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From Hulls to Caves: Insights into the Introduction and Expansion of Non-Indigenous Marine Bivalves of the Genera *Isognomon* and *Malleus* in the Eastern Mediterranean Sea

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Abstract

Although the Eastern Mediterranean Sea is a hotspot for marine bioinvasions, the accurate identification and monitoring of non-indigenous species (NIS) remain impeded by the ambiguous morphologies of species and limited regional genetic data. This study applied an integrative approach, combining morphological identification with DNA barcoding, to assess the taxonomy and expansion of bivalves from the genera *Isognomon* and *Malleus* in the Eastern Mediterranean Sea. Specimens were collected from a broad range of habitats, including marinas, ship hulls, reefs, and marine caves. Phylogenetic analyses revealed two distinct *Isognomon* species in the region: *I. bicolor*, frequently associated with artificial substrates and showing evidence of multiple introductions, and *I.* aff. *legumen*, restricted to cryptic natural habitats. A single species of *Malleus* cf. *regula* was also detected, clustering with sequences from neighboring Mediterranean regions. The study highlights the limitations of morphology-based taxonomy and the urgent need to enhance genetic reference databases, particularly with sequences from areas of nativity. As NIS increasingly expand from anthropogenic habitats into natural ecosystems, validated data are essential for risk assessment and conservation management.

Keywords: non-indigenous species (NIS); DNA barcoding; marine caves; biofouling

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1. Introduction

The global rate of Non-Indigenous Species (NIS) introductions and their establishment in new ecosystems have escalated in recent decades, following the increased economic growth and globalization [1], particularly in the Mediterranean Sea [2–4], causing significant biodiversity changes [5]. In the Mediterranean Sea, moderate to high impacts of bioinvasions on biodiversity have been documented for 88 marine non-indigenous and cryptogenic species from 16 different phyla, affecting ecosystem services and human health [6]. While Lessepsian migration (i.e., the influx of Indo-Pacific and Red Sea-originated species through the Suez Canal) is the main pathway of introduction to the Eastern Mediterranean, ballast

water disposals and biofouling on ship hulls related to maritime transportation are considered to be the second most important pathway, in which targeted policies and mitigation measures can be effectively implemented [7,8].

Among the invaders of the Eastern Mediterranean Sea are several bivalve species of the genus *Isognomon* [Lightfoot], 1786, and *Malleus* Lamarck, 1799. The genus *Isognomon* of the family Isognomonidae Woodring, 1925 (1828) includes 16 distinct species [9] whose nomenclature has undergone several revisions due to the high number of genetically distinct cryptic (classified as a single species due to their identical morphology) and pseudocryptic (species with almost identical morphology due to physical complexities) species and their widespread distribution [10]. The genus *Malleus*, of the family Malleidae Lamark, 1818, includes eight morphologically similar species [11] that have often been mis- determined, especially within the genus *Isognomon*, due to their similar morphological characteristics. Both genera include species with confirmed introductions and expansion in parts of the Mediterranean, but their ecological impacts remain uncertain; they are not yet classified as highly invasive according to the EU Invasive Alien Species of Union Concern, but their continued spread requires monitoring due to the general regional trend of NIS introductions and spread [10].

Several species of the genus *Isognomon* are recent invaders, and one is spreading rapidly in the Mediterranean. The first confirmed record of *Isognomon* in the Mediterranean Sea dates back to 2016 and was reported as *I. legumen* (Gmelin, 1791) based on its morphological features, and was collected in 2015 from the Shikmona marine reserve, south of Haifa [12,13]. Subsequent records were then reported across the region. Specifically, it was documented in the Aegean Sea [14–16], the Levantine coast of Turkey [17], the Italian Ionian coast [18], Cyprus [19,20] and Albania [21]. The same species was also reported as *I. australicus* (Reeve, 1858) from the Ionian islands of Greece [22] and as *I.* aff. *australicus* in southern Crete and Cyprus [23].

Isognomon bicolor (C.B. Adams, 1845), is a species native to the tropical western Atlantic/Caribbean and is considered to have arrived in the Mediterranean Sea through commercial shipping [10]. *Isognomon bicolor* is a prominent species with a high invasive potential and dispersal ability, and has been associated with disruption to native communities (e.g., intertidal sandstone reefs), also causing economic impacts (e.g., marine activities) [24–27]. In Brazil, where it was first reported, *I. bicolor* was primarily found in ports, suggesting that the most possible pathways of introduction are ballast waters and/or ship fouling [28,29]. However, the species has also colonized natural habitats such as rocky beaches and intertidal crevices [30,31] dominating over native species.

On the other hand, the first report of the genus *Malleus* in the Mediterranean Sea belongs to the species *Malleus regula* (Forsskål, 1775), dating back to 1929. Initially, it was reported from the coasts of Tyre in Lebanon and the bay of Saint-Georges near Beirut and Syria [32], indicating that this species entered through the Suez Canal. The species was subsequently recorded in Israel [33] and Cyprus [34], the Gulf of Antalya in Turkey [35] and Egypt [36], gradually expanding in the Eastern Mediterranean. Although reports by [37] from Libya were later shown to be misidentifications, referring in fact to *I. legumen* [16], the presence of *M. regula* in Greece was confirmed from Astypalaia Island [15]. The latest record of *M. regula* is from the Gulf of Taranto, Italy [38] and of *I. bicolor* from the south Aegean [39], Tunisia [40], the Tyrrhenian Sea [41], and Egypt [42].

1.1. Taxonomic Complexity and Morphological Similarity

Isognomon legumen, *I. bicolor*, and *Malleus regula* can be characterized as cryptic species due to their morphological similarity to one another and other congeneric species. This has led to several misidentifications over the years when relying only on the conchological

characteristics. Specifically, both *I. australicus* and *M. regula* were previously misclassified as *I. legumen* in Mediterranean records [16,39], while *I. bicolor* can also be easily confused with *I. alatus* (Gmelin, 1791) [24]. Additionally, both *I. australicus* and *I. bicolor* share common morphological characteristics, such as the irregular shell outline, which can be affected by the substrate where they grow, making the identification challenging. However, *I. australicus* exhibits prominent radial ribs and occasional tubular spines (more evident in juveniles), characteristics which are absent in the concentric-lamellae-dominated *I. bicolor* [19,39,43], whose juveniles show a set of ridge-like pits along the hinge. In adults, color can be another distinguishing feature: while *I. australicus* has a uniformly pale, corneous exterior with nacreous interiors, *I. bicolor* has a distinctive bicoloration—yellowish near the umbones grading to dark brown or purplish edges, with a bright, silvery nacre [43]. Despite misidentifications in the past, *M. regula* can be distinguished from the others by its spatulate "hammer" shell, reduced lateral wings, and concentric growth lines' pattern [16,44,45], in contrast with *I. legumen*, which has a wide series of short ligamental pits resembling tooth-like structures, *M. regula* lacks both pits and hinge teeth.

1.2. Ecology and Habitat

Habitat is an essential factor in species expansion and settlement. All three species attach by byssus to hard substrates. *Isognomon* aff. *legumen* is typically found in cryptic habitats such as below overhangs or even inside empty shells, favoring sheltered microhabitats in contrast to the more exposed, high-energy shore occurrences typical of *I. bicolor* [19]. The latter species prefers hard substrates but inhabits the more exposed intertidal and shallow subtidal zone. In both its native and introduced range (e.g., Brazil and the Mediterranean), it is often found in clusters, colonizing cracks and crevices on high-turbidity rocky coasts or encrusting pier pilings, and buoys in harbors, sometimes outcompeting native bivalves [24]. *Malleus regula* also prefers cryptic environments such as coral reefs and rocky bottoms, as well as artificial environments such as under floating platforms in marinas, from the low intertidal to about 50–60 m depth [36].

1.3. Integrative Approaches to Address Taxonomic Challenges

Despite the increasing effort and reports of *Isognomon* and *Malleus* species in the Eastern Mediterranean, their exact identity, distribution, and ecological preferences remain unresolved. Their taxonomically challenging morphology still leads to misidentifications, leading to confusion and mis-documentation of their invasion steps, and as a result, delaying effective monitoring and risk assessment studies. Only lately have a few studies begun to combine both morphological and molecular data, while very few include diverse habitats.

For instance, the *16S rRNA* gene marker was used to assess the preliminary identification of *Isognomon* sp. and *Malleus* sp. specimens from different localities in the Southern Mediterranean Sea, and concluded that the primarily identified *Isognomon* specimens belonged to the Atlantic species *I. bicolor* [10,39]. More recently, the systematics of the genus *Isognomon* in the Mediterranean Sea was revised using both morphological and molecular approaches and showed the presence of two species only: *I. bicolor*, distributed in the Central and Eastern Mediterranean, and the Indo-Pacific *I. australicus*, only from the Eastern Mediterranean, which was suggested to be renamed as *Isognomon* aff. *legumen* [39,45].

Unravelling species identities is of major concern when studying NIS, especially for those with high dispersal abilities, especially in hard-to-reach shaded environments (e.g., marine caves). Utilizing genetic methods such as DNA barcoding could help to decrypt taxonomically problematic high-risk species as a prerequisite to monitor their expansion, understand their pathways, and eventually predict their next steps.

This study aimed to conduct an integrative survey of *Isognomon* and *Malleus* bivalves in the Eastern Mediterranean Sea, combining morphological identification with DNA barcoding using mitochondrial *16S rRNA* and *COI* markers. The specific objectives were to: (1) document new occurrence records from both artificial and natural habitats, including hard-to-access environments such as marine caves; (2) expand the molecular reference database for these taxa; (3) clarify their current status in the surveyed region; and (4) evaluate species' habitat preferences and potential pathways of introduction.

2. Materials and Methods

2.1. Field Sampling Sites & Habitats

A total of 39 specimens belonging to Isognomonidae and Malleidae families were collected from October 2020 to December 2024, in the framework of multiple projects and sampling campaigns in Greece and Cyprus. The sampling locations were from artificial environments such as ship hulls, ports, and marinas to natural environments such as marine caves and rocky reefs (Table 1, Figure 1). In ports and marinas, the specimens were collected by scraping off hard substrates from the ship hulls while SCUBA diving. During the years 2020–2023, and in the framework of the "ECOHULLCLEAN" program (Integrated System for Underwater Ecological Hull Cleaning of Vessels), only two juvenile specimens were collected during the cleaning of 30 different ship hulls in total. Both specimens were collected from the bulk carrier GINGO (IMO: 9182710) in Lavrio (Attica, Greece), which had previously anchored in the Suez Canal and anchorages in Sri Lanka, India, and a port in South Africa. Samples from marine caves and rocky reefs were all detached from hard substrates while SCUBA diving. Specimens were obtained through opportunistic sampling, with divers collecting target taxa whenever they were encountered during surveys. In marine cave habitats, collections were made across a depth range of approximately 1–16 m, ranging from 1 m in Agia Paraskevi Cave (Chalkidiki) to 16 m in Purple Cave (Evia Island). The same approach was applied across all habitat types, with sampling conducted at depths of 1–2 m in marina environments and 3–4 m on reef sites. All the collected specimens were preserved in 99% ethanol for morphological and molecular identification.

Table 1. Specimen number, habitat type, geographic location, and sampling date for each collected specimen. GR: Greece, CY: Cyprus. More details can be found in Table S1 of the Supplementary Material.

Specimen Code	Habitat	Geographic Location	Sampling Date
sp. 1: Glifada marina	Marina	Saronikos Gulf (GR)	1 April 2023
sp. 2: Achata 2 cave	Cave	Karpathos Island (GR)	5 August 2020
sp. 3: Blue 2 cave	Cave	Kastellorizo Island (GR)	10 August 2020
sp. 4: Daskalogiannis tunnel	Cave	Crete Island (GR)	12 June 2021
sp. 5: Blue 2 cave	Cave	Kastellorizo Island (GR)	10 August 2020
sp. 6: Blue 1 cave	Cave	Crete Island (GR)	24 May 2021
sp. 7: D8 cave	Cave	Cape Greco (CY)	31 July 2021
sp. 8: D8 cave	Cave	Cape Greco (CY)	31 July 2021
sp. 9: Blue 2 cave	Cave	Kastellorizo Island (GR)	10 August 2020
sp. 10: Blue 2 cave	Cave	Kastellorizo Island (GR)	10 August 2020
sp. 11: Blue 2 cave	Cave	Kastellorizo Island (GR)	10 August 2020
sp. 12: D8 cave	Cave	Cape Greco (CY)	31 July 2021
sp. 13: D8 cave	Cave	Cape Greco (CY)	31 July 2021
sp. 14: D8 cave	Cave	Cape Greco (CY)	31 July 2021
sp. 15: Agia Roumeli reef	Reef	Crete Island (GR)	5 June 2023
sp. 16: Schisma cave	Cave	Crete Island (GR)	6 June 2023
sp. 17: Achata 2 cave	Cave	Karpathos Island (GR)	7 June 2023
sp. 18: Panteleimonas cave	Cave	Saria Island (GR)	9 June 2023
sp. 19: Heraklion marina	Marina	Crete Island (GR)	1 September 2022
sp. 20: Seal's 2 cave	Cave	Rhodes Island (GR)	9 August 2020

Table 1. *Cont.*

Specimen Code	Habitat	Geographic Location	Sampling Date
sp. 21: Gingo ship hull	Ship hulls	Saronikos Gulf (GR)	20 October 2020
sp. 22: Gingo ship hull	Ship hulls	Saronikos Gulf (GR)	20 October 2020
sp. 23: Rhodes marina	Marina	Rhodes Island (GR)	22 September 2020
sp. 24: Zakynthos marina	Marina	Zakynthos Island (GR)	18 April 2024
sp. 25: Liakouri beach	Reef	Crete Island (GR)	31 May 2023
sp. 26: Liakouri beach	Reef	Crete Island (GR)	31 May 2023
sp. 27: Purple cave	Cave	Evia Island (GR)	8 October 2024
sp. 28: Heraklion marina	Marina	Crete Island (GR)	10 December 2024
sp. 29: Agia Paraskevi	Cave	Chalkidiki (GR)	18 July 2024
sp. 30: Alikes 2 cave	Cave	Crete Island (GR)	19 November 2024
sp. 31: Alikes 2 cave	Cave	Crete Island (GR)	19 November 2024
sp. 32: Alikes 2 cave	Cave	Crete Island (GR)	19 November 2024
sp. 33: Siphoni cave	Cave	Crete Island (GR)	20 November 2024
sp. 34: Siphoni cave	Cave	Crete Island (GR)	20 November 2024
sp. 35: Elephant cave	Cave	Crete Island (GR)	16 December 2022
sp. 36: Elephant cave	Cave	Crete Island (GR)	16 December 2022
sp. 37: Seal's 2 cave	Cave	Rhodes Island (GR)	9 August 2020
sp. 38: Seal's 2 cave	Cave	Rhodes Island (GR)	9 August 2020
sp. 39: Reef cave	Cave	Crete Island (GR)	12 June 2021

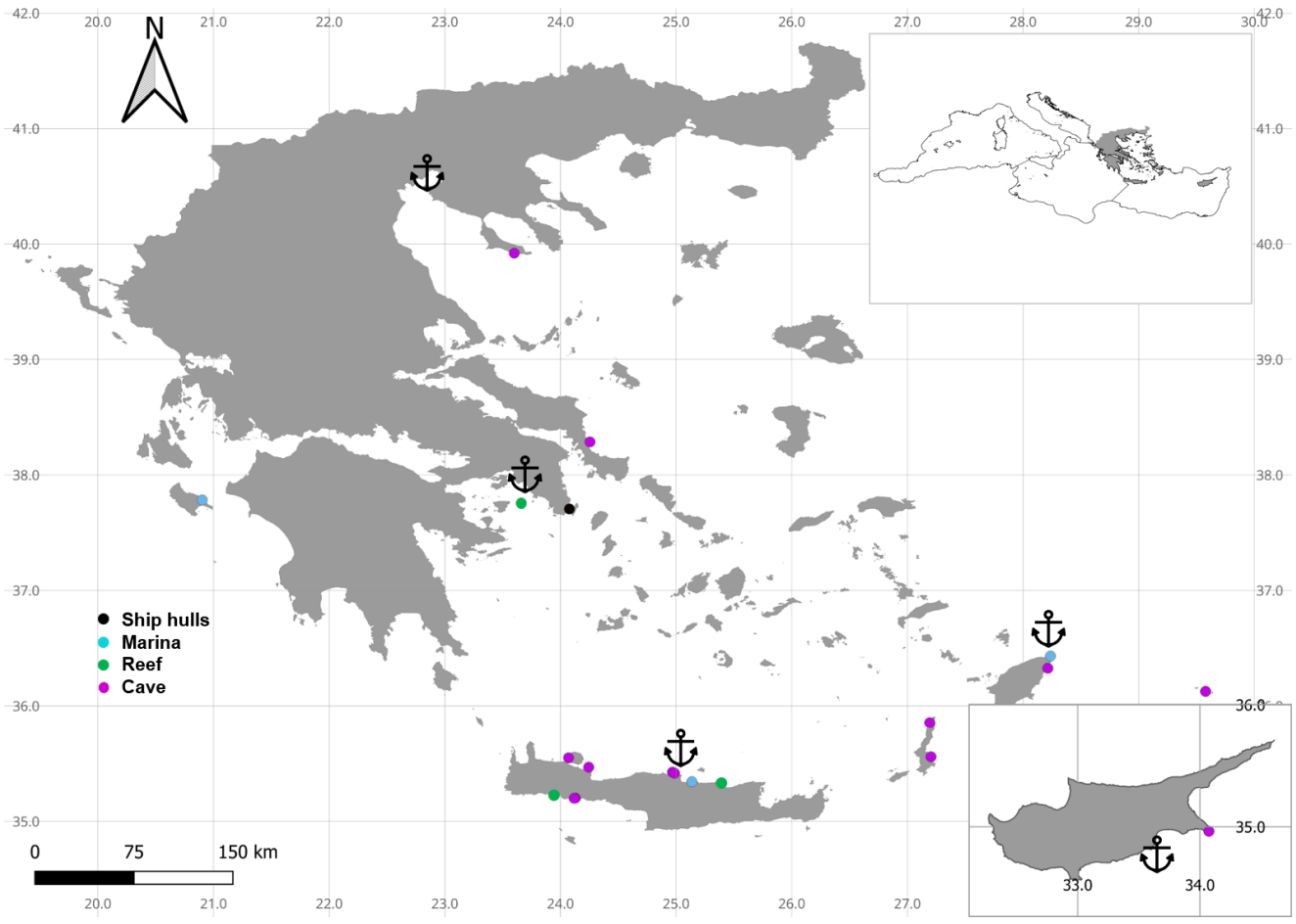

Figure 1. Map of the sampling locations in Greece and Cyprus. Areas and habitat types where samples were collected are depicted by differently colored circles (Ship hulls, cave, marina, reef). Anchors mark locations of major ports of Greece and Cyprus. Coordinates of the sampling locations can be found in Table S1 of the Supplementary Material.

2.2. Morphological Identification and Imaging

Specimens were initially sorted and examined morphologically. Small-sized individuals were photographed through a Zeiss SteREO Discovery V12 stereomicroscope, while larger specimens were photographed with an Olympus System Tough TG-7, accompanied by proper scaling.

The morphological identification was performed following the keys and published literature [16,19,38,39,43–45]. Exterior sculpture, shell thickness and coloration, hinge and ligament were used as main identifiers of the different specimens. *Isognomon* specimens were usually either oval to oblong and very flat, with thin but rigid valves, or more elongated in shape and relatively thicker and harder with smaller valves. In some specimens, the exterior exhibited coarse, wavy lamellar sculpture and variable, often pale cream to purplish bicoloration, while others presented a creamy or brown/grey coloration with present ligament pits. *Malleus* specimens, presented mostly a different shell structure, more elongated with thin transparent valves, from brownish to purple coloration, with no hinge teeth.

2.3. DNA Extraction and Sequencing

DNA was extracted from the abductor muscle tissue of each specimen using the DNeasy® Blood & Tissue kit (Qiagen, Hilden, Germany), following the manufacturer's instructions with minor adjustments. The mitochondrial *16S rRNA* gene fragment was amplified using the primers 16SAR-L (5′-CGCCTGTTTATCAAAAACAT-3′) and 16SBR-H (5′-CGGTCTGAACTCAGATCACGT-3′) [46]. The mitochondrial *COI* gene was amplified using the primers LCO1490 (5′-GGTCAACAAATCATAAAGATATTGG-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) [47].

The PCR reaction for most specimens was performed in a total volume of 12.5 µL containing the following: 1 µL of DNA (10-20 ng), 6.75 µL of ddH_2O, 2.5 µL of MyTaq Red Reaction Buffer (5×), 0.25 µL MyTaq™ Red DNA Polymerase (meridian BIOSCIENCE, Cincinnati, USA), and 0.5 µL of each primer (10 µM). PCR amplification for the *16S* gene was performed using the following program: initial denaturation for 3 min at 94 °C, followed by 38 denaturation cycles at 94 °C for 30 s; primers' annealing at 45 °C for 30 s and extension at 72 °C for 40 s; final extension for 10 min at 72 °C. For the *COI* gene, only QIAGEN Multiplex PCR Master Mix (2×) (QIAGEN, Hilden, Germany) was used, following the program: initial denaturation for 15 min at 95 °C, followed by 36 denaturation cycles at 94 °C for 30 s; primers' annealing at 45 °C for 90 s and extension at 72 °C for 90 s; final extension for 10 min at 72 °C. The repetitive PCR reactions for specimens with technical difficulties were performed in a total volume of 12.5 µL consisting of the following: 1 µL of DNA (~ 10 ng), 4.25 µL of dd H_2O, 6.25 µL of PCRBIO Taq Mix (2×) (PCRBIOSYSTEMS, London, UK) or QIAGEN Multiplex PCR Master Mix (2x) (QIAGEN, Hilden, Germany), and 0.5 µL of each primer (10 µM). All reactions were performed in a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA).

Amplified gene PCR products were excised from the gel and cleaned using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). Sanger sequencing reactions were performed bidirectionally using BigDye™ Terminator v3.1 Cycle Sequencing Kit and were electrophoresed on an ABI 3730xl DNA Analyzer (Applied Biosystems™, Waltham, MA, USA). All resulting sequences were confirmed by sequencing both strands (forward and reverse directions).

2.4. Phylogenetic Analysis

The obtained sequences were initially compared with existing sequences in GenBank using Basic Local Alignment Tools v2.17.0 (BLAST) analysis at The National Center for

Biotechnology Information (NCBI), accessible at https://www.ncbi.nlm.nih.gov (accessed on 21 August 2025). The sequences were aligned using the AliView software v1.30.

Maximum Likelihood phylogenetic analyses were performed using IQ-TREE v3 [48]. For each dataset, the best-fit nucleotide substitution model was selected independently using ModelFinder based on the Bayesian Information Criterion (BIC), and tree inference was conducted under the corresponding model. Branch support was assessed with 1000 ultrafast bootstrap replicates. For the *16S rRNA* gene, the best-fit nucleotide substitution model for the Isognomonidae family was TPM3+G4, whereas for the Malleidae family it was TPM3u+R2 according to the Bayesian Information Criterion (BIC). For the *COI* gene, the best-fit model selected by BIC was TN+F+I+G4 for Isognomonidae and HKY+F+I for Malleidae.

Moreover, datasets for both genes (*16S, COI*) were further analyzed with Bayesian inference (BI) in MrBayes 3.2.7 software [49]. The GTR+G model was used, running two MCMC of 1,000,000 generations, sampled every 1000. The BI trees were visualized using FigTree v.1.4.5 [50] (Figure S2 in Supplementary Material). Two *Brachidontes pharaonis* (P. Fischer, 1870) sequences (*16S*: MF345840, *COI*: MF345914) were used in all of the analyses as outgroups. In all *COI* gene trees, due to the bigger number of sequences available, species were limited to haplotypes to get depicted easier. Phylogenetic tree visualization and annotation were carried out using the Interactive Tree Of Life (iTOL).

3. Results

Bivalve specimens collected from various localities in the Eastern Mediterranean Sea were identified as belonging to three NIS (Figure 2). Specifically, ten individuals were identified as *I. bicolor* (Figure 3), seventeen as *I.* aff. *legumen* (Figure 4) and twelve as *M.* cf. *regula* (Figure 5). Of the ten specimens identified as *I. bicolor* (Figure 3), two were found in marinas, two on ship hulls, two on rocky reefs, and four in marine caves (Figure 2). All seventeen *I.* aff. *legumen* specimens were found exclusively in marine caves (Figure 2). Lastly, among the twelve *M.* cf. *regula* specimens (Figure 4), three were collected from marinas, one from a rocky reef, and eight from marine caves. A summary of species occurrences by habitat type is presented in Figure 2.

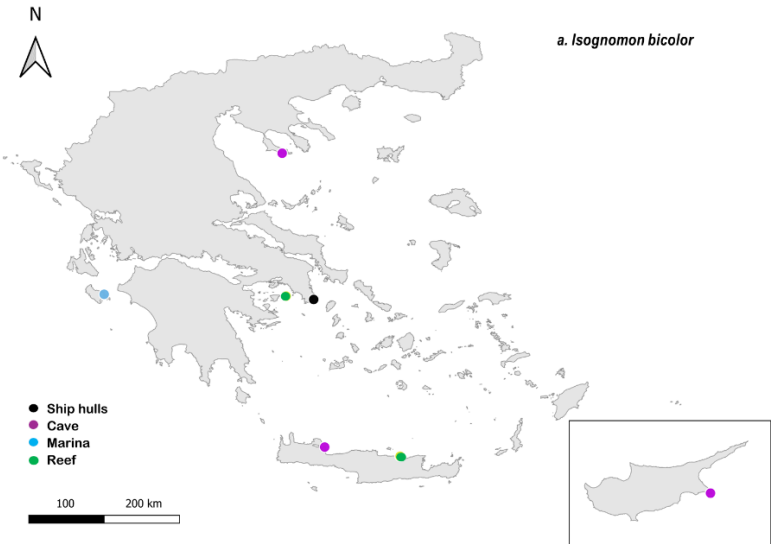

Figure 2. *Cont.*

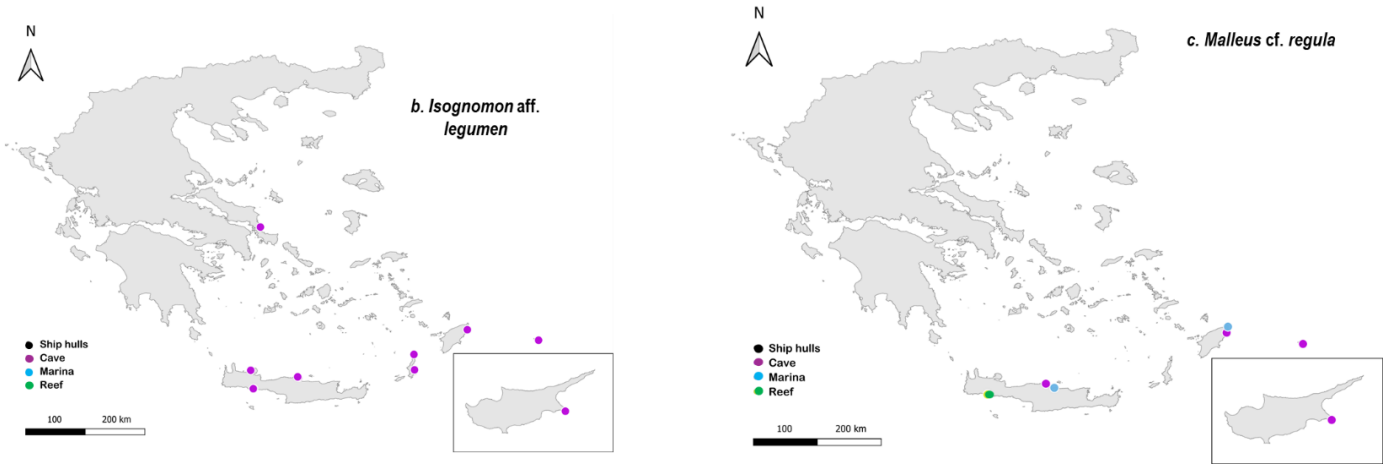

Figure 2. Habitat types across sampling locations for the species (**a**) *Isognomon bicolor*; (**b**) *Isognomon* aff. *legumen*; (**c**) *Malleus* cf. *regula*. Colored circles represent habitat types: marine caves (purple), rocky reefs (green), marinas (blue), and ship hulls (black).

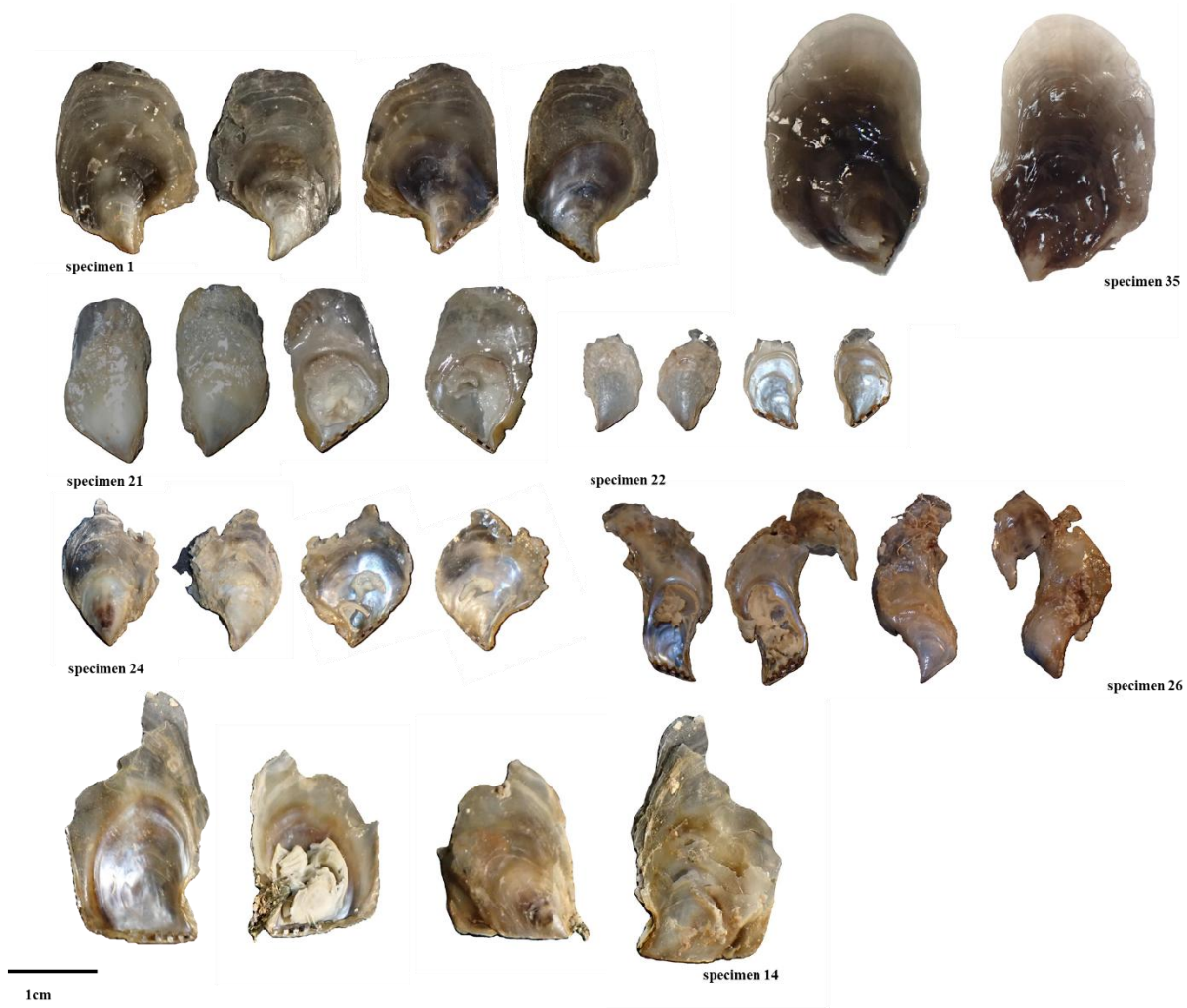

Figure 3. *Isognomon bicolor* specimens. Specimens 21 and 22 were collected from the hull of the GINGO vessel; specimens 1 and 24 from marinas; and specimens 14, 26, and 35 from marine caves in Cyprus, Chalkidiki and Crete (Table 1). Scale bar = 1 cm.

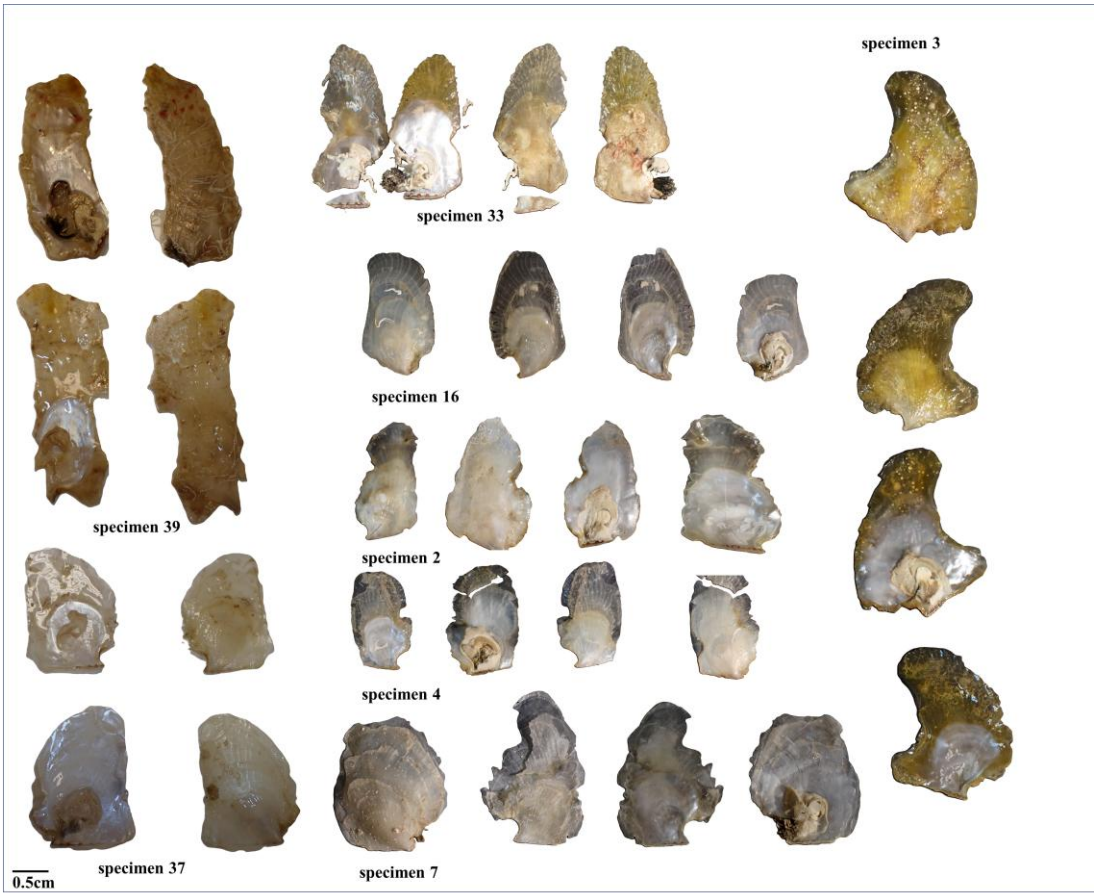

Figure 4. *Isognomon* aff. *legumen* specimens. All specimens were collected from marine caves. Specimens 2, 3 and 4 were collected from marine caves in Karpathos, Kastellorizo and Crete, respectively; specimen 7 was collected from Cyprus, specimen 16, 33, 39 from Crete, while specimen 37 was collected from Rhodes (Table 1). Scale bar = 0.5 cm.

Molecular Analysis

Isognomon bicolor: Ten specimens (specimen codes: 1, 14, 21, 22, 24, 25, 26, 29, 35, 36) were identified as *I. bicolor* based on more than 99% BLAST similarity to sequences in GenBank (e.g., HQ329406.1; PP034416–419.1; OK104096–097.1) [10,19,51]. These specimens presented less than 94% similarity to other congeneric species such as *I. recognitus* (Mabille, 1895) (GenBank: KT317424.1), and less than 82% similarity to *I. ephippium* (Linnaeus, 1758) (GenBank: KY081325.1) and *I. alatus* (GenBank: KC429251.1), supporting their assignment to *I. bicolor*.

Isognomon aff. *legumen*: Seventeen specimens (IDs: 2, 3, 4, 6, 7, 9, 13, 16, 17, 18, 27, 31–34, 37, 39) were assigned to *I.* aff. *legumen*, sharing more than 99% similarity to GenBank sequences PP034420.1, PP034421.1, and HQ329409.1, and less than 87% similarity to other *Isognomon* species.

Malleus cf. *regula*: Twelve specimens (IDs: 5, 8, 10–12, 15, 19, 20, 23, 28, 30, 38) matched *Malleus* sequences (e.g., OK166813.1, OK104098.1) with more than 99% identity and less than 89% similarity to other *Malleus* species. Due to limited molecular representation of *Malleus* spp. in public databases, these specimens were provisionally assigned as *Malleus* cf. *regula*.

All resulting sequences have been deposited in GenBank under accession numbers PV955598-PV955637 for the *16S rRNA* gene and (pending accession numbers) for the mitochondrial *COI* gene. Both phylogenetic approaches, Maximum Likelihood and Bayesian Inference, produced consistent tree topologies and relationships between species.

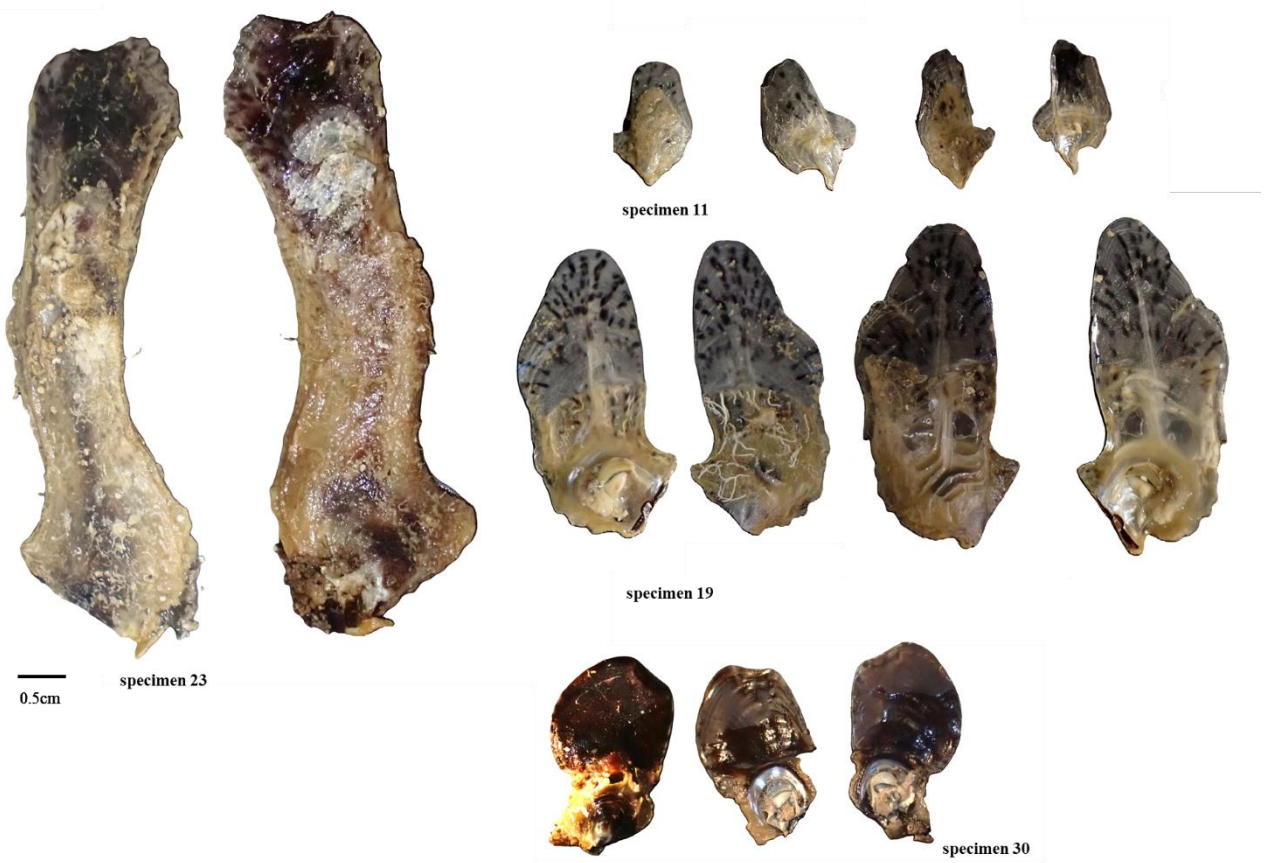

Figure 5. *Malleus* cf. *regula* specimens. Specimens 19 and 23 were collected from marinas in Crete and Rhodes, and specimens 11 and 30 from marine caves in Kastellorizo and Crete, respectively (Table 1). Scale bar = 0.5 cm.

4. Discussion

This study presents an integrative assessment of non-indigenous bivalves of the genera *Isognomon* and *Malleus* in the Eastern Mediterranean Sea, combining morphological identification with molecular tools to overcome long-standing taxonomic uncertainties. By leveraging an extended and spatially broad sampling effort across both natural and artificial habitats, this research captures a more complete picture of these species' current distribution and ecological preferences. Our approach included cryptic and understudied natural environments, such as marine caves and areas beneath rocks, which are often overlooked in marine biodiversity surveys, as well as heavily disturbed artificial habitats (i.e., ports and marinas), which form key entry points and stepping stones for the establishment and further spread of NIS. The species *I. bicolor* and *I.* aff. *legumen* constitute new records for the marine cave habitat in the Mediterranean Sea [52]. The integrative methodology applied here provides critical insights not only into species identification and phylogenetic relationships but also into their potential pathways of introduction and patterns of habitat colonization.

4.1. Taxonomic Complexity and Morphological Plasticity

The integration of taxonomic and molecular approaches offers more reliable insights into the identification and origin of NIS, particularly in cases where taxonomic classification remains uncertain. This is especially relevant for the bivalve genera *Isognomon* and *Malleus*, whose systematics are still controversial and in need of revision supported by molecular evidence. For instance, some of the *I.* aff. *legumen* specimens analyzed in this study (specimens 2, 3, 6, 9, 37, and 39) were previously assigned to *I.* cf. *australicus*, while

four *M.* cf. *regula* specimens (5, 10, 11, and 38) had been assigned to *M. regula* in a previous study of ours [53]. These earlier assignments were based solely on morphological examination and the literature available at that time (e.g., [16,54]).

In addition, the genera *Isognomon* and *Malleus* present significant taxonomic challenges due to their pronounced morphological plasticity, particularly in shell shape and size. These traits are largely influenced by environmental factors such as substrate type, hydrodynamic conditions, salinity, and exposure [55,56]. Shell morphology in both genera adapts to local microhabitats, resulting in considerable phenotypic variation that complicates identification at both species and genus levels. This variability is a major source of taxonomic confusion, especially in juvenile specimens, which often exhibit incompletely developed and highly variable morphological features.

For instance, *I. australicus* and *M. regula* have historically been misidentified as *I. legumen* in Mediterranean records [54], underscoring the limitations of shell morphology-based taxonomy. A critical issue lies in the overlap of diagnostic characters, many of which likely reflect environmental adaptation rather than genetic divergence. Occupation of structurally complex substrates, like caves or reefs, could further enhance phenotypic plasticity, increasing the likelihood of misidentification and obscuring the true taxonomic identity of these NIS.

It is important to distinguish between true cryptic species and phenotypic plasticity when interpreting morphological variation. Cryptic species are typically defined as morphologically indistinguishable but genetically distinct evolutionary lineages [57]. In other words, they are separate species that have been hidden under one name due to a lack of obvious morphological differences. Phenotypic plasticity is the capacity of one genotype (one species or population) to produce different phenotypes depending on environmental conditions [58]. The morphological variability observed in the present study is not accompanied by clear genetic divergence in the markers examined and, therefore, is interpreted as intra-specific variation rather than evidence of cryptic speciation. Nevertheless, the possibility of undetected genetic structuring cannot be entirely excluded given the resolution limits of the markers used, and further investigation using higher-resolution genomic approaches would be valuable.

4.2. Phylogenetic Trees and Species' Spread

The phylogenetic hypotheses inferred by the Maximum Likelihood and Bayesian Inference analyses demonstrated complete agreement regarding the evolutionary relationships. The phylogenetic analysis of the *16S rRNA* gene for *Isognomon* (Figure 6a) revealed six well-supported clades. The first clade includes the majority of specimens from Greece and Cyprus, which cluster with *Isognomon* sp. from Mexico and *Isognomon* aff. *legumen* specimens from Cyprus, with strong bootstrap support (99%). All 17 specimens assigned to *I.* aff. *legumen* were found exclusively in cryptic natural environments such as marine caves, in agreement with earlier observations suggesting that *I.* aff. *legumen* prefers such habitats, as opposed to *I. bicolor* [19].

A distinct monophyletic clade contains ten *Isognomon* specimens from Greece and Cyprus, clustering with *I. bicolor* sequences from Florida, Israel, and Italy, supporting their identification as *I. bicolor*. The species in this clade seem to have a broader distribution in contrast to *Isognomon* aff. *legumen* specimens, possibly assisted by human activities. More specifically, specimens 21 and 22, which were collected from fouling material on the hull of ship GINGO, form a small though distinct sub-clade within this group. This pattern suggests either the presence of a separate haplotype reflecting regional variation or distinct biogeographic populations introduced via different pathways. *Isognomon bicolor* seems to be favored in disturbed environments, such as ports and marinas, with its presence

confirmed in samples from Glifada and Zakynthos marinas. However, molecular analysis also identified *I. bicolor* in natural environments in Cyprus (specimen 14), Crete (specimens 25, 26, 35, and 36), and Chalkidiki (specimen 29) (Table S1 in Supplementary Material, Figures 6a and 7a), suggesting ongoing expansion into less disturbed habitats.

The second clade in the *16S* tree includes only *I. radiatus* from Florida, while the third comprises four *I. recognitus* individuals from Mexico. Additional clades are comprised of *I. ephippium* and *I. alatus* species, both from the Caribbean.

The phylogenetic tree of the mitochondrial *COI* gene (Figure 7a) provides more information on phylogenetic relationships within the genus *Isognomon*, likely due to the broader availability of *COI* barcodes in GenBank and BOLD databases. The tree resolves a larger number of clades such as *I. legumen* (China), *I. lobata* Reeve, 1858 (Australia), *I. quadrangularis* (Reeve, 1858) (Australia, Japan), *I. spathulata* (Reeve, 1858) (Australia, Japan), *I.* sp., *I. recognitus* (Costa Rica), *I. bicolor* (Florida, Gingo ship hulls), *I. ephippium* (China), *I. ephippium* (Australia), *I. acutirostris* (Lamarck, 1819), *I. nucleus* (Lamarck, 1819) (South Africa), *I. nucleus* (China), *I. alatus* (Caribbean), *I.* sp. (Caribbean, Florida), *I. perna* (Linnaeus, 1758) (USA, China), *I. isognomum* (Linnaeus, 1758) (Singapore) and a mixed clade of *I. australicus/aff.legumen/legumen*. The clade of *I. australicus/aff.legumen/legumen* is differentiated from the *I. legumen* clade, indicating either (i) misidentification of one or both clades, (ii) that the second clade contains misidentified *I. legumen* or *I.* aff. *legumen* specimens, or (iii) that the second clade contains specimens originating from the Pacific and Atlantic Oceans and can be assigned as *I.* aff. *legumen*, until further sequences from Australia become available to reconstruct the tree.

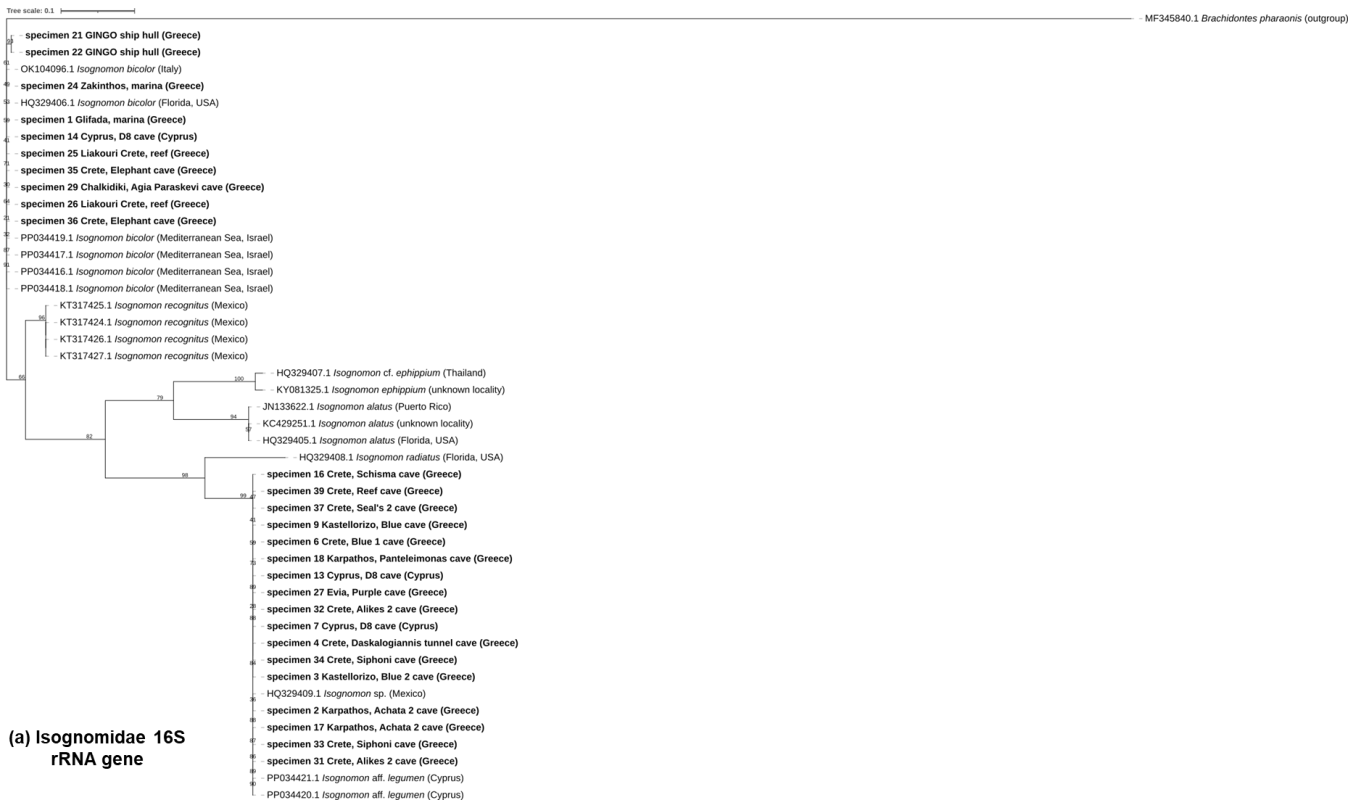

Figure 6. *Cont.*

Figure 6. Phylogenetic positioning based on *16S rRNA* gene and of the families of (**a**) Isognomonidae and (**b**) Malleidae, in relation to outgroup taxa from other bivalve families. Evolutionary relationships were inferred using the Maximum Likelihood method under the best-fit nucleotide substitution model for the Isognomonidae family which was TPM3+G4, whereas for the Malleidae family it was TPM3u+R2 according to the Bayesian Information Criterion (BIC). *Brachidontes pharaonis* (MF345840.1) was used as an outgroup in the Isognomonidae and Malleidae trees.

Figure 7. Phylogenetic positioning based on *COI* gene and of the families of (**a**) Isognomonidae and (**b**) Malleidae, in relation to outgroup taxa from other bivalve families. Evolutionary relationships were inferred using the Maximum Likelihood method under the models selected by BIC which was TN+F+I+G4 for Isognomonidae and HKY+F+I for Malleidae. *Brachidontes pharaonis* (MF345914.1) was used as an outgroup in the Isognomonidae and Malleidae trees. *COI* trees contain only haplotypes of each species, to reduce the excessive amount of duplicate and triplicate sequences from the same localities.

Specimens 21 and 22, collected from fouling material on the ship GINGO, again form a distinct sister clade very close to *I. bicolor*. This finding is consistent with the results on the

16S tree, where a second haplotype appears inside the clade of *I. bicolor*, suggesting firstly the transportation of the species most probably via shipping into the Mediterranean and the existence of multiple biogeographic populations in the genus with genetic differences. Shipping has been previously suggested as a putative mechanism for transportation of *I. bicolor* for its ability to attach as ship fouling [59]. The species has been recorded to attach to empty shells of the barnacles *Megabalanus* spp. [60], as observed also in our study in specimens 21 and 22. However, tracking of the route of GINGO vessel over a year, from November 2019 to October 2020, indicates that the native area of *I. bicolor* (Western Central Atlantic Coast), was not visited, leading to the conclusion that *I. bicolor* could have already existed, in the ports that GINGO stopped before reaching its destination, such as the Suez Canal, and therefore suggesting secondary dispersion. Another plausible explanation for this finding could be the co-existence of a very similar and closely related species of *I. bicolor*, supported by the grouping of the two specimens in the same clade, yet separate from the rest of the *I. bicolor* specimens. The most probable scenario is that those two specimens belong to a subspecies or an isolated population that is separated genetically from others of the same species, due to geographic barriers.

Regarding *Malleus*, phylogenetic analyses of both *16S rRNA* (Figure 6b) and *COI* genes (Figure 7b) showed that all specimens in this study belong to the species *Malleus* cf. *regula*, clearly separated from other known *Malleus* species. The family Malleidae is underrepresented in global sequence databases compared to *Isognomon* species. However, phylogenetic grouping of our specimens within a distinct clade containing *Malleus* sequences from neighboring Italy and Cyprus supports their assignment to this taxon. Given the limited database representation, we designate our samples according to the closest identification available. These results are consistent with previous studies [10], as all of our specimens represent one haplotype and form a separate clade grouping with *Malleus* sp. (OK104098) and *M.* cf. *regula* (OK166813), distant from the clade of *M. regula* and *M. albus* Lamarck, 1819 from Australia.

As with *I. bicolor*, *M.* cf. *regula* appears to follow a pattern of initial establishment in anthropogenically impacted areas (e.g., marinas) followed by expansion into more natural habitats. Unfortunately, the genetic information is not yet enough to provide total insight into the species' introduction and expansion. The limited genetic data currently available restricts a complete understanding of its introduction pathways and population structure. Further sequencing and broader geographic sampling are needed to fully resolve the phylogenetic placement and invasion dynamics of both *Isognomon* and *Malleus* in the Mediterranean.

4.3. Methodological and Taxonomic Limitations

Several limitations must be taken into consideration in interpreting the findings of this study. Firstly, specimens were collected in small numbers, especially from hard-to-reach areas (e.g., ship hulls, marine caves), leading to a limited sample size, therefore preventing further quantitative comparisons and introducing regional biases in invasion patterns and community composition. Secondly, taxonomic uncertainty persists for *Isognomon* aff. *legumen* and *Malleus* cf. *regula* [10,16,19], so until formal taxonomic revision and genetic confirmation are available, species-level assignments for these taxa should be considered as interim. Finally, the phylogenetic analyses relied on standard mitochondrial markers (*16S rRNA* and *COI* genes) to ensure compatibility with the existing sequence data available for these species. While these markers are widely used for species identification, they offer limited resolution for invasion pathways and dispersal patterns over shorter timescales [61–63], especially when compared to nuclear markers.

In this study, our primary objective was to identify broad phylogeographic patterns and assess whether sampled individuals correspond to known lineages previously reported from native and introduced ranges. Therefore, the results do not constitute direct estimates of migration. Future studies could benefit from more sensitive genetic markers, such as microsatellites or single-nucleotide polymorphisms (SNPs), which offer higher resolution. Despite these limitations, the records presented here provide valuable baseline information on NIS associated with maritime vectors in the Eastern Mediterranean.

4.4. Planktonic Larval Dispersal and Connectivity

Planktonic larval dispersal is an additional key factor facilitating the spread and connectivity of bivalve populations. Larval duration, together with local hydrodynamic conditions, influences dispersal potential and the establishment of new populations. The planktonic phase supports demographic and genetic connectivity among otherwise isolated sessile populations, promoting persistence and range expansion [64]. Pelagic larval duration (PLD) determines dispersal opportunity: longer PLDs generally increase transport potential, though realized connectivity is often reduced by mortality and retention processes [65,66].

In Mediterranean bivalves, dispersal patterns help explain regional connectivity and invasive success, particularly in semi-enclosed basins such as the Eastern Mediterranean [66,67]. However, the relationship between PLD and gene flow is species-specific—oceanographic barriers or behavior may limit connectivity even in long-PLD species, while short-PLD species can remain connected through stepping-stone habitats [65]. In this context, the similarities among sampled populations may result from both larval dispersal and human-mediated transport (e.g., shipping). Considering larval dispersal processes offers a more mechanistic way to interpret the observed phylogeographic patterns. This also highlights the need for future studies that combine genetic data with larval ecology models.

4.5. The Role of Reference Databases in Molecular Identification of NIS

DNA barcoding plays a critical role in the identification of NIS, especially in cases involving juvenile or morphologically ambiguous specimens such as individuals 21 and 22 in our study. However, its effectiveness is directly dependent on the completeness as well as the accuracy of the reference databases. This limitation is particularly evident in taxa like *Isognomon*, where database representation remains uneven and incomplete. Many species in the Isognomonidae family do not yet carry sequences for the *16S gene* or *COI* gene. Notably, only five out of the 16 species in this family are currently represented by *16S rRNA* gene sequences, and 13 by the mitochondrial *COI* gene. Recently, *I. legumen* was reported in Albania [21] and Tunisia [40] based solely on morphological identification, so the absence of corresponding molecular data meant these records could not be reflected in our phylogenetic analysis.

The reliability and taxonomic resolution of DNA-based identifications are closely tied to the quality and breadth of the reference database used. A well-curated, taxonomically diverse reference library for each ecoregion allows for more accurate species assignments, more informative evolutionary inferences, and greater confidence in the results. In contrast, limited or poorly curated databases may increase uncertainty, even when working with high-quality sequence data [68]. Ultimately, the effectiveness of any DNA analysis is totally dependent on how comprehensive and robust the reference databases are, like GenBank and the Barcode of Life Data System (BOLD).

This research highlights a collaboration of scientists from different areas of expertise, utilizing samples from diverse sampling campaigns and habitats. By joining efforts with

experts in molecular methods and bioinvasion research, this study was able to correct long-standing morphology-based errors. This research validates the need for integration of methods, combining morphological identification with DNA barcoding, to resolve taxonomic issues in highly variable bivalve genera like *Isognomon* and *Malleus*. We documented the expanding distribution of three NIS (*I. bicolor*, *I.* aff. *legumen*, and *M.* cf. *regula*) across both anthropogenic and natural, difficult-to-reach environments often neglected by standard monitoring. Our phylogenetic analyses suggest the presence of potentially different introduction pathways for the *Isognomon bicolor* species and evidence of intraspecific variation among *I. bicolor* samples associated with hull fouling, while the remaining samples appear to represent a single haplotype. Furthermore, the data provides no clear support for the presence of cryptic species; instead, the observed morphological variability in both families is more plausibly attributable to phenotypic plasticity, which may complicate reliable identification. Molecular analyses expose significant deficiencies in current genetic databases, underscoring the urgent need for robust, geographically diverse reference sequences. To effectively monitor the introduction and expansion of NIS across all different habitat types, future strategies must integrate collaborative sampling, high-quality genetic databases, and open-access data sharing.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d18020127/s1, Table S1: Unique specimen codes, accession numbers for the *16S* and *COI* genes, specific habitat, exact geographic location (GR: Greece, CY: Cyprus), area, date of samplings, collector names, and comments; Figure S1: Phylogenetic positioning based on *16S rRNA* gene of the families of (a) Isognomonidae and (b) Malleidae, in relation to outgroup taxa from other bivalve families. Evolutionary relationships were inferred using Bayesian Inference (BI). The GTR+G model was used, running two MCMC of 1,000,000 generations, sampled every 1000. *Brachidontes pharaonis* (MF345840.1) was used as an outgroup in the Isognomonidae and Malleidae trees; Figure S2: Phylogenetic positioning based on (*COI* mitochondrial gene for species of the families of (a) Isognomonidae and (b) Malleidae, in relation to outgroup taxa from other bivalve families. Evolutionary relationships were inferred using Bayesian Inference (BI). The GTR+G model was used, running two MCMC of 1,000,000 generations, sampled every 1000. *Brachidontes pharaonis* (MF345914.1) was used as an outgroup in the Isognomonidae and Malleidae trees. *COI* trees contain only haplotypes of each species, to reduce the excessive amount of duplicate and triplicate sequences from the same localities.

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