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Molecular phylogeny of Didemnidae (Ascidiacea: Tunicata)

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Didemnidae is the largest family of tunicates within Aplousobranchia, with about 578 species. This family comprises eight genera: *Atriolum*, *Clitella*, *Didemnum*, *Diplosoma*, *Leptoclinides*, *Lissoclinum*, *Polysyncraton*, and *Trididemnum*. Morphological and molecular data suggest that Didemnidae is monophyletic, but the monophyly of each didemnid genus and their phylogenetic relationships are still poorly understood. This study aimed to evaluate the monophyly of six of the eight didemnid genera and assess their phylogenetic relationships based on mitochondrial (*COI*) and nuclear (*18S*) sequences. All genera were recovered as monophyletic except *Trididemnum*. *Didemnum* comprises two clades that differ one from the other by the presence of an atrial lip, the number of testicular lobes, and the number of ampullae in larvae. These morphological differences indicate that *Didemnum* could be split into two genera. Morphological evidence and previous taxonomists have suggested a close relationship between *Didemnum* and *Polysyncraton* as well as between *Diplosoma* and *Lissoclinum*. We re-evaluate these hypotheses, which are supported by our *18S* sequences and concatenated data.

ADDITIONAL KEYWORDS: Aplousobranchia – clades – *Didemnum* – mitochondrial DNA – nuclear DNA

INTRODUCTION

Ascidiacea is the largest taxon of tunicates, including about 3000 species divided into three orders: Stolidobranchia, Phlebobranchia, and Aplousobranchia (Lambert, 2005). Within Aplousobranchia, the most diverse of the three orders, Didemnidae Giard, 1872, is the largest family, with about 578 species distributed worldwide (Shenkar & Swalla, 2011); however, it is more speciose in tropical waters (Monniot, Monniot & Laboute, 1991). Using morphological or molecular data, a small number of studies have investigated the phylogenetic relationships between the Aplousobranchia families and lend support to the monophyly of Didemnidae (Turon & López-Legentil, 2004; Moreno & Rocha, 2008).

All taxonomic classifications proposed for this family have been based on morphological features. The current classification recognizes eight genera within Didemnidae: *Atriolum* Kott, 1983; *Clitella* Kott, 2001; *Didemnum* Savigny, 1816; *Diplosoma* Macdonald, 1859; *Leptoclinides* Bjerkan, 1905; *Lissoclinum* Verrill, 1871; *Polysyncraton* Nott, 1892; and *Trididemnum* Della Valle, 1881 (Kott, 2001). The first two genera account for only ten valid species, while most of the diversity is included in the other genera, especially *Didemnum* and *Polysyncraton*. Within Didemnidae, the taxonomic classification has changed during the last century due to conflicts about the validity of genera (Van Name, 1945; Kott, 1962, 2001; Monniot & Monniot, 1972).

The genera within Didemnidae are distinguished mainly by the presence or absence of an atrial siphon, the number of stigmata rows, the shape of the vas deferens, the number of testicular lobes, and the presence or

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absence of spicules (Monniot, Monniot & Laboute, 1991; Kott, 2001). The assessment of the phylogenetic relationships within didemnids has been hindered because the zooids are small and the distinguishing morphological features are limited, making them difficult to work with.

Initial hypotheses on evolutionary relationships in this group were based on the similarity of morphological features, according to a purely gradist approach (Kott, 1962; Monniot, 1984; Lafargue & Wahl, 1987). However, phylogenetic relationships between didemnid genera remain controversial and have not been resolved. The main objectives of this study are to assess the monophyly of Didemnidae genera using both mitochondrial and nuclear markers and to evaluate the evolution of the morphological characters in light of these data.

MATERIALS AND METHODS

Samples

The present study analysed six of the eight currently accepted genera. The specimens were obtained from the biological collection (ColBIO) at the University of São Paulo's Oceanographic Institute (IOUSP) as well as from the Invertebrate Zoology collections in the Florida Museum of Natural History (FLMNH) at the University of Florida (UF) (Table 1).

Table 1. List of species included in the phylogenetic analysis and the accession numbers of the sequences obtained in the present study

Species	Locality	Voucher No.	Accession No.	
			COI	*18S*
Outgroup				
Distaplia sp.	Ceará, Brazil	ColBIO TL 02	KU221227.1	KU174394.1
Ingroup				
Didemnum apuroto	Moorea, French Polynesia	UF 813; UF 814	KU221211.1; KU221212.1	_
Didemnum cineraceum	Ceará, Brazil; São Paulo, Brazil	ColBIO TL 230; TL 839	KU221193.1; KU221192.1	_
Didemnum cuculliferum	Moorea, French Polynesia	UF 676; UF 724	KU221199.1; KU221219.1	_
Didemnum fragile	Moorea, French Polynesia	UF 746; UF 839	KU221209.1; KU221201.1	_
Didemnum granulatum	Moorea, French Polynesia	UF 573; UF 601; UF 1408	KU221196.1; KU221204.1; KU221215.1	_
Didemnum granulatum	Fernando de Noronha, Brazil	ColBIO TL 671; TL 686	KU221190.1; KU221191.1	_
Didemnum ligulum	Espírito Santo, Brazil; Moorea, French Polynesia	ColBIO TL 796; UF 613; UF 694; UF 1387	KU221210.1; KU221198.1; KU221200.1; KU221202.1	_
Didemnum cf. *ligulum*	Fernando de Noronha, Brazil	ColBIO TL 649	_	KU170205.1
Didemnum membranaceum	Moorea, French Polynesia	UF 526; UF 720; UF 1389	KU221216.1; KU221225.1; KU221213.1	_
Didemnum mutabile	Moorea, French Polynesia	UF 689; UF 691	KU221207.1; KU221223.1	_
Didemnum psammatodes	Ceará, Brazil	ColBIO TL 27	KU221189.1	_
Didemnum sordidum	Moorea, French Polynesia	UF 597; UF 612; UF 734	KU221195.1;KU221197.1 ;KU221208.1	_
Diplosoma listerianum	Moorea, French Polynesia	UF 649	KU221221.1	_
Diplosoma simile	Moorea, French Polynesia	UF 697	KU221218.1	_
Diplosoma sp.	Espírito Santo, Brazil	ColBIO TL 817	KU221188.1	KU170204.1
Diplosoma sp.1	Rio de Janeiro, Brazil	ColBIO TL 729	KU221184.1	_
Diplosoma sp.2	Moorea, French Polynesia	UF 644	KU221220.1	_

Table 1. *Continued*

Species	Locality	Voucher No.	Accession No.	
			COI	*18S*
Diplosoma sp.3	Moorea, French Polynesia	UF 680; UF 759	KU221228.1;KU221226.1	_
Lissoclinum ravarava	Moorea, French Polynesia	UF 1398	KU221214.1	_
Lissoclinum patella	Moorea, French Polynesia	UF 781	_	KU174393.1
Lissoclinum vareau	Moorea, French Polynesia	UF 606	KU221205.1	_
Lissoclinum verrilli	Gulf of Mexico, USA	UF 1244	KU221194.1	_
Polysyncraton poro	Moorea, French Polynesia	UF 664; UF 682	KU221217.1;KU221222.1	_
Polysyncraton sp.	Ceará, Brazil; Espírito Santo, Brazil	ColBIO TL 432; TL 810	KU221187.1;KU221186.1	KU170203.1; KU170202.1
Trididemnum maragogi	Saint Peter and Paul Archipelago, Brazil	ColBIO TL 234	KU221183.1	_
Trididemnum maragogi	Rocas Atoll, Brazil	ColBIO TL 449	_	KU170201.1
Trididemnum fetia	Moorea, French Polynesia	UF 629	KU221206.1	KU174392.1
Trididemnum pigmentatum	Moorea, French Polynesia	UF 696	KU221224.1	_
Trididemnum tomarahi	Moorea, French Polynesia	UF 1424	KU221203.1	KU174391.1
Trididemnum sp.	Fernando de Noronha, Brazil	ColBIO TL 538	KU221185.1	_

Note: Museum and collection abbreviations: UF: University of Florida, USA; ColBIO: Biological collection, Brazil.

The specimens were collected between 2009 and 2012 from the Gulf of Mexico, the Brazilian coast, and adjacent oceanic islands, and Moorea Island, French Polynesia, to depths up to 40 m. Fragments from each colony were subsampled after collection, preserved in 90% ethanol, and stored at –20 °C. Vouchers for each sample are available at their respective institutions (Table 1). Species were identified using specialized literature and the usual dissection and microscopy procedures (Van Name, 1945; Monniot & Monniot, 1987; Kott, 2001). Some sequences were obtained from the Smithsonian's Laboratory (Washington, DC) as part of the Moorea Biocode Project. Additional sequences for *COI* and *18S* rDNA genes of the family Didemnidae were obtained from GenBank and included in the analysis (Table 2). The species *Cystodytes dellechiajei* (Della Valle, 1877) and *Cystodytes* sp. (family Polycitoridae) were selected as outgroups for *COI* and *18S* analysis, respectively. *Distaplia* sp. (family Holozoidae) was used as an outgroup for concatenated (*COI* + *18S*) analyses. These species were selected as outgroups for the phylogenetic analyses because they are considered to be closely related to the family Didemnidae (Berrill, 1936; Lafargue, 1983).

DNA Amplification and Sequencing

Genomic DNA was extracted using two methods. Some extractions were performed using the DNeasy Blood and Tissue kit and the QIAamp DNA Mini kit (Qiagen) in order to isolate DNA from about 20 to 40 dissected thoraxes, following the manufacturer's protocol. DNA was quantified using a Nanodrop 2000 (Thermo Scientific), and up to 100 ng of DNA was used in the Polymerase Chain Reactions (PCR). Other extractions were performed using a 10% solution of Chelex 100 (Walsh, Metzger & Higuchi, 1991) and 1.0 µL of the supernatant was used for PCR.

PCR amplifications were carried out in a 25 µL volume using 12.5 µL of Amplitaq Gold 360 Master Mix (Applied Biosystems), 1.0 µL of each primer (10 µM), DNA as specified above, and nuclease-free water. Fragments of *COI* were amplified using the primers LCO1490 and HCO2198 (Folmer *et al.*, 1994) and Tun F and Tun R (Stefaniak *et al.*, 2009) and the primers Did F and Did R, newly designed based on the Folmer primers as modified by Geller *et al.* (2013), and full mitochondrial genomes available on GenBank for *Didemnum vexillum* (KM259616.1) and *Diplosoma listerianum* (FN313539.1) (Table 3). The

Table 2. List of species and the accession number of the *COI* and *18S* rDNA sequences from GenBank

Species	Gene *COI*	Gene *18S*
Outgroup		
Cystodytes sp.	_	FM244842.1
Cystodytes dellechiajei	AY523068.1; AY523072.1	_
Ingroup		
Didemnum albidum	EU419432.1	EU337058.1
Didemnum granulatum	JQ780685.1; JQ780688.1	_
Didemnum molle	_	AB211071.1
Didemnum multispirale	KC017430.1	_
Didemnum psammatodes	EU742661.1	_
Didemnum vexillum	JQ663512.1	JF738070.1
Didemnum sp.	_	AJ579862.1
Didemnum sp.B	_	EU337061.1
Diplosoma listerianum	KF309664.1; KF309605.1	_
Diplosoma ooru	_	AB211100.1
Diplosoma spongiforme	AY600972.1	_
Diplosoma simile	AB723718.1	AB211106.1
Diplosoma simileguwa	_	AB211107.1
Diplosoma virens	_	AB211114.1
Leptoclinides madara	KC017427.1	AB211070.1
Lissoclinum bistratum	_	AB211081.1
Lissoclinum fragile	JF506180.1	_
Lissoclinum patella	KJ009374.1; KJ009371.1	_
Lissoclinum punctatum	_	AB211091.1
Lissoclinum timorense	_	AB211094.1
Lissoclinum verrilli	JX099361.1	_
Polysyncraton lacazei	AY600986.1	_
Trididemnum cyanophorum	JF506187.1; KJ632947.1	_
Trididemnum maragogi	KR604728.1	_
Trididemnum paracyclops	_	AB211077.1
Trididemnum solidum	JF506186.1	_

primers Did F and Did R were designed to improve amplification success, especially for *Didemnum*, *Lissoclinum*, and *Trididemnum*, for which previously available primers were not producing good results. PCR cycle conditions for *COI* using the primers LCO1490 and HCO2198 were as follows: initial denaturation for 2 min at 94 °C, followed by 1 min at 94 °C, 1 min at 39 °C, and 1.5 min at 72 °C for 35 cycles, and a final extension for 7 min at 72 °C. The following were the cycle conditions for *COI* using the primers Tun F and Tun R: initial denaturation for 4 min at 95 °C, followed by 1 min at 94 °C, 1 min at 39 °C, and 1.5 min at 72 °C for 40 cycles, with a final extension for 10 min at 72 °C. Lastly, the cycle conditions for *COI* using the primers Did F and Did R were as follows: initial denaturation for 2.5 min at 95 °C, followed by 40 s at 94 °C, 40 s at 44 °C, and 1 min at 72 °C for 40 cycles, with a final extension for 10 min at 72 °C. The primers 18S1 and 18S4 were used to amplify partial *18S* rDNA (Table 3). Cycle conditions for the *18S* gene followed Tsagkogeorga *et al.* (2009).

For sequencing, samples were purified with the ExoSAP-IT enzyme (USB Scientific) following the manufacturer's protocol and sequenced on an ABI3730 (Applied Biosystems) automatic sequencer. Other samples were sequenced at the Interdisciplinary Center for Biotechnology Research (University of Florida) and at the Smithsonian Institution. All sequences obtained in this study were deposited in GenBank (see Accession numbers in Table 1).

Phylogenetic Inference

The obtained DNA sequence chromatograms were assembled and edited using Geneious R7 and aligned using MAFFT v7.017 with the L-INS-i algorithm (Katoh & Standley, 2013). The phylogenetic analyses were conducted independently on *COI* and *18S*, as well as on the concatenated data set. *COI* sequences

Table 3. Primers used in amplification and sequencing for the genes *COI* and *18S* rDNA

Markers	Primers	References
COI	LCO1490 – 5′GGTCAACAAATCATAAAGATATTGG3′ HCO2198 – 5′TAAACTTCAGGGTGACCAAAAAATCA3′	Folmer *et al.* (1994)
COI	TunF – 5′TCGACTAATCATAAAGATATTAG3′ TunR – 5′AACTTGTATTTAAATTACGATC3′	Stefaniak *et al.* (2009)
COI	DidF – 5′TATCIACIAATCATAAAGATATTGG3′ DidR – 5′CTTCTYCYGRWGGRTCAAAAARCT3′	Present study
18S rDNA	18S1 – 5′CCTGGTTGATCCTGCCAG3′ 18S4 – 5′GATTAAAGAAAACATTCTTGGC3′	Tsagkogeorga *et al.* (2009)

were checked for the absence of indels and stop/invalid codons. Trees were obtained using Maximum Likelihood (ML) inference in PhyML (Guindon *et al.*, 2010) and Bayesian Inference (BI) in MrBayes v3.2.2 (Ronquist *et al.*, 2012). We used jModelTest v2.1.4 to select the best-fit evolutionary model (GTR + gamma) for each data set using the Akaike's Information Criterion (AIC) (Guindon & Gascuel, 2003; Darriba *et al.*, 2012).

To assess the support for the inferred topologies, 1000 bootstrap replicates were used with the ML inference. For BI, four independent Metropolis Coupled Markov Chain (MCMC) runs with 5 million generations were used. The trees were sampled every 1000 generations, and the first 25% of the sampling trees were discarded as burn-in. We used the Geneious plugin to check that the MrBayes analyses had reached stationarity (average standard deviation of split frequencies was 0.003) and that the independent runs have converged. The proportion of trees supporting the majority rule consensus tree among the sampled posterior distribution was computed. The trees obtained were visualized using Figtree v1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/) (Rambaut, 2012).

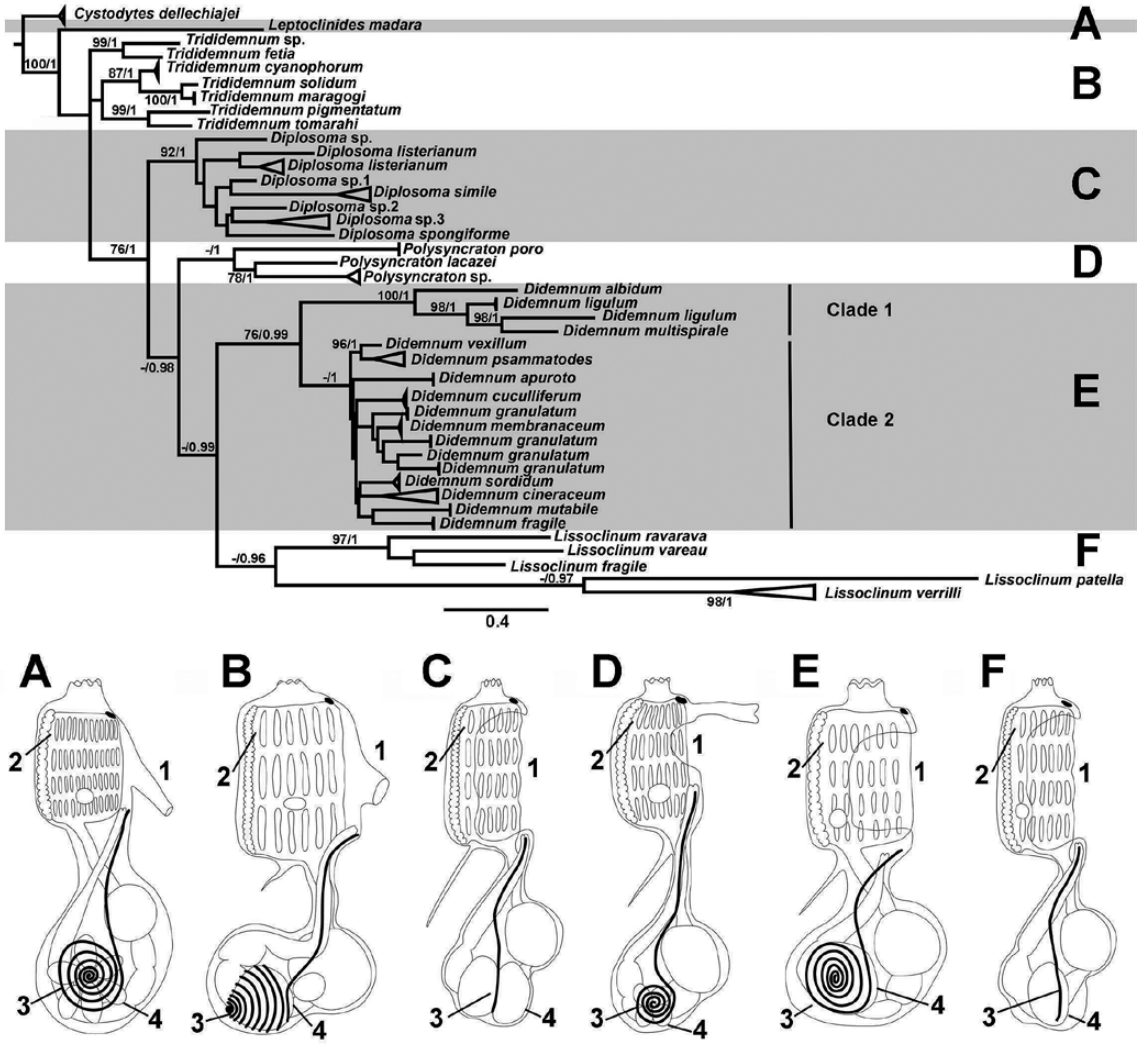

Figure 1. Molecular phylogenetic hypothesis of the family Didemnidae based on partial mitochondrial *COI* sequences. Numbers on and below the main branches represent the bootstrap values for maximum likelihood (ML) (>70%) and posterior Bayesian probabilities (BP) (>0.95), respectively. Genera of Didemnidae represented in this study: A) *Leptoclinides*, B) *Trididemnum*, C) *Diplosoma*, D) *Polysyncraton*, E) *Didemnum*, and F) *Lissoclinum*. Adapted from Monniot, Monniot & Laboute, (1991) and Moreno & Rocha, (2008). Morphological characters: 1 – Atrial siphon (open or tubular), 2 – Number of stigmata rows (three or four), 3 – Form of the vas deferens (coils or straight) and, 4 – Number of testicular lobes (undivided or divided or more than 2 follicles).

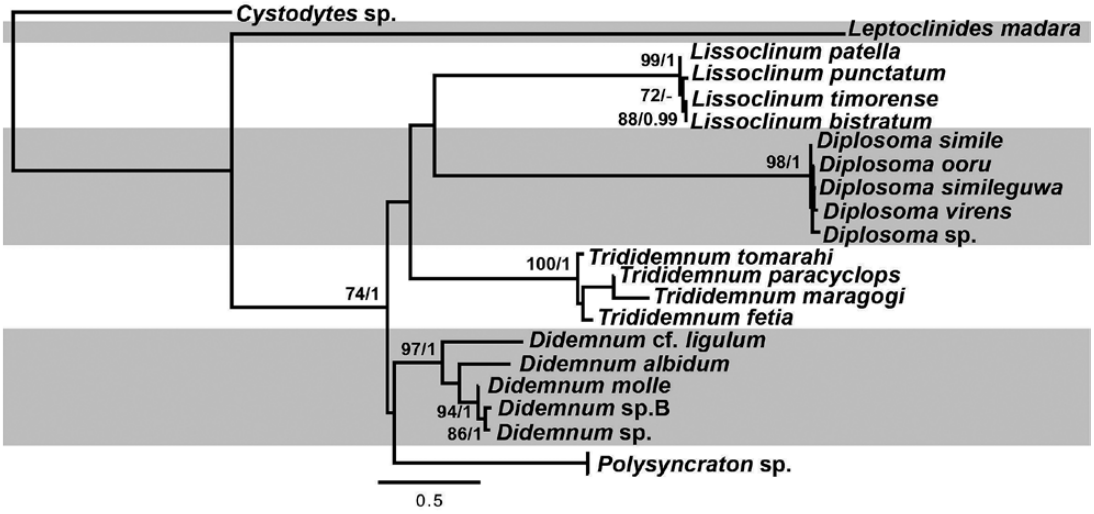

Figure 2. Molecular phylogenetic hypothesis of the family Didemnidae based on nuclear *18S* rDNA sequences. Numbers on or below main branches represent the bootstrap values for maximum likelihood (>70%) and posterior Bayesian probabilities (>0.95), respectively.

We also inferred the phylogenetic relationships using the amino acid sequences from *COI*. We converted the nucleotides to amino acids using the ascidian mitochondrial code (transl_table=13). The alignment was analysed with RAxML v8.2.4 (Stamatakis, 2014) using rapid bootstrapping (100 replicates) and a subsequent ML search (option: –fa). We used the automatic detection of the best model for protein substitution (–m PROTGAMMAAUTO).

All trees reported in this study have been deposited in TreeBASE: http://purl.org/phylo/treebase/phylows/study/TB2:S19582.

RESULTS

The same topologies were obtained with ML and BI algorithms for *COI*, *18S* (Figs 1 and 2), and concatenated analyses (Fig. S1); thus only the ML trees are shown. Analyses with *COI*, *18S*, and the concatenated data set recovered all of the genera traditionally accepted for Didemnidae as monophyletic except for *Tridemnum*, which was not fully resolved with the *COI* data (DNA sequences, Fig. 1; and amino acid tree, Fig. S2).

All analyses supported *Didemnum*, the largest didemnid genus, as monophyletic (76%/0.99 with *COI* in Fig. 1, 97%/1 with *18S* in Fig. 2, and 99%/1 with the concatenated data set in Fig. S1, for bootstrap values/posterior probabilities). Two major clades were recovered within *Didemnum*. One clade included *Didemnum albidum* Verrill, 1871, *Didemnum multispirale* Kott, 2001, and *Didemnum ligulum* Monniot, 1983 (Fig. 1), while the other comprised all of the other species of *Didemnum* included in this study.

Diplosoma was shown to be monophyletic in both Likelihood and Bayesian analyses (bootstrap values of 92%, 98%, and 100% for *COI*, *18S* and the concatenated data sets, respectively, and posterior probability of 1 for the three data sets, Figs 1, 2, S1).

Lissoclinum was also determined to be monophyletic, but was not fully supported. The monophyly was supported with high posterior probability in the BI analyses for both markers (0.96 for *COI* and 1 for *18S*) (Figs 1 and 2) and the concatenated data set (1) (Fig. S1). This monophyly was also recovered by ML analysis for both genes and concatenated data; however, for the *COI* gene, the bootstrap support value was low (33%, value not shown in Fig. 1). Two clades in *Lissoclinum* were identified for the *COI* tree. The first clade included three species: *Lissoclinum ravarava* Monniot & Monniot, 1987, *Lissoclinum vareau* Monniot & Monniot, 1987, and *Lissoclinum fragile* Van Name, 1902 with high support values (BS = 97%, PP = 1). The second clade comprised two species: *Lissoclinum patella* Gottschaldt, 1898 and *Lissoclinum verrilli* Van Name, 1902, with high posterior probability (0.97) (Fig. 1). The monophyly of *Polysyncraton* was recovered for *COI*, with a moderate bootstrap value (69%, value not shown) and high posterior probability (1) (Fig. 1).

The results concerning the *Tridemnum* monophyly were conflicting. This genus was recovered as monophyletic using *18S* and concatenated data only, with strong support for both analyses (BS = 100%, PP = 1, Figs 2, S1). However, the monophyly was not recovered for *COI*, for which a polytomous arrangement was obtained (Fig. 1). The results showed that the species *Leptoclinides madara* Tokioka, 1953 is a

sister to the rest of the family on *COI*, *18S*, and concatenated phylogenies (Figs 1, 2, S1).

Our results showed a single clade that included the genera *Didemnum*, *Lissoclinum*, *Diplosoma*, and *Polysyncraton* for *COI*. For this clade, the bootstrap support was moderate (76%) and the posterior probability was high (1) (Fig. 1). The same topology was recovered with *18S* and concatenated data and included *Tridemnum* (Figs 2, S1). The bootstrap value was also moderate (74%) and the posterior probability high (1) for *18S* (Fig. 2), as were the bootstrap value and the posterior probability for concatenated data (91%, 1) (Fig. S1).

The results concerning the relationships within Didemnidae had low bootstrap and posterior probabilities with the *18S* gene and concatenated data in both analyses (values not shown). Despite these low values, the tree topologies were the same, and these results suggest a close relationship between *Didemnum* and *Polysyncraton*, as well as between *Diplosoma* and *Lissoclinum*. The relationships within Didemnidae using *COI* showed the same tree topology in both analyses. However, support values were discordant. In the BI analyses, this relationship was strong with *Diplosoma* + *Polysyncraton* + *Didemnum* + *Lissoclinum* (1; 0.98 and 0.99, respectively) (Fig. 1). The results for ML analyses had low support values. Therefore, reliable evolutionary relationships between didemnid genera were not recovered.

DISCUSSION

This study provides the first molecular phylogeny to include six of the eight genera of Didemnidae. The monophyly of all included genera is well supported by the analyses carried out here, except for *Tridemnum*. Overall, the recovered molecular phylogeny of Didemnidae is supported by morphological evidence.

The spiralled sperm duct and the spherical-/star-shaped spicules with diverse radial structures, along with esophageal budding and a flexed gut loop, are considered the most distinctive synapomorphies for Didemnidae. Few studies have focused on the evolution of this family, the largest within ascidians, and almost all of them disagree on how didemnids originated. Kott (2001) suggests that didemnids arose from a common ancestor shared with a holozoid or diazonid ascidian, based on the overall similarity between zooids of *Atriolum* and *Hypodistoma*, but this particular scenario was not corroborated by any other study. Romanov (1989 *apud* Kott, 2001) proposed a didemnid ancestor devoid of spicules and with a straight sperm duct and placed *Diplosoma* as sister to all other didemnids. The genetic evidence, however, does not corroborate this hypothesis since all lineages stemming from the Didemnidae basal node in all reconstructions have spicules. In line with this, Lafargue (1983) suggested *Cystodytes* as sister to the Didemnidae, pointing to a shared origin for spicules in both taxa. Indeed, many Polycitoridae, namely, *Cystodytes*, *Eucoelium*, and *Polycitorella*, have rounded globular spicules in the tunic, and an evolutionary path towards a typical didemnid spicule seems plausible. Furthermore, *Cystodytes* also has a conspicuous bladder cell layer in the tunic, as in most didemnids, but is absent in other aplousobranchs, as noticed by Van Name (1945). Some species of this genus also have associations with *Prochloron* (a photosynthetic prokaryote), a typical feature shared with didemnids (Oliveira *et al.*, 2013). Finally, the hypothesis of Tunicata phylogeny presented by Tsagkogeorga *et al.* (2009), which was based on *18S* sequences, also placed *Cystodytes* as the sister group of Didemnidae. However, fossil didemnid spicules are among the oldest records of colonial ascidians and indicate the existence of this family since the late Triassic (Varol & Houghton, 1986), while spicules similar to those of polycitorids are known only from the early Eocene to Recent (Buge & Monniot, 1972). Further assessments with a more comprehensive taxon sampling and focusing Aplousobranchia as a whole are needed to elucidate how Didemnidae is related to other genera and families.

Leptoclinides is sister to the rest of the didemnid genera. This is based on at least three pieces of evidence. First, this genus was recovered as a sister to all other genera in all analyses carried out. In addition, *Leptoclinides* has two morphological features (i.e. tubular atrial siphon and multiple testicular lobes) that are apparently retained from the ancestral aplousobranch. Furthermore, this genus is also unique among the didemnids by having high levels of vanadium, which is more typical of phlebobranchs (Hawkins *et al.*, 1983). Therefore, molecular, morphological, and chemical evidence support the hypothesis that *Leptoclinides* is sister to remaining didemnid genera.

The genus *Didemnum*, the most diverse and common within Didemnidae, is confirmed as monophyletic with moderate and high support in both analyses. The tree topology based on *COI* data resolves two clades within *Didemnum* (Fig. 1). Morphologically, Clade 1 species (*D. albidum*, *D. multispirale*, and *D. ligulum*) are characterized by the presence of an atrial lip, one or two testicular lobes, and larvae with multiple ampullae. All these features are absent in clade 2 species. In line with these findings, Monniot (1984) acknowledged the taxonomic value of the number of larval ampullae. This same author noted the presence of *Didemnum* species with an atrial lip and up to three testicular follicles, but argued that these characters were shared by *Didemnum* and

Polysyncraton, and hence their division would be merely arbitrary (Monniot, 1993). Clade 2 species have morphological characters typical of *Didemnum candidum* Savigny, 1816, the type species for the genus. According to Lafargue (1974), *D. candidum* has a single testis, no atrial lip, and larvae with three papillae and four ampullae. Therefore, our results make the case for creating a new genus to accommodate *Didemnum* species that have either an atrial lip, one or two testicular lobes, larvae with multiple ampullae, or any combination of these characters. As the most speciose genus, a split in and reorganization of *Didemnum* may be welcome, but the prevalence of the characters mentioned here and their association with the clades identified in this study need to be clarified before making such taxonomic decision.

The monophyly of *Polysyncraton* is generally supported by our data. The validity of this genus has always been disputed in the literature, based on the argument that its distinction from *Didemnum* is purely artificial (Van Name, 1921, 1945; Berrill, 1950; Monniot, 1984, 1993, 1995). *Polysyncraton* and *Didemnum* have always been considered closely related (Eldredge, 1966; Lafargue, 1975b) and few morphological characters can be used to distinguish between these two genera. Additionally, the absence of gonads and larvae makes it difficult to tell them apart (Kott, 2001). Morphologically, two diagnostic characters for *Polysyncraton* remain. These are a testis with three or more lobes and the structure of the atrial opening, usually with a conspicuous languet. Eldredge (1966) also noticed the trend towards a larger number of ampullae in the larvae, in comparison with *Didemnum*. Our results confirm the validity of *Polysycraton*, as firmly asserted by other taxonomists (Eldredge, 1966; Kott, 2001), but its relationships with other genera are still unclear.

Diplosoma and *Lissoclinum* are the only genera within Didemnidae with a straight sperm duct – without the typical coils present in other didemnids. The monophyly of both genera was well supported in the reconstructions, especially with *18S* and concatenated data. Their relationships with other genera, on the other hand, were not convincingly resolved. The analysis of Yokobori *et al.* (2006), which also used *18S*, did not fully support *Lissoclinum* as a monophyletic group. *Diplosoma* and *Lissoclinum* genera can be distinguished from one another only by the presence of spicules in *Lissoclinum* (Eldredge, 1966; Lafargue, 1975a). In fact, spicules are nearly universally present in Didemnidae. Although sometimes regarded as poorly effective (Lindquist, Hay & Fenical, 1992), didemnid spicules seem to be relevant as a form of predation deterrence. In contrast, *Diplosoma* species are not only devoid of spicules, but their tunics are usually soft and mucosal. Therefore, these animals must rely on other defence mechanisms, such as toxic or unpalatable chemical compounds.

Finally, *Tridemnum* is the only genus not to have its monophyly confirmed. Monophyly was recovered with *18S* and the concatenated data, but not with *COI*. The data set for *18S* had fewer species, but included species that appeared in different clades with *COI* (e.g. *Tridemnum fetia*, *Tridemnum maragogi*, and *Tridemnum tomarahi*). Additionally, the position of this genus in the phylogeny remains uncertain as it lacks proper support in both data sets. One interpretation is that the *COI* topology is the product of some artefact and does not reflect the phylogenetic history of this genus. In fact, *COI* has shown inconsistent results for Tunicata (Stach & Turbeville, 2002; Pérez-Portela *et al.*, 2009). Nevertheless, *COI* did show results consistent with previous morphological hypotheses for all other didemnid genera, which suggests that *COI* is useful for the recovery of didemnid relationships. Therefore, it is possible that the failure of *COI* to recover a monophyletic *Tridemnum* was due to phylogenetic history. Morphologically, *Tridemnum* differs from other didemnid genera in the reduction of the pharynx, having only three rows of stigmata. A posteriorly directed tubular atrial siphon, similar to those of *Leptoclinides*, is also common in *Tridemnum* species, but this is not a universal character for this genus. Therefore, further studies with additional molecular markers and morphological characters are needed to clarify its status.

According to our results, relationships within Didemnidae are not fully resolved due to conflicting topologies between the markers used and the lack of support for some taxa. However, the weakly supported relationship between *Didemnum* and *Polysyncraton*, as well as between *Diplosoma* and *Lissoclinum,* as indicated by *18S* data, has already been postulated by others following a gradist approach (Van Name, 1945; Lafargue, 1983; Kott, 2001). The position of *Tridemnum* is especially unresolved and remains a relevant unanswered question. Future assessments with larger taxon sampling allied with a more comprehensive molecular data set will improve knowledge about the evolution of this remarkable group of deuterostome animals.

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SUPPORTING INFORMATION

Figure S1. Molecular phylogenetic hypothesis of the family Didemnidae based on the concatenated sequences (mitochondrial *COI* + nuclear *18S* rDNA). Numbers on or below main branches represent the bootstrap values for Maximum Likelihood (>70%) and Bayesian Inference (>0.95), respectively.

Figure S2. Molecular phylogenetic relationships of the family Didemnidae based on amino-acid sequences from *COI*. Number on or below main branches represent the bootstrap values for Maximum Likelihood (>70%).