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Molecular Data Confirm the Occurrence of the Allochthonous *Gambusia holbrooki* (Pisces: Poeciliidae) in Sicily and the Maltese Archipelago

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Abstract: A major threat to biodiversity is represented by Invasive Alien Species (IAS), particularly on freshwater ecosystems, which are already heavily altered by human activities. Two of the most pernicious IAS are the eastern and western mosquitofish, i.e., *Gambusia holbrooki* and *G. affinis*. These two poeciliids are morphologically very close to each other, and soon after their formal description, *G. holbrooki* was considered a subspecies of *G. affinis*. In the following years, several studies proved that these two entities belonged to two different species; nevertheless, it was only at the end of the 1990s that their separate taxonomic status was re-established. In the 1920s and 1930s, both *G. holbrooki* and *G. affinis* were asynchronously introduced from the United States into Europe and subsequently translocated globally as biocontrol agents of the malaria vector (i.e., the larvae of the *Anopheles* mosquitoes), with dramatic consequences for the inland water native fauna. However, due to taxonomic uncertainties and nomenclatural instability, for years, there were doubts about which *Gambusia* species had been introduced in different regions. The first available molecular studies confirmed the occurrence of *G. holbrooki* in Europe, but no evidence confirming the occurrence of *G. affinis* was found. Despite this, some records report the occurrence of western mosquitofish in Italy and Malta. Considering the negative effects that the mosquitofish has on the native biota, it is of paramount importance to know the precise biological diversity of the native and non-native species to better implement environmental management strategies to properly preserve the already-fragile waterbodies. Therefore, to check for the possible occurrence of *G. affinis* in Italy and Malta, we conducted extensive sampling in Sicily (Italy) and in the Maltese archipelago, aiming to verify the identity of *Gambusia* populations occurring in the study area. Based on sequences of the mitochondrial cytochrome b gene, we consistently observed the occurrence of only *G. holbrooki* in the investigated area, finding, almost exclusively, the most common haplotype known for the species in the whole invaded range (i.e., "HOL1").

Keywords: cyprinodontiformes; cytochrome b gene; inland water; invasive alien species; mediterranean area; mtDNA; eastern mosquitofish; biological invasions



Academic Editors: Lei Zhou, Junjie Wang, Chuanbo Guo and Michael Wink

Received: 20 November 2024

Revised: 8 January 2025

Accepted: 8 January 2025

Published: 13 January 2025

Citation: Vecchioni, L.; Liuzzo, M.; Sciberras, A.; Sciberras, J.; Formosa, J.; Deidun, A.; Giacalone, G.; Arizza, V.; Arculeo, M.; Marrone, F.; et al.

Molecular Data Confirm the Occurrence of the Allochthonous *Gambusia holbrooki* (Pisces: Poeciliidae) in Sicily and the Maltese Archipelago. *Diversity* **2025**, *17*, 48. <https://doi.org/10.3390/d17010048>

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1. Introduction

Biological invasions by non-native species are among the most significant causes of biodiversity loss and ecosystem alteration [1–5]. In fact, the introduction of invasive alien species (hereafter IAS) through commercial trade represents a key factor in transforming biocenoses and natural ecosystems ([6] and references therein). Over centuries, humans have frequently introduced, deliberately or unwarily, non-native species worldwide for various purposes such as sport fishing, ornamental reasons and biocontrol attempts (e.g., [1,7]). In this context, inland waters are by far the most sensitive ecosystems to the introduction and spread of IAS, mainly due to the overexploitation of these habitats linked to human activities as well as the high dispersal ability of non-native species, the eradication of which, once established, is often difficult or impossible [8,9]. Furthermore, the occurrence of non-native species involves multi-faceted impacts, since they can predate on or outcompete native species (e.g., [10,11]) and lead to phenomena of niche displacement [12] or parasite spill-over [13], thus significantly compromising the stability of the native communities that inhabit the invaded waterbodies.

Native to the United States, the eastern and western mosquitofishes (also known as “plague minnows”) of the genus *Gambusia* Poey, 1854, i.e., *G. holbrooki* Girard, 1859 (Figure 1) and *G. affinis* (Baird & Girard, 1853) are small, viviparous poeciliid fishes inhabiting both fresh and brackish water. *Gambusia* usually reaches sexual maturity in 3–8 weeks, with females that can have multiple broods with an average clutch size of 5 to 100 over a single breeding season ([14] and references therein), thus having great population expansion potential. At the beginning of the 20th century, they were introduced worldwide as biocontrol agents (e.g., [14–20]) in an attempt to control the mosquitoes of the genus *Anopheles* Meigen, 1818, i.e., the dispersal vectors of malaria. Employing mosquitofishes was one of the best solutions found by Howard [21] and other investigators (see [16] and reference therein) to control and limit the spread of mosquito populations, i.e., the vectors of the disease. Therefore, from the beginning of the 20th century onwards, *Gambusia* spp. were imported from the USA and introduced to Europe [14,22], leading to one of the worst global invasions occurring in inland waters (e.g., [9,18,23–30]).

Eastern and western mosquitofishes are two phylogenetically and morphologically closely related poeciliid species. After their description, due to their alleged resemblance, *Gambusia holbrooki* was considered a subspecies of *G. affinis*, the latter also including another subspecies, namely *G. affinis patruelis*, later synonymised with *G. affinis affinis* (see [14] and references therein). Several years later, the distinct species status of the two taxa was re-established [31,32]. However, due to taxonomic uncertainties and nomenclatural instability, for years, there were doubts about which mosquitofish species were translocated throughout the world and where exactly they had been introduced. Although some studies correctly identified the species since its first introduction in Europe, it was only after the molecular study of Vidal et al. [33] (but see also [34–36]) that it was ascertained that *G. holbrooki*, the eastern mosquitofish, was the *Gambusia* species most frequently introduced in Europe.

In Italy, both *Gambusia holbrooki* and *G. affinis* were acclimated and introduced (cf. [37–40]). Despite the increasing amount of evidence attesting the impact of *Gambusia* spp. on native biocenoses (e.g., [41–48]), the reputation of the mosquitofish as an effective predator of mosquito larvae remained undiminished, thus leading to its official or informal active introduction into waterbodies throughout the country (e.g., [49]).

Nowadays, due to their known impact on native biocenoses, both *Gambusia affinis* and *G. holbrooki* are considered invasive alien species of Union concern according to the EU Regulations [50] and [51]; accordingly, their breeding, trade, introduction in the wild and translocation are prohibited across the entire European Union. However, unidentified

mosquitofishes of unknown origin can still often be found for sale in pet shops and street markets as well as on the internet; accordingly, it is possible that multiple, independent introduction events posterior to those of the first half of the 20th century occurred and still occur to this very day.

In light of the likely occurrence of *Gambusia affinis* in Italy and possibly in Slovenia ([39,52–56], but see also [57]), and of the possibility that multiple introductions from different source areas took place in the past and in recent times in our study area, we aimed to establish, through an extensive sampling effort, which *Gambusia* species actually occur in the Sicilian and Maltese inland waters. Considering the fact that the mosquitofish negatively affects the inland water's biota (e.g., [45,58]), the accurate and precise knowledge of the biological diversity of both the native and non-native species of both islands is in fact of paramount importance for the future environmental management of the heavily endangered aquatic ecosystems [59]. Furthermore, a reliable taxonomic assessment is also crucial from a legislative and management point of view, especially when dealing with groups of cryptic species or those that are difficult to identify on a solely morphological basis.



Figure 1. Illustrations of female and male specimens of *Gambusia holbrooki* (modified from: Comes, [60]).

2. Materials and Methods

Prior to sampling in the Sicilian and Maltese freshwater waterbodies, an in-depth bibliographic research work through the use of the databases of SCOPUS (<https://www.scopus.com>, last accessed on 13 November 2024), Google Scholar (<https://scholar.google.it/>, last accessed on 13 November 2024) and ResearchGate (<https://www.researchgate.net/>, last accessed on 13 November 2024), was conducted to assess the current knowledge on the presence and distribution of *Gambusia* on both investigated areas, thus allowing us to correctly plan the subsequent sampling sessions. The query structure used to gather information was based on the following keywords: “mosquitofish”, “*Gambusia*”, “*Gambusia holbrooki*”, “*Gambusia affinis*”, “Sicil*”, “Malt*” “Italy”.

Mosquitofish samples were collected throughout mainland Sicily (Italy) and Malta (Figure 2). In the frame of this work, permanent natural and artificial ponds, dams, reservoirs and mouth rivers were sampled using a 250 µm mesh-sized hand net. In addition to the samples collected in Sicily, *Gambusia* samples from “Lago di Canterno” (province of Frosinone, Latium) and “Passo Campalto” (province of Venice, Veneto) were also in-

vestigated as comparative materials (see Table 1 for further details). Collected *Gambusia* individuals were preserved in situ in 96% ethanol for subsequent identification in the laboratory. Collected samples are now deposited at the Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, Italy, under the responsibility of LV.

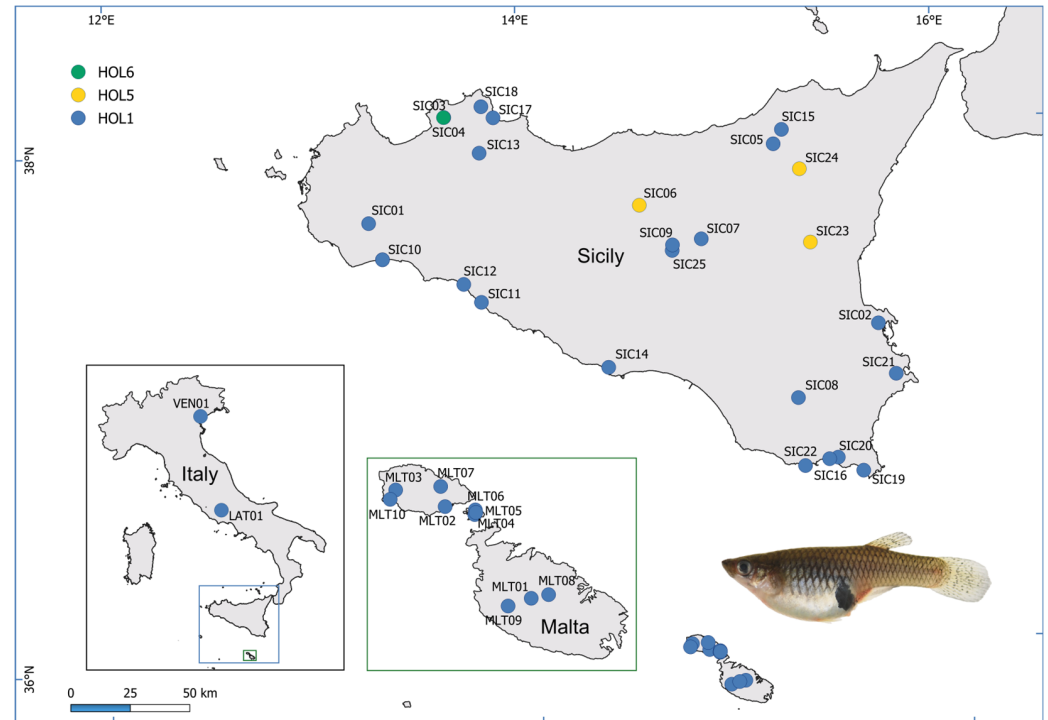


Figure 2. Distribution of *Gambusia* spp. samples collected in Peninsular Italy, Sicily and Malta. Haplotype nomenclature of *G. holbrooki* follows [33]. See Table 1 for the coordinates and codes of the sampled sites. Photo of *G. holbrooki* by Robert Aguilar, Smithsonian Environmental Research Center (https://www.flickr.com/photos/serc_biodiversity/12597857535/, last accessed on 13 November 2024). Map created using the Free and Open Source QGIS software v. 3.30.2.

Table 1. Geographic origins of the collected *Gambusia holbrooki* samples and GenBank accession numbers (A.N.'s). Geographical decimal coordinates are reported according to the WGS84 datum. Haplotype nomenclature of *G. holbrooki* follows [33].

Geographic Origin	Voucher Code	Latitude N	Longitude E	Cytb A.N.	Holotype
Lago di Canterno, Frosinone, Latium, Italy	LAT01	41.75715	13.25021	PQ737554	Hol1
Affluente diga Trinità, Trapani, Sicily, Italy	SIC01	37.72381	12.76003	PQ737565	Hol1
Augusta, Siracusa, Sicily, Italy	SIC02	37.24082	15.16481	PQ737566	Hol1
Bevaio Accitella Cinisi, Palermo, Sicily, Italy	SIC03	38.12096	13.13615	PQ737567	Hol6
Bevaio Piano Margi, Palermo, Sicily, Italy	SIC04	38.12113	13.13779	PQ737568	Hol1
Biviere di Cesarò, Messina, Sicily, Italy	SIC05	37.95524	14.71244	PQ737569	Hol1
Blufi, Palermo, Sicily, Italy	SIC06	37.74841	14.05694	PQ737570	Hol5
Diga Nicoletti, Enna, Sicily, Italy	SIC07	37.60611	14.34488	PQ737571	Hol1
Diga Santa Rosalia, Ragusa, Sicily, Italy	SIC08	36.97299	14.76598	PQ737572	Hol1

Table 1. Cont.

Geographic Origin	Voucher Code	Latitude N	Longitude E	Cytb A.N.	Holotype
Diga Villarosa, Enna, Sicily, Italy	SIC09	37.58888	14.20618	PQ737573	Hol1
Foce fiume Modione, Trapani, Sicily, Italy	SIC10	37.58288	12.82062	PQ737574	Hol1
Foce fiume Platani, Agrigento, Sicily, Italy	SIC11	37.40364	13.28470	PQ737575	Hol1
Foce fiume Verdura, Agrigento, Sicily, Italy	SIC12	37.47560	13.20380	PQ737576	Hol1
Lago di Piana degli Albanesi, Palermo, Sicily, Italy	SIC13	37.97835	13.30099	PQ737577	Hol1
Licata, Agrigento, Sicily, Italy	SIC14	37.13070	13.87576	PQ737578	Hol1
Longi, Messina, Sicily, Italy	SIC15	38.00865	14.75579	PQ737579	Hol1
Foce Marina di Modica, Ragusa, Sicily, Italy	SIC16	36.70968	14.78165	PQ737580	Hol1
Botanical Garder, Palermo, Sicily, Italy	SIC17	38.11206	13.37431	PQ737581	Hol1
Private fish tank, Palermo, Sicily, Italy	SIC18	38.15670	13.31826	PQ737582	Hol1
Pantano Baronello, Siracusa, Sicily, Italy	SIC19	36.67759	15.05427	PQ737583	Hol1
Pantano Gariffi, Ragusa, Sicily, Italy	SIC20	36.73414	14.93803	PQ737584	Hol1
R.N.O. "Fonte Ciane", Siracusa, Sicily, Italy	SIC21	37.04199	15.23482	PQ737585	Hol1
Rio Favara, Ragusa, Sicily, Italy	SIC22	36.73022	14.89691	PQ737586	Hol1
Salinelle del fiume Simeto, Catania, Sicily, Italy	SIC23	37.56857	14.86396	PQ737587	Hol5
Stagno di Balze Sottane, Bronte, Sicily, Italy	SIC24	37.85265	14.83128	PQ737588	Hol5
Torrente Morello, Enna, Sicily, Italy	SIC25	37.56775	14.20349	PQ737589	Hol1
Passo Campalto, Venezia, Veneto, Italy	VEN01	45.48085	12.29839	PQ737590	Hol1
Chadwick Lakes, Rabat village, Malta island	MLT01	35.88415	14.38234	PQ737555	Hol1
Pond, Ghaj il-Papri, Għajnsielem village, Gozo island, Malta islands	MLT02	36.02276	14.28793	PQ737556	Hol1
Pond, Ghajn Abdul, Gozo island, Malta islands	MLT03	36.04878	14.20874	PQ737557	Hol1
Pond, Għajnsielem village, Comino island, Malta islands	MLT04	36.01609	14.33691	PQ737558	Hol1
Vegetative pond, Għajnsielem village, Comino island, Malta islands	MLT05	36.00977	14.33558	PQ737559	Hol1
Reservoir, Għajnsielem village, Comino island, Malta islands	MLT06	36.01256	14.33750	PQ737560	Hol1
Reservoir, Nadur village, Gozo island, Malta islands	MLT07	36.05017	14.28233	PQ737561	Hol1
Pond, San Anton Palace, Attard village, Malta islands	MLT08	35.89632	14.44913	PQ737562	Hol1
Pond, Ta' Qali, Attard village, Malta island	MLT09	35.89274	14.42058	PQ737563	Hol1
Sarraflu pond, Kerċem village, Gozo island, Malta islands	MLT10	36.03662	14.19906	PQ737564	Hol1

A map showing the geographic locations of the sampled sites was produced using QGIS software v. 3.30.2 (<https://www.qgis.org/>, last accessed on 13 November 2024).

A single individual from each site was processed for molecular analyses. Total genomic mosquitofish DNA was obtained through direct DNA extraction, starting with 40–50 mg of muscular tissue from each collected specimen, using the BIORON GmbH "Ron's Tissue DNA Mini Kit", following the manufacturer's instructions. Afterwards, the

extracted DNA was amplified by Polymerase Chain Reaction (PCR). The primers “CytBF1” and “CytBR1” [33] were used to amplify a fragment of the mitochondrial cytochrome b gene (*Cytb*), following the procedure described by Vidal et al. [33]. To check for the correct amplification of the *Cytb* fragment, 4 µL of each PCR product was used to perform electrophoresis on 1% agarose gel at 90 V for 30 min and then visualised with a UV transilluminator. Before sequencing, PCR products were purified using the Exo-SAP-IT[®] kit (Affymetrix USB, USA). Sequencing was performed by MacroGen Europe (Milan, Italy) using an ABI 3130xL sequencer (Applied Biosystems), using the same *Cytb* primers used previously for the PCRs. Obtained chromatograms were analysed and manually proofread using MEGA11 software [61]. The novel *Cytb* sequences of *Gambusia* were deposited in GenBank (see Table 1 for their Accession Numbers, A.N.’s). Furthermore, aiming to compare the 37-novel produced *Cytb* sequences with a selection of those publicly available, 86 *Gambusia* spp. *Cytb* sequences (including at least one sequence for each known haplotype of *G. holbrooki* and *G. affinis*) and a sequence of *Belonesox belizanus* Kner, 1860 (used as an out-group) were downloaded from GenBank and included in the analyses (see Figures 3 and S1 for their A.N.’s). All *Cytb* sequences were aligned with MEGA11 software through the ClustalW method. Novel *Cytb* sequences were translated into amino acids to check for any possible presence of frameshifts or stop codons, eventually highlighting the presence of sequencing errors or pseudogenes.

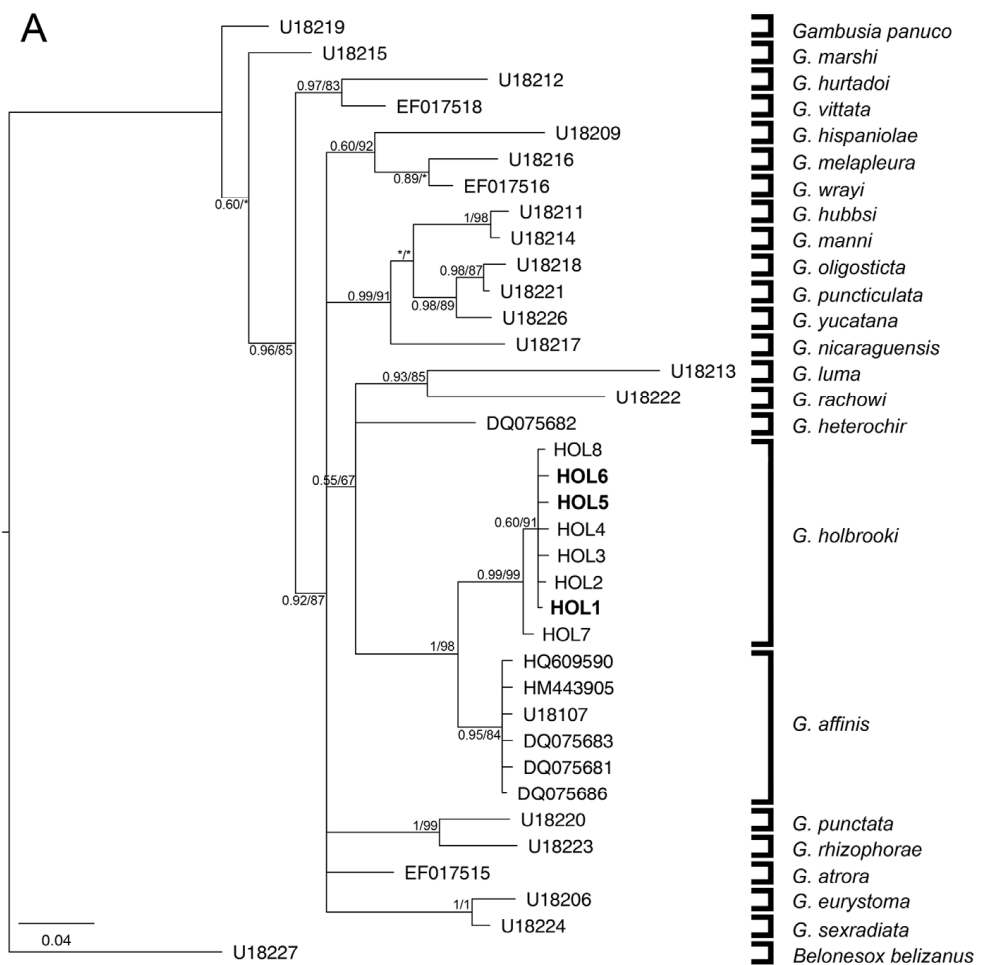


Figure 3. Cont.

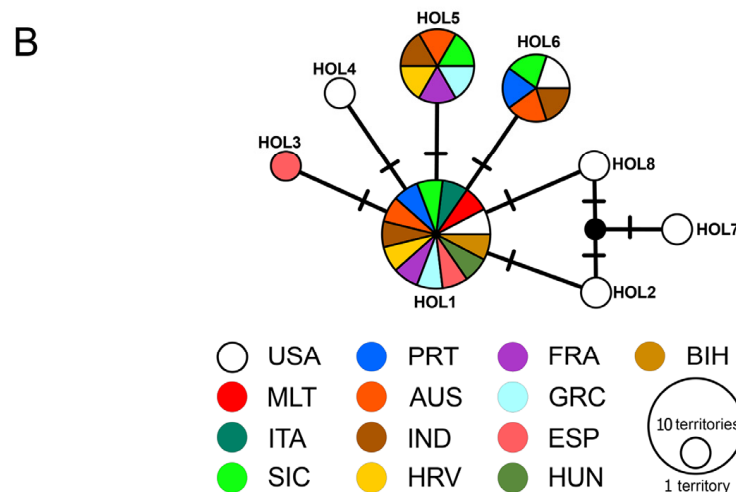


Figure 3. (A): Bayesian phylogram of *Gambusia* spp. haplotypes based on a 305 bp long *Cytb* fragment. *Belonesox belizanus* was used as an outgroup to root the tree. Node supports are reported as posterior probabilities (BI)/bootstrap values (ML). Asterisks indicate support values lower than 50. Square brackets assort the samples according to their current taxonomy. *Gambusia holbrooki* haplotypes found in our novel samples are reported in bold. Haplotype nomenclature of *G. holbrooki* follows [33]. (B): Median-joining haplotype network based on *Cytb* *G. holbrooki* sequences. Dashes indicate substitution steps. Each circle represents a haplotype, and its size is proportional to its frequency. Haplotype nomenclature of *G. holbrooki* follows [33]. Three-letter countries are reported according to the [62]. SIC: Sicily.

The molecular identification of the studied specimens and reconstruction of the phylogenetic relationships among taxa were performed based on the Bayesian Inference (BI) and Maximum Likelihood (ML) analyses using the MrBayes v. 3.2.7 [63] and PhyML v. 3.0 [64] software packages, respectively. The best evolutionary model was chosen using PartitionFinder v. 1.0.1 [65]. Both the BI and ML analyses were performed under a General Time Reversible sequence model of evolution for molecular data with a proportion of gamma and invariant sites (GTR+I+ Γ ; nst = 6). Two independent Markov Chain Monte Carlo (MCMC) analyses were run with 1,000,000 generations (temp.: 0.2; default priors). Twenty-five percent of the initial trees were conservatively discarded as “burn in”. Effective Sample Size values above 200 were obtained for all parameters analysed.

The haplotype network based on novel and already-published *Cytb* *Gambusia holbrooki* sequences, including one sequence for each known haplotype (*sensu* [33]) in each country available, was built using the software PopART v. 1.7 ([66]—<https://popart.maths.otago.ac.nz/>, accessed on 13 November 2024), implementing the Median-joining network algorithm [67].

3. Results

The bibliographic review showed that, at the beginning of the 20th century, mosquitofishes were introduced to Sicily and the rest of Italy, as well as neighbouring countries (49 and reference therein), as an agent against the malaria-bearing mosquitoes of the genus *Anopheles* [68]. Sicily was among the first Italian regions where the mosquitofish were translocated: the first batch of individuals arrived in Catania in 1927 [68,69]; these belonged to a stock originally collected in Edenton (NC, USA), i.e., within the known distribution range of the eastern mosquitofish, *Gambusia holbrooki*. During those years, dozens of fish farms were instituted, and these started mass-producing mosquitofish to be translocated to more than 100 Sicilian waterbodies (see pag. 84 in [68]). To date, mosquitofish have been reported in several localities across the Sicilian mainland (e.g., [48,60,70–86]), but their

local distribution is still severely underestimated. Vidal et al. [33], based on mtDNA sequences, attested the occurrence of *G. holbrooki* in the only Sicilian locality they investigated (i.e., Catania). Presumably, during those years, a similar introduction pathway occurred in Malta as in most European countries; unfortunately, no evidence of this pattern was found in the frame of the conducted bibliographic review work. In fact, no information is available about the historical introduction of mosquitofish to Malta. However, the Maltese islands have also experienced malaria (e.g., [87,88]), and there is evidence of the presence of mosquitoes of the genus *Anopheles* on the islands until at least 1943, when the genus probably became locally extinct [89]. It is thus likely that mosquitofishes were introduced to the Maltese archipelago in the same period as in Sicily and the other circum-Mediterranean areas. Nowadays, mosquitofishes are widespread in the Maltese archipelago (e.g., [90–92]), most likely due to multiple, independent introduction events originating from unknown source populations.

Due to some uncertainties in *Gambusia* taxonomy and identification, it is not clear which species were actually introduced and which ones established self-sustaining populations in the study area. As a result, although the available data suggests a general prevalence of *G. holbrooki* across most of Europe (e.g., [33,93]), some evidence also points to the occurrence of sparse populations of *G. affinis* as well (e.g., [53]). Therefore, the identity of *Gambusia* populations inhabiting currently uninvestigated areas is still to be ascertained (cf. [33,54,57]).

The sampling activities led to the collection of *Gambusia* individuals from 37 sites, specifically 25 sites in Sicily and 10 in Malta; two further sampling sites are related to the comparative samples collected in Veneto and Latium (peninsular Italy). See also Table 1 and Figure 2.

Overall, 37 *Cytb* *Gambusia* sequences were obtained and included in the analyses (see Table 1 and Figures 3 and S1). The length of the *Cytb* PCR product ranged from 345 to 356 bp. After having trimmed out the tails of the sequences, a properly aligned *Cytb* alignment of 305 bp was obtained (the alignment is available from the corresponding author on request). We used the BLAST tool (through the web interface available at <https://blast.ncbi.nlm.nih.gov/>, accessed on 13 November 2024) to preliminarily identify the species. The queries led us to 98 to 100% identity matches of the sequences with published sequences ascribed to *G. holbrooki*. The phylogenetic trees obtained based on BI and ML analyses and rooted on *Belonesox belizanus* showed a congruent topology in accordance with the current taxonomy of the analysed species (Figures 3A and S1). All novel Sicilian and Maltese *Gambusia* sequences clustered together with the already-published *Cytb* *G. holbrooki* sequences.

In addition, from the molecular analyses conducted, we noticed that one of the sequences available on GenBank (i.e., A.N. KF013226), labelled as *Gambusia affinis*, is actually *G. holbrooki* (see Figure S1), thus highlighting a misidentification of the specimen by the authors of the sequence (i.e., [94]).

The Median-joining haplotype network based solely on the analysed *Cytb* *Gambusia holbrooki* sequences revealed the occurrence of three already-known haplotypes in our Sicilian and Maltese specimens (Figure 3B). All the Maltese and 23 of the Sicilian sequences matched with the haplotype “HOL1” detected by Vidal et al. [33], whereas the other three Sicilian *G. holbrooki* sequences matched the haplotypes “HOL5” and “HOL6” (see Figure 3B and Table 1 for further information).

4. Discussion

Based on the novel molecular data obtained within this work, the only mosquitofish species occurring in Sicily and Malta proved to be the eastern mosquitofish, *Gambusia*

holbrooki. In fact, in all 37 sites sampled within the study area, only this species was found; this finding is in accordance with the available historical information about the origin of the Sicilian populations of the species, whereas it contrasts with the report of *G. affinis* in Malta [91], which thus needs to be revised.

The mitochondrial marker used in the present study (i.e., *Cytb*) highlights a low genetic diversity among samples, allowing us to detect the presence of three haplotypes, corresponding to “HOL1” (for 34/37 of the specimens examined), “HOL5” and “HOL6” (*sensu* [33], see also Figure 3). It is not surprising that the most frequent haplotype found is the one designated “HOL1”, since this is the most common haplotype found throughout the invaded range of the species, as already reported in the literature [24,27,30,33,95,96]. Moreover, Vidal et al. [33] reported that the occurrence of the “HOL1” haplotype is related to the first introduction event of the mosquitofish from Edenton (North Carolina, United States) to Spain, from where it was then translocated to Italy and to the rest of the world (see also [95]). As for the other two haplotypes found (i.e., “HOL5” and “HOL6”), these can be linked to subsequent, independent introduction events that occurred in France and Portugal, respectively, then followed by the likely translocation of individuals to Sicily [24,27,30,33].

Although the introduction of mosquitofishes into Europe at the beginning of the 20th century was aimed at protecting public health in an attempt to stem the spread of the malaria vector (i.e., anopheline mosquitoes), it led to dramatic environmental impacts that still afflict our fragile aquatic ecosystems. In fact, differing from what was initially thought (e.g., [21]), the efficiency of mosquitofish in limiting mosquitoes’ proliferation is not particularly high. Moreover, in natural ecosystems with complex and well-structured biocenoses, the introduction of mosquitofishes might even have the opposite effect, since it may indirectly increase the survival rate of mosquitoes through the elimination of efficient native competitors (e.g., *Aphanius fasciatus* Valenciennes, 1821) [78] or even invertebrate predators of mosquito larvae [97].

Nowadays, malaria has been mostly eradicated from most of the circum-Mediterranean countries, due in part to wetland reclamations and the use of chemical insecticides (e.g., “Paris green”, see [39,68]). Despite this, mosquitofish have long been sold in Europe, and they are still often available in local shops or through the web, under the belief that these poeciliid species are effective solutions for controlling and/or eradicating mosquitoes from waterbodies.

The trade and informal translocation of *Gambusia* spp. have further contributed to the uncontrolled spread of the species in inland waters, dramatically altering the conditions of these ecosystems and damaging their native invertebrate and vertebrate biota. Several studies highlighted how the occurrence of mosquitofish actively alters the delicate equilibria of the invaded waterbodies, for example, by competing with native species such as fish or amphibians (e.g., [45,58]).

In the study area, only a few native fish species inhabit inland waterbodies. These are *Aphanius fasciatus*, *Salariopsis fluviatilis* (Asso, 1801), *Salmo trutta* s.l. Linnaeus, 1758 and *Syngnathus abaster* Risso, 1827 [98–101]. Conversely, a rich and diversified non-native fish fauna is widespread in both Sicilian and Maltese inland waters [82,92,102]. Among the native fish species that are most negatively affected by the competition with the eastern mosquitofish, there is the “Mediterranean toothcarp” *A. fasciatus*, which is an endemic cyprinodontid to the Mediterranean basin, listed in Annex II of the Berne Convention (the Convention on the Conservation of European Wildlife and Natural Habitats) and in Annex II of the EU Habitats Directive (Council Directive 92/43/EEC). Accurate and updated data about the distribution of *Gambusia holbrooki* and *A. fasciatus* in Sicily and the Maltese Archipelago are currently missing, but the two species currently co-exist syntopically, at

least in the wetlands and river mouths of eastern and south-eastern Sicily (e.g., [76]; *pers. obs.*). However, in accordance with what was reported by Monti et al. [46] for a site in Central Italy, in most Sicilian and Maltese localities, *G. holbrooki* seems to have displaced *A. fasciatus* to saline or even hyperaline waterbodies. The Mediterranean toothcarp is particularly sensitive not only to the presence of *G. holbrooki* but also to habitat degradation of coastal wetlands. For these reasons, the species is experiencing a dramatic decline of its populations throughout its known range [46,76,78].

In conclusion, the new molecular data pointed out the sole occurrence of *Gambusia holbrooki* in both Sicily and the Maltese archipelago. Furthermore, although the commercialisation of the species is prohibited in Europe, the sale of the species has still been observed locally. Consequently, in order to protect the autochthonous species of the Sicilian and Maltese inland waters, more attention should be paid by local authorities to prevent the commercialisation, breeding and translocation of mosquitofish. In addition, specific monitoring activities should be implemented to produce a comprehensive distribution map of this invasive alien species, aiming to protect the endangered native biota. Moreover, further actions should be taken to verify the impact of this species in Sicily. Understanding trophic interactions is particularly important in zoology [103,104] especially for the evaluation of the effect of non-native species [105,106], this is one of the unknown aspects of the impact of Sicilian populations of *G. holbrooki* that deserves to be further investigated.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/d17010048/s1>: Figure S1: Bayesian phylogram of *Gambusia* spp. based on a 305 bp long *Cytb* fragment. *Belonesox belizanus* was used as outgroup to root the tree. Node supports are reported as posterior probabilities (BI)/bootstrap values (ML). Asterisks indicate support values lower than 50. Square brackets assort the samples according to their current taxonomy. Three-letter countries are reported according to the [62]. SIC: Sicily. Novel *G. holbrooki* sequences are reported in bold.

Author Contributions: Conceptualisation, M.A., A.D. and F.M.; methodology, F.P.F., M.L., A.S., J.S., J.F., G.G., F.M. and L.V.; validation, F.P.F., F.M. and L.V.; formal analysis, L.V.; writing—original draft preparation, L.V., F.P.F. and F.M.; writing—review and editing, L.V., M.L., A.D., A.S., M.A., V.A., G.G. and F.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the fund “NextGenerationEU” of the European Union (D.M. 737/2021—CUP B79J21038330001).

Institutional Review Board Statement: Not applicable.

Data Availability Statement: The data presented in this study are available in GenBank at <https://www.ncbi.nlm.nih.gov/genbank/>, accessed on 13 November 2024 (A.N.'s: PQ737554-PQ737590).

Acknowledgments: We wish to thank Luca Altavilla, Simone Costa, Gina Curmi, Francesco Lillo, Angelica Rallo and Fabio Stoch for the help they provided us with the collection of some samples.

Conflicts of Interest: The authors declare no conflicts of interest.

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