# Molecular phylogeny endorses the relationship between carnivorous and filter-feeding tunicates (Tunicata, Ascidiacea)

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Submitted: 12 April 2011
Accepted: 17 August 2011
doi:10.1111/j.1463-6409.2011.00493.x

Tatián, M., Lagger, C., Demarchi, M. & Mattoni, C. (2011). Molecular phylogeny endorses the relationship between carnivorous and filter-feeding tunicates (Tunicata, Ascidiacea).—*Zoologica Scripta*, *40*, 603–612.

The phylogeny of the tunicates (animals considered the closest relatives to the vertebrates) is not yet completely defined, especially the evolutionary relationships within the class. Molecular studies do not include particular benthic deep-sea species that show morphological changes in the evolution from filter feeding into a carnivorous-feeding habit. According only to morphological features, these animals are considered as a part of the Class Ascidiacea (Family Hexacrobylidae), but also as a different class, Sorberacea, belonging to the Phylum Tunicata. In this study, we present a phylogenetic analysis based on 18S rDNA sequences, which clearly included these animals in Ascidiacea but in the Family Molgulidae, faster-evolving ascidians with a high evolution rate. This finding supports the idea that carnivory in Molgulidae represents a more recent adaptation to life in the ocean deep bottoms, where organisms have to adapt themselves to a less plentiful particulate organic carbon supply. Based on molecular and morphological evidence, we propose the following new synonymy: Hexacrobylidae Seeliger 1906 = Molgulidae Lacaze-Duthiers, 1877.

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## Introduction

Tunicates have occupied an important role relative to chordate and vertebrate evolution (Stach & Turbeville 2002; Bourlat *et al.* 2006; Vienne & Pontarotti 2006; Delsuc *et al.* 2008) as their chordate affinities were discovered by Kowalevsky (1866). Despite their key position in the tree of life, the phylogenetic affinities within the tunicates are still incomplete (Tsagkogeorga *et al.* 2009). Specifically, little is known about the evolutionary relationships of deep-sea tunicates, which still remain controversial.

The subphylum Tunicata comprises mostly filter-feeding animals. It includes the classes Thaliacea, Appendicularia (pelagic species) and Ascidiacea (benthic species). Ascidians are the largest and most diverse class within Tunicata, comprising 2.815 described species found in all marine habitats from shallow water to the deep sea (Shenkar & Swalla 2011).

Hexacrobylidae (Order Aspiraculata), a group of benthic predatory tunicates, are found in bathyal and abyssal areas. Bourne (1903) and Sluiter (1905) placed these predatory tunicates in the Family Molgulidae (Order Stolidobranchia, Class Ascidiacea), owing to the presence of a kidney and molgulid-type gonads. However, Seeliger (1906) established Hexacrobylidae (Order Aspiraculata) as a new family and order.

Strong modifications to the oral siphon (development of prehensile oral lobes) and the reduction of the pharynx enabled *Hexacrobylus* to adopt a predatory lifestyle on large

single prey that is very different from filter-feeding relatives (Millar 1959). These characteristics and differences in the nervous system and the position of the kidney were considered by Monniot *et al.* (1975) to raise Hexacrobylidae to a class of Tunicata, different from Ascidiacea: the Sorberacea. According to Kott (1989), there are not enough reasons to elevate Hexacrobylidae to a class of Tunicata separated from the Ascidiacea. Unlike Monniot *et al.* (1975), she believed the position of the neural complex (a dorsal ganglion and a gland) to be superficial, as it is located in the body wall beneath the epidermis between the atrial and oral siphons, as in all families of Ascidiacea. The position of the kidney (on the right or across the ventral region) varies according to the development of the gut. Only two valid genera were recognized within Hexacrobylidae by Kott (1989). In a revision of the class, Monniot & Monniot (1990) assumed that the systematic position of this group was uncertain; nevertheless, they supported Sorberacea with four genera and 12 species.

Recent phylogenomic studies revealed the tunicates as the closest living relatives of vertebrates, instead of cephalochordates, as traditionally accepted (Bourlat *et al.* 2006; Vienne & Pontarotti 2006; Delsuc *et al.* 2008; Tsagkogeorga *et al.* 2009). The phylogeny of the Tunicata was reconstructed by Stach & Turbeville (2002) using molecular (mitochondrial cytochrome oxidase I and 18S rRNA sequences) and morphological characters. More recently, Turon & López-Legentil (2004) and Tsagkogeorga *et al.* (2009) further added to the existing phylogeny. However, phylogenetic analyses with deep-sea ascidians are rare and no studies have been conducted on species in the Family Sorberacea/Hexacrobylidae. Only one report has described the phylogenetic position of a deep-sea ascidian *Megalodicopia hians* belonging to the Family Octacnemidae (Kurabayashi *et al.* 2003). Therefore, we sequenced 18S rRNA [the most widely used in previous molecular phylogenetic analyses: see Stach & Turbeville (2002); Tsagkogeorga *et al.* (2009)], of a single species belonging to Sorberacea/Hexacrobylidae. Our objective is to understand the phylogenetic relationship between deep-sea predatory tunicates and their filter-feeding counterparts.

## Materials and methods

### *Samples collection*

Ascidian samples were trawled, using an Agassiz (AGT) trawl, from a depth of 3000 m during the ANTXXII/3 cruise of RV 'POLARSTERN' (ANDEEP project) between 21 January and 6 April 2005 at the Weddell Sea, Antarctica [Kapp Norvegia (70°40′S; 14°43′W)]. Three individuals were collected and photographed to record their original colour before preservation. Two individuals were fixed in ethanol 96% for molecular analyses and one specimen in buffered formalin seawater 4% for species identification. All individuals were then identified, using a stereoscope and compound microscope [following the dissection and staining procedure recommended by Monniot & Monniot (1990)] at the Facultad de Ciencias Exactas, Físicas y Naturales, Universidad Nacional de Córdoba, Argentina.

### *DNA extraction and amplification*

DNA was extracted using the extraction protocol of QIAGEN DNeasy® kit (Qiagen, Hilden, Germany) from the tissue of gonads and mantle (muscle) of two individuals. Three overlapping 18s rRNA fragments of about 950, 900 and 850 bp were amplified with primer pairs 1F–5R, 3F–18Sbi and 18Sa2.0–9R, respectively, as described by Giribet *et al.* (1996, 1999). All amplifications were made using a Px2 Thermal Cycler (Thermo™). All 25-μL reaction tubes of PuReTaq Ready-To-Go™ PCR Beads contained 2 μL of DNA (10 ng/μL), 1 μL of each direction of primer (10 μM) and 21 μL of sterile water. The thermocycling program consisted of an initial denaturation step of 94 °C for 5 min, followed by 39 cycles of 15 s at 94 °C, 30 s at 50 °C, 15 s at 72 °C and followed by a final 7 min extension at 72 °C. Amplified fragments were sequenced in both directions by Macrogen Inc. (Korea). Sequencing results were analysed and assembled using the program DNABaser 2.0 (© Cubic Design, 2006).

The final sequences obtained, identical of 1770 bp each, are deposited in the NCBI GenBank database, with the following accession numbers: JN565043 and JN565044.

### *Matrix assembly and alignment*

To compare our DNA sequences to those of other tunicate taxa, we downloaded (March 2010) 18S sequences of all tunicates registered in GenBank. After deleting pseudogenes and repeated sequences of single species, our data set comprised 119 tunicates and 10 outgroups (see Supporting Information Table S1 for a complete list of species and GenBank accession numbers). Our outgroups were chosen to represent all the other deuterostomate phyla including Xenoturbellida, absent in previous analyses of ascidian relationships (Bourlat *et al.* 2006; Vienne & Pontarotti 2006; Delsuc *et al.* 2008; Tsagkogeorga *et al.* 2010). Echinoderms have never been regarded as closely related to tunicates; therefore, we chose a sea urchin to root the tree. Because the main goal of this work was to find the most suitable placement for the sorberacean/hexacrobylid species and considering that inference of phylogenetic relationships between the deuterostomates requires different types of data (i.e. morphology and many more genes), discussion of the results will comprise only the ingroup (tunicates). The sequences were aligned using MAFFT 5.3

(Katoh *et al.* 2002) and accessed online at http://align.bmr.kyushu-u.ac.jp/mafft/software/. Algorithm L-ins-i, a scoring matrix 20PAM/$k$ = 2, gap opening cost of 1.53, and offset value 0 were used for the alignment. Ambiguously aligned sites and sites including gaps in more than 50% of the sequences were excluded using Gblocks (Castresana 2000) using the following parameters: minimum number of sequences for a conserved position = $n/2$ (where $n$ = number of taxa), minimum number of sequences for a flanking position $\approx 0.70 \times n$, maximum number of contiguous non-conserved positions = 8, minimum length of a block = 10, allowed gap positions = with half. Owing to the fast evolutionary rate of aplousobranch species (Tsagkogeorga *et al.* 2009), we assembled the following two sequence matrices: (i) maximized the diversity of species, including the 118 tunicate species, our sample and outgroups; (ii) excluded the fast-evolving aplousobranch species, keeping only species of *Pycnoclavella* and *Clavelina* as representatives of the group. Following this approach, we could have the maximum number of choices to relate to the sorberacean/hexacrobilid species during the analysis of the complete matrix, avoiding the influence of the fast-evolving aplousobranch species when analysing the second, incomplete, matrix. Final size was 129 species × 1370 pb (596 parsimony informative characters) for the complete data set (matrix A), and 99 species × 1504 pb (514 parsimony informative characters) for the smaller matrix (B). Both alignments are available from the authors upon request.

### *Phylogenetic analyses*

Phylogenetic analyses for both matrices were performed using parsimony and maximum likelihood (ML) approaches. Parsimony analyses were conducted using the program TNT 1.1 (Goloboff *et al.* 2008; available at http://www.zmuc.dk/public/phylogeny/TNT/), using equal weights, inactivating parsimony uninformative characters (command: 'xinact') and treating gaps as a missing state (command: 'nsates nogaps'). Optimal trees were searched using random addition sequences of Wagner trees, followed by the TBR algorithm, making 500 replications and saving up to 10 trees per replica (command sequence: 'hold 5000; mult=tbr replic 500 hold 10;'). The resulting trees were used as starting points for a round of TBR branch swapping (command: 'bbreak=TBR'). We estimated support values for the clades using jackknife and bootstrapping on group frequencies (see Goloboff *et al.* 2008). We used a probability of alteration $P$ = 0.36 for jackknife calculations. For both re-sampling measures, we performed 500 pseudoreplicates of 10 random addition sequences each followed by TBR swapping, keeping up to 10 trees (string of commands 'mult: noratchet repl 10 tbr hold 10; resample jak repl 1000). Results from all the most parsimonious trees were expressed through strict consensus trees.

Maximum likelihood analyses were performed using Genetic Algorithm for Rapid Likelihood Inference (GARLI: Zwickl 2006), accessing the GARLI Web service at http://www.molecularevolution.org (Bazinet *et al.* 2007). The analysis settings were as follows: one thousand replicates, beginning with a random starting tree, using the general time-reversible model (GTR, as demonstrated by Tsagkogeorga *et al.* 2009; is the best fitting model for this gene in ascidians) and a rate heterogeneity model gamma, estimating the remaining parameters (base frequencies and proportion of invariant sites). The support of the clades was calculated using 500 replicates of boostrap resampling. Cladograms with bootstrap percentages were obtained from the 50% majority rule consensus of the 500 reconstructed trees using the program Mesquite 2.71 (Maddison & Maddison 2009).

We tested the results for a possible long-branch attraction artefact that can be present in parsimony and ML analyses on groups that showed relatively long branches (Felsenstein 1978; Pol & Siddall 2001) by following the procedure defined by Siddall & Whiting (1999), removing one of the long-branch groups, rerunning the analysis and doing the same with the other long-branch group.

## Results

Characteristics of the dissected ascidians were in accordance with those corresponding to the species *Oligotrema lyra* (Monniot & Monniot, 1973) (Fig. 1). Parsimony analysis of the matrix A (complete dataset) found 3456 equally parsimonious trees, of 3307 steps (Ci: 0.358; Ri: 0.835). The analysis of matrix B (reduced dataset) found 2520 trees of 2426 steps (Ci: 0.372; Ri: 0.776). Strict consensus of the

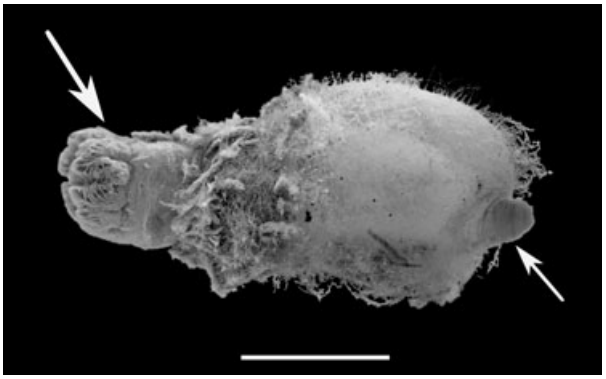

**Fig. 1** *Oligotrema lyra*. External appearance. Large arrow indicates the enlarged, lobed, oral siphon; small arrow shows the atrial siphon. Scale bar: 1 cm.


**Fig. 2** Phylogeny of Tunicata resulting from parsimony analysis of 18S rRNA sequences of 99 species (excluding derived aplousobranch species), now including the carnivorous species *Oligotrema lyra* (arrow). Other predator tunicate, the octacnemid *Megalodicopia hians*, is indicated by an asterisk. Numbers above branches show bootstrap/jackknife percentages, respectively.

cladograms obtained from matrix B with the jackknife and bootstrap support can be seen on Fig. 2; consensus of the matrix A can be seen in Supporting Information Fig. S1.

Maximum likelihood analysis of matrix A found a tree (Fig. 3) similar to that obtained under parsimony (−ln $L$ = 19240.32) and the same for matrix B (−ln $L$ = 15887.66) (see Supporting Information Figs. S2 and S3).

In all the analyses, *O. lyra* was placed inside the Family Molgulidae, being the family itself strongly supported (100% in all the analyses). In most analyses, *O. lyra* was sister to *Molgula occidentalis* (weakly supported), both species being the basalmost in the Molgulidae clade. Only in the ML analysis of the complete matrix (A), *O. lyra* was grouped with *Eugyra* and *Bostrichobranchus* species, this clade sister to *M. occidentalis*.

As Molgulidae and Appendicularia showed relatively long branches (Fig. 3), we tested the results for a possible long-branch attraction artefact. Both analyses (without


 


**Fig. 3** Maximum likelihood (ML) tree from analysis of 18S rRNA sequences of 99 tunicate species (excluding derived aplousobranch species), now including the carnivorous species *Oligotrema lyra* (arrow). Other predator tunicate, the octacnemid *Megalodicopia hians*, is indicated by an asterisk. Numbers below branches show bootstrap percentages.

molgulids and without appendicularids) showed the same result, being both long-branch groups alone placed as a sister group to Pyuridae–Styelidae clade each time.

## Discussion

### *General comments*

The main groups and relationships between clades revealed that Aplousobranchia, Phlebobranchia and Thaliacea species were related and were sister to the Appendicularia and Stolidobranchia, similar to Tsagkogeorga *et al.* (2009). Inside Stolidobranchia, Pyuridae was a paraphyletic family that included Styelidae (Fig. 2). Unlike Tsagkogeorga *et al.* (2009), our study did not resolve a placement for *Ciona savignyi* (Fig. 2). This species could be a sister to Perophoridae species in ML analysis, but not sister to *Ciona intestinalis*. This strange connection observed in our analysis could be related to the weak phylogenetic signal present in *C. savignyi*, a sequence that was much shorter (1093 bp) than that of most of the remaining species (complete 18S gene was around 1800 bp). Perhaps, some of the signal in the sequence was removed when processing the matrix with Gblocks program, as we used slightly stronger options (favoring strong data) than Tsagkogeorga *et al.* (2009), resulting in smaller matrices (i.e. less characters). Our resulting cladograms showed good support values, and most of the groups were consistently recovered in all the analyses. However, it is important to recognize that more genes (that show different evolution rates, to provide information at different levels of the tree) and additional morphology are required for a more robust phylogenetic hypothesis for the tunicates. A previous study has shown that even the use of the complete mitochondrial genome in tunicates could lead to ambiguous results (Stach *et al.* 2010).

### *Sorberacea/Hexacrobylidae placement*

Traditionally, the Family Hexacrobylidae was considered a part of Ascidiacea, but a major taxonomic change was proposed by Monniot *et al.* (1975), who proposed a new class, Sorberacea, for these deep-sea animals. Since then, diverse opinions were expressed, adhering (Monniot & Monniot 1990) or rejecting that decision (Kott 1989, 1992, 2005, 2009; Sanamyan & Sanamyan 2006). Phylogenetic studies based on morphological characters revealed that Sorberacea/Hexacrobylidae formed the sister group of the stolidobranchiate ascidians (Stach & Turbeville 2002). There was only one unambiguous synapomorphy uniting the Sorberacea/Hexacrobylidae with Styelidae+Pyuridae+Molgulidae: the position of the neural gland dorsal to the cerebral ganglion, despite the variability of that character. While the pleurogonid position of the gonads was a probable synapomorphy of that clade, the homology of the kidney in both Sorberacea/Hexacrobylidae and Molgulidae awaits further investigations (Stach & Turbeville 2002). The presence of minute protrusions in the cuticular surface of one hexacrobylid species, *Sorbera unigonas*, similar in size and shape to those usually seen in Stolidobranchia, was considered by Hirose *et al.* (1992) to be proof of phylogenetic affinity, particularly as these protrusions occur in limited families and related species. Although the protrusions proved to have some features of certain phylogenetic significance in many cases, those of *S. unigonas* are much higher than those of the Molgulidae (about 80 nm in the former, instead of 30–50 nm in the latter). According to Hirose *et al.* (1992), this difference in protrusion size might suggest the somewhat distant phylogenetic position between *Sorbera* and the Molgulidae. For that reason, these authors preferred the traditional systematic treatment for these groups, rejecting the inclusion of *Sorbera* in Molgulidae.

Our results confirm that 18S rDNA provided a clear view of the evolution of major tunicate lineages similar to previous studies (Swalla *et al.* 2000; Stach & Turbeville 2002; Tsagkogeorga *et al.* 2009). The present phylogenetic analyses include *O. lyra* in Ascidiacea and, specifically, in the Molgulidae clade. In our opinion, the status of Sorberacea, as a different class, and of Hexacrobylidae as a family belonging to the Order Aspiraculata cannot be supported any longer. This view is in agreement with what was proposed by Kott (1989), based on morphological features. For her, 'the justification for the Family Hexacrobylidae separate from the Molgulidae is somewhat problematical. It is retained as a reflection of the apparently close relationship between its two genera rather than an indication of the phylogenetic distance from the Molgulidae'.

Although not recovered in all the analyses (see 'Results'), the apparent relation between *O. lyra* and *M. occidentalis* is somewhat surprising, as the latter is a shallow water tropical species. But *M. occidentalis* was recovered basal to other *Molgula* species in previous studies using complete 18S sequences (Stach & Turbeville 2002; Tsagkogeorga *et al.* 2009) and as well as related to *Bostrichobranchus* and *Eugyra* when analysing an 18S fragment (Huber *et al.* 2000).

*Molgula occidentalis* is found closer to the equator than most of the molgulid species, which are, in general, found closer to the North Pole than to the equator. Nevertheless, a few warm species exist such as *M. occidentalis* and *Bostrichobranchus digonas*. Members of the Family Molgulidae are particularly widely distributed and not limited by usual zoogeographic boundaries (Kott 1969). Although most Molgulidae live on shallow bottoms, some members of the family (*Eugyra*, *Pareugyrioides*, *Molgula*, *Molguloides)* represent part of the Antarctic deep-sea tunicates, reaching depths as deep as 5800 m (Monniot & Monniot 1982).

  

Despite the fact that we used incomplete sequences of 18S for *Bostrichobranchus* and *Eugyra* (around 980 bp), their basal placement, together with *O. lyra* and *M. occidentalis* in the molgulid clade, appears supported. However, to clarify these relationships would require more data (i.e. morphology, more genes).

The chordate phylogeny portrays tunicates as highly derived chordates with specialized developmental modes and lifestyles (Delsuc *et al.* 2008). According to Swalla & Smith (2008), six separate tunicate clades, corresponding to traditionally recognized groupings, are supported by molecular data: the ascidian clades, Aplousobranchia, Phlebobranchia, Stolidobranchia, Molgulidae and the pelagic tunicates, Appendicularia and Thaliacea. Closely related molgulid species have very similar genomes but different gene expression patterns and corresponding differences in the larval body plan. The ascidian phylogeny generated using 18S rRNA sequences shows four distinct clades in Molgulidae, and each clade contains species with anural larvae (Hadfield *et al.* 1995). According to these authors, the tailless larvae are likely to have evolved four times independently within this ascidian family. The polyphyletic loss of the larval tail allows examination of ecological and/or biological conditions that may contribute to the selective advantage of a major alteration in the ascidian larval body plan (Huber *et al.* 2000). Unfortunately, larvae for predatory deep-sea molgulid species, which would help us providing information useful to infer relationships of this clade, are completely unknown.

The different length of the branches leading to different clades suggests that tunicates have unequal evolutionary rates. The molgulid ascidian 18S rRNA genes evolve faster than the rest of the stolidobranchian ascidian (Huber *et al.* 2000). The fast evolutionary rate in molgulids could be related to the high adaptability shown by the members of the family; i.e., they are able to fix on soft bottoms and, thus, to colonize deep-sea environments where these kinds of substrates predominate.

Molgulidae have also been reported as a monophyletic clade within stolidobranch ascidians that also include the families Pyuridae and Styelidae (Stach & Turbeville 2002). Stolidobranchia appeared as unambiguously monophyletic in reconstructions performed by Tsagkogeorga *et al.* (2009). Nevertheless, these authors found that branch lengths of the molgulids were longer than those of other stolidobranch families in neighbor-joining analysis using partial 18S rRNA sequences. According to Swalla *et al.* (2000), the ascidian Family Molgulidae forms a natural clade that might be best described as a separate order within the Ascidiacea. Molgulidae species are solitary, most of them having a single hermaphroditic gonad above the intestinal loop on the left side and above a kidney on the right side. Disposition of gonads at both sides of the body is a character that relates Molgulidae with Styelidae and Pyuridae (Perrier 1898). The presence of a plicated pharynx defines the Order Stolidobranchia (Lahille 1886) that includes Molgulidae, Styelidae and Pyuridae (the lack of true folds can be found both in some styelids and molgulids). In our opinion, the disposition of gonads and structure of the pharynx are strong reasons to retain Molgulidae in the Order Stolidobranchia.

The inclusion of *O. lyra* in Molgulidae turned this family even more diverse, with macrophagous species adapted to feed on prey items. Ascidians are mostly filter feeders that utilize particulate matter, mainly phytoplankton, and consequently play an important role in the coupling of pelagic and benthic systems (Riisgård *et al.* 1995). The plasticity shown by ascidians allows these animals to live under different conditions and to flourish in all the seas. Perhaps, one of the most relevant features that describe this plasticity is the adaptation to the intake of other food different than seston, which means a change from the typical filter-feeding habit to the capture of small zooplankton and benthic preys. That particular feeding mode can be found in only a few ascidians. Species belonging to the abyssal Family Octacnemidae (Order Phlebobranchia) have macrophagous feeding habits, under a mixed diet (Monniot & Monniot 1975, 1991; Monniot 1984). They possess an enlarged oral siphon, which is the main organ for prey capture, a great development of sense organs and reduction of the pharynx, which is a vestigial organ in the Antarctic species *Cibacapsa gulosa* Monniot & Monniot (1983), the only octacnemid known that seems to be exclusively carnivorous (Monniot & Monniot 1983; Lescano *et al.* 2010). Other Phlebobranchia are adapted to macrophagia: the species *Fimbrora calsubia* Monniot & Monniot (1991). That species also develops the mantle musculature and the nervous system in the area of the long oral lobes, despite the preservation of filter-feeding features, i.e., the well-developed pharynx, suggesting a mixed diet in that species (Monniot & Monniot 1991). *In situ* observations of the octacnemid *Megalodicopia hians* revealed the utilization of water currents for feeding (Okuyama *et al.* 2002). Different is the case of exclusive carnivorous tunicates such as *O. lyra*, which are able to capture a wide variety of preys but catching them actively, using their lobed oral siphon. If, as supposed previously by Millar (1959), modifications shown by comparatively unrelated *Hexacrobylus* and *Octacnemus* are explained by convergence, the acquisition of these modifications (mainly development of oral lobes and reduction of the pharynx) are adaptations for a carnivorous habit, probably following isolation in ocean trenches (Kott 2005).

According to Monniot & Monniot (1990), sorberaceans are widely distributed: its absence in some areas is proba-

bly related to insufficient collection. Usually, genera and even species present a cosmopolitan distribution. From these, *O. lyra* have been previously found in the NW Atlantic, the East Atlantic (from the south to the north) and the SW Indian Ocean. The species have also been found in the Pacific Ocean (Kott 1989) and tropical areas (Monniot & Monniot 2003). Although this species was previously recorded in Antarctica (Kott 1989), here, we report the first record of *O. lyra* at the Weddell Sea. In relation to depth, the finding was in the known depth range for *O. lyra*, 1500–5000 m (Monniot & Monniot 1990).

The present result contributes to a better understanding of the phylogeny of tunicates and reinforces the inclusion of the species, traditionally considered as Sorberacea/Hexacrobylidae, in Ascidiacea, within the Order Stolidobranchia, Family Molgulidae. According to Kott (1989), the Family Hexacrobylidae, which included two genera *Oligotrema* and *Asajirus*, shows characters in common with Molgulidae. These are the morphology of the pharynx and the gut, the presence of a kidney, the arrangement of the gonads, the test thin but tough and fibrous. Additionally, *Oligotrema* and *Asajirus* show several unique synapomorphies, as the enlarged oral siphon provided by six large and branched oral lobes, the reduced pharynx and the small atrial siphon without lobes that strongly support a close relationship between both genera (Kott 1989). The proximity of both genera justifies the expectation that the inclusion of *O. lyra* in Molgulidae represents the evolutionary placement of both genera.

The balance of morphological and molecular evidence does not, in our opinion, warrant continued recognition of the Family Hexacrobylidae, which renders Molgulidae paraphyletic. We therefore propose the following new synonymy: Hexacrobylidae Seeliger (1906) = Molgulidae Lacaze-Duthiers, 1877. That decision implies the inclusion of carnivorous species within Molgulidae. More findings about the biology of carnivorous deep-sea molgulids, e.g., larval development, will improve our understanding about the evolution of this enigmatic group of tunicates.

## Acknowledgements

This study was supported by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Instituto Antártico Argentino (IAA), Alfred Wegener Institute (AWI) and Universidad Nacional de Córdoba (UNC). This work was partially funded by PICTO ANPCyT-DNA No 36326 and SECyT (05/I602), Universidad Nacional de Córdoba. We express our gratitude to Dr. Pablo López-González and Dr. Angelika Brandt, who gently gave us the samples. We thank Dr. José Ochoa (Universidade de São Paulo) who helped with the molecular analysis and two anonymous referees whose suggestions improved the manuscript. Comments on language by Jenn Dijkstra were welcome.

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1**. Strict consensus of the 3456 cladograms obtained by parsimony analysis (length: 3307 steps; Ci: 0.358; Ri: 0.835) of matrix A (18S rRNA sequences from 119 tunicate species and outgroups). Values above branches indicate bootstrap/jackknife percentages. Placement of *Oligotrema lyra* marked by an arrow.

**Fig. S2**. Maximum Likelihood (ML) tree from analysis of 18S rRNA sequences of 119 tunicate species, now including the carnivorous species *Oligotrema lyra* (arrow). Other predator tunicate, the octacnemid *Megalodicopia hians*, is indicated by an asterisk.

**Fig. S3**. Majority rule consensus tree for the 500 bootstrap replicates, in the Maximum Likelihood analysis of matrix A (119 tunicate species and outgroups). Values below nodes represent bootstrap percentages.

**Table S1**. Species sampling, taxonomy and sequence accession numbers. The Table indicates the taxonomy and the species sampling used in the present study with associated GenBank® sequence Accession Numbers.

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