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Molecular characterization of 18S rDNA partial sequence in *Microcosmus* (Stolidobranchiata, Pyuridae)

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Abstract

We present a 18S rDNA based molecular phylogeny of two species of the genus *Microcosmus* (*M. sulcatus* and *M. claudicans*) sampled in the Mediterranean, to investigate their phylogenetic position relative to species of the order Stolidobranchiata. The analysis is based on partial sequences (739 bp) of the 18S rDNA. Among the 18 variable sites found between the two species, 4 correspond to transitions (ts), 14 to transversions (tv) and 4 to deletions/insertions. In the considered Stolidobranchiata, we found 4.3% overall mean number of nucleotide differences and 0.06 (S.E. ±0.01) Kimura 2-parameter distance. The mean number of nucleotide differences between *Microcosmus* spp. and other Stolidobranchiata species was of 6% and 0.08 (S.E. ±0.01) Kimura 2-parameter distance. A molecular phylogeny obtained by Maximum Parsimony corroborates results of the traditional taxonomy.

Keywords: *Microcosmus*; 18S rDNA; Tunicata; phylogenesis.

Introduction

Tunicata represents a subphylum of Chordata widely distributed in the Mediterranean Sea subdivided into three classes: Appendicularia, Asciidiacea and Thaliacea.

Asciidiacea, or ascidians, are by far the largest and most diverse extant group, with over 2500 described species in 14 families (Satoh 1994).

The order Stolidobranchia contains three families of ascidians, the Molgulidae, the Styelidae, and the Pyuridae (HADFIELD et al. 1995; HUBER et al. 2000; SWALLA et al. 2000; SWALLA 2001). According to traditional taxonomy the genus *Microcosmus* (Heller 1877) is placed in the family of Pyuride.

Though *Microcosmus* formed a sister group of *Pyura* in traditional taxonomy, no molecular phylogenetic tree was created to confirm or contradict this relationship.

Recent extensive molecular phylogenetic reconstruction was developed by STACH and TURBEVILLE (2002), who confirmed the monophyly of Stolidobranchiata as regards the families Styelidae, Molgulidae, and Pyuridae, but there have been no data on any species of the *Microcosmus* genus. Therefore, it is interesting to resolve the molecular phylogeny of *Microcosmus* by analysing the same markers used by Stach and
Turbeville.

In the present study we aim to characterise, through 18S rDNA gene sequences, two morphologically distinct species of *Microcosmus* found in the Mediterranean sea i.e. *M. sulcatus* and *M. claudicans*, and to clarify their relative phylogenetic position within tunicates.

*Microcosmus sulcatus* (Coquebert 1797) is exclusively found in the Mediterranean Sea and it is an edible species well known among fishermen. *Microcosmus claudicans* (Savigny 1816), on the other hand, is quite small and rare. Below are presented some distinctive morphological and ecological traits of the two species:

* M. sulcatus is a species of relatively large dimensions with a hard tunic with many folds, tawny red or brilliant red in colour, siphons with four red lobes, branchial chamber with 7 folds on every side and sex-separated gonads (SALFI 1931; RIEDL et al. 1991).

* M. claudicans has an ovoid body, covered by sand or other deposit materials. Its tunic is thick and hard, with a wrinkled surface and reddish brown in colour. It has a hermaphrodite, undivided gonad for each side of the body; the dorsal folds are fused in one unique membrane, siphons have reddish-yellow longitudinal bands and the branchial cambers are supplied with 7–8 and 8–9 folds on the right and the left respectively (SALFI 1931; RIEDL et al. 1991).

**Materials and Methods**

Ten samples of each species were collected in the Tyrrhenian Sea (Gulf of Naples) at a depth of about 10 metres. *Microcosmus* individuals in this area can be found on rocky or on organic substrates.

Specimens were randomly collected by snorkelling immersion in the upper intertidal zone and immediately frozen in liquid nitrogen. Once in the laboratory, they were transferred at -80° C until further analysis.

Total DNA was extracted from 1g of muscular tissue, taken from each individual, with a standard proteosane K and phenol/chloroform extraction protocol (SAMBROOK et al. 1989).

18S rDNA sequence is the most widely used in previous molecular phylogenetic analyses of Urochordates (HADFIELD et al. 1995; SWALLA et al. 2000; WADA 1998; WADA et al. 1992; WADA & SATOH 1994; STACH & TURBEVILLE 2002), therefore allowing straightforward data integration.

The partial 18S rDNA was amplified by polymerase chain reaction (PCR) using the following primers (STACH & TURBEVILLE, 2002):

- 18S 607-626, 5’ TCTGGTCCCAG-CAGCGCGG 3’
- 18S 1338-11324r, 5’ GAACGGCCAT-GCACCACC 3’.

All PCR reactions were performed in a 50µl volume with 1.7 u of Taq polymerase (Sigma), 100 ng of DNA, 5 µl 10x thermal polymerase buffer (Sigma), 0.2 mM MgCl₂, 200 µM dNTPs, and 300 pmol of each primer. The cycling conditions were 35 cycles of 1 min at 94° C, 1 min at 59° C and 1 min 30 s at 72° C, followed by a final step of 5 min at 72° C.

PCR products were purified with a Qiaquick PCR purification kit (Qiagen) and submitted to direct sequencing by use of BigDye™ Terminator Cycle Sequencing chemistry (Applied Biosystem product), following the manufacturer’s protocols. The sequences were run and analysed with an ABI3100 automated sequencer (Perkin-Elmer).

Sequence alignment and phylogenetic analyses were conducted using the software MEGA, version 2.1 (KUMAR et al. 2001).

**Results and Discussion**

The utilised primers amplified 739 nt of 18S rDNA for *M. sulcatus* (GeneBank Accession Number DQ149330) and 735 nt for *M. claudicans* (GeneBank Accession Number...
**Fig. 1:** Variable sites in 18S rDNA partial sequences of some Stolidobranchiata species.

| Species                | Sequence 1 | Sequence 2 | Sequence 3 | Sequence 4 | Sequence 5 |
|------------------------|------------|------------|------------|------------|------------|
| *M. sulphatus*         | GTCGGGATGA | TTCCT-GTC  | ACGTTA     |            |            |
| *M. claudicans*        |            |            |            | AC.....T.T | ...C..-...CA.... |
| *Pyura*                |            | ..CCCCA..T CGGGGCGTCG | GATG.G | | | |
| *Botryllus*            | G.....A.  | ..T.TGG.TC. | ..C..GTCG | GATG..     | | |
| *Botrylloides*         | G.....A.  | ..T.TGC..C. | ..C..GTCG | GATG..     | | |
| *Pyura*                |            | ..G...A.   | ..T.TGG..G | ..C..GTCG  | GATG..     | |
| *Pyura*                |            | ..G...A.   | ..T.TGG..G | ..C..GTCG  | GATG..     | |
| *Cnemidocarpa*         | G.....A.  | ..T.TGG..G | ..C..GTCG  | GATG..     | | |
| *Bostrichobranchus*    | ..GC CGGT..GTG CTGATCT..-C...GGGTTTACCC ..C-PTTGCAGA | | | | |
| *Eugyra*               | ..GC CGGT..GTG CTGATCT..-C...GGGTTTACCC ..C-PTTGCAGA | | | | |
| *Molgula*              | G.T..ATG. CGCG..C..C...-T T..GAC..TTT..G..G.-.T.GG.. | | | | |
| *M. sulphatus*         |            |            |            |            |            |
| *M. claudicans*        | GATGGAGCTC | TTC-CT-GTC | ACGTTA     |            |            |
| *Pyura*                |            |            |            | AC.....T.T | ...C..-...CA.... |
| *Botryllus*            | G.....A.  | ..T.TGG.TC. | ..C..GTCG | GATG..     | | |
| *Botrylloides*         | G.....A.  | ..T.TGC..C. | ..C..GTCG | GATG..     | | |
| *Pyura*                |            | ..G...A.   | ..T.TGG..G | ..C..GTCG  | GATG..     | |
| *Pyura*                |            | ..G...A.   | ..T.TGG..G | ..C..GTCG  | GATG..     | |
| *Cnemidocarpa*         | G.....A.  | ..T.TGG..G | ..C..GTCG  | GATG..     | | |
| *Styela*               | G.....A.  | ..T.TGG..G | ..C..GTCG  | GATG..     | | |
| *Symplegma*            | G.....A.  | ..T.TGG..G | ..C..GTCG  | GATG..     | | |
| *Bostrichobranchus*    | ..GC CGGT..GTG CTGATCT..-C...GGGTTTACCC ..C-PTTGCAGA | | | | |
| *Eugyra*               | ..GC CGGT..GTG CTGATCT..-C...GGGTTTACCC ..C-PTTGCAGA | | | | |
| *Molgula*              | G.T..ATG. CGCG..C..C...-T T..GAC..TTT..G..G.-.T.GG.. | | | | |

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From the 10 samples collected, not all tissue gave good quality sequences that could be considered for a comparative analysis. In fact, the results referred to only three sequences for species, and in these we did not observe any significant intraspecific variation. Further investigations into this feature, also using new markers such as Cox1, are in progress.

The nucleotide composition of the sequences averaged 21.2% C, 25.1% T, 23.2% A and 30.5% G. There were 16.6% polymorphic sites, 10% Parsimony informative sites and 6% singleton mutations. Alignment identified 4 deletions/insertions and 18 variable sites between the two species, corresponding to 4 transitions (ts) and 14 transversions (tv) with $ts:tv = \kappa = 0.3$. This ratio did not deviate significantly from neutral expectation (1:2, $\chi^2 = 0.0072$, d.f. = 1, $P > 0.05$), suggesting that there is no selection acting on the _M. sulcatus_ and _M. claudicans_ 18S rDNA genes.

The sequences of the two _Microcosmus_ species, were analysed with sequences of 17 Stolidobranchiata species, representative of all genera of the families: a) Molgulidae, with L12379 (GeneBank Accession Number) _Bostrichobranchus algonus_, L12414 _Eugya arenosa_, L12420 _Molgula citrina_, AB013016, b) Pyuridae, with _Halocynthia roretzi_, AF165827 _Herdmania curvata_, AJ250772 _Pyura vittata_, and _Styelidae_, with _Botryllus fuscus_, AF008424 _Botryllus scalaris_, AJ250775 _Cnemidocarpa clara_, AJ250774 _Dendrodoa aggregata_, L12440 _Pelonaia corrugata_, AF165825 _Polycarpa pomaria_, L12442 _Styela clava_, AF165826 _Symplegma reptans_.

In the considered Stolidobranchiata, we found 4.3% overall mean number of nucleotide differences and a 0.06 (S.E. ±0.01) Kimura 2-parameter distance. The mean number of nucleotide differences between _Microcosmus_ spp. and other Stolidobranchiata species was of 6% and 0.08 (S.E. ±0.01) Kimura 2-parameter distance (Fig. 1).

The phylogenetic analysis was carried out by Maximum Parsimony using as outgroups two Phlebobranchiata species (Gene Bank Accession Number: AF165820 _Ascidia zaraf_, AJ250778 _Ciona intestinalis_).

The strict consensus of the 42 most parsimonious trees obtained suggests that _Microcosmus_ genus is placed within Stolidobranchiata (Fig. 2). Differences between _M. claudicans_ and _M. sulcatus_ are supported.

In order to fully resolve the phylogenetic relationship of the _Microcosmus_ species we provided some molecular information that might be integrated with morphological and ecological knowledge. This is a preliminary study, but further intra- and inter-specific investigations of the other _Microcosmus_ species, will be useful to describe the diversity patterns in this group, with important implications in developing appropriate conservation management strategies for coastal ecosystems.

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This short article is devoted to the memory of Mario Milone, recently disappeared.

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Fig. 2: Phylogenetic relationship of Stolidobranchiata. Strict consensus of 42 most parsimonious trees. Bootstrap values were calculated for 1,000 replicates using heuristic search strategy with $n = 1,000$ random addition sequence replicates. 

Ascidia zara and Ciona intestinalis represent two species of Phlebobranchiata used as outgroups.

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