

## Mediterranean Marine Science

---

Vol 7, No 2 (2006)

---



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

D. FULGIONE, D. RIPPA, A. DE LUCA, M. MILONE

doi: [10.12681/mms.165](https://doi.org/10.12681/mms.165)

---

#### To cite this article:

FULGIONE, D., RIPPA, D., DE LUCA, A., & MILONE, M. (2006). Molecular characterization of 18S rDNA partial sequence in *Microcosmus* (Stolidobranchiata, Pyuridae). *Mediterranean Marine Science*, 7(2), 5–10.  
<https://doi.org/10.12681/mms.165>

**Mediterranean Marine Science**  
Volume 7/2, 2006, 5-10

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

**D. FULGIONE<sup>1</sup>, D. RIPPA<sup>2</sup>, A. DE LUCA<sup>3</sup> and M. MILONE**

<sup>1</sup> Dipartimento di Biologia Strutturale e Funzionale, Università di Napoli Federico II.  
Via Cinthia MSA, Napoli

<sup>2</sup> Dipartimento delle Scienze Biologiche sezione di Zoologia. Università di Napoli Federico II.  
Via Mezzocannone 8 – 80134 Napoli

<sup>3</sup> Museo di Anatomia Veterinaria. Università di Napoli Federico II

e-mail: fulgione@unina.it

---

**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.  $\pm 0.01$ ) 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.  $\pm 0.01$ ) 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 (Sato 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 Pyuridae.

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 Styelyidae, 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 chambers 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 mus-

cular tissue, taken from each individual, with a standard proteinase 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-CAGCCGCGG 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 MgCl<sub>2</sub>, 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

```

[
[
[
#M.sulcatus
#M.claudicans
#Pyura
#Botryllus
#Botrylloides
#Cnemidocarpa
#Dendrodoa
#Halocynthia
#Herdmania
#Pelonaia
#Polyandrocarpa
#Polycarpa
#Styela
#Symplegma
#Bostrichobranchnus
#Eugyra
#Molgula

[
[
[
#M.sulcatus
#M.claudicans
#Pyura
#Botryllus
#Botrylloides
#Cnemidocarpa
#Dendrodoa
#Halocynthia
#Herdmania
#Pelonaia
#Polyandrocarpa
#Polycarpa
#Styela
#Symplegma
#Bostrichobranchnus
#Eugyra
#Molgula

[
[
[
#M.sulcatus
#M.claudicans
#Pyura
#Botryllus
#Botrylloides
#Cnemidocarpa
#Dendrodoa
#Halocynthia
#Herdmania
#Pelonaia
#Polyandrocarpa
#Polycarpa
#Styela
#Symplegma
#Bostrichobranchnus
#Eugyra
#Molgula

[
[
[
#M.sulcatus
#M.claudicans
#Pyura
#Botryllus
#Botrylloides
#Cnemidocarpa
#Dendrodoa
#Halocynthia
#Herdmania
#Pelonaia
#Polyandrocarpa
#Polycarpa
#Styela
#Symplegma
#Bostrichobranchnus
#Eugyra
#Molgula

```

**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 genera 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 *Polycarpa misakiensis*, L12441 *Polycarpa pomaria*, L12442 *Styela clava*, AF165826 *Sympyema reptans*.

In the considered Stolidobranchiata, we found 4.3% overall mean number of nucleotide differences and a 0.06 (S.E.  $\pm 0.01$ ) 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.  $\pm 0.01$ ) 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.

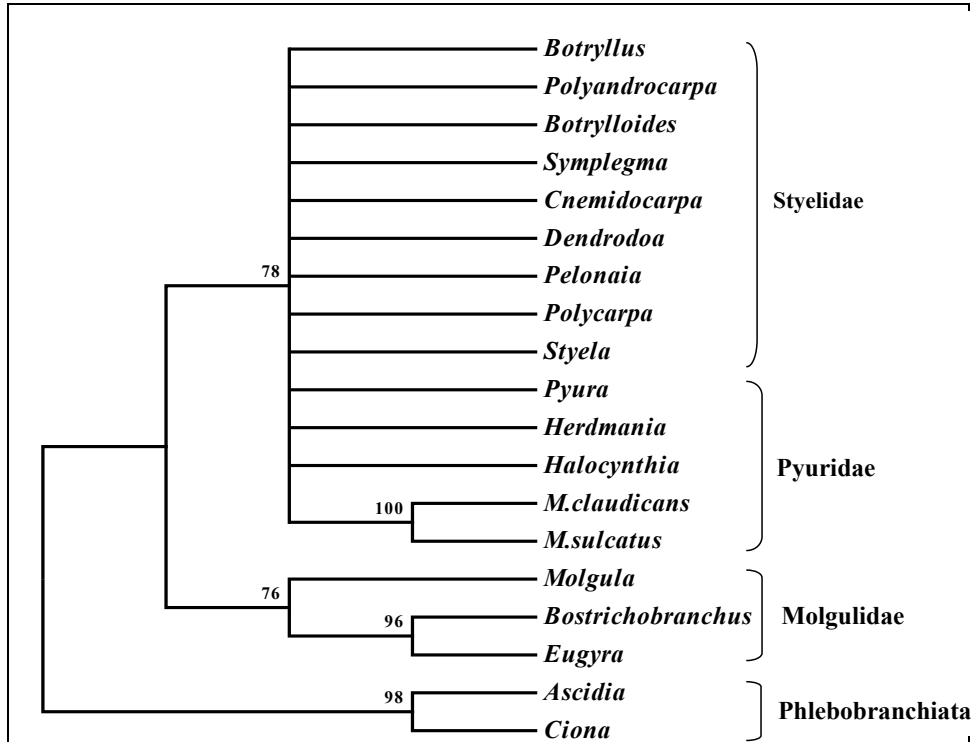
In order to fully resolve the phylogenetic relationship of the *Microcosmus* species we provided some molecular information that might be integrated with morphological and ecological knowledge. This is a preliminary study, but further intra- and inter-specific investigations of the other *Microcosmus*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

## Acknowledgements

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

## References

- HADFIELD, K.A., SWALLA, B.J. & JEFFRY, W.R., 1995. Multiple origins of anural development in ascidians inferred from rDNA sequences. *Journal of Molecular Evolution*, 40: 413 - 427.
- HUBER, J.L., BURKE DA SILVA, K., BATES, W.R. & SWALLA, B.J., 2000. The evolution of anural larvae in molgulid ascidians. *Semin. Dev. Biol.* 11: 419 - 426.
- KUMAR, S., TAMURA K., JAKOBSEN I.B. & NEI, M., 2001. MEGA2: Molecular Evolutionary Genetics Analysis software. *Bioinformatics*, 17 (12): 1244 - 1245.
- RIEDL, R., ABEL, E., KOHLMAYER, J., KUSEL, H., MATTHES, D., ROSSI, L., SALVINI-PLAWEN, L. & SARÀ, M., 1991. Fauna und Flora des Mitter-



**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.

- Imeeres. Verlag Paul Parley, Hamburg–Berlin, Germany.
- SALFI, M., 1931. Gli Ascidiacei del Golfo di Napoli. *Pubblicazioni della Stazione Zoologica di Napoli*, 11: 293-360 [in Italian].
- SAMBROOK, J., FRITSCH, E.F. & MANIATIS, T., 1989. *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- SATO, N. 1994. *Developmental biology of ascidians*. Cambridge University Press, New York.
- STACH, T. & TURBEVILLE, J.M., 2002. Phylogeny of Tunicata inferred from molecular and morphological characters. *Molecular Phylogenetics and Evolution*, 25: 408 - 428.
- SWALLA, B.J., CAMERON, C.B., CORLEY, L.S. & GAREY, J.R., 2000. Urochordates are monophyletic within the Deuterostomes. *Systematic Biology*, 49: 52 - 64.
- SWALLA, B.J. 2001. Phylogeny of the urochordates: implications for chordate evolution. In *Biology of Ascidians: Proceedings of the First International Symposium on the Biology of Ascidians*, Hokkaido University, Sapporo, Japan, June 2000. H. Sawada, H. Yokosawa & C. Lambert (Eds). Springer-Verlag, Tokyo. pp. 219 - 224.

- WADA, H., MAKABE, K.W., NAKAUCHI, M. & SATOH, N., 1992. Phylogenetic relationship between solitary and colonial ascidians, as inferred from sequence of the central region of their respective 18S rDNA. *Biological Bulletin*, 183: 448 - 455.
- WADA, H. & SATOH, N., 1994. Details of the evolutionary history from invertebrates to vertebrates, as deduced from the sequenced of 18S rDNA. *Proceeding of National Academic Science USA*, 91: 1801 - 1804.
- WADA, H., 1998. Evolutionary history of free-swimming and sessile lifestyles in urochordates as deduced from 18S rDNA molecular phylogeny. *Molecular Phylogenetics and Evolution*, 15(9): 1189 - 1194.

*Accepted in April 2007*