heterogenous steep rocky substrates (Whorff *et al.,* 1995; Domaneschi & Martins, 2002) and rock crevices (Moyses, 2005; Zamprogno *et al.,* 2010), seem to be important for the invasive success of *I. bicolor*, which preferentially settles inside biogenic secondary substrates (Rocha, 2002). Such observations, as well as suggested associations in invaded environments with native macroalgae (López & Coutinho, 2010) and vermetid reef habitat (Breves & Junqueira, 2017), substantially agrees with our sampling data. This habitat preference suggests that *I. bicolor*, despite the high invasiveness, cannot be considered a pioneering species in substratum colonization (Rocha, 2002) and does not display a clear opportunistic behaviour (Benthotage *et al*., 2020). Ballast water and/or fouling of international commercial vessels (Breves-Ramos *et al.*, 2010; Fernandes *et al.,* 2016) and relocation of oil or gas platforms from the Caribbean coast (Oliveira & Creed, 2008; Breves-Ramos *et al.,* 2010) have been considered possible vectors of *I. bicolor* to Brazilian waters, although others have reported on the role of rafting by floating debris (Breves *et al*., 2014). This latter suggestion agrees with the known use of floating macroalgae by *I. bicolor* as a secondary dispersal pathway (Dias *et al.,* 2013) and also explains its stranding in south England as an epibiont on a plastic spool, almost simultaneously with other Caribbean molluscan species (Holmes *et al*., 2015). Such mechanisms for west to east Atlantic dispersal of southern, warm water taxa (Holmes *et al.,* 2015), cannot be separated from the Mediterranean spreading of tropical neuston communities, which occasionally even reach the cold Liguro-Provençal basin (Betti *et al.,* 2017). Moreover, the northward shift of the 15°C February isotherm divide, providing a warmer corridor which directly connects the Strait of Gibraltar with the eastern Mediterranean (Bianchi *et al.,* 2012), allows tropical taxa to bypass most of the western Mediterranean Sea (Di Silvestro *et al.,* 2010), rapidly reaching the most favourable Levantine Basin throughout the Mid-Mediterranean Jet Current. Genetic investigations have evidenced that these patterns of invasion have occurred frequently in other taxa; for example, the warm Atlantic Heterobranchia *Aplysia dactylomela* Rang, 1828, settled in the eastern Mediterranean before spreading westward and northward (Valdés *et al*., 2013). Similarly, secondary dispersal from Aegean might be responsible for the Ionian colonization by *I. bicolor* which, according to our data, initially settled in the eastern coasts of Sicily (Scuderi & Viola, 2019, as *I. legumen*), and subsequently spread northward throughout the Strait of Messina, rapidly colonizing the Southern Tyrrhenian coasts, according to the local sea-surface circulation (Vetrano *et al.,* 2010). We thus agree with Por (2009), who considered the attention towards the Lessepsian migrants disproportionately greater than the increasing settlement by tropical Atlantic newcomers through the Straits of Gibraltar. Among others, a consequence of this is that when non-native species belonging to tropical genera or families first appear in the Eastern Mediterranean they may be classified according to the most similar Indo-Pacific taxon, implicitly excluding other possibilities. Such Lessepsian prejudice, allowing a cascade of misidentifications, could preclude early warning of potential threatening invaders, such as *I. bicolor*. This invasive species, which in Brazilian coasts has caused profound changes in the native rocky coast communities reaching densities up to 800 individuals/100 cm^2 (Magalhães, 1999; Rapagnã, 2004; Breves-Ramos *et al.,* 2010), was locally responsible for reducing 50% of the native barnacle population, *Tetraclita stalactifera* Lamarck, 1818 (López, 2003), and probably competes with *Perna perna* (Linnaeus 1758), an edible mussel of commercial interest (Rapagnã 2004; Breves-Ramos *et al.,* 2010). In this respect, the observation that this invasive species does not settle on bare substrate, appearing only at late succession stages (Rocha, 2002), is not reassuring, and thereby represents a potential threat for protected species and habitats, such as the Mediterranean vermetid reefs (Milazzo *et al.,* 2016), whose analogues in Brazilian waters are competing with *I. bicolor* colonization (Breves & Junqueira, 2017).

The spreading of *Malleus* sp.1 in the Mediterranean, confirmed by a sample from Cyprus, strongly questions previous records regarding the establishment of *M. regula* in Greek and Turkish Aegean waters (Giannuzzi-Savelli *et al*., 2001; Crocetta *et al*., 2017), in the Ionian Sea (Prato & Rubino in Kousteni *et al*., 2019), as well as recent records from Sicily (Bini & Pinasco, 2021). In the meantime, these observations cast doubts about both historical and recent reports from the Levantine Basin. Whilst we wait to understand the true identity of this undetermined taxon through molecular and morphological screening of the entire genus, its spread needs to be carefully monitored, since it represents a potential threat for biodiversity conservation in the Mediterranean.

Conclusions

Increasing anthropogenic introductions and climate change have expanded the frequency of alien invasions and the variety of ecoregion sources of potential invaders. Invasive patterns, which were previously taken for granted, such as the Lessepsian migration, must be reconsidered with stronger critical attention. This changing scenario highlights an even stronger need for accurate taxonomic identification, from which more reliable predictions of settlement patterns and potential ecosystem damage can be derived. Despite the occurrence of an increasing number of marine alien species in the Mediterranean Sea, taxonomical identification of these invasive taxa is often still difficult when based on morphological characters alone. This uncertain identification often leads to an incorrect estimation of the number of alien species and their true routes of introduction. This is particularly true for bivalves characterized by a high variation in their shells, leading to uncertainty in morphological identification. This is the cases for two alien bivalves assigned to Isognomonidae and Malleidae families characterized by their confused Sea of origin and their controversial and changing nomenclature. The variable phenotype of their shells has historically produced several cases of misidentification, which has negatively affected our knowledge on their geographical distribution to date. In this respect, molecular approaches using DNA barcoding and phylogenetic analyses are fundamental tools to avoid the misidentification and related hasty conclusions, mainly when traditional taxonomy fails. In this work, the 16S rRNA marker was utilized for the first time to assess the identification of *Isognomon* sp. and *Malleus* sp. specimens from different localities of the southern Mediterranean Sea. The first taxon is now assigned as the Atlantic species *I. bicolor*, whilst the unassigned second taxon should be referred as *Malleus* sp.1 instead of the Indo-Pacific *M. regula*. Obviously, indispensable tools such as GenBank are still far from offering the full coverage required for our current needs, which is why a greater effort should be devoted to integrating known families or genera suspected to be more involved in invasive processes.

Acknowledegements

We would like to thank Estefania Rodriguez and Lily Berniker of the American Museum of Natural History (AMNH, New York, USA) for sending the photos of *Isognomon bicolor* and *Malleus regula* specimens. We thank Daniele Salvi for the constructive discussion on molecular analyses. We are extremely grateful with Josephine Paris for the English revision of the manuscript. Part of the sampling was carried out during the “Monitoring of Non-Indigenous Species, NIS,” starting in the framework of the PRA 2008/2009 program “Settlement dynamics and colonization of allochthonous assemblages in the Capo Peloro Lagoon” (Strait of Messina, central Mediterranean) (PhD school contribution 2016). GF wish to thank University of Salento and the Italian Ministry of Education, University and Research (PON 2014-2020, grant AIM 1848751-2, Linea 2) for support.

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