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Dolioletta advena sp. nov., a New Species of Doliolid (Tunicata, Thaliacea) from the Adriatic Sea

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Abstract: The Adriatic Sea, as a part of Mediterranean, is one of the best investigated areas in the world regarding zooplankton. Nevertheless, in the last decade four new species of gelatinous zooplankton were described from the Adriatic Sea. Whether these species are newcomers or they were simply overlooked is still under investigations. Here we provide a description of a new species of Doliolida from the genus *Dolioletta*, *Dolioletta advena* sp. nov., found in the Adriatic Sea in August 2021, in a period of high sea temperatures and salinities, suggesting its thermal predilection. Its blastozooids dominated the studied doliolid blastozooid communities in the South Adriatic, except in the 50–100 m depth layer at a coastal Lokrum station. Blastozooids of *D. advena* sp. nov. possess unique morphological features which easily distinguish it from other doliolid species: the most prominent feature being the curved digestive tract where the intestine does not form a tight coil as in other *Dolioletta* species. The placement of this species in the genus *Dolioletta* is corroborated by COI phylogenetic analysis which showed that *D. advena* sp. nov. forms a well-supported monophyletic clade with *Dolioletta gegenbauri* (81% bootstrap support). In addition to *D. advena* sp. nov. COI sequence, we provide COI sequences of five doliolid and one pyrosomatid species, which will greatly improve the availability of thaliacean sequences for metabarcoding studies. The origin of *D. advena* sp. nov. is unknown, but given the fact that doliolids are well investigated in the Mediterranean Sea, it is likely that it arrived with sea currents either through the Suez Canal or the Strait of Gibraltar. Future investigations will confirm or reject this hypothesis.

Keywords: new species; *Dolioletta advena* sp. nov.; Doliolida; Thaliacea; Adriatic Sea; Mediterranean

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1. Introduction

The class Thaliacea consists of the following three orders of holoplanktonic tunicates: Doliolida, Pyrosomatida and Salpida. Doliolids possess complex life cycle with alternations between sexual and asexual generations and inhabit mostly warm and temperate waters with cosmopolitan distribution [1]. Historically they have been most extensively studied in the Mediterranean and the southeastern continental shelf of the USA [1]. Despite large amount of research on doliolids in the Mediterranean (e.g., [2–8]), doliolids in the Adriatic have been relatively poorly investigated with few published papers scattered throughout the last one-hundred years [9–16]. The Adriatic itself is a semi-enclosed sea which is defined by large variations in depth, yearly sea temperature and zooplankton species abundance and richness between its northern and southern parts [13,17–20]. It exhibits complex physical dynamics which shape plankton community dynamics. In winter the Adriatic is under the influence of strong bura winds which cause cooling and vertical convection at the basin scale, reaching up to more than 600 m depth in its southern part [20]. The wind-driven vertical convection in winter triggers a spring plankton bloom [20–22]. On a decadal scale, thermohaline properties of the Adriatic are modified by the BiOS mechanism (Bimodal Oscillating System). BiOS is a phenomenon in which the changes in the direction of circulation in the North Ionian Gyre (NIG) determine the prevailing type of water entering the Adriatic. During its cyclonic phase the NIG brings more saline and warmer waters from the East Mediterranean and during its anticyclonic phase it brings less salty and colder waters from the West Mediterranean into the Adriatic [23,24]. In recent decades it has been shown that advection of allochthonous gelatinous zooplankton species into the Adriatic is also under the influence of the Bimodal Oscillating System (BiOS) [25]. The phase of the BiOS not only determines the prevailing origin of allochthonous species (either East or West Mediterranean) entering the Adriatic, but it also influences the abundance contribution of already established allochthonous species within their respective taxonomic groups at the yearly level [25]. In addition to decadal variations in temperature and salinity due to the BiOS, there is a constant upward trend of both parameters in the Adriatic due to climate change [21,26], favoring the establishment of thermophilic species [25,27,28].

Genetic investigations of doliolids have been rare and only three species have been sequenced so far [29–34], with only the *Doliolum nationalis* COI sequence published [30]. The scarcity of doliolid barcoding sequences largely hampered metabarcoding studies of doliolid distribution and diversity [35].

Here we present a description of *Dolioletta advena* sp. nov., a first doliolid species described in the last 17 years [36] and the first described doliolid epipelagic species in more than 50 years. *Dolioletta advena* sp. nov. was found in August 2021 at a coastal as well at an open sea station in the South Adriatic. The species is morphologically as well as genetically distinct with the shape of its digestive tract as its most prominent feature.

2. Materials and Methods

Dolioletta advena sp. nov. individuals were collected at two stations in the South Adriatic: a coastal Lokrum station (42°37′21″ N, 18°06′05″ E) of 100 m maximum depth on 18 August 2021 and an open sea station P-1200 (42°13′01″ N, 17°42′50″ E) of 1200 m maximum depth on 23 August 2021 (Figure 1). The temperature and salinity of the water column at both stations were measured using a Sea-Bird SeaCAT SBE 19plus instrument (Sea-Bird Scientific, Bellevue, WA, USA). Zooplankton at the Lokrum station was sampled using a 200-µm closing Nansen net (56.5 cm diameter, 340 cm length) in two layers, 0–50 m and 50–90 m, while at station P-1200 zooplankton was sampled with a larger 200-µm Nansen net (113 cm diameter and 450 cm length) in one layer, 0–100 m. Samples were taken in duplicate tows. Zooplankton from one tow was put in 10% formalin-seawater solution, for the zooplankton composition analysis, and zooplankton from the other tow was put in 96% ethanol for a genetic analysis after straining over 53-µm mesh in order to remove seawater. The ethanol samples were stored at −20 °C for long-term storage. Formalin-fixed zooplankton samples were processed under an Olympus SZX-16 stereomicroscope (Tokyo, Japan) in order to determine doliolid abundance and community composition. The abundance of doliolids was expressed as individuals per cubic meter, ind. m^{-3}. For the morphological analysis, the collected organisms were stained with a few drops of 0.1% Janus Green B dye (Sigma-Aldrich, St. Louis, MO, USA) dissolved in seawater [12] until a sufficient level of coloration had been achieved. After dyeing, the animals were removed from the dyeing solution and placed in clear 10% formalin-seawater solution. In order to collect various doliolid and pyrosomatid species for phylogenetic reconstruction, opportunistic samplings were performed on 8 April 2016 (P-1200), 16 April 2021 (Lokrum), 16 September 2021 (Lokrum) and on 18 October 2021 (Table 1).

DNA was isolated from whole animals fixed in 96% ethanol. Each animal was photographed before DNA isolation. After removing ethanol, individual animals were put in acetone to speed up the drying process and were subsequently dried at 55 °C for half an hour. DNA was isolated and purified using the ammonium-acetate procedure followed by isopropanol precipitation. The DNA isolation procedure and purification is described in detail in Garić and Batistić, 2022 [37]. The resulting DNA pellet was dissolved in 40 µL of TE buffer.

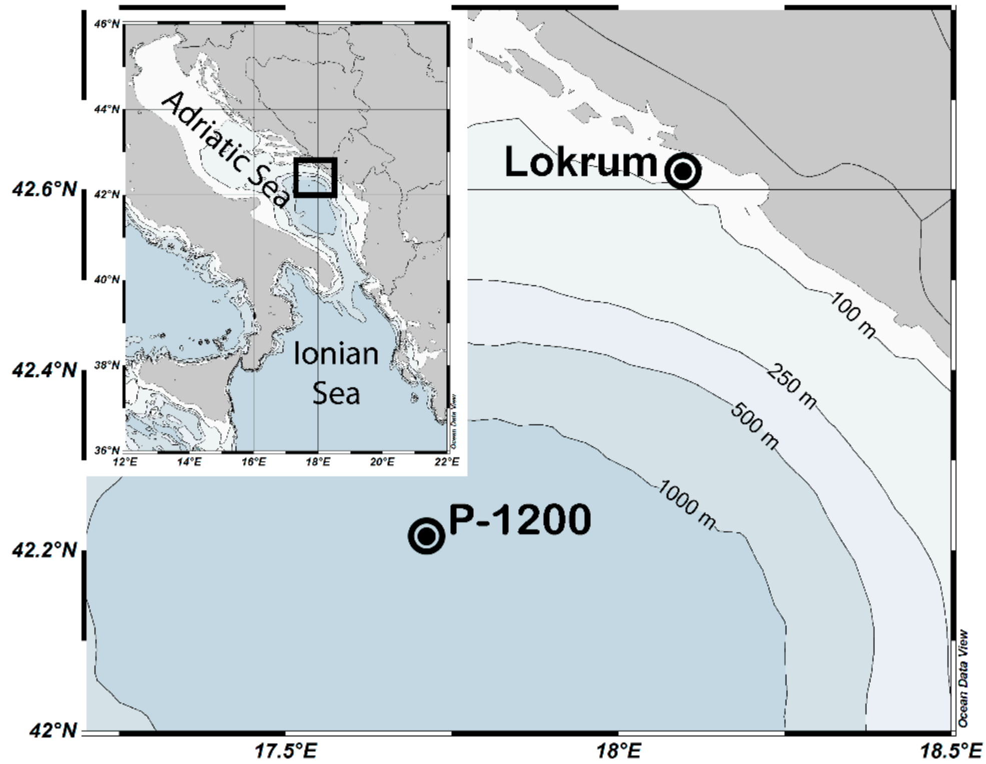

Figure 1. Position of stations Lokrum (42°37′21″ N, 18°06′05″ E) and P-1200 (42°13′01″ N, 17°42′50″ E) in the South Adriatic.

Table 1. List of sequenced pyrosomatid and doliolid species, GenBank accession numbers of their respective COI sequences, date of collection of animals, stations at which they were collected and primers used for COI amplification.

Species	Isolate	GenBank Acession	Date of Collection (DD-MM-YYYY)	Station	Forward COI Primer	Reverse COI Primer
Pyrosoma atlanticum	pyrver	OP437488	08-04-2016	P-1200	coi-70f	od1140coir
Dolioletta advena sp. nov.	gega	OP437493	23-08-2021		dollco	dol1150coir
	gegb	OP437492	23-08-2021			
	dcgen	OP437494	18-08-2021	Lokrum		
Dolioletta gegenbauri	gegpr2	OP437491	16-09-2021			
Doliolina krohni	dkroh	OP437490	18-08-2021			
Doliolina muelleri	dcfmul1	OP437489	18-08-2021			
Doliolum denticulatum	dolden11	OP437487	16-04-2021			
Doliolum nationalis	dolnat11	OP437495	18-10-2021			jgHCO2198

A fragment of mitochondrial cytochrome oxidase subunit I (COI) was amplified from five sequenced species using newly designed primer pair dollco (5′-TTTTCTACGAATCATAAAGATATCAG-3′) and dol1150coir (5′-TGAGCTACCACGTAGTAGGTATCATG-3′) (Table 1). This primer pair was designed to have an identical sequence to the corresponding portion of the COI gene sequence of *Doliolum nationalis* (accession number AB176541) published by Yokobori et al. [30]. The position of the forward dollco primer was selected to correspond to the position of Folmer's LCO1490 primer [38], while the position of the reverse dol1150coir primer was selected to be at the position of about 1100 nucleotides downstream, which is a fairly conserved position within COI detected by aligning

Doliolum nationalis COI sequence (accession number AB176541) [30] with *Salpa fusiformis* (accession number LC333181) [39] and *Salpa thompsoni* (accession number LC333180) [39] COI sequences. In addition to the dollco and doll1150coir primer pair, we designed the reverse primer od1140coir (5′-GCGACAACATAGTAGGTATCATG-3′) as a variant of primer dol1150coir in order to target a wider range of species. The theoretical annealing temperature of newly designed primers was calculated using Oligo Analyzer 1.0.3 software (Freeware, Teemu Kuulasmaa, Finland). They were also checked for likelihood of hairpin structures and dimer formation using the same software. For COI amplification of species *Pyrosoma atlanticum*, primers coi-70f (5′-ATGTCTACTAATCATAAAGATATT-3′) [12] and od1140coir were used, while for species *Doliolum nationalis* primers dollco and jgHCO2198 (5′-TAIACYTCIGGRTGICCRAARAAYCA-3′) [40] were used (Table 1). PCR was performed in 25 μL of a mix containing 1× PCR buffer, 0.2 mM of each dNTP, 3 mM $MgCl_2$, 0.2 μM of each primer, 1.2 U of Taq polymerase (NEB #M0273) and 0.5 μL of template DNA. All amplifications were performed using the same PCR program, as follows: 2 min denaturation step at 94 °C, followed by 40 cycles of 94 °C for 15 s, 45 °C for 1 min, 68 °C for 2 min and a final extension step at 68 °C for 5 min. PCR products were sequenced from both directions by the company Macrogen (Amsterdam, The Netherlands) with the same primers as used for PCR. The obtained sequences were assembled in BioEdit (Ibis Biosciences, Carlsbad, CA, USA) [41]. All obtained sequences were translated using the ascidian mitochondrial genetic code in order to check for stop codons in order to detect possible pseudogenes and were then deposited at GenBank (Table 1).

A total of 13 COI sequences belonging to 12 thaliacean species (6 doliolid species, one pyrosomatid and 5 salpid species) were aligned using the ClustalW sequence alignment software with default options [42]. The resulting alignment length (Alignment S1 in Supplementary Material) was 620 bases long. Phylogenetic analysis was performed using MegaX software [43] and on all codon positions. The in-software option for optimal model selection was used to determine the optimal model of evolution. The maximum likelihood method was used for phylogenetic reconstruction with the Hasegawa–Kishino–Yano model with 4 discrete gamma categories as the optimal model of evolution. The resulting tree was tested with 1000 bootstrap replicates.

3. Results

3.1. Temperature and Salinity at Stations P-1200 and Lokrum

In August 2021, the water column was stratified at both stations with a pronounced thermocline at about 20 m (Figure 2). The thermocline was slightly deeper at the Lokrum station than at station P-1200. Above 20 m depth, the average temperature at station P-1200 was 25.60 °C and at station Lokrum it was 26.74 °C. The average salinity was 39.15 and 38.66 PSU, respectively. Maximum temperatures were 27.80 at P-1200 at the surface and 26.73 °C at 2 m depth at Lokrum station. Maximum salinities were recorded at the surface at P-1200 being 39.32 PSU. At the Lokrum station, the maximum of salinity was in the layer from 49 to 53 m depth being 39.01 PSU. In the layer from the surface to 50 m depth, the average temperature at station P-1200 was 20.48 and at station Lokrum it was 21.14 °C. The salinities in the same layer were 39.00 and 38.92 PSU, respectively. In the layer from 50 to 95 m depth the temperature and salinity at both stations were similar and fairly constant: 15.15 °C and 38.96 PSU at station P-1200 and 15.72 °C and 38.99 PSU at the Lokrum station.

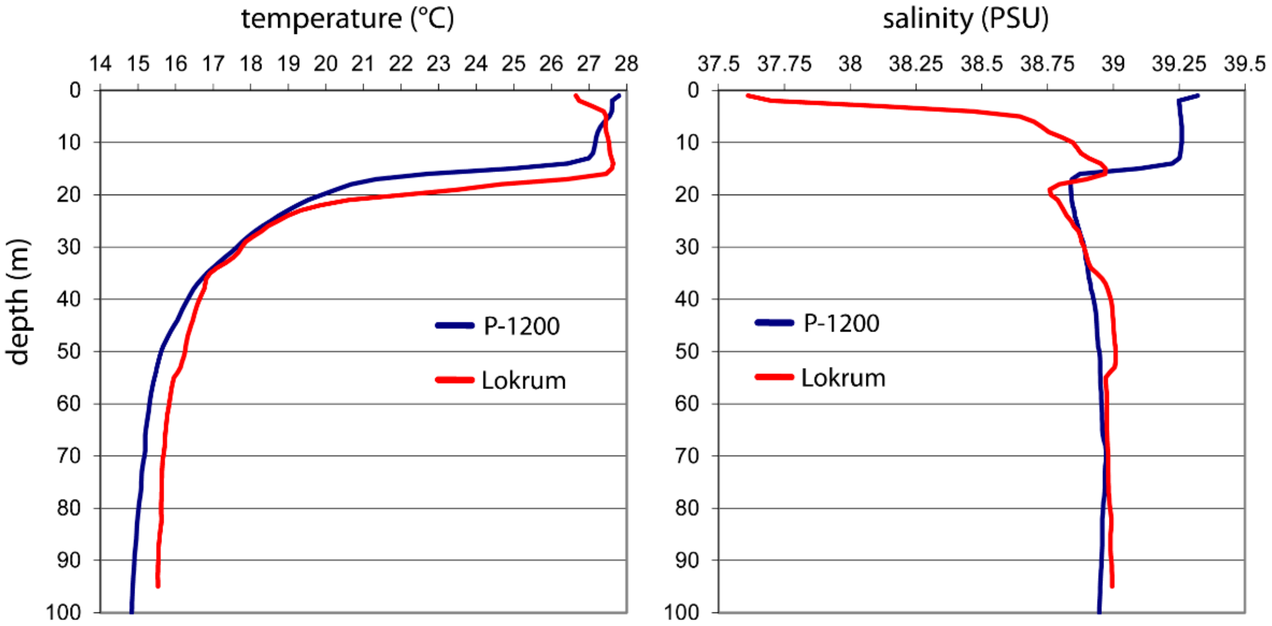

Figure 2. Temperature and salinity at stations P-1200 and Lokrum in August 2021.

3.2. Abundance and Composition of Doliolid Communities at Stations P-1200 and Lokrum

In August 2021, at station P-1200 in the 0–100 m depth layer, blastozooids of only *Dolioletta advena* sp. nov. and *Doliolina krohni* were present, the latter being in very low numbers, 0.01 ind. m^{-3} (Table 2). At the Lokrum station, blastozooids of *Dolioletta advena* sp. nov. were the most abundant blastozooids in the 0–50 m depth layer, 2.24 ind. m^{-3}, and *Doliolina muelleri* blastozooids were in low numbers, 0.32 ind. m^{-3}. In the 50–90 m depth layer, blastozooids of *Doliolina muelleri* were abundant, 2.80 ind. m^{-3}, while *Dolioletta advena* sp. nov. blastozooids were in low numbers, 0.80 ind. m^{-3}. *Dolioletta* nurses and oozooids were present at all depths and stations, while *Doliolina* nurses and oozooids were present only at the Lokrum station, 50–90 m depth layer, and in high abundance, 13.60 ind. m^{-3}, coinciding with the high abundance of *Doliolina muelleri* blastozooids.

Table 2. Abundance and species composition of doliolids at stations P-1200 (on 23 August 2021) and Lokrum (on 18 August 2021). Abundances are expressed as number of individuals per m^3.

Stations	**P-1200**	**Lokrum**	
Layers / **Species**	**0–100 m**	**0–50 m**	**50–90 m**
Dolioletta advena sp. nov. blastozooids	0.80	2.24	0.80
Dolioletta sp. oozooids + nurses	0.54	0.48	1.60
Doliolina krohni blastozooids	0.01	0	0.80
Doliolina muelleri blastozooids	0	0.32	2.80
Doliolina sp. oozooids + nurses	0	0	13.60
Doliolida larvae	0	0	3.20
Doliolida ova	0	1.28	0
Doliolida unidentified	0.38	0	0

3.3. DNA Analysis

DNA was isolated and the COI gene fragment was amplified and sequenced from three individuals of *Dolioletta advena* sp. nov., one gonozooid and two phorozooids (Table 1, Figure 3), and one blastozooid of each *Dolioletta gegenbauri*, *Doliolum nationalis*, *Doliolum denticulatum*, *Doliolina muelleri*, *Doliolina krohni* and *Pyrosoma atlanticum* (Table 1).

The three obtained COI sequences of *D. advena* sp. nov. were 1057 nucleotides long and they were highly similar, but they were not identical. The percentage difference between isolates gega and gegb was highest, being only 0.85%.

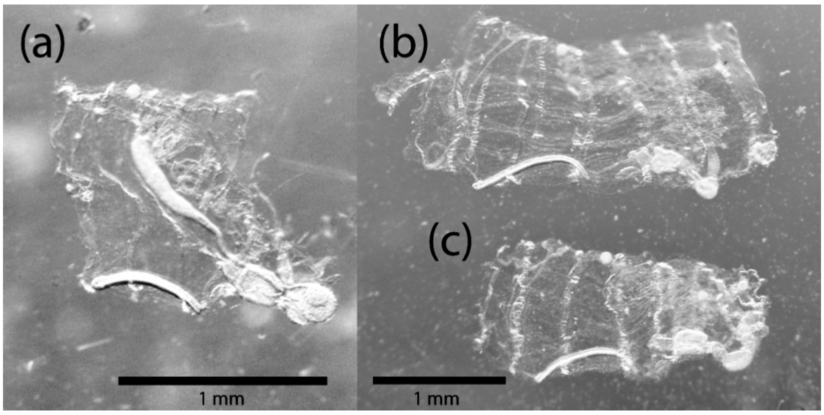

Figure 3. Photographs of *Dolioletta advena* sp. nov. individuals from which DNA was isolated and COI sequences were obtained. (**a**) gonozooid, isolate dcgen, (**b**) phorozooid, isolate gega, (**c**) phorozooid, isolate gegb.

Phylogenetic reconstruction showed that doliolids formed well-supported clade (94% bootstrap support). *Doliolina* as a monophyletic genus was not supported, while *Doliolum nationalis* and *Doliolum denticulatum* formed a well-supported monophyletic group (78% bootstrap support), as well as *D. advena* sp. nov. and *Dolioletta gegenbauri* (81% bootstrap support) (Figure 4).

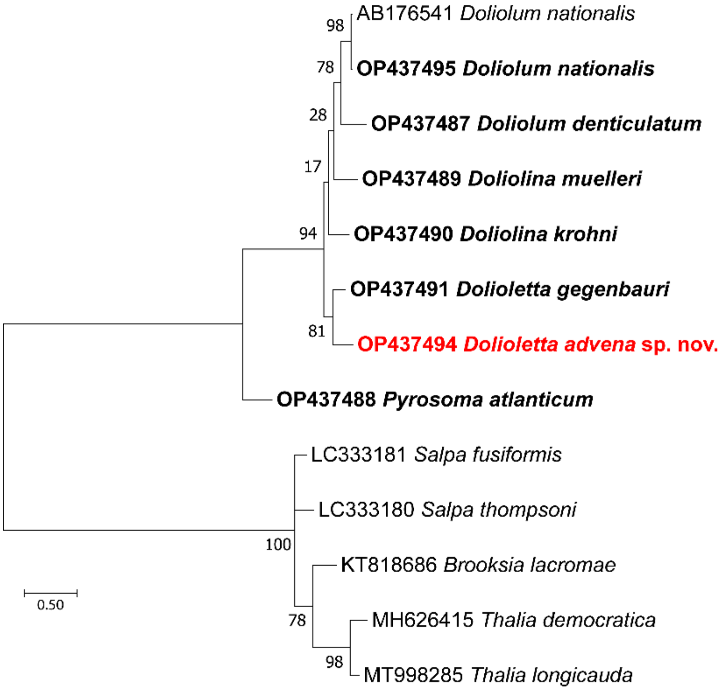

Figure 4. Doliolid COI phylogenetic ML tree with the position of *Dolioletta advena* sp. nov. (in red). Five salpid sequences are used as the outgroup. Names in bold represent species sequenced during this investigation, while all other sequences are obtained from GenBank. Numbers next to nodes represent the percentage of bootstrap support for respective nodes.

3.4. Species Description

Class: Thaliacea Van der Haeven, 1850

Order: Doliolida Delage & Hérouard, 1898
Suborder: Doliolidina Godeaux, 1996
Family: Doliolidae Bronn, 1862
Genus: *Dolioletta* Garstang, 1933
Dolioletta advena sp. nov. Garić & Batistić
Figures 3, 5 and 6.

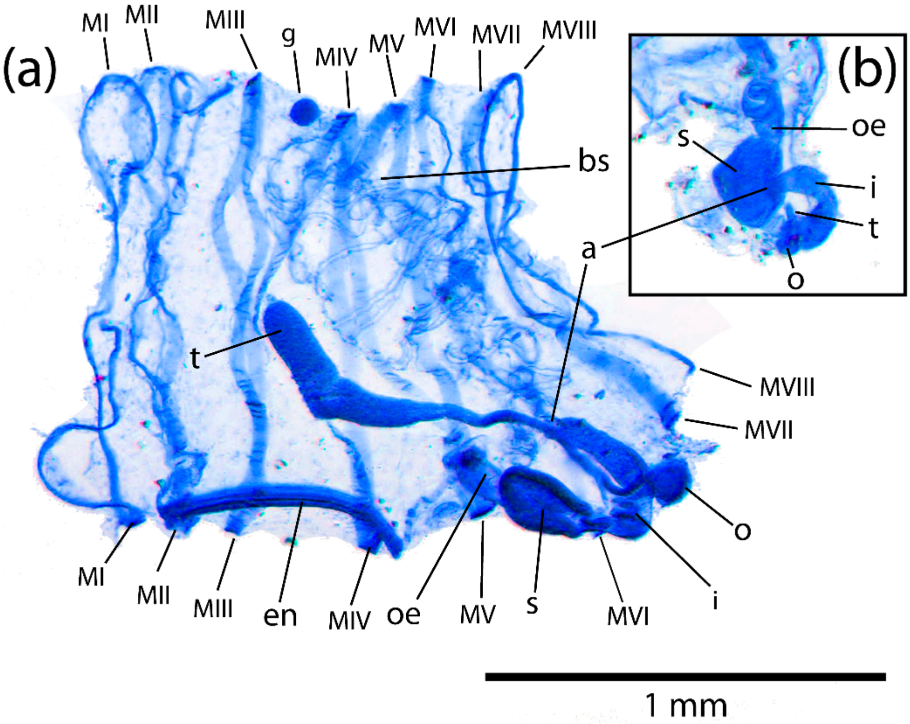

Figure 5. (**a**) Photograph of Janus Green B stained holotype of *Dolioletta advena* sp. nov. (**b**) Dorsal view of unrelated Janus Green B stained individual of *D. advena* sp. nov. which was dissected so that digestive system is exposed. MI to MVIII—muscle bands, en—endostyle, g—ganglion, t—testis, o—ovary, bs—branchial septum, oe—oesophagus, s—stomach, i—intestine, a—anal aperture.

Material examined: 10 gonozooids and 86 phorozooids.

Holotype: Gonozooid 1.41 mm in length with developed gonads. Holotype is deposited in Natural History Museum Dubrovnik under inventory number: PMD-2415.

Publication LSID: http://zoobank.org/56A31891-1124-4369-A2A8-60DAF74B9D21

Nomenclature act LSID:http://zoobank.org/331F5A65-5D05-4675-AB6D-EA8CEB2A9E37

Diagnosis: *Dolioletta advena* sp. nov. does not possess a tightly coiled intestine, which so far has been a unique feature of the *Dolioletta* species. Instead, after forming a right turn, the intestine sharply turns left so that the anal aperture is facing the left body wall (Figures 5 and 6). The position of the ganglion in *D. advena* sp. nov. is between MIII and MIV, but close to MIV, unlike other *Dolioletta* species where the ganglion is positioned close to MIII or midway between MIII and MIV as in *Dolioletta mirabilis* (Figure 6). The branchial septum reaches the ganglion dorsally, as in other *Dolioletta* species, while ventrally it reaches MV, or slightly anteriorly. The endostyle in blastozooids of *D. advena* sp. nov. extends from MII 2/3 to MIV 1/3, sometimes reaching MII anteriorly, which is similar to other *Dolioletta* species (Figure 6). In gonozooids of *D. advena* sp. nov., the ovary is round, while the testis is hockey-stick shaped, pointing dorsally. The testis reaches MIV, but it does not seem to surpass it (Figure 5), which is a unique characteristic of this species. Gonozooids of species *Dolioletta gegenbauri* and *Dolioletta tritonis* possess testis which surpasses MII, while *Dolioletta mirabilis* and *Dolioletta valdivie* possess coiled testis (Figure 6). The size of the holotype was 1.41 mm (gonozooid), while the maximum recorded size of a gonozooid was 1.95 mm. Maximum recorded size of a phorozooid was 2.05 mm. Four individuals were examined for the number of gill slits which varied between 17 and 19.

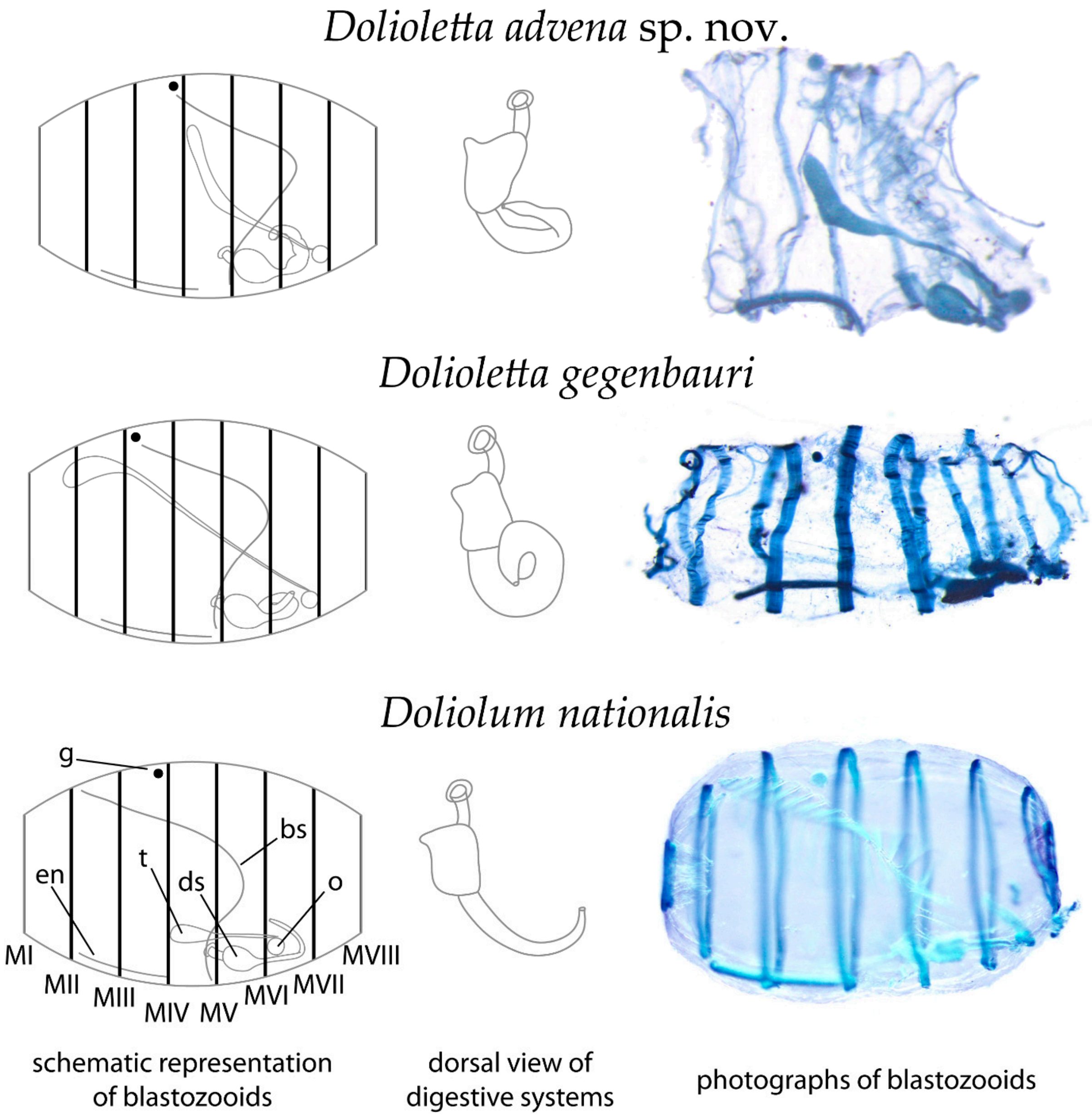

Figure 6. Schematic representation of blastozooids of three doliolid species, *Dolioletta advena* sp. nov. (holotype), *Dolioletta gegenbauri* and *Doliolum nationalis*, ventral view of their respective digestive systems and photographs of Janus Green stained blastozooids. MI to MVIII—muscle bands, en—endostyle, g—ganglion, t—testis, ds—digestive system, bs—branchial septum, o—ovary.

Etymology: *Dolioletta advena* sp. nov. is named based on its possible foreign origin in the Adriatic, advenus in Latin meaning stranger or foreigner.

Remarks: Due to the unique shape of the intestine in *Dolioletta advena* sp. nov., it does not conform to the diagnoses of genus *Dolioletta* published by previous authors [44,45], nor to any existing genus. We do not believe that this warrants creation of a new genus for this species. Therefore, we decided to place it in the genus *Dolioletta* based on genetic evidences as well as on the position of branchial septum which reaches ganglion dorsally. The position of the branchial septum in relation to the ganglion is a determining feature of genus *Dolioletta* so far not recognized by previous authors. The diagnoses of the genus

Dolioletta provided by Garstang (1933) [44] (alimentary canal forming a close dextral coil in the middle of the cloacal floor with median anus) and Godeaux (2003) [45] (coiled digestive tube, variable length of endostyle, brain behind M III, branchial septum extending between M II and M IV, tubular testis of various shapes), should be amended in the following way: alimentary canal forming a close dextral coil or it makes $\frac{3}{4}$ right turn so that the anal aperture is facing the left body wall, branchial septum dorsally reaching ganglion.

4. Discussion

Doliolids are a group with a relatively low number of species. According to WoRMS there have been so far only 24 doliolid species known to science [46]. With the description of *Dolioletta advena* sp. nov., the number of known doliolid species has grown to 25. There have been eight doliolid species so far recorded in the Mediterranean, as follows, with *Dolioletta advena* sp. nov. the ninth: *Doliolum nationalis*, *Doliolum denticulatum*, *Doliolina muelleri*, *Doliolina krohni*, *Doliolina intermedia*, *Dolioloides rarum*, *Doliopsis rubescens* and *Dolioletta gegenbauri* [45]. Species *Dolioloides rarum* was considered to be probably extinct in the Mediterranean by Godeaux [45,47], because, according to him, it had not been recorded since the work of Sigl (1912) [10] from the early years of the 20th century. Conversely, Katavić [9] recorded *Dolioloides rarum* in the Adriatic in the mid-70s [9], and it has been found in the Adriatic again in 2014 by Garić and Batistić (2016) [12]. *Doliopsis rubescens* is a species that was never recorded in the Adriatic, but it was abundant in the Mediterranean until the beginning of the 20th century [45,47]. Afterwards, it was never recorded again in the Mediterranean [45,47]. *Doliolina intermedium* is known in the Mediterranean only from nurses as reported by Godeaux [4,5]. Godeaux considered this finding uncertain since only blastozooids of this species were found [47]. Nevertheless this species was included in later Mediterranean doliolid checklists [1,45,48]. *Dolioletta advena* sp. nov. does not possess the typical tightly-coiled intestine which has been so far the main distinguishing character of the genus *Dolioletta*, nevertheless, the position of the branchial septum in relation to the ganglion, as well as COI phylogenetic reconstruction led us to conclude that it is indeed a member of genus *Dolioletta* and does not warrant a separate genus.

Dolioletta advena sp. nov. was found in the South Adriatic from the shore to the open sea in August 2021. Its peak of abundance was in the top 50 m at the coastal station where the temperature was over 21 °C. The distribution and abundance of *D. advena* sp. nov. suggests that it is a thermophilic species. Its highest abundance was recorded at the Lokrum station in the 0–50 m layer, which would indicate that it is an epipelagic species that prefers inshore waters. Due to the low number of samples examined, future investigations are needed to confirm these indications. One of the big challenges when new or allochthonous species are found is identifying the origin of newcomers. The task is hampered by scarce distribution data due to the limited number of studies and the overall lack of taxonomists, especially from the Indian Ocean. For species which are present in the Atlantic as well as in the Mediterranean, the route of arrival could be inferred using genetic methods if genetic structuring between the basins is present. For a genetic approach to be successful, there should be a good coverage of barcoded taxa from multiple basins. At this point only 31% of all copepods are barcoded [35] and with this study the percentage of doliolid COI barcoded species has risen from 4 to 24%. The last possibility, especially for fragile gelatinous taxa, is that the species has simply been overlooked by lack of effort, expertise or inadequate sampling methods as in the case of *Fritillaria ragusina* [49]. For the Adriatic, the scarcity of data on doliolids [9–16] is compensated by extensive investigations in the Mediterranean [2–8]. Therefore, it is likely that *D. advena* sp. nov. would have been found by earlier researchers had it been present in the Mediterranean, especially bearing in mind its morphological distinctness and its ease of identification. This points to the possibility that it arrived in the Adriatic, and probably the Mediterranean, from either the Atlantic, through the Straits of Gibraltar, or the Red Sea, through the Suez Canal. The high salinities at the time of collection, in excess of 39 PSU, could indicate that in the year 2021 a cyclonic regime of BiOS was in place, which could indicate that *D. advena* sp. nov. arrived from the East Mediterranean into the Adriatic, hence favouring the Red Sea origin hypothesis. Until more researches are conducted, the origin of this species is speculative. *D. advena* sp. nov. is a new addition to a streak of new gelatinous species for science described from the Adriatic Sea in little more than a decade [12,37,49,50]. For a well-researched sea such as the Adriatic, as a part of the Mediterranean, five newly described species of gelatinous zooplankton in little more than a decade, out of which two are quite large scyphomedusae, is somewhat unexpected. This could indicate that changes in Adriatic zooplankton fauna, influenced by hydroclimatic changes, are picking up pace and points to the need and importance of systematic monitoring projects in such a rapidly changing sea.

So far, sequences of only *Doliolum nationalis* (18S, COI), *Doliolum denticulatum* (18S) and *Dolioletta gegenbauri* (18S) were available from GenBank, which was insufficient to provide any insight of phylogenetic relationships between doliolid genera [30,32,33]. In order to provide genetic context for this newly described species, and also to provide reference sequences for metabarcoding projects, we sequenced COI gene fragment of seven thaliacean species, out of which the COI sequences of six species were not already present in GenBank. The phylogenetic reconstruction showed that all three thaliacean orders (Doliolida, Pyrosomatida and Salpida) are well supported. Within Doliolida, genera *Doliolum* and *Dolioletta* were reconstructed with good bootstrap support, while genus *Doliolina* was not supported in COI phylogeny. Genus *Doliolina* possesses a primitive branchial septum [44] and digestive system which forms a vertical U-loop, which has also been speculated to be a primitive feature [51]. Given that fact, it is possible that the genus *Doliolina* is not monophyletic, but that should be confirmed or rejected by sequencing more doliolid species or more genetic markers.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d14110959/s1, Alignment S1: COI alignment of 12 thaliacean species.

Author Contributions: Specimen collection, genetic analysis, R.G.; morphological analysis, species description, writing, R.G. and M.B. All authors have read and agreed to the published version of the manuscript.

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