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Morphological Characteristics and DNA Barcoding of the Rare Blanket Octopus *Tremoctopus violaceus* (Cephalopoda: Tremoctopodidae) in the Adriatic Sea

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Abstract: Tremoctopods are epipelagic argonautoid octopods characterized by their expanded dorsal webs and strong sexual size dimorphism, with dwarfed males. The scarcity of taxonomic features attributed to this genus presents a challenge, and there is growing evidence of species misidentification in *Tremoctopus* genus on a molecular level. In this study, we investigated four female specimens of blanket octopus *Tremoctopus violaceus* caught by purse seine fishing in the Central Eastern Adriatic Sea in 2019. Individuals had smooth, firm and muscular bodies, dark bluish purple on the dorsal and iridescent silvery on the ventral side, with dorsal mantle lengths of 113, 82, 80 and 78 mm. The constructed phylogenetic trees based on the 16S ribosomal RNA and cytochrome c oxidase subunit I sequences of investigated Adriatic specimens and publicly available sequences showed strong support for the *T. violaceus* clade, consisting of individuals collected from the Adriatic Sea, Mediterranean Sea and Atlantic Ocean, with the exclusion of Indo-Pacific clade most probably corresponding to *T. gracilis*. To fully understand the life-history traits of *Tremoctopus* species, future research should focus on DNA-based methods for correct species identification combined with morphological characters, geographic distribution and ecological information.

Keywords: octopods; 16S rRNA; COI; phylogeny; morphometry; Adriatic

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1. Introduction

Tremoctopods are epipelagic argonautoid octopods characterized by their expanded dorsal webs and strong sexual size dimorphism, with males being much smaller than females and serving only for storage and transfer of spermatophores [1]. Within the monogeneric family Tremoctopodidae Tryon, 1879 four species have been currently recognized: *Tremoctopus violaceus* Delle Chiaje, 1830, *Tremoctopus gelatus* Thomas, 1977, *Tremoctopus gracilis* (Souleyet, 1852) and *Tremoctopus robsonianus* T. W. Kirk, 1884 (*Tremoctopus robsoni* (sic) Kirk, 1884) [2]. The genus *Tremoctopus* is rather poorly investigated, and most reports are based on only few specimens, mostly strandings or occurrence records. The life history of these species is not yet fully understood, and the scarcity of taxonomic features presents an additional challenge for investigation. *Tremoctopus gelatus* is easily distinguished morphologically due to its gelatinous transparent body with few chromatophores, while others have a highly pigmented muscular body. One morphological discriminative character between *T. violaceus* and *T. gracilis* is male hectocotylus, firstly introduced by Thomas [3], though small tremoctopod males are hardly ever seen or collected. In most cases, live or stranded females are observed and often discriminated based on the chromatic web pattern of their dorsal arms [4]. Difficulties in identification arise when dealing with specimens caught during fishing operations when arms and interbranchial membrane (arm web), usually get greatly damaged. Furthermore, a lot of findings are juvenile females, and according to Thomas [3], juveniles of these two species are morphologically quite indistinguishable.

The genus *Tremoctopus* has a worldwide distribution, but at the moment this is mostly based on morphological species identification: *T. robsonianus* is limited to the south-west Pacific Ocean, *T. gelatus* inhabits the Indian Ocean, the eastern Pacific Ocean and the western Atlantic Ocean, *T. violaceus* is widespread through the Atlantic Ocean, Gulf of Mexico, Caribbean Sea and Mediterranean Sea, and *T. gracilis* is found in the Indo-Pacific waters and the Mediterranean Sea (see references in [1]). Findings of *T. gracilis* in the Mediterranean waters [5,6] suggest two possibilities: either this Indo-Pacific species is in fact a Lessepsian migrant carried in ballast waters; or it has been misidentified as *T. violaceus* [7].

Presently, based on molecular data, there is growing evidence of species misidentification in the *Tremoctopus* genus, especially between *T. violaceus* and *T. gracilis* [8,9]. Most recently, Jiménez-Badillo et al. [9] provided one 16S sequence of *T. violaceus* from the Southwestern Gulf of Mexico (Atlantic Ocean) representing the westernmost known occurrence of this species. Additionally, Agus et al. [8] molecularly identified two specimens of *T. violaceus* from the Sardinian waters in the Mediterranean Sea and one from the Southern Adriatic Sea, providing detailed morphological and meristic analysis, as well as age estimation using upper beaks. The authors used the mitochondrial cytochrome c oxidase subunit I (COI) and mitochondrial 16S rRNA (16S) gene to show that the investigated specimens belonged to the same species, i.e., *T. violaceus sensu stricto*. Furthermore, the authors emphasized that misidentifications in public repositories are due to the lack of distinguishing taxonomic characters that could easily separate *T. violaceus* and *T. gracilis*.

The violet blanket octopus *T. violaceus*, although known in the Mediterranean [8,10–14], is rather sporadically recorded in the Adriatic Sea (Table S1). The earliest mention we were able to track back of this species in the Adriatic Sea is by Nardo in 1847 [15], who considered it to be very rare in the Adriatic, and reported that it appeared on the fish market (possibly in Venice) but was almost unknown to local fishermen. Earliest records are reported by Kolombatović in 1888 [16], who mentions three specimens caught near the city of Split and the islands Vis and Hvar without additional details. Surprisingly, the same author later reports on the records of five additional specimens (it is unclear whether those include the previous three), but provides no details except for one individual caught near Split in July 1904, for which a detailed description is given [17]. In 1936, Kramer [18] reported a huge number of blanket octopuses in the Northern Adriatic (Rovinj). Afterward, there were no published records until 1991, when Bello [19] found remains of *T. violaceus* in the stomach of the swordfish caught in the Southern Adriatic. The last documented record of a blanket octopus in the Eastern Adriatic Sea is that of a live stranded female found in the Central Eastern Adriatic (Šunj Bay of island Lopud) in April 2000 [20]. Here, we report on the collection of four tremoctopod specimens from the Central Eastern Adriatic and complement it with molecular identification and phylogenetic analyses of genus *Tremoctopus* that will further clarify the relationships between *T. violaceus* and *T. gracilis* in their geographical distribution area.

2. Materials and Methods

2.1. Morphological and Biological Analysis

In July 2019, four individuals of blanket octopus were caught by purse seine during commercial fishing (fishing vessel "Davorin") at 0.7 nm North of the Cape Kabal of Island Hvar in the Central Eastern Adriatic Sea (Figure 1). Specimens were frozen on board and sent for identification to the Institute of Oceanography and Fisheries (Split, Croatia).

Morphological identification was performed by following diagnostic features presented in Finn [1]. Prior to measurement, individuals were thawed at room temperature. All body measurements were taken to the nearest 1 mm, and total body weight (BW) was recorded with a precision of 0.01 g. The following measurements were taken: total length (TL); dorsal mantle length (DML); head length (HL); and head width (HW). The beaks and radulae were surgically removed from the buccal mass and stored in 70% ethanol. Beak measurements were taken by digital caliper to an accuracy of 0.01 mm, following

Clarke [21]. The following morphometric features were measured: upper hood length (UHL); upper crest length (UCL); lower hood length (LHL); and lower crest length (LRL). Statoliths and stylets were surgically extracted from the cephalic cartilage and mantle, respectively, and stored in 70% ethanol.

Figure 1. Map of the Adriatic Sea showing sampling location, 1 (43.259167° N, 16.520556° E) of *Tremoctopus violaceus* in 2019.

The stomach weight (SW) was recorded. Stomach contents were preserved in 70% ethanol and examined under a dissecting microscope. Found prey remains were identified to the lowest taxonomic level possible. A subjective fullness index of the stomach content was applied, with 0 indicating empty, 1 containing a very scarce amount remaining and 2 containing significant remains to full repletion [22].

Due to the extreme sexual dimorphism of this genus, i.e., dwarfed males with DML to at least 15 mm, sex was easily determined macroscopically. Thawed specimens were dissected to determine the maturity stage according to the MEDITS maturity scale for cephalopods [23]. Reproductive system weight (RW), i.e., ovary with oviducts and oviducal glands, and ovary weight (OW) were recorded.

All photomicrographs were taken using an Olympus DP25 microscope digital camera mounted on Olympus SZX10 light microscope.

2.2. DNA Extraction, PCR Amplification and Sequencing

From sampled individuals, a small piece of mantle tissue was preserved in 96% ethanol for molecular analysis and stored at +4 °C. Total genomic DNA was extracted following a modified protocol from Martínez et al. [24]. Briefly, cell lysis buffer (0.2% SDS, 0.01 M TrisBase, 0.01 M EDTA, 0.15 M NaCl) with proteinase K (0.2 mg/mL) was used to dissolve a small piece of tissue overnight at 55 °C. After centrifugation (10 min at 13.000 rpm) to precipitate the debris, proteins were salted out from supernatant by the addition of NaCl (2.2 M in a final solution), kept on ice for 10 min and centrifuged at 13.000 rpm for 10 min. DNA was precipitated with an equal volume of cold isopropanol, washed in 75% ethanol, dried at 37 °C and resuspended in TE buffer (0.01 M Tris-HCL, 0.0125 M EDTA, pH = 8).

Partial sequences of two mitochondrial genes, cytochrome c oxidase subunit I gene (COI; 1100 bp) and 16S ribosomal RNA gene (16S; 480 bp) were analyzed. COI gene was amplified using primer pair LCO1490: 5′-GGTCAACAAATCATAAAGATATTGG-3′ [25] and H7005: 5′-CCGGATCCACNACRTARTANGTRTCRTG-3′ [26]. 16S gene was amplified using primers 16S ar: 5′-CGCCTGTTTATCAAAAACAT-3′ and 16S br: 5′-CCGGTCTGAACTCTGATCAT-3′ [27].

Standard PCR reactions were prepared in a final volume of 25 µL, which included DNAse/RNAse free water, 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.4 µM of each primer, 1 U of GoTaq® G2 Hot Start Polymerase (Promega Corporation, Madison, WI, USA) for COI and Sigma Taq Polymerase (MiliporeSigma, Burlington, MA, USA) for 16S gene. The DNA template used for amplification was set to 25 and 75 ng for COI and 16S, respectively.

The PCR cycling conditions used were as follows: for COI, 2 min at 95 °C, with 5 cycles of 1 min at 95 °C, 1 min at 45 °C, 1 min at 72 °C plus 30 cycles of 1 min at 95 °C, 1 min at 50 °C, 1 min at 72 °C with a final extension of 5 min at 72 °C; and for 16S, the cycle conditions were 3 min at 94 °C, with 5 cycles of 30 s at 94 °C, 1.5 min at 45 °C, 1 min at 72 °C plus 35 cycles of 30 s at 94 °C, 1 min at 50 °C and 1 min at 72 °C, followed by a final extension of 7 min at 72 °C. Successful PCR amplifications were verified using 1% agarose gel-electrophoresis and sent for sequencing. Amplicons were sequenced using ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems), as provided by Macrogen Europe sequencing service. Large amplicons (COI, 1100 bp) were sequenced in both directions.

2.3. Molecular and Phylogenetic Analyses

DNA sequences of 4 sampled individuals were processed, quality trimmed, assembled if necessary (COI) and aligned using Geneious Prime v2023.4 (https://www.geneious.com (accessed on 15 March 2023); Auckland, New Zealand) [28]. Obtained 16S sequences were deposited to GenBank (https://www.ncbi.nlm.nih.gov/genbank (accessed on 15 March 2023)) under Accession numbers OQ561743–OQ561746 and COI sequences under OQ561768–OQ561771. In total, 8 16S and 12 COI publicly available sequences of the *Tremoctopus* genus from GenBank were used for the analysis of genetic diversity, as well as phylogenetic analysis (Accession numbers in Table S2). Genetic diversity was measured using the DnaSP program 6.12.03 [29] by estimating the number of haplotypes (H), polymorphic sites (S), nucleotide diversity (π) [30] and the mean number of differences between sequence pairs (k) [31]. Reconstruction of the phylogenetic tree was performed in Geneious Prime v2023.4 software using the Bayesian inference method [32]. The best-fit evolutionary models HKY + F + G4 for 16S and GTR + F + I for COI, according to Bayesian information criterion (BIC), were determined using IQ-TREE web server with auto model selection [33–36]. The tree was rooted with *Octopus vulgaris* (for 16S: AJ252778; for COI: HQ908426) as the outgroup species and was subsequently visualized and graphically processed using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree (accessed on 15 March 2023); Edinburgh, UK). Genetic distances were calculated to quantify sequence divergences among individuals using Kimura 2-parameter model with 1000 bootstrap iterations in MEGA 11 [37,38].

3. Results

3.1. Morphological and Biological Analysis

All examined tremoctopods were females. Body and beak measurements of all analyzed specimens are presented in Table 1. Individuals had smooth, firm and muscular bodies, with dark bluish-purple on the dorsal and iridescent silvery coloration on the ventral side (Figure 2). All specimens were in a rather good condition, except for minor damage to the arms and, unfortunately, major damage to the distinguished web between the first two pairs of arms. A part of the web was best preserved in the largest female (ID 1) (Figure 3A). All individuals had two easily distinguishable pairs of cephalic water pores, with one ventral pair located near the funnel (Figure 3D), and the other placed dorsally between the eyes (Figure 3C). The funnel was not embedded in head tissue, and reached

beyond the level of the eyes. The gills of all specimens had 13 filaments (Figure 3E). In the nuchal region, posterior to the eyes, there were 8 nuchal folds. The arms were unequal in size with the arm formula of II > I > IV > III (Table 1) with elevated biserial suckers, arranged in a single row at the distal part of the arm (Figure 3B). Two dorsolaterally positioned stylets were recovered from the mantle wall (Figure 3G), and two statoliths were dissected from the cephalic cartilage (Figure 3H). The radula of examined individuals consisted of 7 longitudinal teeth rows with tricuspid central (rachidian) tooth (Figure 4).

Table 1. Measurements of *Tremoctopus violaceus* specimens collected in the Central Eastern Adriatic Sea in 2019. (NO—not observed).

Specimen ID	**1**	**2**	**3**	**4**
Sex	Female	Female	Female	Female
Body weight (g)	257.68	93.94	81.55	74.72
Dorsal mantle length (mm)	113	82	80	78
Total length (mm)	412	267	238	251
Head length (mm)	41	30	27	30
Head width (mm)	36	31	34	27
Stomach weight (g)	3.60	1.51	2.35	1.59
Stomach fullness index	1	0	2	2
Maturity	mature	developing	developing	developing
Reproductive system weight (g)	11.66	0.62	0.55	0.59
Ovary weight (g)	8.31	0.32	0.22	0.25
Arm length I (left/right) (mm)	191/185	129/NO	97/101	138/140
Arm length II (left/right) (mm)	300/292	175/172	NO/146	168/165
Arm length III (left/right) (mm)	139/135	97/91	76/80	76/82
Arm length IV (left/right) (mm)	143/147	105/105	95/106	130/100
Upper hood length (mm)	7.30	5.62	5.48	5.06
Upper crest length (mm)	12.04	9.68	9.10	8.86
Lower hood length (mm)	5.43	3.99	3.91	3.35
Lower crest length (mm)	8.68	6.25	6.05	5.99

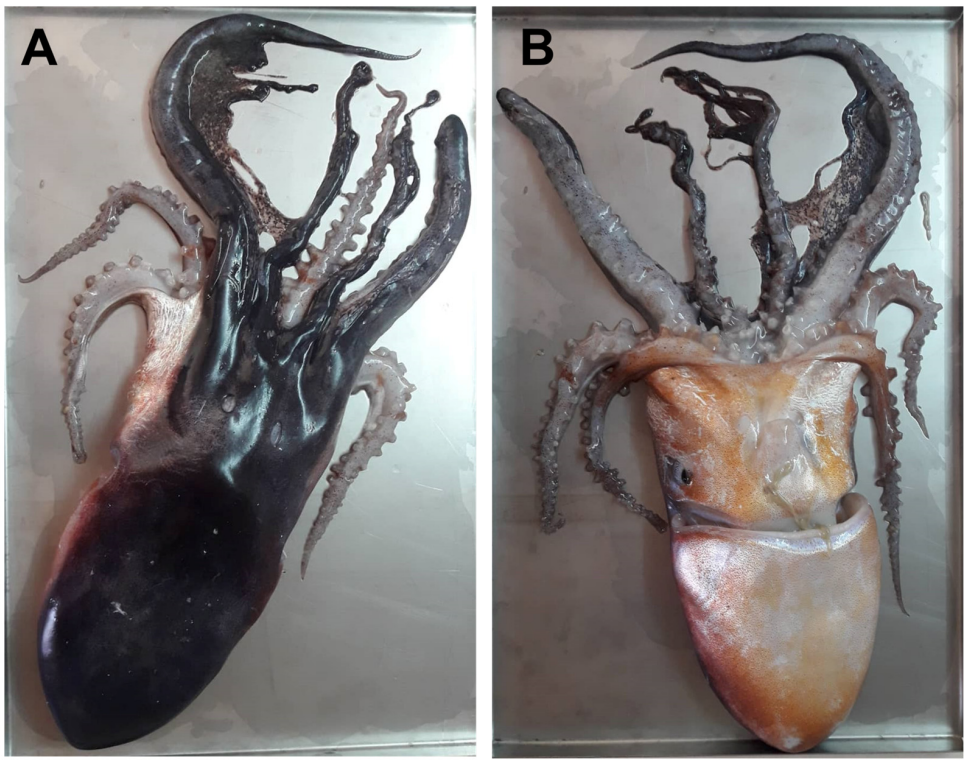

Figure 2. *Cont.*

Figure 2. Photographs of fresh *Tremoctopus violaceus* female caught in the Central Eastern Adriatic Sea: (**A**) dorsal, (**B**) ventral and (**C**) lateral view. (Photo: Rino Stanić).

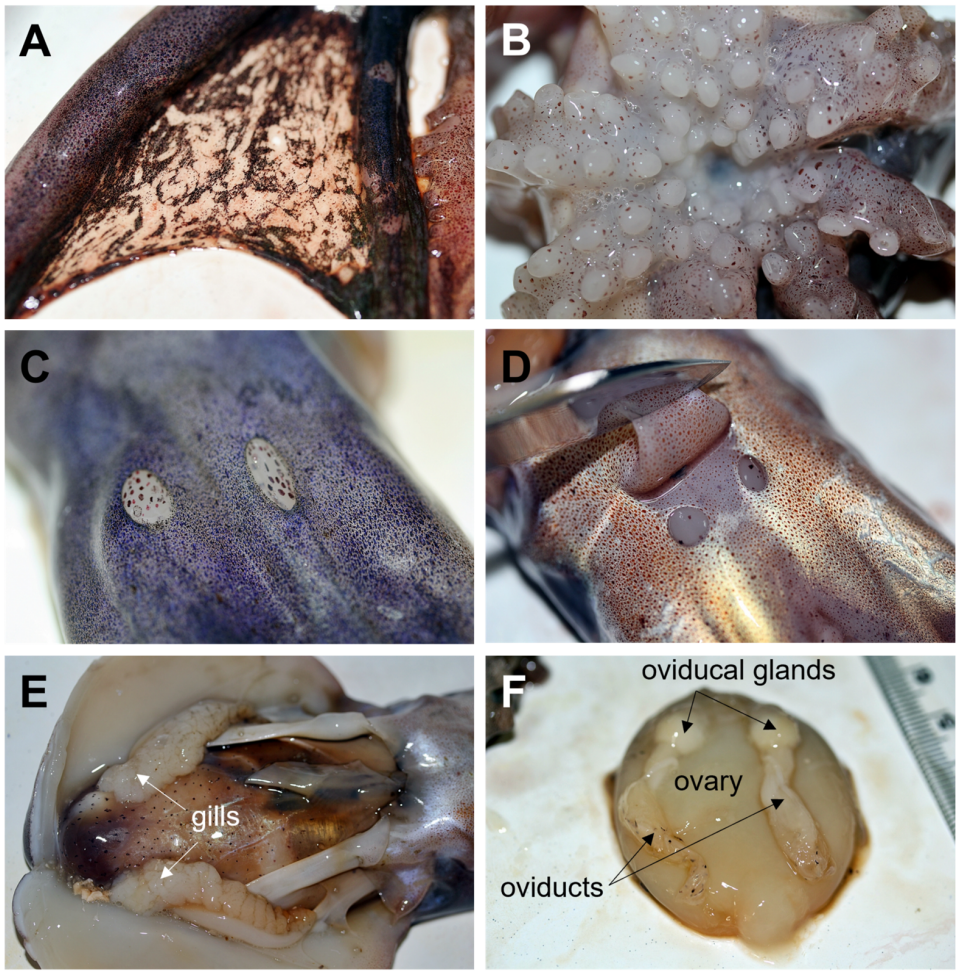

Figure 3. *Cont.*

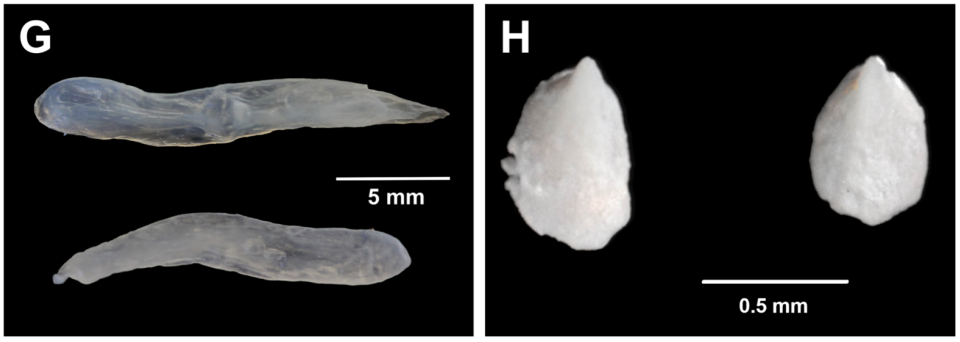

Figure 3. *Tremoctopus violaceus* from the Central Eastern Adriatic Sea: (**A**) dorsal arm web detail; (**B**) biserial suckers on arms; (**C**) pair of dorsal water pores; (**D**) pair of ventral water pores; (**E**) mantle cavity; (**F**) reproductive tract; (**G**) stylets; and (**H**) posterior view of statoliths.

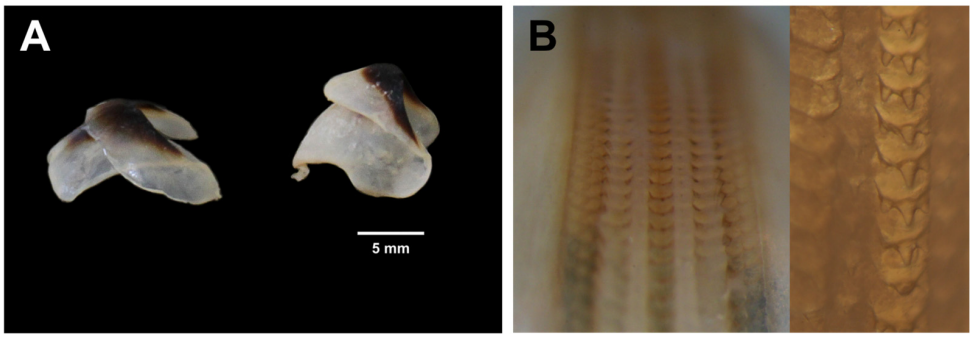

Figure 4. *Tremoctopus violaceus* from the Central Eastern Adriatic Sea: (**A**) lateral view of lower (left) and upper (right) beaks; and (**B**) radula showing enlarged detail (right) of central tricuspid teeth.

The stomach content analysis revealed high levels of prey digestion. In one individual (ID 1), few non-identified fish scales were found, while the full stomachs of two individuals (ID 3 and ID4) contained hard remains from only one prey, i.e., the jaws of Polychaeta from the Nereididae family (Table 1).

A mature female (ID1) had a large ovary with enlarged oviducts and oviducal glands (Figure 3F). The other three females were in the developing stage; ovaries had a visible granular structure with a clearly visible oviduct meander.

3.2. Molecular and Phylogenetic Analysis

From all 4 investigated specimens, amplicons were successfully obtained. Alignment for 16S rRNA and COI data set was 504 and 988 bp in length, respectively. Our 16S rRNA data set of 4 individuals showed only one haplotype with no variable polymorphic sites. Furthermore, no polymorphisms were detected in the full 16S data set composed by combining our 16S sequences with those publicly available from GenBank (Table S2), i.e., the Southern Adriatic Sea (1 sequence), Mediterranean Sea (2 sequences) and from the Atlantic Ocean (1 sequence) into a joint alignment.

Our COI data set of 4 individuals revealed 3 variable polymorphic sites, of which all were singletons. Overall, 4 haplotypes were detected, with each haplotype represented by one individual, haplotype diversity (Hd) of 1.000 (stdev = 0.177; variance = 0.03125) and a low nucleotide diversity (π) of 0.00152. The estimated average number of nucleotide differences (k) was 1.500, and Tajima's D was −0.7544 (not significant, $p > 0.10$). DNA polymorphisms were also detected in the full COI dataset that combined publicly available COI sequences from the southern Adriatic (1 sequence) and from the Atlantic Ocean (2 sequences) (Table S2). In this full data set, there were 4 variable polymorphic sites, including 1 singleton and 3 parsimony informative sites. Five haplotypes were identified with high haplotype diversity of 0.905 (stdev = 0.103; variance = 0.01067), low nucleotide

diversity of 0.00325 and an estimated average number of nucleotide differences of 1.810. Tajima's D was 0.51788 (not significant, $p > 0.10$). One haplotype was shared by 2 Atlantic individuals.

The constructed phylogenetic trees based on the 16S and COI sequences of investigated Adriatic specimens and those obtained from Genbank are presented in Figures 5 and 6, respectively. Both Bayesian inferred trees showed strong support for the *T. violaceus* clade, consisting of individuals collected from the Adriatic Sea, the Mediterranean Sea and the Atlantic Ocean.

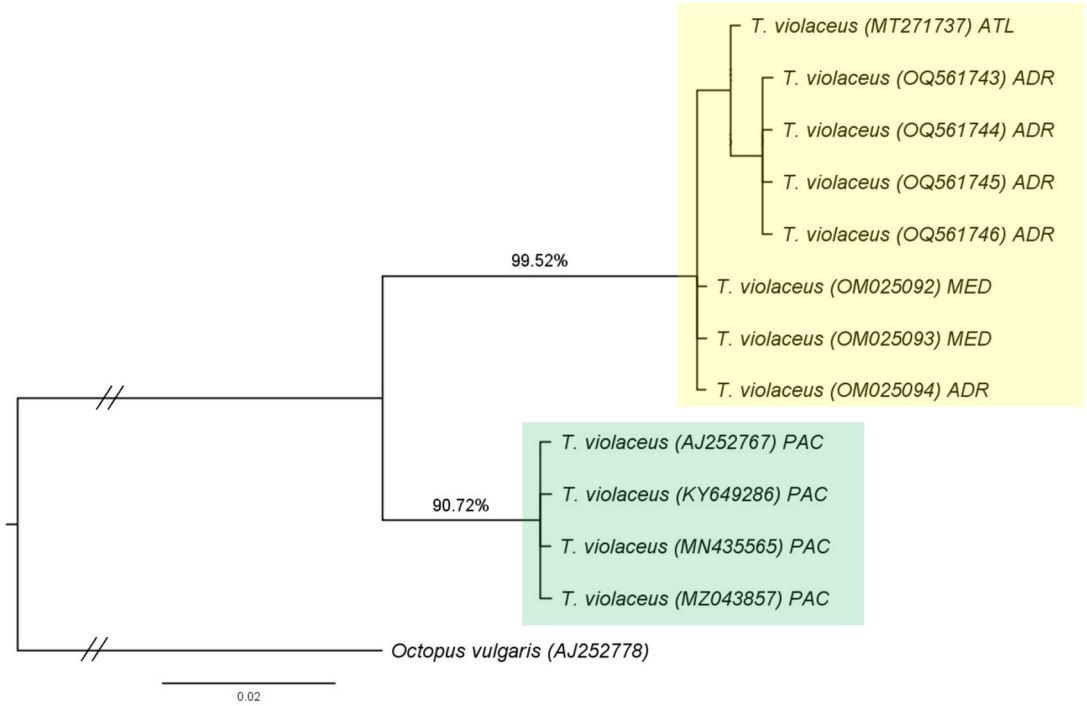

Figure 5. Phylogenetic tree of the genus *Tremoctopus* based on Bayesian Inference (BI) of the mitochondrial 16S ribosomal RNA gene. The values at the nodes show the posterior probabilities (%) of BI analyses. Only values $\geq$ 90% are shown. Represented sequences are from this study (OQ561743–OQ561746) and those available from GenBank. The clade highlighted in yellow represents *T. violaceus* from the Adriatic/Mediterranean/Atlantic area (ADR/MED/ATL) and in green *T. violaceus* from the Pacific area (PAC), probably misidentified and corresponding to *T. gracilis*.

The phylogenetic tree of the full 16S data set showed the presence of two clades: one clade with *T. violaceus* specimens from the Adriatic/Mediterranean/Atlantic area (ADR/MED/ATL); and another clade consisting of 4 specimens assigned as *T. violaceus* from the Pacific Ocean (PAC), with 99.52% and 90.2% posterior probability support, respectively (Figure 5). Pairwise genetic distances between ADR/MED/ATL and PAC sequences ranged from 5.73 to 6.76% (Table S3), indicating that sequences from the Pacific are most probably misidentified and are corresponding to *T. gracilis*.

Tree topology of COI, due to the availability of *T. gracilis* and *T. robsonianus* sequences, was more informative, and illustrated three major clades: one clade of *T. violaceus* from Adriatic/Mediterranean/Atlantic area (100% posterior probability support); and one clade of *T. gracilis* from Indian and Pacific Ocean (99.76% posterior probability support), which also included other sequences from the Pacific area most likely misidentified as *T. violaceus*, and one clade consisting of *T. robsonianus* individuals (100% posterior probability support) (Figure 6). Sequence AF377978 [39] deposited as *T. violaceous* clustered together with *T. gracilis* sequences from the Indian and Pacific Oceans.

Interspecific genetic distances between species, as supported by our phylogenetic analyses, ranged from 8.47% to 9.60% for *T. violaceus* and *T. gracilis*, 14.56% to 15.95% for

T. violaceus and *T. robsonianus* and from 14.51% to 15.13% for *T. gracilis* and *T. robsonianus* (Table S4). Intraspecific distances were an order of magnitude lower: on average 0.32% for *T. violaceus* and 0.35% for *T. gracilis*.

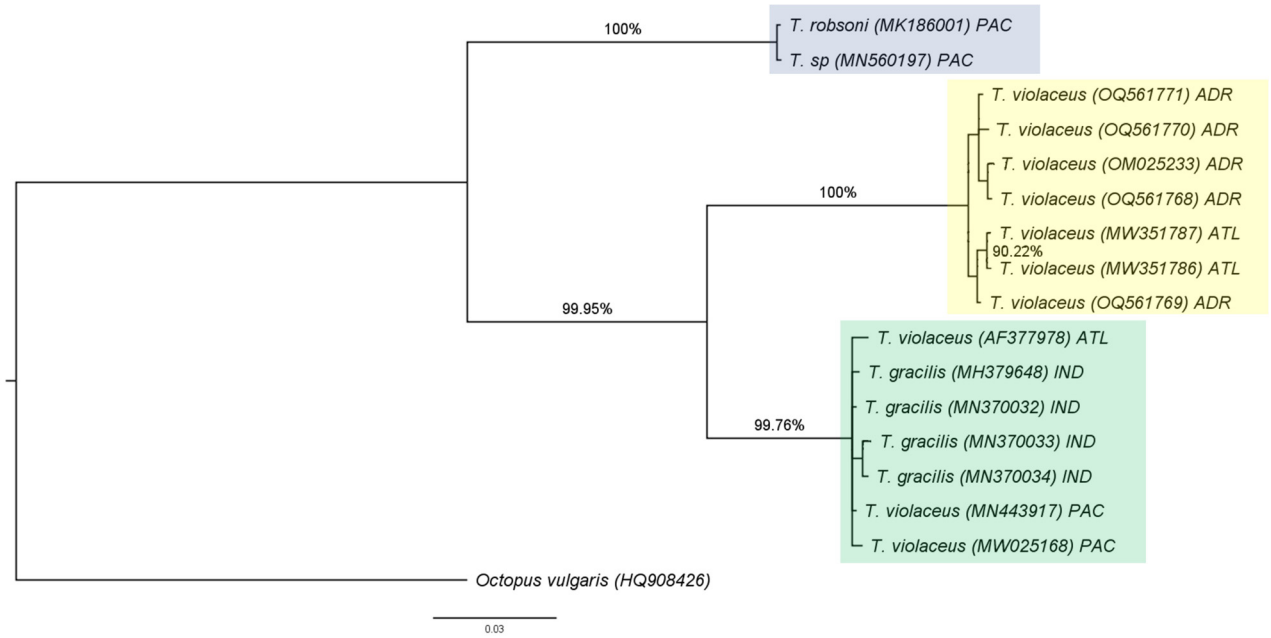

Figure 6. Phylogenetic tree of the genus *Tremoctopus* based on Bayesian Inference (BI) of the mitochondrial cytochrome c oxidase subunit I gene. The values at the nodes show the posterior probabilities (%) of BI analyses. Only values ≥ 90% are shown. Represented sequences are from this study (OQ561768–OQ561771) and those available from GenBank. Clade highlighted in blue are *T. robsonianus* (*T. robsoni* (sic)) individuals from the Pacific, while yellow represents *T. violaceus* from the Adriatic/Mediterranean/Atlantic area (ADR/MED/ATL) and green *T. gracilis* and *T. violaceus* from the Indo-Pacific area (IND/PAC), with sequences referenced as *T. violaceus* probably misidentified and corresponding to *T. gracilis*.

4. Discussion

Considering the paucity of data for blanket octopus *Tremoctopus violaceus* throughout its distribution area, it remains unclear how rare these octopods are. Judging by the recent increase in the number of records both in the Mediterranean and the Adriatic Sea, it may not be a case of rarity, but a case of the probability of encounter. In fact, after the catch of specimens analyzed in this study, about twenty additional individuals were observed by one of the authors (R.S.) in the fishery landings from the same area in the same period. Pelagic octopods are found exclusively in the surface layer of the sea, therefore not collected by trawling, but rather by pelagic fishing gears, i.e., purse seiners, as is the case with our specimens. According to Bello [40], the assessment of the structure of the oceanic community is highly dependent on the selectivity of the fishing gear, and there are severe limitations in collecting pelagic cephalopods by nets. Results from the midwater trawl indicate that adults and subadults of some cephalopods are very rare, while juveniles are much more numerous. However, studies of large predator diets demonstrate the opposite [19,41,42]. Romero et al. [41] recorded 81 individuals of *T. violaceus* from the stomach contents of large fish predators (mainly blue-fin tuna) in the Central Mediterranean Sea (Southern Tyrrhenian Sea and Strait of Messina) between 2002 and 2008, suggesting that these pelagic octopods are present and not so rare in the Mediterranean basin. Additionally, our specimens of *T. violaceus* caught in purse seine would likely go unnoticed if they were not identified during the landing of the catch by one of the authors, who is familiar with the species and its rarity, indicating that they would likely be overlooked through regular fishing operations.

In this study, we investigated four female specimens of blanket octopus *T. violaceus* in the Central Eastern Adriatic Sea. The presented external and internal morphological and morphometric characteristics complement available data from the specimens found in the Adriatic [8,17,20], Mediterranean [8,11–14] and Atlantic waters [3,9,43]. Most papers report the findings of only one female, usually accidentally caught or found stranded on shore, although sightings of more animals are often observed by fishermen. Biagi and Bertozzi [11] reported that every night during 20 days of fishing activity, an average of 10 to 20 sub-adult females of *T. violaceus* were caught, but unfortunately, the authors came into possession of only one individual. Interestingly, males of *T. violaceus* have never been recorded in the Adriatic and Mediterranean Seas. From these waters, the smallest reported individual measured 33 mm DML from the Adriatic [20], and the largest 165 mm DML was found in an octopus trap at a depth of about 6 m in the Mediterranean waters [8]. Dorsal mantle lengths of our analyzed individuals were 113, 82, 80 and 78 mm, which is in a similar range to previous records [8,9,12,13,43].

Previous diet studies of *T. violaceus* report on a variety of items, such as fish scales, cephalopod flesh, polychaete jaws, algae and *Posidonia* [1,3,8,13]. Of these, our specimens had only fish scales and polychaete jaws. Unlike the findings of Agus et al. [8] and Quetlas et al. [13], who reported *Posidonia* and algae in the stomachs of blanket octopus from the Mediterranean area, our specimens were not strandings and were actively caught. As Quetlas et al. [13] suggested, remains such as algae and *Posidonia* could have been accidentally ingested by the moribund specimens, which could explain this difference. Regarding maturity stages, the results of this study are congruent with previous reports, indicating that females above 100 mm DML are always sexually mature. According to Laptikovsky and Salman [12], *T. violaceus* is an intermittent terminal spawner, which lays eggs during the first 1–2 weeks of the 0.5–1 month-long spawning period.

Since *T. violaceus* is mostly reported during the summer months, Bello [19] suggested that the species occurrence in the Adriatic waters could be related to hydrological properties, namely extreme seasonal temperatures. During the winter, the Adriatic's cold surface water prevents warm water species from entering the basin, unlike during the summer when increased surface layer temperature (above 25 °C) favors the spread of thermophilic organisms. *Tremoctopus violaceus* is an epipelagic species that does not descend below the thermocline [3,44]. The presence of *T. violaceus* in the Adriatic Sea could also be linked to the bimodal oscillating system (BiOS) that occurs in the Northern Ionian Sea, and influences thermohaline oscillations of the Adriatic-Ionian system. Namely, this system influences the hydrographic properties of the Adriatic water masses through a complex oscillating system characterized by cyclonic and anticyclonic regimes, which in certain periods favor waters of Atlantic and Levantine origin, respectively. These circulation regimes could influence periodic biodiversity patterns also reflected through the appearance of some rare species [45,46].

Voight [47] pointed out that the rarity of specimens of extant taxa is a significant limiting factor in understanding octopod evolution. Cephalopod systematics is significantly based on morphometric variables (i.e., counts, indices) due to the lack of hard structures, though they are seldom diagnostic and species-specific [48]. Recently, cephalopod studies emphasized the importance of combining DNA barcoding-based identification with careful morphological analysis [49,50]. *Tremoctopus violaceus* and *T. gracilis* are morphologically similar; in fact, Jiménez-Badillo et al. [9] molecularly identified their Atlantic specimen as *T. violaceus*, stating that the individual fully conformed morphologically to the description by Orsi Relini [51]. Unfortunately, at the moment there are not enough collected specimens to support strong morphometric analysis in combination with DNA identification. The results of our study complement existing data, and provide valuable molecular 16S and COI sequences to support species identification in the genus *Tremoctopus*. Our phylogenetic analysis showed tree topology with three highly supported clades within the genus *Tremoctopus* and further consolidated the findings of Agus et al. [8] and Jiménez-Badillo et al. [9].

Based on currently available sequences, all specimens collected in the Indo-Pacific area clustered under *T. gracilis* with strong support. It seems that sequences MW025168, MN443917, KY649286, MZ043857, MN435565, AJ252767 and AF377978 deposited under the name *T. violaceus* are misidentified and are in fact *T. gracilis*. Following the '10 times rule' proposed by Hebert et al. [52], our calculated COI intraspecific genetic distances were over ten times smaller than interspecific distances between *T. violaceus* and *T. gracilis*, delineated as proposed by our phylogenetic analysis. Separation of Indo-Pacific specimens as different species, i.e., *T. gracilis*, is thus supported, similarly to the conclusions of Agus et al. [8]. Moreover, Gebhardt and Knebelsberger [53] reported that intraspecific distances calculated as uncorrected p-distances in cephalopods are usually below 3%.

So far, *T. gracilis* in the Mediterranean was identified exclusively based on morphology and record reports [5,6]. Interestingly, GenBank sequence AF377978, misidentified as *T. violaceus*, lacks information for a geographical locality, but according to the locale/voucher data in Carlini et al. [39], the specimen originates from the Atlantic Ocean (Key Biscayne, Florida). Therefore, if the location data of this specimen is correct, it indicates the presence of *T. gracilis* in Atlantic waters. From there this species might have entered the Mediterranean basin through the Strait of Gibraltar, explaining the above-mentioned records of *T. gracilis* in the Mediterranean. Another possible migration route for *T. gracilis* into the Mediterranean could be through the Suez Canal from the Red Sea or via ballast waters. However, present molecular evidence does not support *T. gracilis* occurrence in the Mediterranean. As Jiménez-Badillo et al. [9] stated, there is a need for more studies considering interspecific and intraspecific geographic dispersion to solve the molecular phylogeny of the *Tremoctopus* genus. Therefore, to clarify the alien nature of *T. gracilis* in the Mediterranean and Adriatic Sea, future efforts should focus on increasing data quantity, i.e., the number of species sampled and the number of specimens per species, with combined analysis of morphological and genetic data.

An important factor in DNA identification is the choice of genetic markers, as well as the choice of primer pairs. Ideal primers are responsible for the high efficiency and specificity of the amplification reaction, which will ensure good amplicon quality [54]. Universal Metazoan COI primers LCO1490 and HCO2198 [25] have been shown to have great versatility and applicability; however, they do not seem to perform well for this octopodid squid. Agus et al. [8] reported problems with PCR amplification and sequencing of the COI gene from *T. violaceus*, assigning it to poor DNA quality due to poor tissue preservation and ending with only one good sequence out of three possible. The authors also suggested the region might contain a pseudogene complicating its analyses. We have experienced similar difficulties with using COI LCO1490 and HCO2198 primers, and finally opted for another pair, LCO1490 [25] and H7005 [26]. Using a degenerate primer (H7005) might help amplify regions of greater divergence, which seems to be the case for *T. violaceus*. The latter combination efficiently amplified the target region with very little prior optimization. Obtained sequences were of very good quality, and easily translated to amino acid sequences. In addition, the target amplicon overlaps with the standard barcoding area (same forward primer), and covers a larger region (cca 1000 bases), providing more informative sites for phylogenetic analyses and genetic diversity estimations. We recommend this primer combination for the COI metabarcoding of *Tremoctopus* species.

To fully understand the life-history traits of *Tremoctopus* species, more studies are needed, even if these are based on very small sample sizes or even single specimens, which would allow more informative meta-analyses in the future. All available resources must be used, and data integrated through citizen science initiatives, collaborations with fishermen, local people, students, media and scientific investigators to help build information funds on pelagic and deep-sea cephalopods. Videos and photos of these animals often appear in the media, and from the published literature, it is obvious that fishermen occasionally encounter them during commercial fishing. Therefore, education on sampling procedures,

reporting and supplying preparatory kits for tissue sampling would help in collecting valuable information that would clarify their lifestyle, or at least taxonomy.

In conclusion, the results of this study provide much-needed reference for the taxonomy and biodiversity of pelagic cephalopod fauna. Prior to this study, there were only 3 sequences of *T. violaceus* in Genbank of Mediterranean origin for the 16S rRNA gene (1 Adriatic), and only one COI sequence of Adriatic/Mediterranean origin, meaning this adds valuable knowledge for future species identification and taxonomy. When dealing with rare species, every record is important and contributes to general knowledge of species biology. Additionally, some aspects of this species distribution and identification are becoming clarified and supported with genetic evidence. Future research should focus on DNA-based methods (molecular information) for correct species identification, combined with morphological characters, geographic distribution and ecological information.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15060794/s1, Table S1: Records of *Tremoctopus violaceus* in the Adriatic Sea; Table S2: List of GenBank Accession Numbers of *Tremoctopus* genus sequences used in this study; Table S3: Pairwise K2P genetic distances between 16S sequences of the genus *Tremoctopus*; and Table S4: Pairwise K2P genetic distances between COI sequences of the genus *Tremoctopus*.

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