Molecular Phylogenetics and Evolution 50 (2009) 560–570

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Phylogeny of the families Pyuridae and Styelidae (Stolidobranchiata, Ascidiacea) inferred from mitochondrial and nuclear DNA sequences

R. Pérez-Portela [a,b,*], J.D.D. Bishop [b,c], A.R. Davis [d], X. Turon [e]

[a] Eco-Ethology Research Unit, Instituto Superior de Psicologia Aplicada (ISPA), Rua Jardim do Tabaco, 34, 1149-041 Lisboa, Portugal
[b] Marine Biological Association of United Kingdom, The Laboratory Citadel Hill, PL1 2PB Plymouth, UK
[c] School of Biological Sciences, University of Plymouth, PL4 8AA Plymouth, UK
[d] School of Biological Sciences, University of Wollongong, Wollongong, NSW 2522, Australia
[e] Centre d'Estudis Avançats de Blanes (CEAB, CSIC), Accés a la Cala St. Francesc 14, Blanes, Girona E-17300, Spain

ARTICLE INFO

Article history:
Received 22 July 2008
Revised 10 November 2008
Accepted 15 November 2008
Available online 27 November 2008

Keywords:
Pyuridae
Styelidae
Stolidobranchiata
Molecular phylogeny
Coloniality
COI
18S rDNA

ABSTRACT

The Order Stolidobranchiata comprises the families Pyuridae, Styelidae and Molgulidae. Early molecular data was consistent with monophyly of the Stolidobranchiata and also the Molgulidae. Internal phylogeny and relationships between Styelidae and Pyuridae were inconclusive however. In order to clarify these points we used mitochondrial and nuclear sequences from 31 species of Styelidae and 25 of Pyuridae. Phylogenetic trees recovered the Pyuridae as a monophyletic clade, and their genera appeared as monophyletic with the exception of *Pyura*. The Styelidae, on the other hand, appeared as a paraphyletic group split into several clades. One of them was formed by solitary oviparous species, of which the Pyuridae were a sister group. A second clade included the colonial genera *Botryllus*, *Botrylloides* and *Symplegma*. The remaining colonial and solitary genera formed several poorly resolved clades. One of the more species genus, *Polycarpa*, was shown to be polyphyletic, and the species *Styela plicata* grouped into two genetically distant clades suggesting the existence of two cryptic species. The internal phylogeny of Styelidae has bearings on the origin of coloniality in this family. We suggest to abandon the traditional division of colonial forms into social and compound species and use instead the categories of aggregated colonies that do not have common vascular systems, and integrated colonies, that do possess such systems. Our molecular results indicate that there have been several independent acquisitions of coloniality in the Styelidae, and that viviparity may be a pre-adaptation for a colonial life-style.

1. Introduction

In the last two decades, molecular techniques have been applied to questions addressing the evolution of the deuterostomes (e.g. Turbeville et al., 1994; Cameron et al., 2000; Swalla et al., 2000; Bourlat et al., 2003; Blair and Hedges, 2005). The phylogeny of the Phylum Chordata, originally divided into three subphyla, Vertebrata, Cephalochordata and Urochordata, has also been intensely studied in order to clarify the mechanisms of chordate evolution (Winchell et al., 2002; Zeng and Swalla, 2005).

New phylogenomic approaches have recently overturned conventional thinking about the relationships within chordates (Philippe et al., 2005; Bourlat et al., 2006; Delsuc et al., 2006; Dunn et al., 2008; Blair and Hedges, 2005). One of the most recent molecular phylogenies has suggested that the Subphylum Urochordata (Tunicata), represented by three different classes, Ascidiacea, Thaliacea and Larvacea, should be raised to the phylum level (Zeng and Swalla, 2005) but the subject is still under discussion since there are discrepancies between phylogenomic analyses and results from mitochondrial and rRNA data. Clarifying the phylogeny of Urochordata may be a critical step in understanding the evolution of the chordate body plan as well as the vast morphological and life-style differences within this animal group. Unfortunately, only a few works have addressed particular questions about the internal phylogeny of the Urochordata and, while some interesting relationships such as the inclusion of thaliaceans within ascidians have been uncovered (Swalla et al., 2000; Stach and Turbeville, 2002; Zeng and Swalla, 2005), other important questions, such as the placement of the Appendicularia, remain unresolved (Stach and Turbeville, 2002; Zeng et al., 2006).

The class Ascidiacea comprises three different orders and more than 17 families with a diversity of biological features. For most of these taxa, phylogenetic relationships remain poorly resolved (Turon and López-Legentil, 2004). Within the Ascidiacea, the Order

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[*] Corresponding author. Address: Eco-Ethology Research Unit, Instituto Superior de Psicologia Aplicada (ISPA), Rua Jardim do Tabaco, 34, 1149-041 Lisboa, Portugal. Fax: +351 21 8860954.

E-mail addresses: rportela@ispa.pt, rocio_perez@ub.edu (R. Pérez-Portela), jbis@mba.ac.uk (J.D.D. Bishop), adavis@uow.edu.au (A.R. Davis), xturon@ceab.csic.es (X. Turon).

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doi:10.1016/j.ympev.2008.11.014

Stolidobranchiata is one of the most important groups as it is species-rich and exhibits high morphological plasticity and complexity. To date, molecular and morphological data support the monophyly of the Stolidobranchiata uniting the traditionally recognized families Pyuridae, Styelidae and Molgulidae (Berrill, 1950; Kott, 1985; Monniot et al., 1991; Swalla et al., 2000; Zeng et al., 2006). However, the internal classification of this order remains under discussion. Whereas the Molgulidae has emerged as a well-supported monophyletic family, the relationships among the families Styelidae and Pyuridae have been poorly resolved and the phylogenies obtained inconclusive (Wada et al., 1992; Huber et al., 2000; Stach and Turbeville, 2002; Zeng et al., 2006). There are strong morphological evidences that Pyuridae and Molgulidae are related, with the latter having probably originated from the former (Berrill, 1950). However, Swalla et al. (2000) showed that the families Pyuridae and Styelidae formed a robust clade, separated from Molgulidae, but with both families being either paraphyletic or polyphyletic. In the molecular phylogeny reconstructed by Zeng et al. (2006), including five pyurids and 12 styelids, the family Pyuridae appeared either as a paraphyletic or a monophyletic group depending on the algorithms of reconstruction applied. Consequently, the relationships and internal phylogeny of these two families are not yet fully resolved.

Styelidae and Pyuridae show great complexity of the general body plan. Styelid body organization in particular has by far the greatest range of variation among ascidians, and styelids can resemble in one way or another species of almost any other family, including both solitary and colonial species as well as intermediate morphologies (Monniot et al., 1991). One currently accepted systematic arrangement of the family comprises three subfamilies, the Styelinae including solitary forms, the Polyzoinae including colonies whose zooids do not form systems, and the Botryllinae grouping colonial species that do form systems (Kott, 1985). On the other hand, the pyurid body plan may well be (together with Molgulidae) the most differentiated among ascidians (Berrill, 1950; Monniot et al., 1991). Pyuridae consists exclusively of simple, usually large, oviparous ascidians. Both styelids and pyurids feature stalked and unstalked forms. Being raised above the substratum on stalks can have important benefits for spatial competition and feeding activity (Young and Braithwaite, 1980; Kott, 1989; Monniot et al., 1991). It is not known whether this adaptation has appeared many times independently or whether there are evolutionary affinities between some or all of the stalked forms within families.

Another key question that can be addressed if a sound phylogenetic framework can be established is the origin of coloniality in Styelidae. Ascidians comprise both solitary and colonial forms. Colonial species include most of the Aplousobranchiata while solitary forms dominate the Phlebobranchiata and Stolidobranchiata. The ancestral ascidian may have been a colonial or a solitary form (Van Name, 1921; Garstang, 1928; Berrill, 1955; Kott, 1985), but it is clear that the colonial life-style in stolidobranchs is independently acquired (Kott, 1985; Wada et al., 1992) and differs from that of aplousobranchs and phlebobranchs in the type of budding and colony structure (Berrill, 1951; Nakauchi, 1982).

Colonial forms, all of them showing both sexual and asexual reproduction, are often divided into social and compound species. This classification, dating back to Milne Edwards (1841), distinguishes between colonies where the zooids are embedded in a common tunic (compound species) and those in which zooids are more or less connected basally but generally retain their individuality (social species). However, this classification is problematic, as there are intermediate forms, even in a single species. From the point of view of colony integration it is more relevant to consider whether zooids posses common vascular connections, which is a hallmark of strong colonial integration and the acquisition of colony specificity (Koyama and Watanabe, 1986; Satoh, 1994; Bishop and Sommerfeldt, 1999). Common vascular systems are found in the phlebobranch Perophoridae (social colonies) and the stolidobranch Botryllinae (compound colonies) (Brien, 1948). Most of the Polyzoinae would qualify as social colonies, and they do not have in general vascular connections between zooids. However, some genera included within Polyzoinae, such as *Symplegma* and *Metandrocarpa*, feature common vascular systems (Abbot, 1953; Watanabe and Newberry, 1976; Mukai et al., 1978). The vascular system may be important in clarifying the phylogeny of colonial styelids. We propose to adopt a more biologically meaningful classification of colonial species, with names partly derived from Mukai et al. (1978), into "integrated" colonial forms and "aggregated" colonial forms, depending on the presence or absence of permanent vascular connections among individuals, respectively. Mapping these colonial categories into a phylogenetic scheme might help unravel the evolution of coloniality in the Styelidae. Zeng et al. (2006) obtained results consistent with the appearance of coloniality just once, but their analysis included only three genera of colonial species, and a broader taxonomic sampling is necessary.

Variability and complexity in the body structure of ascidians has frustrated the establishment of clear relationships between families and genera using traditional morphological data. In this sense, molecular analyses provide us with a new and independent source of information for interpretation of the relationships among groups at several taxonomic levels. Phylogenies based on DNA have addressed interesting aspects of ascidian evolution, such as the independent origin of anural larval development within the order Stolidobranchiata (Hadfield et al., 1995; Huber et al., 2000) and the placement of the family Cionidae, previously included in the Phlebobranchiata, within the Aplousobranchiata (Turon and López-Legentil, 2004). Further, these phylogenies have clarified issues regarding some family-level arrangements (e.g. Pérez-Portela and Turon, 2008).

To date 18S rDNA and mtDNA (cytochrome genes) sequences have been the most widely used markers in molecular phylogenies in tunicates (e.g. Wada et al., 1992; Wada, 1998; Swalla et al., 2000; Stach and Turbeville, 2002; Turon and López-Legentil, 2004; Pérez-Portela et al., 2007; Zeng et al., 2006). However, only rarely have both types of marker been combined in a study (Stach and Turbeville, 2002; Zeng et al., 2006). In this work, we have assembled COI mtDNA sequence data along with sequences of the nuclear 18S rDNA to address specific taxonomic and phylogenetic questions about the Styelidae and Pyuridae. Using these two markers and a broader taxonomic sampling than in previous works, our goals were to clarify the relationships and the internal arrangement of Pyuridae and Styelidae and to study the evolutionary relationship between solitary and colonial species in Styelidae. Additionally, we were interested in analyzing the affinities between stalked and unstalked species found within the Styelidae and Pyuridae.

2. Material and methods

2.1. Ascidian samples

Eighty specimens of ascidians belonging to 17 species of the family Pyuridae and 19 species of Styelidae (Stolidobranchiata) were collected from the Mediterranean Sea, Atlantic, Pacific and Indian Ocean by SCUBA diving (see localities in Table 1). Colonial and solitary ascidians were removed from the tunic and preserved in absolute ethanol at −20 °C until processed. We added to these data