





Article

# Cytochrome *b* Sequencing as a Tool for Identification of Morphologically Similar Mediterranean Gobies *Gobius incognitus* and *Gobius bucchichi* (Actinopterygii: Gobiidae)

Katarína Čekovská<sup>1</sup>, Radek Šanda<sup>2</sup>, Eva Ašenbrennerová<sup>2</sup>, Abderrahmane Kassar<sup>3</sup> , Dimitris Zogaris<sup>4</sup>, Anna Maria Pappalardo<sup>5</sup>, Ali Serhan Tarkan<sup>6,7</sup>, Ekaterina Vasil'eva<sup>8</sup> , David Santos<sup>1,†</sup>  and Jasna Vukić<sup>1,\*</sup> 

<sup>1</sup> Department of Ecology, Faculty of Science, Charles University, Viničná 7, CZ-128 44 Prague, Czech Republic; chalupek@natur.cuni.cz (K.Č.); davidsantos226@gmail.com (D.S.)

<sup>2</sup> Department of Zoology, National Museum of the Czech Republic, Václavské Nám. 68, CZ-115 79 Prague, Czech Republic; radek.sanda@nm.cz (R.Š.); kyalova.e@seznam.cz (E.A.)

<sup>3</sup> LCVRM, Ecole Nationale Supérieure des Sciences de la Mer et de l'Aménagement du Littoral, Campus Universitaire de Dély Ibrahim Bois des Cars, B.P. 19, Algiers 16047, Algeria; a.w.kassar@gmail.com

<sup>4</sup> Department of Ichthyology and Aquatic Environment, University of Thessaly, GR-38446 Volos, Greece; zogarisd@gmail.com

<sup>5</sup> Department of Biological, Geological and Environmental Sciences, University of Catania, Via Androne 81, 95124 Catania, Italy; pappalam@unict.it

<sup>6</sup> Department of Basic Sciences, Faculty of Fisheries, Muğla Sıtkı Koçman University, Muğla 48000, Turkey; serhantarkan@gmail.com

<sup>7</sup> Department of Ecology and Vertebrate Zoology, Faculty of Biology and Environmental Protection, University of Lodz, PL-90136 Lodz, Poland

<sup>8</sup> Zoological Museum, Moscow State University, Moscow 121069, Russia; vas\_katerina@mail.ru

\* Correspondence: jasna.vukicova@natur.cuni.cz

† Current address: Marine and Environmental Sciences Centre (MARE), Faculty of Sciences, University of Lisbon, PT-2750-374 Cascais, Portugal.



**Citation:** Čekovská, K.; Šanda, R.; Ašenbrennerová, E.; Kassar, A.; Zogaris, D.; Pappalardo, A.M.; Tarkan, A.S.; Vasil'eva, E.; Santos, D.; Vukić, J. *Cytochrome b* Sequencing as a Tool for Identification of Morphologically Similar Mediterranean Gobies *Gobius incognitus* and *Gobius bucchichi* (Actinopterygii: Gobiidae). *J. Mar. Sci. Eng.* **2023**, *11*, 2289. <https://doi.org/10.3390/jmse11122289>

Academic Editor:  
Jose Pedro Andrade

Received: 30 October 2023  
Revised: 22 November 2023  
Accepted: 28 November 2023  
Published: 2 December 2023



**Copyright:** © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

**Abstract:** Despite being one of the most speciose fish families in the Mediterranean Sea, knowledge about the diversity of gobies (Actinopterygii: Gobiidae) in this sea is still unsatisfactory, as documented by recent descriptions of a number of new species. Although very common in shallow water, *Gobius incognitus* Kovačić & Šanda, 2016, had escaped attention until 2016, when it was discovered. Due to its overall superficial morphological similarity, *G. incognitus* used to be confused with a much rarer species, *Gobius bucchichi* Steindachner, 1870, which was considered one of the most common shallow-water gobies in the Mediterranean Sea. In this work, we tested the suitability of the genetic data (mitochondrial gene encoding *cytochrome b*) for identifying and distinguishing between these two goby species, and assessed the congruency between the distribution records based on genetic data and those based on morphological identification. We analysed material of 304 specimens of *G. incognitus* and *G. bucchichi* from 49 localities covering a considerable part of the Mediterranean Sea, Black Sea, and the Atlantic Ocean near Gibraltar, representing 19 geographically well-separated areas. We detected 270 sequences of *G. incognitus*, and only 34 of *G. bucchichi*. In both species, a high haplotype variability was observed. The sequence species identity matched morphological identification for all specimens for which vouchers were available. The mean uncorrected p-distance between *G. incognitus* and *G. bucchichi* was 13%, while the mean intraspecific distances were much lower (0.63% and 0.68%, respectively). We found 79 fixed mutations between these two species. Data on distribution based on genetic identification are completely congruent with published results based on morphological identification. The results of this study support molecular methods as a reliable tool for distinguishing morphologically similar fish species, which is particularly useful when only tissue is available for determination.

**Keywords:** diversity; distribution; gobies; Mediterranean; genetic identification

## 1. Introduction

Gobies (Gobiidae) are one of the most speciose fish families worldwide [1]. At the same time, they are one of the most species-rich families of the Mediterranean marine fishes [2]. Despite this fact, gobiid diversity in the Mediterranean Sea is still far from being completely known. At the family level, the native gobiid fishes from the Mediterranean Sea are considered either members of a single family, Gobiidae [1], or of two sister families, Gobiidae and Oxudercidae [3]. The knowledge about the diversity of the gobies in the Mediterranean Sea is still unsatisfactory, as documented by a description of a number of new species in the last years, e.g., [4–9]. Usually, new species were first discovered in a small geographic area, but subsequently, they were found in even geographically considerably distant localities [10–15], indicating that they are rather widespread, and that the knowledge about their distribution could be improved by increased research effort [16]. Furthermore, some of the new species were described from greater depths or are typical cryptobenthic species that are difficult to sample by conventional fishing methods, but also by SCUBA divers (e.g., by active sampling methods or by a photographic evidence). Thus, it is more difficult to record them despite their often widespread presence. However, one of the recently described species was discovered in a very shallow water, where it is very common and abundant, and its late discovery is only due to its overall superficial morphological similarity with another, well-known species, which used to be considered one of the most common shallow-water gobies in the Mediterranean Sea region [17]. This species pair is a long-time known *Gobius bucchichi* Steindachner, 1870 and a newly described *Gobius incognitus* Kovačić & Šanda, 2016. Although cursorily very similar (Figure 1), both species are in fact readily distinguishable by morphological character [18]; they can even be reliably identified from photographs [19]. Both species also seem to be genetically considerably distinct [4,18]. Data on the distribution of both species were recently comprehensively reviewed [20]. While the newly described *G. incognitus* is widespread throughout the Mediterranean Sea, reaching the Atlantic Ocean in the Gibraltar straight region [20], *G. bucchichi* was confirmed only in a few scattered areas in the northern part of the Mediterranean Sea (in the Adriatic, Ionian, and Aegean Seas), and the Marmara and Black Seas [20]. However, the studies differentiating both species and providing reliable data about the exact distribution of either of these species were based mostly on morphological evidence: either on morphological examination or on photographic evidence [18,20–27], while the genetic methods were used only scarcely and on very limited amounts of material [4,18,28]. Even though these two gobies have no economic value, their biogeography is of particular interest, especially in the case of *G. bucchichi*, which seems to have a unique distribution pattern among the Mediterranean gobies [20]. Moreover, the conservation status of both species needs an urgent update. In the international IUCN Red List of threatened species, only *G. bucchichi* is included, and is evaluated as Least Concern [29], while *G. incognitus* has not yet been evaluated. However, the conservation status of *G. bucchichi* [29] is based on data that include both species, and thus is no longer valid.

Molecular methods were used in numerous studies on gobies from the Mediterranean Sea and in general from Europe. Most of the studies focused on taxonomy or distribution, e.g., [4–9,11,12,14,15,18,28,30–32]. Several studies deal with phylogenetic relationships, e.g., [33–36], and also the population genetic structure and phylogeography were investigated for several species, e.g., [37–43].

The aims of this work were to test the suitability of the genetic data (mitochondrial marker *cytochrome b*) for the identification and distinguishing between the goby species *G. bucchichi* and *G. incognitus*, and to assess the congruency between the distribution records based on genetic data and those based on morphological identification, which are, for a large part, based on citizen science [20]. Genetics has been proven to be a useful tool for the identification of taxonomically complicated gobiid species complexes in the Mediterranean region [30]. Moreover, if proved to be reliable, this type of identification can be applied also

in the case when voucher material, apart from a tissue, is absent, or on specimens that are too damaged to be used for unambiguous morphological identification.



**Figure 1.** *Gobius incognitus* from Hvar Island, Sveta Nedjelja, Croatia (loc. 23 in Table 1) (upper) and *Gobius buchichi* from Selce, Croatia (loc. 19 in Table 1) (lower) photographed in an aquarium. Both specimens are deposited in the collection of the National Museum of the Czech Republic in Prague and were morphologically determined. Photo: R. Šanda.

## 2. Materials and Methods

Tissue samples for molecular analyses (fin clips) were preserved in 96% ethanol. Genomic DNA was extracted with the Geneaid Genomic DNA Mini Kit (Tissue) (Geneaid Biotech, New Taipei City, Taiwan). Mitochondrial gene *cytochrome b* (*cyt b*) was used for the identification, considering the relatively high divergence between both species on this marker observed by Kovačić & Šanda [18]. *Cyt b* was confirmed to be a useful marker for species delimitation in gobies, e.g., [9,18,32], reconstruction of phylogenetic relationships between species e.g., [31,34–36], as well as for studying phylogeography and population genetics e.g., [37,38,40,42,43]. *Cyt b* was amplified by polymerase chain reaction (PCR) using PPP Master Mix (Top-Bio, Praha, Czech Republic) following primers and protocols described in Šanda et al. [44]. Sequencing was performed by MacroGen Inc using newly designed primers (GbuchR1: 5'-TGGGGGAAAAGAGGGCAAGG-3'; GbuchF1: 5'-GGCTTCTCCGTTGACAATGC-3'). These primers were designed based on previously published *cyt b* sequences of both species [18,34] using Geneious Prime 2021.1 (<https://www.geneious.com>, accessed on 20 January 2022).

In total, 304 sequences of *G. buchichi* and *G. incognitus* originating from 49 localities were analysed (Table 1, Figure 2). Thirty-nine localities covered only new material, seven localities were based on published data supplemented by newly analysed specimens, while three localities were based solely on the published data (Table 1). The newly analysed material was morphologically identified following [2,18] if the voucher specimens were available (Table 1).

All sequences were visually checked and manually edited using Chromas v.2.6.4, and aligned in Bioedit v.7.2.6.1 [45]. New sequences were collapsed to haplotypes in Fabox [46], and the haplotypes were deposited in GenBank under the accession numbers OR834513–OR834734. The dataset included three sequences, which were considerably shorter than most of the sequences due to problematic sequencing. These sequences were used only for the identification, which was unambiguous in each case, while for the analyses only the long sequences were used. The dataset was further extended by available

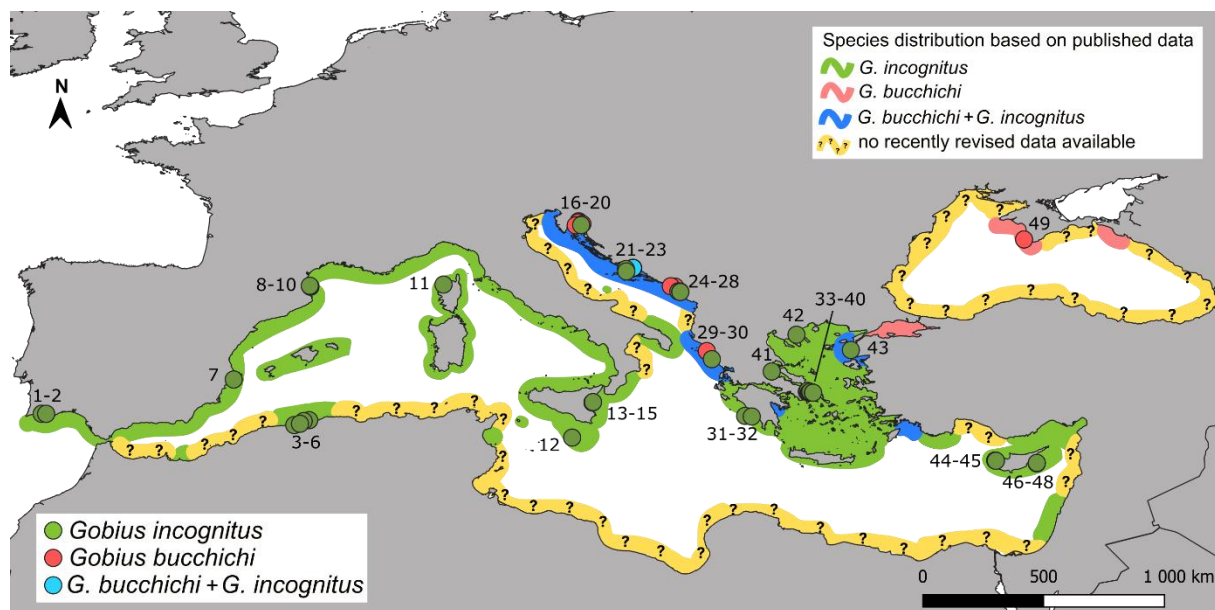
published sequences of *cyt b* of both species (FJ526784 [34] and KR811029-KR811059 [18]) (Table 1). Sequences of both species published by Kovačić & Šanda [18] were taken as a reference, because they include sequences of type material of *G. incognitus* and morphologically confirmed specimens of *G. bucchichi* based on redescription of this species.

**Table 1.** Overview of the analysed material of *G. incognitus* and *G. bucchichi*. For each included locality, geographic coordinates and sea subarea are provided. Sea subareas names follow Kovačić et al. [20]. Locality numbers correspond to the numbers of localities in Figure 2. Voucher specimens were available for all localities, where new material was included, except for the locality marked with \* (loc. 43). NE—north-eastern, W Med—western Mediterranean. Published sequences originated from <sup>A</sup> [18] and <sup>B</sup> [34].

Detected Species on Locality	Sea Subarea	Locality	Country	Coordinates	No. in Map	New Sequences	Published Sequences
<i>Gobius incognitus</i>	NE Atlantic	Gale	Portugal	37.076972, -8.310722	1	7	
<i>Gobius incognitus</i>	NE Atlantic	Sesmarias, Praia de Evaristo	Portugal	37.074006, -8.303575	2	6	
<i>Gobius incognitus</i>	African W Med	Bou Ismail	Algeria	36.650767, 2.691153	3	4	
<i>Gobius incognitus</i>	African W Med	Tipaza	Algeria	36.594200, 2.449131	4	5	
<i>Gobius incognitus</i>	African W Med	Algiers, Sidi Fredj	Algeria	36.764291, 2.848401	5	17	
<i>Gobius incognitus</i>	African W Med	Algiers, Ain Benian	Algeria	36.802050, 2.898339	6	19	
<i>Gobius incognitus</i>	Spanish W Med	Benidorm	Spain	38.533982, -0.129395	7	4	
<i>Gobius incognitus</i>	Gulf of Lion	Banyuls-sur-Mer	France	42.481817, 3.136032	8	10	6 <sup>A</sup>
<i>Gobius incognitus</i>	Gulf of Lion	Banyuls-sur-Mer, Paulliles beach	France	42.505759, 3.123662	9	7	3 <sup>A</sup>
<i>Gobius incognitus</i>	Gulf of Lion	Port Vendres	France	42.523300, 3.109700	10	1	
<i>Gobius incognitus</i>	Corsican shelf	Corsica, Lumio	France	42.574444, 8.805278	11	25	
<i>Gobius incognitus</i>	Maltese shelf	Gozo Island, Ramla	Malta	36.061869, 14.284042	12	2	
<i>Gobius incognitus</i>	Ionian Sea	Sicily, Aci Castello	Italy	37.555419, 15.149383	13	9	
<i>Gobius incognitus</i>	Ionian Sea	Sicily, Aci Trezza	Italy	37.562758, 15.163661	14	9	
<i>Gobius incognitus</i>	Ionian Sea	Sicily, Capo Mulini	Italy	37.574014, 15.173408	15	8	
<i>Gobius bucchichi</i>	Adriatic Sea	Kraljevica	Croatia	45.267660, 14.562256	16	1	1 <sup>A</sup>
<i>Gobius bucchichi</i>	Adriatic Sea	Krk Island, Omišalj	Croatia	45.218911, 14.550908	17		2 <sup>A</sup>
<i>Gobius bucchichi</i>	Adriatic Sea	Krk Island, Glavotok	Croatia	45.094134, 14.436694	18		1 <sup>B</sup>
<i>Gobius bucchichi</i>	Adriatic Sea	Selce	Croatia	45.152293, 14.718928	19	6	1 <sup>A</sup>
<i>Gobius incognitus</i>	Adriatic Sea	Krk Island, Sveti Marak	Croatia	45.105944, 14.668500	20	2	
<i>Gobius bucchichi</i> + <i>Gobius incognitus</i>	Adriatic Sea	Brač Island, Sumartin	Croatia	43.285934, 16.878829	21	10 (5x <i>G. inc.</i> , 5x <i>G. buc.</i> )	6 <sup>A</sup> ( <i>G. inc.</i> )
<i>Gobius incognitus</i>	Adriatic Sea	Hvar Island, Smočiguzica	Croatia	43.233889, 16.574722	22	1	
<i>Gobius incognitus</i>	Adriatic Sea	Hvar Island, Sveta Nedjelja	Croatia	43.134433, 16.591381	23	14	
<i>Gobius bucchichi</i>	Adriatic Sea	Boka Kotorska, Kostanjica	Montenegro	42.485403, 18.669678	24	6	
<i>Gobius bucchichi</i>	Adriatic Sea	Boka Kotorska, Strp	Montenegro	42.503989, 18.669453	25	4	
<i>Gobius incognitus</i>	Adriatic Sea	Budva, Jaz beach	Montenegro	42.283836, 18.809034	26	1	2 <sup>A</sup>
<i>Gobius incognitus</i>	Adriatic Sea	Kamenovo	Montenegro	42.273917, 18.887667	27	5	
<i>Gobius incognitus</i>	Adriatic Sea	Sveti Stefan	Montenegro	42.257180, 18.892118	28		5 <sup>A</sup>
<i>Gobius bucchichi</i>	Ionian Sea	outflow of Butrint lagoon	Albania	39.743614, 20.018654	29	1	5 <sup>A</sup>
<i>Gobius incognitus</i>	Ionian Sea	Sivota	Greece	39.399800, 20.234664	30	2	
<i>Gobius incognitus</i>	Ionian Sea	Romanos, Peloponnesos	Greece	36.985361, 21.651634	31	1	
<i>Gobius incognitus</i>	Ionian Sea	Petalidi, Peloponnesos	Greece	36.959142, 21.934967	32	1	
<i>Gobius incognitus</i>	Aegean Sea	Euboia Island, Petalii Islands	Greece	38.014764, 24.280875	33	3	
<i>Gobius incognitus</i>	Aegean Sea	Euboia Island, Marmari	Greece	38.048808, 24.318328	34	4	
<i>Gobius incognitus</i>	Aegean Sea	Euboia Island, Kalamitsi beach	Greece	37.971372, 24.365764	35	2	
<i>Gobius incognitus</i>	Aegean Sea	Euboia Island, Mnima peninsula	Greece	37.978775, 24.399581	36	4	
<i>Gobius incognitus</i>	Aegean Sea	Euboia Island, Agia Pelagia Islet	Greece	37.997008, 24.398186	37	1	
<i>Gobius incognitus</i>	Aegean Sea	Euboia Island, Karystos	Greece	38.009667, 24.430804	38	3	
<i>Gobius incognitus</i>	Aegean Sea	Euboia Island, Kastri Beach	Greece	37.973022, 24.537658	39	1	
<i>Gobius incognitus</i>	Aegean Sea	Euboia Island, Mirmigia rocks	Greece	37.972336, 24.548042	40	1	
<i>Gobius incognitus</i>	Aegean Sea	Raches	Greece	38.876144, 22.785700	41	3	
<i>Gobius incognitus</i>	Aegean Sea	Gavradia	Greece	40.440278, 23.839722	42	18	
<i>Gobius incognitus</i>	Aegean Sea	between Geyikli and Dalyan *	Turkey	39.789722, 26.156111	43	22	
<i>Gobius incognitus</i>	Levantine Sea	Akamas, Fontana	Cyprus	35.090108, 32.303844	44	3	
<i>Gobius incognitus</i>	Levantine Sea	Akamas, George Island	Cyprus	35.074958, 32.335222	45	8	
<i>Gobius incognitus</i>	Levantine Sea	Cavo Greco, Tunnel—cave	Cyprus	34.963592, 34.073078	46	7	
<i>Gobius incognitus</i>	Levantine Sea	Cavo Greco, chappel	Cyprus	34.976053, 34.076781	47	2	
<i>Gobius incognitus</i>	Levantine Sea	Cavo Greco, Cyclops cave	Cyprus	34.985631, 34.076928	48	1	
<i>Gobius bucchichi</i>	Black Sea	Sevastopol	Ukraine	44.494094, 33.533650	49	1	



Reconstruction of the phylogenetic relationships of the analysed material was estimated by Bayesian Inference (BI). Only unique haplotypes collapsed in Fabox [46] were used for the phylogenetic reconstruction. Sequences of *Gobius niger* Linnaeus, 1758 (FJ526782 [34]), *Gobius fallax* Sarato, 1889 (FJ526785 [34]), and *Gobius couchi* Miller & El-Tawil, 1974 (FJ389196 [47]) were used for comparison as well, while *Pomatoschistus minutus* (Risso, 1810) (FJ526776 [34]) and *Economidichthys pygmeus* (Holly, 1929) (KF415544 [33]) were used as outgroup. The appropriate model of nucleotide substitution was determined in jModeltest v.2.1.9 [48] based on Bayesian information criterion (BIC). BI was assessed in MrBayes v.3.2.2 [49] under the GTR+I+G model selected by JModeltest. Two independent runs, each consisting of four Markov Chain Monte Carlo (MCMC) methods, were simultaneously run for 5 million generations. Sampling of the trees was performed every 1000 generations. TRACER v.1.7.0 [50] was used to analyse the trace files generated by MCMC runs and visualise the convergence of chains. The first 25% of trees were discarded as burn-in and kept trees were used for the construction of a 50% majority-rule consensus tree. The final tree was visualised in FigTree v.1.4.4 [51] and edited in Inkscape version 0.92.3 [52]. Program MEGA 10 [53] was used to calculate genetic distance (uncorrected p-distances) between and within the species. Fabox [46] was used to visualise the variable sites and assess fixed mutations between the two species.



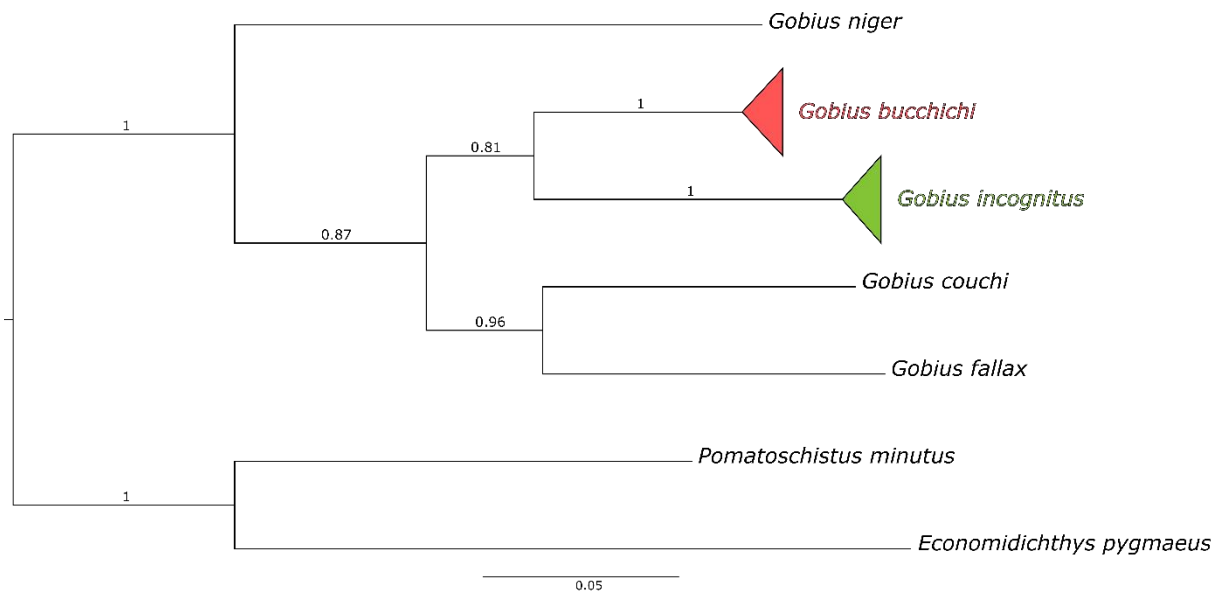
**Figure 2.** Map showing the localities from which material was analysed (circles) and distribution range of *G. incognitus* and *G. bucchichi* based on Kovačić et al. [20]. Locality numbers correspond to those in Table 1.

### 3. Results

#### 3.1. Genetic Distinctiveness

In this work, we used altogether 304 sequences of *cyt b*, of which 272 were new and 32 were published. For *G. incognitus*, there were 270 sequences, of which 22 were published and 248 were newly obtained. Two new sequences of *G. incognitus* were shorter and were used only for identification. The remaining 258 sequences were 1077 base pairs long and represented 208 different haplotypes. For *G. bucchichi*, there were 34 sequences, of which 10 were published and 24 were new. A single new sequence of *G. bucchichi* was shorter and was used only for the identification. The remaining 33 sequences were 1077 base pairs long and they represented 29 different haplotypes. Altogether, 79 sites with fixed mutations discriminating between both species were detected.

The phylogenetic reconstruction analysis showed each of the included *Gobius* species as very distinct (Figure 3). The sequence species identity matches the morphological identification for all specimens, for which vouchers were available. All calculated genetic divergences are summarized in Table 2. The mean uncorrected p-distance between *G. incognitus* and *G. bucchichi* was 13% ( $\pm 0.8\%$  S.E.), while the mean intraspecific distances were much lower (*G. incognitus* 0.63%  $\pm 0.086$  S.E.; *G. bucchichi* 0.68%  $\pm 0.11\%$  S.E.). The maximum observed intraspecific genetic distance between *G. incognitus* sequences was 1.76%, while for *G. bucchichi* it was 1.21%.



**Figure 3.** The 50% majority-rule consensus Bayesian phylogenetic reconstructions based on mitochondrial *cytochrome b* sequence data. Numbers on branches represent posterior probabilities.

**Table 2.** Genetic distance (uncorrected p-distances, in %) based on *cytochrome b* sequences for the analyzed *Gobius* species. Mean genetic distances between species are below diagonal, S.E. above diagonal. On the diagonal, mean intraspecific genetic divergence is shown, with S.E. in parentheses. Numbers in parentheses after species name in the first column are numbers of analyzed sequences for species. N/A—not applicable.

	<i>G. incognitus</i>	<i>G. bucchichi</i>	<i>G. couchi</i>	<i>G. falax</i>	<i>G. niger</i>
<i>G. incognitus</i> (258)	0.63 ( $\pm 0.086$ )	0.8	0.9	1.1	1.1
<i>G. bucchichi</i> (30)	13	0.68 ( $\pm 0.11$ )	1	1.1	1.2
<i>G. couchi</i> (1)	16.7	14.9	N/A	1.1	1.1
<i>G. falax</i> (1)	16.6	15.3	14.3	N/A	1.2
<i>G. niger</i> (1)	19.4	18	18.9	19.7	N/A

### 3.2. Distribution

We analyzed material of *G. incognitus* and *G. bucchichi* from 49 localities covering considerable parts of the Mediterranean Sea, Black Sea, and the Atlantic Ocean near Gibraltar (Figure 2, Table 1). Some localities are close to each other, within a distance of only a few km, and the samples cover 19 geographically well-separated areas (Figure 2).

*Gobius incognitus* is widespread, occurring throughout the Mediterranean, and in the Atlantic Ocean near Gibraltar (Figure 2, Table 1). However, the documented distribution of *G. bucchichi* is much more restricted (Figure 2, Table 1). Genetic data confirmed its presence from three areas in the northern coastal region of the Adriatic Sea, one in the north-western part of the Ionian Sea, and from the Crimean region of the Black Sea. Both species were recorded sympatrically in four areas (Figure 2), though mostly from different localities

within the area (Table 1, Figure 2). However, a syntopic presence (i.e., at the same locality) of both species was revealed at a single locality (Table 1, Figure 2).

#### 4. Discussion

The mitochondrial marker *cyt b* was confirmed to be a highly reliable tool for the genetic identification of morphologically similar and often confused gobies *G. incognitus* and *G. bucchichi*. Both species showed very high haplotype variability, relatively uncommon in coastal species, whose dispersal capability is limited by local oceanographic patterns [54]. However, *G. incognitus* and *G. bucchichi* can be genetically easily unambiguously identified, given that the average interspecific genetic divergence between them is almost 20 times higher than the average intraspecific divergence, and the number of fixed mutations between species on *cyt b* sequence data is nearly 80. Even the highest observed value of intraspecific distance (1.76% in *G. incognitus*) is six to seven times lower than the lowest interspecific genetic distance between both studied species. In general, gobies of the genus *Gobius* are easily identifiable by mtDNA markers, as proved by Iglésias et al. [4], where many species were analyzed by *cytochrome c oxidase*, and minimum genetic distance between species was 8–9% (with the only exception of the *Gobius auratus* complex), while the intraspecific distance was always several times lower. The overall high genetic divergence between species of the genus *Gobius* could be a result of the considerably long evolutionary history of this genus; the oldest paleontological record of *Gobius* based on complete skeleton fossil is dated to 20 MY ago [55]. It is probable that the evolution of the lineages leading to the extant species started in the very early diversification of the genus, allowing many mutations to accumulate over time. Nevertheless, the intraspecific genetic distance within the Mediterranean gobies on mtDNA markers can also be relatively high, commonly reaching 1.5–2% [15,18,37], and rarely even 5% [4,9], so molecular identification of genetically more similar species of gobies may be challenging. However, as this is not a case of *G. bucchichi* and *G. incognitus*, even the advanced genetic methods for identification of the species presence from environmental samples (eDNA approach) are applicable to these species, but also actually to most of the gobies from the Mediterranean Sea. The data from eDNA could potentially fill in the gaps in the knowledge on distribution of many goby species. A limitation of the use of the mtDNA markers for the species identification is that potential hybrids or mito–nuclear discordance cannot be detected, as mtDNA is uniparentally inherited. *Gobius incognitus* and *G. bucchichi* occur sympatrically, even syntopically, this work, [20]); their natural hybridisation, although not reported, cannot be a priori excluded. So far, the hybridisation of gobies from the European seas was reported only in the genus *Pomatoschistus* [56,57]. The hybridisation between European marine gobies can be assumed to be rare owing to their complex mating system, which includes nest building and species-specific sound production [58,59], which can serve as important prezygotic barrier through strong auditory-based assortative mating.

*Gobius bucchichi* was for a long time considered a widespread species in the Mediterranean and Black seas, present also in the European Atlantic near the Gibraltar strait up to Algarve coast of Portugal [17]. However, this assumption was actually based on the distribution of two morphologically similar species. After the discovery and description of *G. incognitus* by Kovačić & Šanda [18], the distribution of both species was questioned. Almost a decade after the description of *G. incognitus*, we can state that only several publications have provided unambiguous distribution data, accompanied by appropriate identification [4,18,20–28], while other works, even those recently published, continue to report only *G. bucchichi* [60–62], probably being unaware of the description of *G. incognitus*.

Kovačić et al. [20] have summarized the available information on the distribution of both *G. incognitus* and *G. bucchichi* and confirmed that distribution range of *G. incognitus* covers a considerable part of the Mediterranean Sea coastal region, and the European Atlantic near the Gibraltar strait up to Algarve (Figure 2), as previously suggested by Kovačić & Šanda [18]. In contrast, *G. bucchichi* was confirmed for a very restricted and scattered area (Figure 2). It has been reported only from the northern part of the Adriatic

Sea, from Italy to Montenegro, from a few localities in the northern Ionian Sea in Albania and Greece, from one locality in the south-western Aegean Sea in Greece, south-eastern and south-western part of the Aegean Sea in Turkey, from the Sea of Marmara, and from the two areas of the northern Black Sea in Ukraine and Russia [20]. The range of both species in the Mediterranean Sea completely overlaps, while for the Black Sea and Marmara Sea, only the presence of *G. bucchichi* was confirmed [20]. Our results for distribution based on genetic data, often including a considerable number of analyzed specimens per locality/geographic area, are completely congruent with the revision of Kovačić et al. [20]. In our data, *G. bucchichi* was recorded only from the northern side of the Adriatic Sea, westernmost Ionian Sea, and from the Crimean coast in the northern Black Sea (Figure 2, Table 1), while *G. incognitus* was detected throughout the whole Mediterranean Sea and the southern Portuguese Atlantic Ocean coast (Figure 2, Table 1).

However, for the large areas of the previously reported range of *G. bucchichi* [17], respectively for large portions of the Mediterranean coastal region in general, we are still awaiting clarification on which of the two species is present (Figure 2). This applies to almost all of African Mediterranean coastal region, as *G. bucchichi* was reported only from Libya [63] and Egypt [64], but this was not supplemented by morphological or molecular evidence; moreover, *G. bucchichi* had been previously reported from Morocco [65] and was recently confirmed to be *G. incognitus* instead [20]. Data are missing also for part of the Levantine coastal region of Turkey [20] and the coastal regions of Syria and Lebanon, from where neither of the species was reported (Syria [66]), or only *G. bucchichi* was listed (Lebanon [60,62]). The Black Sea is another area with unsatisfactory data on the presence of *G. incognitus* and *G. bucchichi*. *Gobius bucchichi* is the only unambiguously confirmed species from this region and, moreover, only from a small part of the northern coast [20] (our data). Nevertheless, at least one of these species is apparently widespread around the Black Sea, as there are reports on *G. bucchichi* from Turkey [67], Russia [68,69], Ukraine [70,71], as well as Bulgaria [72]. Finally, the lack of distribution data for the Italian Adriatic coastal region (Figure 2) is surprising, as it was considered a part of the distribution range of *G. bucchichi* by Miller [17]. Kovačić et al. [20] assumed that this gap in distribution may be real, as the area is well studied, and it could be a result of lack of suitable habitats in this region.

Further research should focus not only on the clarification of the distribution of both species, but especially on their ecology, including microhabitat use of each species and their niche separation, since nothing is known about how the species coexist/interact in areas of sympatric and syntopic occurrence.

The results of this study suggest an alternative, easier, and reliable identification method of morphologically similar fish species. A high divergence in *cyt b* between *G. incognitus* and *G. bucchichi*, including numerous sites with fixed mutations (79 in total), enables clear distinctions between the two goby species at the mtDNA level. This relatively fast and low-cost method complements an array of molecular methods, such as multiplex PCR assays, melt curve analysis real-time PCR, or restriction fragment length polymorphism, which are successfully used for fish identification [73]. Importantly, molecular tools could also aid potential conservation efforts of less common species, for example, in this particular case, of *G. bucchichi*. At the same time, our results demonstrate how insufficient our knowledge on the life in the Mediterranean Sea remains, and point to the great potential of molecular methods to achieve even a partial improvement.

**Author Contributions:** Conceptualization, R.Š. and J.V.; methodology, K.Č., E.A., J.V. and R.Š.; software, K.Č., E.A., J.V. and R.Š.; validation, J.V. and R.Š.; formal analysis, J.V. and R.Š.; investigation, K.Č., J.V. and R.Š.; resources, J.V., R.Š., A.K., D.Z., A.M.P., A.S.T., E.V. and D.S.; data curation, R.Š. and J.V.; writing—original draft preparation, J.V., R.Š. and K.Č.; writing—review and editing, A.K., D.Z., A.M.P., A.S.T., E.V. and D.S.; visualization, K.Č., E.A., J.V. and R.Š.; supervision, J.V.; project administration, R.Š. and J.V.; funding acquisition, R.Š. and J.V. All authors have read and agreed to the published version of the manuscript.



**Funding:** This research was funded by the Ministry of Culture of the Czech Republic (DKRVO 2019–2023/6.III.e National Museum, 00023272) and by institutional resources of the Ministry of Education, Youth, and Sports of the Czech Republic. Scientific studies of EV were carried within a State Project of Moscow State University № 121032300105-0.

**Institutional Review Board Statement:** Not applicable. Work conducted on museum material.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Sequences are deposited in GenBank.

**Acknowledgments:** We are grateful to Konstantinos Moustakas and Ioulianos Pantelides (DFMR, Cyprus), Stamatis Zogaris (HCMR, Greece), the Dikelas Dive Center (Karystos, Greece), Marcelo Kovačić (PMR, Rijeka, Croatia), Mihurić Dive Center (Selce, Croatia), and Iain Wilson (Albania) for their support or providing material used in this study. Sampling in Greece and Cyprus was conducted under permission of the Hellenic Ministry of Environment (through collecting licence to HCMR, no. 220965/2583/22-8-2011) and the Cyprus Ministry of Agriculture, Rural Development and Environment, through the Department of Fisheries and Marine Research (no. III2921III/10-11-2017) respectively. Collection of part of the material was supported by the EU FP7 project ASSEMBLE at CCMar/Centre of Marine Sciences of Algarve, Faro, Portugal, and Observatoire oceanologique de Banyuls/Mer, Laboratoire Arago, Banyuls/Mer. Computational resources were provided by the e-INFRA CZ project (MetaCentrum, ID:90254), supported by the Ministry of Education, Youth and Sports of the Czech Republic.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

- Parenti, P. A checklist of the gobioid fishes of the world (Percomorpha: Gobiiformes). *Iran. J. Ichthyol.* **2021**, *8*, 1–480. [[CrossRef](#)]
- Kovačić, M. Checklist of gobies (Teleostei: Gobiidae) of the Mediterranean Sea and a key for species identification. *Zootaxa* **2020**, *4877*, 75–101. [[CrossRef](#)]
- McCraney, W.T.; Thacker, C.E.; Alfaro, M.E. Supermatrix phylogeny resolves goby lineages and reveals unstable root of Gobiaria. *Mol. Phylogenet. Evol.* **2020**, *151*, 106862. [[CrossRef](#)] [[PubMed](#)]
- Iglésias, S.P.; Vukić, J.; Sellos, D.Y.; Soukupová, T.; Šanda, R. *Gobius xoriguer*, a new offshore Mediterranean goby (Gobiidae), and phylogenetic relationships within the genus *Gobius*. *Ichthyol. Res.* **2021**, *68*, 445–459. [[CrossRef](#)]
- Kovačić, M.; Ordines, F.; Schliewen, U.K. A new species of *Speleogobius* (Teleostei: Gobiidae) from the Western Mediterranean Sea. *Zootaxa* **2016**, *4066*, 301–310. [[CrossRef](#)]
- Kovačić, M.; Ordines, F.; Schliewen, U.K. A new species of *Buenia* (Teleostei: Gobiidae) from the western Mediterranean Sea, with the description of this genus. *Zootaxa* **2017**, *4250*, 447–460. [[CrossRef](#)]
- Kovačić, M.; Ordines, F.; Schliewen, U.K. A new species of *Buenia* (Perciformes: Gobiidae) from the western Mediterranean slope bottoms, the redescription of *Buenia jeffreysi* and the first Balearic record of *Buenia affinis*. *Zootaxa* **2018**, *4392*, 267–288. [[CrossRef](#)]
- Kovačić, M.; Ordines, F.; Ramirez-Amaro, S.; Schliewen, U.K. *Gymnesigobius medits* (Teleostei: Gobiidae), a new gobiid genus and species from the western Mediterranean slope bottoms. *Zootaxa* **2019**, *4651*, 513–530. [[CrossRef](#)]
- Kovačić, M.; Šanda, R.; Čekovská, K.; Soukupová, T.; Vukić, J. *Zebrus pallaoroi* sp. nov.: A new species of goby (Actinopterygii: Gobiidae) from the Mediterranean Sea with a dna-based phylogenetic analysis of the *Gobius*-lineage. *Contrib. Zool.* **2021**, *90*, 285–317. [[CrossRef](#)]
- Kovačić, M.; Glavičić, I. The first Adriatic finding of *Speleogobius llorisi* (Actinopterygii: Gobiiformes: Gobiidae). *Acta Ichthyol. Piscat.* **2019**, *49*, 181–184. [[CrossRef](#)]
- Dulčić, J.; Lepen Pleić, I.; Zorica, B.; Bušelić, I.; Šestanović, M.; Kovačić, M. Fish larvae DNA barcoding indicated the potential appearance of rare species: *Buenia massutii* Kovačić, Ordines, and Schliewen, 2017 in the Adriatic Sea. *Acta Adriat.* **2022**, *63*, 45–52. [[CrossRef](#)]
- Seyhan Öztürk, D.; Oruç, A.Ç. The new distribution records of recently described gobies: Aegean Sea records for *Pomatoschistus nanus* Engin & Seyhan Oztürk, 2017 and easternmost records of *Buenia massutii* Kovačić, Ordines & Schliewen, 2017. *J. Mar. Biol. Assoc. U. K.* **2023**, *103*, e40. [[CrossRef](#)]
- Kovačić, M.; Froglija, C. The Adriatic record of *Gobius xoriguer* Iglésias, Vukić & Šanda, 2021 and additional records of *Vanneaugobius dollfusi* Brownell, 1978 (Actinopterygii: Gobiiformes: Gobiidae). *Cybium* **2023**, *47*, 193–196. [[CrossRef](#)]
- Kovačić, M.; Ramirez-Amaro, S.; Farriols, M.T.; Ordines, F. The second record of *Gymnesigobius medits* Kovačić, Ordines, Ramirez-Amaro & Schliewen, 2019, the deepest benthic gobiiform species, and the additional records of *Gobius xoriguer* Iglésias, Vukić & Šanda, 2021 (Actinopterygii: Gobiiformes: Gobiidae). *Fishes* **2023**, *8*, 331. [[CrossRef](#)]
- Kovačić, M.; Šanda, R.; Vukić, J.; Renoult, J.P.; Falzon, M.-A. New records of the recently described *Pomatoschistus nanus* Engin & Seyhan, 2017 (Teleostei: Gobiidae). *Cybium* **2023**, *2017*, 1–4. [[CrossRef](#)]

16. Kovačić, M.; Šanda, R.; Kirinčić, M.; Zanella, D. Geographic distribution of gobies (Gobiidae) in the Adriatic Sea with thirteen new records for its southern part. *Cybium* **2012**, *36*, 435–445. [[CrossRef](#)]
17. Miller, P.J. Fishes of the North-eastern Atlantic and the Mediterranean. In *Gobiidae*; Whitehead, P.J.P., Bauchot, M.-L., Hureau, J.-C., Nielsen, J., Tortonese, E., Eds.; Unesco: Paris, France, 1986; pp. 1019–1085.
18. Kovačić, M.; Šanda, R. A new species of *Gobius* (Perciformes: Gobiidae) from the Mediterranean Sea and the redescription of *Gobius bucchichi*. *J. Fish Biol.* **2016**, *88*, 1104–1124. [[CrossRef](#)]
19. Kovačić, M.; Renoult, J.P.; Pillon, R.; Svensen, R.; Bogorodsky, S.V.; Engin, S.; Louisy, P. Identification of Mediterranean marine gobies (Actinopterygii: Gobiidae) of the continental shelf from photographs of in situ individuals. *Zootaxa* **2022**, *5144*, 1–103. [[CrossRef](#)]
20. Kovačić, M.; Renoult, J.P.; Pillon, R.; Bilecenoglu, M.; Tiralongo, F.; Bogorodsky, S.V.; Engin, S.; Kovtun, O.; Louisy, P.; Patzner, R.A.; et al. The delimitation of geographic distributions of *Gobius bucchichi* and *Gobius incognitus* (Teleostei: Gobiidae). *J. Mar. Sci. Eng.* **2023**, *11*, 516. [[CrossRef](#)]
21. Glavičić, I.; Kovačić, M.; Soldo, A.; Schliwen, U. A quantitative assessment of the diel influence on the cryptobenthic fish assemblage of the shallow Mediterranean infralittoral zone. *Sci. Mar.* **2020**, *84*, 49–57. [[CrossRef](#)]
22. Tiralongo, F.; Pillon, R. New distributional records of *Gobius bucchichi* (Pisces, Gobiidae) from the Mediterranean Sea and in situ comparisons with *Gobius incognitus*. *Ann. za Istrske Mediteranske Stud. Ser. Hist. Sociol.* **2020**, *30*, 215–220. [[CrossRef](#)]
23. Tiralongo, F.; Messina, G.; Lombardo, B.M. First data on habitat preference, diet and length-weight relationship of *Gobius incognitus* Kovačić & Šanda, 2016 (Pisces: Gobiidae). *Acta Adriat.* **2020**, *61*, 67–78. [[CrossRef](#)]
24. Soldo, A.; Glavičić, I.; Kovačić, M. Combining methods to better estimate total fish richness on temperate reefs: The case of a Mediterranean coralligenous cliff. *J. Mar. Sci. Eng.* **2021**, *9*, 670. [[CrossRef](#)]
25. Bilecenoglu, M.; Yokes, M.B. Distribution of two closely allied gobies, *Gobius bucchichi* Steindachner, 1870 and *Gobius incognitus* Kovačić & Šanda, 2016, along the Turkish coast. *FishTaxa* **2022**, *23*, 47–53.
26. Kovačić, M.; Kovtun, O.A. The first record of *Gobius incognitus* (Actinopterygii: Gobiiformes: Gobiidae) from Malta. *Acta Adriat.* **2022**, *63*, 53–58. [[CrossRef](#)]
27. Renoult, J.P.; Pillon, R.; Kovačić, M.; Louisy, P. Frontiers in Fishwatching Series-Gobies of the North-eastern Atlantic and the Mediterranean: *Gobius* and *Thorogobius*. *Les Cah. Fond. Biotope* **2022**, *37*, 1–237.
28. Spatafora, D.; Cattano, C.; Aglieri, G.; Quattrocchi, F.; Turco, G.; Quartararo, G.; Dudemaine, J.; Calosi, P.; Milazzo, M. Limited behavioural effects of ocean acidification on a Mediterranean anemone goby (*Gobius incognitus*) chronically exposed to elevated CO<sub>2</sub> levels. *Mar. Environ. Res.* **2022**, *181*, 105758. [[CrossRef](#)]
29. Francour, P.; Bilecenoglu, M.; Bariche, M.; Tunesi, L.; Goren, M. *Gobius bucchichi*. The IUCN Red List of Threatened Species 2011. Available online: <https://www.iucnredlist.org/species/194873/8911208> (accessed on 20 October 2023). [[CrossRef](#)]
30. Milana, V.; Šanda, R.; Vukić, J.; Ciccotti, E.; Riccato, F.; Petrosino, G.; Rossi, A.R. Far from home: Genetic variability of *Knipowitschia* sp. from Italy revealed unexpected species in coastal lagoons of the Tyrrhenian coast. *Estuar. Coast. Shelf Sci.* **2021**, *251*, 107260. [[CrossRef](#)]
31. Vukić, J.; Kovačić, M.; Zogaris, S.; Šanda, R. Rediscovery of *Knipowitschia goerneri* and its molecular relationships with other European northern Mediterranean *Knipowitschia* species (Teleostei: Gobiidae). *Ichthyol. Explor. Freshwaters* **2016**, *26*, 363–372.
32. Vukić, J.; Ulqini, D.; Šanda, R. Occurrence of *Knipowitschia goerneri* Ahnelt, 1991 (Gobiidae) in southern Albania confirmed with molecular tools. *J. Appl. Ichthyol.* **2017**, *33*, 284–290. [[CrossRef](#)]
33. Agorreta, A.; San Mauro, D.; Schliwen, U.; Van Tassell, J.L.; Kovačić, M.; Zardoya, R.; Rüber, L. Molecular phylogenetics of Gobioidae and phylogenetic placement of European gobies. *Mol. Phylogenet. Evol.* **2013**, *69*, 619–633. [[CrossRef](#)]
34. Neilson, M.E.; Stepien, C.A. Escape from the Ponto-Caspian: Evolution and biogeography of an endemic goby species flock (Benthophilinae: Gobiidae: Teleostei). *Mol. Phylogenet. Evol.* **2009**, *52*, 84–102. [[CrossRef](#)] [[PubMed](#)]
35. Medvedev, D.A.; Sorokin, P.A.; Vasil'ev, V.P.; Chernova, N.V.; Vasil'eva, E.D. Reconstruction of phylogenetic relations of Ponto-Caspian gobies (Gobiidae, Perciformes) based on mitochondrial genome variation and some problems of their taxonomy. *J. Ichthyol.* **2013**, *53*, 702–712. [[CrossRef](#)]
36. Tougard, C.; Vukić, J.; Ahnelt, H.; Buj, I.; Kovačić, M.; Moro, G.A.; Tutman, P.; Šanda, R. Quaternary climatic cycles promoted (re)colonization and diversification events in Adriatic sand gobies. *J. Zool. Syst. Evol. Res.* **2021**, *59*, 1037–1052. [[CrossRef](#)]
37. Čekovská, K.; Šanda, R.; Eliášová, K.; Kovačić, M.; Zogaris, S.; Pappalardo, A.M.; Soukupová, T.; Vukić, J. Population genetic diversity of two marine gobies (Gobiiformes: Gobiidae) from the North-eastern Atlantic and the Mediterranean Sea. *J. Mar. Sci. Eng.* **2020**, *8*, 792. [[CrossRef](#)]
38. Gysels, E.S.; Hellemans, B.; Pampoulie, C.; Volckaert, F.A.M. Phylogeography of the common goby, *Pomatoschistus microps*, with particular emphasis on the colonization of the Mediterranean and the North Sea. *Mol. Ecol.* **2004**, *13*, 403–417. [[CrossRef](#)]
39. Larmuseau, M.H.D.; Huyse, T.; Vancampenhout, K.; Van Houdt, J.K.J.; Volckaert, F.A.M. High molecular diversity in the rhodopsin gene in closely related goby fishes: A role for visual pigments in adaptive speciation? *Mol. Phylogenet. Evol.* **2010**, *55*, 689–698. [[CrossRef](#)]
40. Boissin, E.; Hoareau, T.B.; Berrebi, P. Effects of current and historic habitat fragmentation on the genetic structure of the sand goby *Pomatoschistus minutus* (Osteichthys, Gobiidae). *Biol. J. Linn. Soc.* **2011**, *102*, 175–198. [[CrossRef](#)]
41. Mejri, R.; Arculeo, M.; Ben Hassine, O.K.; Lo Brutto, S. Genetic architecture of the marbled goby *Pomatoschistus marmoratus* (Perciformes, Gobiidae) in the Mediterranean Sea. *Mol. Phylogenet. Evol.* **2011**, *58*, 395–403. [[CrossRef](#)] [[PubMed](#)]

42. Tougard, C.; Folly, J.; Berrebi, P. New light on the evolutionary history of the common goby (*Pomatoschistus microps*) with an emphasis on colonization processes in the Mediterranean Sea. *PLoS ONE* **2014**, *9*, e91576. [[CrossRef](#)] [[PubMed](#)]
43. Seyhan-Ozturk, D.; Engin, S. Genetic diversity of marbled goby populations in the Anatolian coasts of the north-eastern Mediterranean. *J. Mar. Biol. Assoc. U. K.* **2021**, *101*, 419–429. [[CrossRef](#)]
44. Šanda, R.; Vukić, J.; Choleva, L.; Křížek, J.; Šedivá, A.; Shumka, S.; Wilson, I.F. Distribution of loach fishes (Cobitidae, Nemacheilidae) in Albania, with genetic analysis of populations of *Cobitis ohridana*. *Folia Zool.* **2008**, *57*, 42–50.
45. Hall, T.A. BioEdit: A user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **1999**, *41*, 95–98.
46. Villesen, P. FaBox: An online toolbox for FASTA sequences. *Mol. Ecol. Notes* **2007**, *7*, 965–968. [[CrossRef](#)]
47. Šanda, R.; Kovačić, M. First record of *Gobius couchi* (Gobiidae) in the Ionian Sea. *Cybium* **2009**, *33*, 249–250. [[CrossRef](#)]
48. Lanfear, R.; Frandsen, P.B.; Wright, A.M.; Senfeld, T.; Calcott, B. PartitionFinder 2: New methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Mol. Biol. Evol.* **2017**, *34*, 772–773. [[CrossRef](#)]
49. Ronquist, F.; Teslenko, M.; Van Der Mark, P.; Ayres, D.L.; Darling, A.; Höhna, S.; Larget, B.; Liu, L.; Suchard, M.A.; Huelsenbeck, J.P. MrBayes 3.2: Efficient bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* **2012**, *61*, 539–542. [[CrossRef](#)]
50. Rambaut, A.; Drummond, A.J.; Xie, D.; Baele, G.; Suchard, M.A. Posterior summarisation in Bayesian phylogenetics using Tracer 1.7. *Syst. Biol.* **2018**, *67*, 901–904. [[CrossRef](#)]
51. Rambaut, A. FigTree Version 1.4.4. 2018. Available online: <https://github.com/rambaut/figtree/releases> (accessed on 2 February 2019).
52. Inkscape Project. Inkscape Version 0.92.3. Available online: <https://inkscape.org> (accessed on 10 June 2022).
53. Tamura, K.; Stecher, G.; Peterson, D.; Filipowski, A.; Kumar, S. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* **2013**, *30*, 2725–2729. [[CrossRef](#)]
54. Francisco, S.M.; Castilho, R.; Lima, C.S.; Almada, F.; Rodrigues, F.; Šanda, R.; Vukić, J.; Pappalardo, A.M.; Ferrito, V.; Robalo, J.I. Genetic hypervariability of a Northeastern Atlantic venomous rockfish. *PeerJ* **2021**, *9*, e11730. [[CrossRef](#)]
55. Reichenbacher, B.; Gregorová, R.; Holcová, K.; Šanda, R.; Vukić, J.; Přikryl, T. Discovery of the oldest *Gobius* (Teleostei, Gobiiformes) from a marine ecosystem of Early Miocene age. *J. Syst. Palaeontol.* **2018**, *16*, 493–513. [[CrossRef](#)]
56. Webb, C.J. Systematics of the *Pomatoschistus minutus* complex (Teleostei: Gobioidae). *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **1980**, *291*, 201–241. [[CrossRef](#)]
57. Berrebi, P.; Trébuchon, M. Distribution and hybridization of two sedentary gobies (*Pomatoschistus microps* and *Pomatoschistus marmoratus*) in the lagoons of southern France. *Sci. Mar.* **2020**, *84*, 355–367. [[CrossRef](#)]
58. Horvatić, S.; Malavasi, S.; Vukić, J.; Šanda, R.; Marčić, Z.; Čaleta, M.; Lorenzoni, M.; Mustafić, P.; Buj, I.; Onorato, L.; et al. Correlation between acoustic divergence and phylogenetic distance in soniferous European gobiids (Gobiidae; *Gobius* lineage). *PLoS ONE* **2021**, *16*, e0260810. [[CrossRef](#)] [[PubMed](#)]
59. Horvatić, S.; Parmentier, E.; Malavasi, S.; Clara Amorim, M.P.; Fonseca, P.J.; Zanella, D. Endemic fish calling: Acoustics and reproductive behaviour of the Neretva dwarf goby *Orsinigobius croaticus*. *Ecol. Evol.* **2023**, *13*, e10673. [[CrossRef](#)] [[PubMed](#)]
60. Bariche, M.; Fricke, R. The marine ichthyofauna of Lebanon: An annotated checklist, history, biogeography, and conservation status. *Zootaxa* **2020**, *4775*, 1–157. [[CrossRef](#)] [[PubMed](#)]
61. Soldo, A.; Bakiu, R. Checklist of marine fishes of Albania. *Acta Adriat.* **2021**, *62*, 63–73. [[CrossRef](#)]
62. Bitar, G.; Badreddine, A. An updated checklist of the marine fishes in Lebanon. An answer to Bariche and Fricke (2020): “The marine ichthyofauna of Lebanon: An annotated checklist, history, biogeography, and conservation status”. *Zootaxa* **2021**, *5010*, 1–128. [[CrossRef](#)]
63. Elbaraasi, H.; Elabar, B.; Elaabidi, S.; Bashir, A.; Elsilini, O.; Shakman, E.; Azzurro, E. Updated checklist of bony fishes along the Libyan coast (southern Mediterranean Sea). *Mediterr. Mar. Sci.* **2019**, *20*, 90–105. [[CrossRef](#)]
64. Ibrahim, M.A.; Soliman, I.A. Checklist of the bony fish species in the Mediterranean waters of Egypt. *Bull. Natl. Inst. Oceanogr. Fish.* **1996**, *22*, 43–57.
65. Bouchereau, J.L.; Durel, J.S.; Guelorget, O.; Louali, L.R. L’ichtyofaune dans l’organisation biologique d’un système paralique marocain: La lagune de Nador (in french). *Mar. Life* **2000**, *10*, 69–76.
66. Ali, M.F. An updated Checklist of Marine fishes from Syria with an emphasis on alien species. *Mediterr. Mar. Sci.* **2018**, *19*, 388–393. [[CrossRef](#)]
67. Keskin, Ç. A review of fish fauna in the Turkish Black Sea. Karadeniz balık faunası. *J. Black Sea/Mediterr. Environ.* **2010**, *16*, 195–210.
68. Vasil’eva, E.D. *Fishes of the Black Sea. Key to Marine, Brackish–Water, Euryhaline and Anadromous Species with Color Illustrations, Collected by S.V. Bogorodsky*; VNIRO Publishing: Moscow, Russia, 2007; pp. 1–237. (In Russian)
69. Parin, N.V.; Evseenko, S.A.; Vasil’eva, E.D. *Fishes of Russian Seas: Annotated Catalogue*; KMK Scientific Press Ltd.: Moscow, Russia, 2014; pp. 1–733.
70. Manilo, L.G. *Ryby Semeystva Bychkovyie (Perciformes, Gobiidae) Morskikh i Solonovatykh vod Ukrainy*; Naukova Dumka: Kyiv, Ukraine, 2014; pp. 1–243. (In Russian)
71. Boltachev, A.R.; Karpova, E.P. *Marine Fishes of the Crimean Peninsula*, 2nd ed.; Biznes-Inform: Simferopol, Ukraine, 2017; pp. 1–376. (In Russian)

72. Vassilev, M.; Apostolou, A.; Velkov, B.; Dobrev, D.; Zarev, V. *Atlas of the Gobies (Gobiidae) in Bulgaria*; Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences: Sofia, Bulgaria, 2012; pp. 1–114.
73. Giantsis, I.A.; Tokamani, M.; Triantaphyllidis, G.; Tzatzani, S.; Chatzinikolaou, E.; Toros, A.; Bouchorikou, A.; Chatzoglou, E.; Miliou, H.; Sarantopoulou, J.; et al. Development of multiplex PCR and melt–curve analysis for the molecular identification of four species of the Mullidae family, available in the market. *Genes* **2023**, *14*, 960. [[CrossRef](#)]

**Disclaimer/Publisher’s Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.