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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 r DNA; Tunicata; phylogenesis.*

Introduction

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

Ascidiacea, 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 Styelydae, 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 proteisane 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' TCTGGTCCCAGCAGCCGCGG 3'
18S 1338-11324r, 5' GAACGGCCATGCACCACC 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 $MgCl_2$, 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 (Quiagen) 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

```
[                                111 1111111111 1111111111 1111111111 ]
[                 237788888 9999999001 2222223333 3333444444 4445555667 ]
[                7570324678 0235678073 0137890345 6789023456 7890678123 ]
#M.sulcatus      CTCTTCTGCT GGCCCGCCTA TTAGCGGCGC TCTCCCCGGT TTTCGCCATG
#M.claudicans    TG.AA.G... .......... ..T....... .......... ..........
#Pyura           ---.....GC A.T....... .C......TT G.CT.-TACG .C--T..G..
#Botryllus       -----A..GC ....G.T..G .N...CATC. GNN.NT.ACC ..C-T..G..
#Botrylloides    -----G..GC A...GTT..G CN.ATC.TT. GNN.NT..AG G.C-T..GC.
#Cnemidocarpa    ---..T..GC ....T...CG CC..TT..T. G.C--T..C- ..C-T..G..
#Dendrodoa       ---..T..GC ....T....G .....A.TT. G.C--T..C- ..C-T..G..
#Halocynthia     G.......G. ..T.A..... --...T..T. G.CT--..CC ..C-T..G..
#Herdmania       G.......G. ..TT..T... .C......CT G.CT.--.TA ..C-T..G..
#Pelonaia        ---..T..GC ....AA...G .C..TT.TT. G.C--T.ACC ..C-T..G..
#Polyandrocarpa  -....T..GC ....G.T.CG ....TC.GC. G.CT.-GA.C ..-.T..G..
#Polycarpa       ---..T..GC ....A....G ....TT.TT. G.C--T.ACC ..C-T..G..
#Styela          ---..T..GC A......... .C..TT.TT. G.C--T.ACC ..C-T..G..
#Symplegma       -.......GC ....G.T..G --..TC.TC. G.C--T..CC ..C-TT.G..
#Bostrichobranchus ---...A.GC CCGT..GTC. .-.CT..-C. GGGGTTTACC .C-TT.TGCA
#Eugyra          ---...A.GC CCGT..GT.. C-.T...-C. GGAGTTTACC .C-TT.TGCA
#Molgula         G.T...ATG. CCGG....C. C-....T-T. GAC.TT.TT. G.-.T.GG..

[                1111111111 1222222222 2222222233 3333333333 3344444444 ]
[                8888899999 9023344445 5578888900 0011222445 5901578888 ]
[                4567845678 9055602582 4512348934 7901369237 9973790134 ]
#M.sulcatus      GTCGGGATGA GACAT-CCAG GTAGGTATCG AGTTTGGCG- TCCCGAGTCT
#M.claudicans    .......... .....C.... .......... .........- ..A..TCGGA
#Pyura           .-.....GA. .T.TGG.TC. .......... GCGCC....- ..-.......
#Botryllus       .G..T..GA. .T.TGC..C. .......... .CGA.....C ..--------
#Botrylloides    .A.....GA. .T.TGG.... .......... .CGN...T.G ..--------
#Cnemidocarpa    .G......A. .T.TGC..C. .......... GCGC.....- ..-.......
#Dendrodoa       .G.....GA. .T.TGC..T. .......... GCGC.....- ..-.......
#Halocynthia     .G......A. .T.TGC.... .......... GCGC.....- ..-.......
#Herdmania       .G..C.GAA. .T.TGT..C. ..G....... GCAC..A..- ..-.A.....
#Pelonaia        .G......A. .T.TGC..C. .......... GCGC.....- ..-.......
#Polyandrocarpa  .G.....GA. .T.TG-.... .......C.. GCGC.A..T- C.-.......
#Polycarpa       .G.....GA. .T.TGC..T. .......... GCGC.....- ..-.......
#Styela          .G......A. .T.TGT..C. .......... GCAC.....- ..-.......
#Symplegma       .G.....GA. .T.TGT..T. .......... GCG......- ..-.......
#Bostrichobranchus .GT..AGAA. TT.T.TT..T CG.T....T. .TG.....A- .T-T......
#Eugyra          .G...AGAA. TT.T.TT..T CG.T...... .TG.....A- .T-T......
#Molgula         TG.C..GC.G TTTTAG..TT CG..AGGGAT C-.C....A- .T-T......

[                4445555555 5555555555 555567]
[                8881111223 3333446777 778891]
[                5682457363 4568166025 793420]
#M.sulcatus      GATGGAGCTC TTC-CT-GTC ACGTTA
#M.claudicans    AC.....T.T ...C..-... CA....
#Pyura           .......... ...C..GTCG GATGA.
#Botryllus       ---------- ---------- ------
#Botrylloides    ---------- ---------- ------
#Cnemidocarpa    ........C. ...C..GTCG GATG..
#Dendrodoa       .......... ...C..GTCG GATG..
#Halocynthia     .......... ...C..CTCG GATG..
#Herdmania       .......... ...C..GT.G GATGA.
#Pelonaia        .......... ..TC..GTCG GATG..
#Polyandrocarpa  .......... ...C..GTCG GATG..
#Polycarpa       .......... ...C..GTCG GATG..
#Styela          .......... ..TC..GTCG GATG..
#Symplegma       .......... ...C..GTCG GATG..
#Bostrichobranchus ..CCTC...T CGAGGCGTCG GATGA.
#Eugyra          ..CCTC...T CGAGGCGTCG GATGA.
#Molgula         ..CCCCA..T CGGGGCGTCG GATG.G
```

Fig. 1: Variable sites in 18S rDNA partial sequences of some Stolidobranchiata species.

DQ149331). 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 = $\kappa \cong 0.3$. 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 genuses of the families: a) Molgulidae, with L12379 (GeneBank Accession Number) *Bostrichobranchus digonas*, L12414 *Eugyra arenosa*, L12420 *Molgula citrina*, AB013016, b) Pyuridae, with *Halocynthia roretzi*, AF165827 *Herdmania curvata*, AJ250772 *Pyura vittata*, and Styelidae, with AF008422 *Botrylloides fuscus*, AF008424 *Botryllus scalaris*, AJ250775 *Cnemidocarpa clara*, AJ250774 *Dendrodoa aggregata*, L12440 *Pelonaia corrugata*, AF165825 *Polyandrocarpa misakiensis*, L12441 *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 zara*, 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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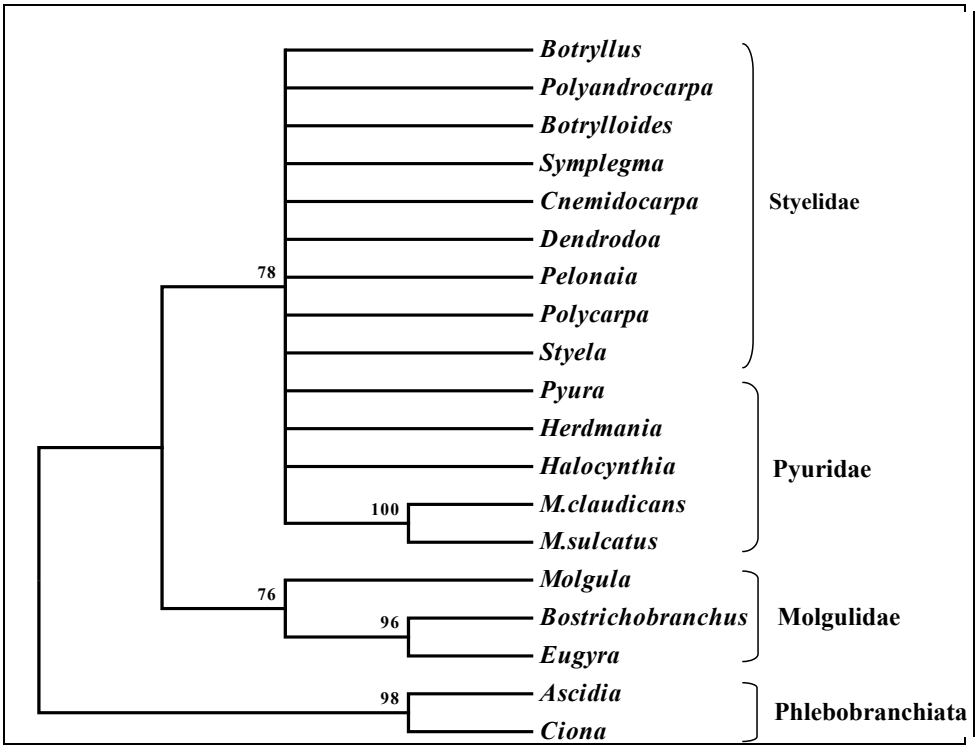

Fig. 2: Phylogenetic relationship of Stolidobranchiata. Strict consensus of 42 most parsimonious trees. Bootstrap values were calculated for 1,000 replicates using heuristic search strategy with n = 1,000 random addition sequence replicates.
Ascidia zara and *Ciona intestinalis* represent two species of Phlebobranchiata used as outgroups.

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