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Short Communication

Back to solitude: Solving the phylogenetic position of the Diazonidae using molecular and developmental characters

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ABSTRACT

The order Aplousobranchia (Chordata, Ascidiacea) contains approximately 1500 species distributed worldwide. Their phylogeny, however, remains unclear, with unresolved family relationships. While most Aplousobranchia are colonial, debates exist concerning the phylogenetic position of families such as the Diazonidae and Cionidae, which exhibit a solitary lifestyle and share morphological characteristics with both Aplousobranchia and Phlebobranchia orders. To clarify the phylogenetic position of the Diazonidae and Cionidae, we determined the complete mitochondrial sequence of the solitary diazonid *Rhopalaea idoneta*. The phylogenetic reconstruction based on the 13 mitochondrial protein coding genes strongly supports a positioning of Diazonidae well-nested within the Aplousobranchia rather than a positioning as a sister clade of the Aplousobranchia. In addition, we examined the regenerative ability of *R. idoneta*. Similar to colonial Aplousobranchia, *R. idoneta* was found to be able to completely regenerate its thorax. *Ciona*, also known to possess high regenerative abilities, is the Aplousobranchia sister clade rather than a member of the Phlebobranchia. Our results thus indicate that the colonial lifestyle was acquired in the Aplousobranchia, starting from a *Ciona*-like solitary ancestor and secondarily lost in Diazonidae representatives such as *Rhopalaea*. The solitary lifestyle of *Rhopalaea* is thus a derived characteristic rather than an ancestral trait.

1. Introduction

The class Ascidiacea (Phylum: Chordata) is the largest and most diverse class of the sub-phylum Tunicata (also known as Urochordata). With nearly 3000 valid species, which include important model organisms such as *Ciona intestinalis* and *Botryllus schlosseri* (Dehal et al., 2002; Voskoboynik et al., 2013), this unique group of invertebrates provides developmental and evolutionary biologists with important insights into evolutionary events at the origin of the vertebrate subphylum. However, phylogenetic relationships between ascidian orders and families, in particular within the Aplousobranchia, as well as the validity of certain lineages, remain to be ascertained.

Nowadays, the majority of phylogenetic studies follow Lahille's (1887) classification of the class Ascidiacea into three orders based on the structure of the adult branchial sac: the order Aplousobranchia with the simplest branchial sac (Aplouso = simple in Greek); the order Phlebobranchia (Phlebo = vessel in Greek), which possesses distinct longitudinal blood vessels in its branchial sac; and the order Stolidobranchia, which is distinguished by the folding of the branchial wall (Stolido = folded, pleated in Greek) (Monniot et al., 1991). Both colonial and solitary lifestyles are reported among all three orders, depending on the particular taxonomic approach considered as valid (Moreno and Rocha, 2008). For example, the phylogenetic position of the solitary *Ciona intestinalis* has been a matter of controversy, as its large branchial sac and its internal longitudinal branchial vessels are typical of solitary phlebobranchs (Monniot, 1991); while its vanadium oxidation state (Hawkins et al., 1983) and the regenerative role of its epicardial tissue imply a closer relationship with aplousobranchs (Kott, 1990).

The family Diazonidae comprises both solitary (i.e., *Rhopalaea*) and colonial (i.e., *Diazona*) species, and shares some primitive characters with the family Cionidae and other Phlebobranchia, such as numerous inner longitudinal branchial vessels, a relatively large

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branchial sac, and a gelatinous translucent test (Kott, 1990). However, the Diazonidae also have Aplousobranchia characteristics: while in solitary phlebobranch species the gut loop is found behind the pharynx with no constriction of the body wall separating the abdomen from the thorax, in members of the family Diazonidae and in Aplousobranchia a true posterior abdomen exists, separating the zooids into two distinct parts (Kott, 1990). This division of the body allows some Aplousobranchia species to lose/degenerate their thorax when the environmental conditions are harsh and regenerate it when conditions improve (Brien, 1930; Turon, 1992). Interestingly, similar thorax regeneration has been mentioned in *R. cloneyi* (Vazquez and Young, 1996).

Previous taxonomic classifications grouped together the genera *Ciona*, *Rhopalaea* and *Diazona* within the family Cionidae, order Phlebobranchia (Monniot et al., 1991) based on their similar branchial sac characteristics (e.g., the presence of inner longitudinal branchial vessels). Kott (1990), on the contrary, placed *Ciona* as the only known genus of the family Cionidae Lahille, 1887, and included *Rhopalaea*, and *Diazona* within the family Diazonidae Seeliger, 1906, order Aplousobranchia. Consequently, according to Kott (1990, 2005), the loss of longitudinal branchial vessels in colonial aplousobranchs and stolidobranchs is a convergent adaptation associated with the reduction in zooid size and does not indicate a phylogenetic affinity.

A broad phylogenetic analysis of Aplousobranchia relationships based on 47 morphological characters has left unresolved the position of *Rhopalaea* and *Diazona*, (Moreno and Rocha, 2008). In this study, the later genera are early offshoots in the ascidian tree, while *Ciona* is placed together with Phlebobranchia and Stolidobranchia representatives. This led Moreno and Rocha (2008) to exclude Cionidae and Diazonidae from the Aplousobranchia. Molecular phylogenetic analyses have not yet resolved the positioning of Diazonidae and Cionidae. The phylogenetic position of Diazonidae has only been investigated based on COI sequences (Turon and López-Legentil, 2004). In this study, *Ciona* is placed as a sister clade of Aplousobranchia and *Rhopalaea* branches within Aplousobranchia, suggesting that Cionidae and Diazonidae are members of the order Aplousobranchia. However, the position of *Rhopalaea* has not been corroborated by high support values except when using the Bayesian criteria. Since Bayesian posterior probabilities have been shown to overestimate branch supports (Douady et al., 2003; Erixon et al., 2003), the study of Turon and López-Legentil (2004) does not exclude the possibility that Diazonidae could be the earliest diverging Aplousobranchia family. Additionally, analysis of complete mt sequences (Rubinstein et al., 2013) and 18S rDNA sequences (Tsagkogeorga et al., 2009) did not confirm the grouping of Cionidae with Aplousobranchia. In these studies, Cionidae is either the sister clade of Phlebobranchia + Thaliacea or the sister clade of Thaliacea. Thus, morphological and molecular data produce contrasting results regarding the phylogenetic position of the families Diazonidae and Cionidae, which still remains uncertain.

Here, we investigate the phylogenetic position of a solitary member of the Diazonidae family, *Rhopalaea idoneta* (Shenkar, 2012), using mitochondrial protein-coding genes. In addition, we also examined the regenerative abilities of this species. *R. idoneta* is a highly abundant species on the coral reefs of Eilat, Red Sea, Israel, found at 5–60 m depth. Field surveys have revealed that it can reach a density of 3 individuals/m^2 at 30 m depth (Koplovitz and Shenkar, 2014). On the coral reefs only the thorax is visible, while the abdomen, which contains the digestive system and gonads, remains completely embedded in the substrate (Fig. 1A). Phylogenies based on complete mitochondrial genome sequences have been shown to successfully resolve deep-level relationships among various metazoan groups, including ascidians (Rubinstein et al., 2013; Singh et al., 2009). Consequently, we sequenced the complete mitochondrial genome of *R. idoneta* and reconstructed the tunicate phylogeny using the 13 mitochondrial protein-coding genes.

Similar to colonial aplousobranch species, some members of the *Rhopalaea* genus are known to possess the ability to regenerate their thorax (Monniot and Monniot, 2001). However, this phenomenon has not been documented to date or studied in depth. We therefore conducted field experiments to further investigate this feature in *R. idoneta*.

2. Material and methods

2.1. Sampling, DNA sequencing and annotations

On February 9th, 2014, five individuals of *Rhopalaea idoneta* were sampled at 33 m depth using SCUBA. The specimens were collected from the natural coral reef in front of the Inter-University Institute in Eilat (29°30′5.69″N, 34°55′2.92″E), under permit number 40201/2014, Israel Nature and Parks Authority. They were preserved in 99% ethanol and deposited at the Steinhardt Museum of Natural History at Tel Aviv University, voucher number SMNHTAU-AS25947.

Total DNA (gDNA) was extracted from a small portion of siphonal tissue following the protocol of Fulton et al. (1995). To reduce the sequencing cost, the DNA of *Rhopalaea* was mixed with DNA of non-tunicate species (which are part of another work). The mixed gDNA was sent for library formation and gDNA sequencing to the Technion Genome Center (Haifa, Israel). A total of ~20,000,000 paired reads (each 100 bp long) were obtained with the Illumina Hiseq 2000 sequencer. Adapter sequences were removed using Cutadapt (Martin, 2011). To assemble the complete mitochondrial genome, we followed the approach of Rubinstein et al. (2013) and used the transcriptome assembler SOAPdenovo-Trans (Xie et al., 2014), rather than a genomic assembler, with a k-mer length of 64. Indeed, genomic assemblers which assume uniform read coverage often fail to assemble complete mitochondrial contigs from mixed gDNA samples (Rubinstein et al., 2013). A Blast search was conducted to identify the mitogenome contig using the COI sequence of *Rhopalaea neapolitana* (AY600983) as query. A coverage analysis was then performed with Geneious Pro (Version 6.1.7, Biomatters, Auckland, New Zealand) using paired-reads. This allowed us to confirm that the contig obtained was circular and to verify the quality of the assembly. The mean coverage was ~100 (SD ~19; minimum 35, maximum 162).

The mitochondrial sequence was annotated following the methods detailed in Rubinstein et al. (2013). The mtDNA sequence of *R. idoneta* was deposited at the EMBL-EBI European Nucleotide Archive under accession number LN877970.

2.2. Phylogenetic analyses

The 13 protein-coding genes of the 35 tunicate mitochondrial genomes available in GenBank (July, 2015) (Supplementary Table S1), and of 18 representative outgroups (Singh et al., 2009), were aligned at the amino acid level using MAFFT under the L-INS-i refinement strategy (Katoh and Standley, 2013). The webserver Guidance (Penn et al., 2010) was used to remove positions with a low confidence score (i.e., below 0.93), as well as positions present in less than 25% of the species. The concatenated alignment of the 13 mitochondrial proteins is available as Supplementary File S2.

Bayesian phylogenetic reconstructions were performed with Phylobayes 3.3b (Lartillot et al., 2009) under the CAT + GTR + Γ mixture model. Three Markov Monte Carlo chains (MCMC) were run for 51,000 cycles and sampled every 10 cycles. After a manual examination of the trace files produced by Phylobayes, the first

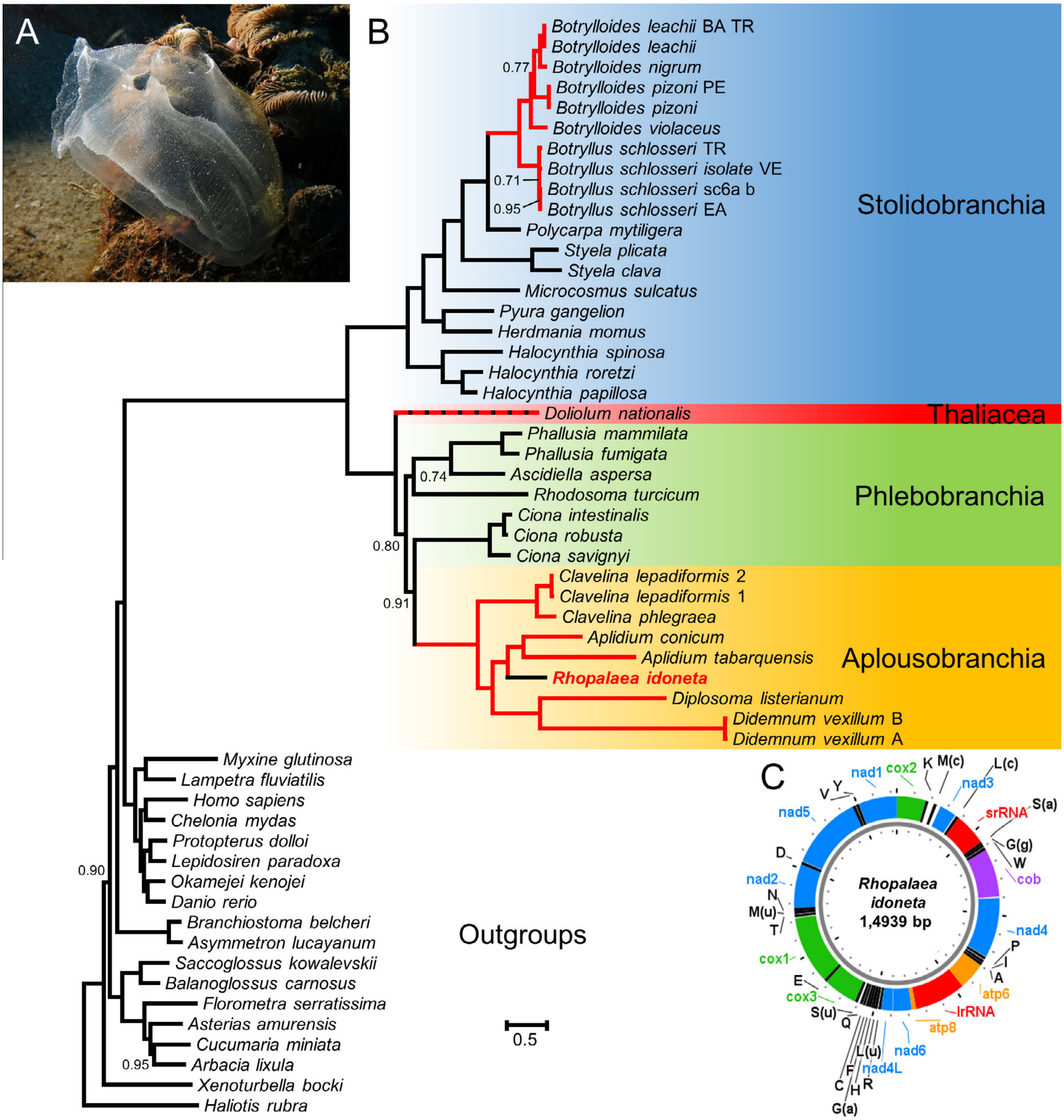

Fig. 1. Mitochondrial evolution. A. *Rhopalaea idoneta*. B. Tunicate phylogenetic relationships. Red branches indicate colonial taxa. The Thaliacea *Doliolum*, whose life cycle alternates between colonial and solitary forms, is indicated by a red and black branch. The phylogeny was inferred from the concatenation of the 13 mitochondrial proteins (3131 amino-acids and 54 taxon). Bayesian consensus tree of 3 independent MCMC runs obtained using the CAT + GTR + Γ mixture model. Most of the branches received maximal support (PP = 1.0). Consequently, branch support values are only indicated for nodes with PP below 0.99. Accession numbers are provided in Table S1. C. Organization of the *Rhopalaea idoneta* mt genome. The tRNA genes are marked in black by their one-letter code, except for G(a), Gly(AGR); G(g), Gly(GGN); L(u), Leu(UUR); L (c), Leu(CUN); M(c), Met(CAU); M(u), Met(UAU); S(a), Ser(AGY); and S(u), Ser(UCN). All genes are transcribed clockwise. rRNAs are in red. ATP synthase genes are in orange. NADH dehydrogenase genes (complex I) are in blue. The cytochrome *b* (complex III) is in purple. Cytochrome *c* oxidase genes (complex VI) are in green. Non-coding regions are marked in white. Species names as in Supplementary Table S1, with *Ciona intestinalis* former *Ciona intestinalis* type B, and *Ciona robusta* former *Ciona intestinalis* type A (Brunetti et al., 2015). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1000 trees were discarded (burn-in). The maximum and average differences, observed at the end of the run were 0.05 and 0.001, respectively, which indicate a correct convergence. We also verified that the parameters “rel_diff” and “effsize” were lower than 0.3 and higher than 50, respectively, which indicates an acceptable run.

2.3. Regeneration experiments

In order to provide additional physiological traits supporting the aplousobranch affinity of *Rhopalaea idoneta*, we investigated whether individuals are capable of regenerating a complete new thorax following its artificial removal. A field experiment was carried out at 30–35 m depth in front of the Interuniversity Institute in Eilat, (29°30′06.2″N, 34°55′01.7″E) between February and June 2014, in which six individuals of uniform size (thorax ~5 cm long) were measured in the field, photographed, and marked with plastic coated metal cords. Subsequently, we manually removed the thorax from each individual, leaving the abdomen buried in crevices or coral branches (Shenkar, 2012). The experiment site was revisited two and three months post-thorax-removal, at which time the individuals were visually examined and photographed. Following the three-month study, we repeated the experiment on the same individuals. The thorax was again removed manually, and the marked individuals were revisited one month later (June 2014).

In order to follow the regeneration progress on a daily basis, in May 2014 we transferred one individual with its associated substrate to a controlled water table at the Interuniversity Institute, and repeated the thorax-removal procedure. The regeneration progress of this individual was followed by photographing on a daily basis for two weeks. The experiment was repeated on the same individual in November 2014. Following thorax removal, siphon development was monitored for ten days.

3. Results

3.1. Mitochondrial genome structure

The mitochondrial genome of *R. idoneta* was found to be 14,939 bp long, which is within the range of other tunicate genomes (Fig. 1C). It possesses the regular tunicate gene content: 13 protein-coding genes, two ribosomal RNA genes, and a total of 24 tRNAs (Supplementary Fig. S1). In agreement with other tunicate mt genomes described (Gissi et al., 2010; Rubinstein et al., 2013), all genes share the same strand orientation. The gene order is extremely rearranged even when compared to closely related species. Indeed, *R. idoneta* possesses a novel gene order which shares only two gene pairs with most of the remaining aplousobranchs (*i.e., trnS(UCN) – cox3* and *trnA-atp6* are shared with all other aplousobranchs, except *Didemnum vexillum* and *Diplosoma listerianum,* respectively).

3.2. Molecular phylogeny

The phylogenetic tree (Fig. 1B) reconstructed under the Bayesian CAT model is well supported except for four nodes with posterior probabilities (PP) below 0.9. Deuterostome relationships agree with the findings of Rubinstein et al. (2013). Among tunicates, the addition of new sequences did not modify the relationships within the Stolidobranchia, in which the Styelidae (*Botryllus*, *Botrylloides*, *Polycarpa*, and *Styela*) remain monophyletic and the Pyuridae paraphyletic, as in Rubinstein et al. (2013). The stolidobranch phylogeny is thus very stable and presents maximal support values (PP = 1.0), except for some relationships among species of the genera *Botryllus* and *Botrylloides*. In contrast, the relationships within the monophyletic clade grouping Aplousobranchia, Phlebobranchia, and Thaliacea (PP = 1.0) are modified compared to Rubinstein et al. (2013): (a) Thaliacea are now placed, with moderate support (PP = 0.8), as sister clade of Aplousobranchia + Phlebobranchia, and not nested within Phlebobranchia; (b) Phlebobranchia are paraphyletic since *Ciona* is placed as a sister group of Aplousobranchia (PP = 0.91), whereas in Rubinstein et al. (2013) *Ciona* is the sister lineage of the Phlebobranchia + Thaliacea clade (although with moderate support, i.e., PP = 0.79); (c) Aplousobranchia are monophyletic (PP = 1.0) and include the solitary *Rhopalaea* (Diazonidae). Moreover, *Rhopalaea* is not of the first diverging Aplousobranchia but is a sister clade of *Aplidium* (Polyclinidae) (PP = 1.0), while the colonial *Clavelina* (Clavelinidae) are the first aplousobranchs to diverge (PP = 1.0).

3.3. Regeneration experiments

Two months following thorax removal, five out of the six marked individuals were found with a fully developed thorax, reaching its original size. During the second thorax-removal round, three out of the five individuals were found with a complete and functioning thorax, one month later. The daily monitoring of the regenerating individual in the lab revealed that new siphons were visible two days after thorax removal, and a fully developed functional thorax, the same size as prior to removal, was visible after only seven days (Fig. 2). The second removal trial, which was conducted after six months on the same individual in the water table, yielded the same results. Throughout the entire monitoring period the regenerating individual exhibited completely healthy and functioning behavior (determined visually by initiating a siphonal contraction response).

4. Discussion

The current study presents a solid support for a positioning of the Diazonidae family, here represented by the solitary species *Rhopalaea idoneta*, as a member of the Aplousobranchia order. The mitochondrial phylogeny (Fig. 1B) reveals that *R. idoneta* is not an early diverging member of the Aplousobranchia, as suggested by Kott (1989), but has a more internal position and is a sister clade of *Aplidium*. Our study thus supports the scenario of an adaptation to a solitary lifestyle from an aplousobranch colonial ancestor. It has been demonstrated that a colonial life-style has evolved several time independently in Stolidobranchia and Phlebobranchia, in particular within Styelidae (Pérez-Portela et al., 2009). Though Zeng et al. (2006) originally suggested the monophyly of colonial Styelidae based on a restricted stolidobranch taxon sample. In the Aplousobranchia *sensu* Kott (1990) we are able to hypothesize that the coloniality is an ancestral trait which originally evolved from a *Ciona*-like solitary ancestor and then reverted to the solitary lifestyle in at least one lineage. Future analysis of colonial representatives of Diazonidae, such as a *Diazona* species, is needed in order to ascertain the monophyletic character of Diazonidae and to definitively confirm its nested position in the Aplousobranchia.

Most aplousobranch species are capable of whole thorax regeneration by the epicardium (Kott, 2005). The epicardium, or epicardial sacs, are paired endodermal sacs that evaginate from the postero-ventral part of the pharynx during embryogenesis (Berrill, 1951; Kott, 1990). In the Phlebobranchia and Stolidobranchia the epicardium has an excretory role. In the Aplousobranchia and *Ciona*, however, the epicardium is a source of stem cells, allowing rapid regeneration and budding (Berrill, 1951; Jeffery, 2015). Similarly to the broad regenerative abilities of *Ciona intestinalis* (Jeffery, 2015), our field experiments confirmed that *R. idoneta* possess the ability to completely regenerate its thorax within only a few days (Fig. 2), presumably by stem cells originating from the epicardium, which extends along the embedded abdomen. Other solitary ascidians from the orders Stolidobranchia and Phlebobranchia are not capable of regenerating a full thorax, as their body is not divided into two distinct body parts as in *Rhopalaea.* Thus, in Stolidobranchia and Phlebobranchia, the thorax is not homologous to the Aplousobranchia thorax since it does not contain the same organs, therefore it cannot be fully removed without damaging the viscera. In the tropical habitat of *R. idoneta*, the rapid regeneration of such a gelatinous thorax may provide an effective mechanism against fish predation, and may further explain the relatively high abundance of this species in the Red-Sea coral reefs (Koplovitz and Shenkar, 2014).

The increase in colonial integration is associated, in ascidians, with a decrease in zooid size (Ryaland and Warner, 1986). Thus, many aplousobranch species are characterized by small and simplified zooids. In the case of the genus *Rhopalaea,* adopting a solitary life-style may have driven the evolution of a large thorax size in order to increase filtration rates resulting in the re-acquisition of a “phlebobranch trait” as longitudinal vessels. For example, *R. idoneta*, *R. macrothorax*, and *R. crassa* are all tropical species characterized by a relatively much larger thorax (5–8 cm long) than those of colonial *Diazona* (10–15 mm long, Tokioka, 1953; Shenkar, 2012).

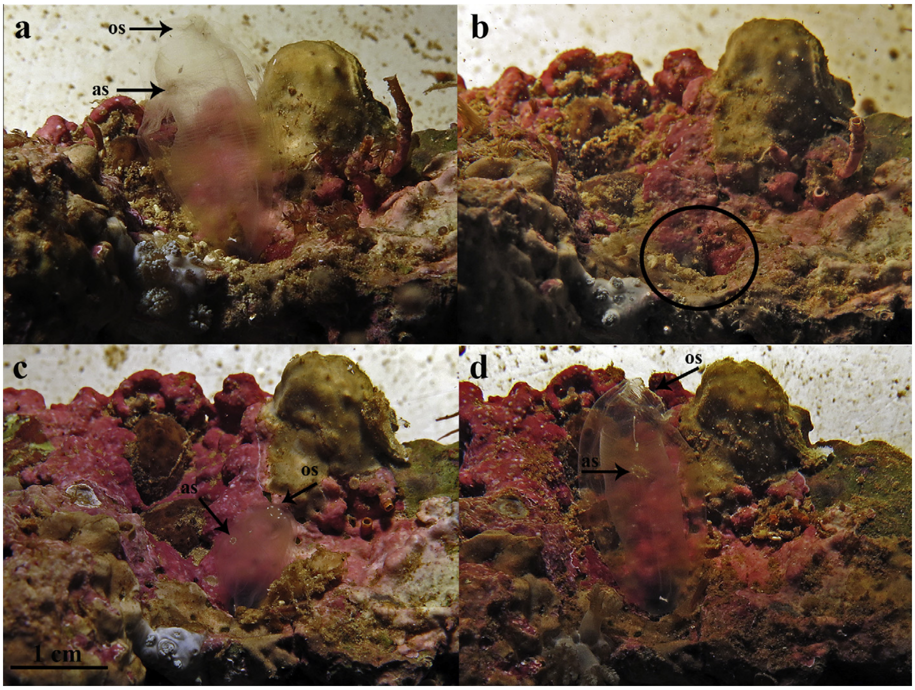

Fig. 2. Regeneration in *Rhopalaea idoneta*. (a) A complete individual before thorax removal. (b) Immediately after thorax removal. Black circle denotes the hole from which the thorax emerged. (c) Newly developing thorax three days post-removal. (d) New and complete thorax nine days post-removal. os – oral siphon; as – atrial siphon, scale bar 1 cm.

The most studied ascidian, *Ciona*, which has long been suggested as the most primitive extant ascidian (Kott, 1989), is currently classified as a phlebobranch (Shenkar et al., 2015). However, our present findings suggest that *Ciona* is in fact a sister clade of Aplousobranchia, as also suggested in the past based both on molecular data (Turon and López-Legentil, 2004) and morphological characters (Kott, 1990, 2005). However, it should be stressed that the relationships among early-diverging Phlebobranchia and Aplousobranchia are unstable, as evident by the low support values and the topological changes observed between studies (see Rubinstein et al., 2013, Fig. 4, for comparison). Thus, the sequencing of additional aplousobranch species, and in particular the sequencing of additional genera belonging to the family Cionidae, as well as the reevaluation of Cionidae morphological characters, may assist in accurately defining its phylogenetic position.

In sum, our results support the assigning of Diazonidae to the Aplousobranchia, and the loss of a colonial lifestyle in *Rhopalaea,* since this species did not diverge early among the Aplousobranchia. The unique phylogenetic position of this large solitary Diazonidae species within a generally colonial clade, together with its wide regenerative ability, makes *Rhopalaea* an interesting model by which to extend our knowledge on the evolution of regeneration and budding in tunicates.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2016.04.001.

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