

## IS THE OCTOMACRIDAE THE SISTER FAMILY OF THE DIPLOZOIDAE ?

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### Summary :

Diplozoidae and Octomacridae are usually considered as sister families. Essentially this is because they are the only polyopisthocotyleans parasitising primary freshwater teleosts. Because of the lack of phylogenetically informative morphological characters to explore the pattern of colonisation of the primary continental freshwater teleosts and in order to understand the appearance of the "natural parabiosis" of Diplozoidae, a molecular phylogeny was inferred by comparing newly obtained partial 28S and 18S rDNA gene sequences of *Eudiplozoon nipponicum* and *Diplozoon homoion* with other already available sequences. The phylogenetic analysis seems to show that Diplozoidae and Octomacridae are not sister groups. Thus, the colonisation of primary freshwater teleosts by these two families could be independent.

**KEY WORDS :** Diplozoidae, Octomacridae, Cyprinidae, Catostomidae, freshwater fishes, molecular phylogeny.

### Résumé : LES OCTOMACRIDAE CONSTITUENT-ILS LE GROUPE FRÈRE DES DIPLOZOIDAE ?

Les Diplozoidae et les Octomacridae sont décrits dans l'ensemble des classifications actuelles des Polyopisthocotylea comme étant des groupes frères. Cela est essentiellement dû au fait qu'ils sont les seuls Polyopisthocotylea parasitant des poissons d'eaux douces primaires. Les données morphologiques et ultrastructurales qui réunissent ces deux familles sont peu nombreuses et discutables. Pour explorer le mode de colonisation des poissons d'eaux douces primaires et pour mieux comprendre l'apparition de la parabiose naturelle des Diplozoidae, nous avons réalisé des séquences partielles de 18S et 28S ADNr pour deux espèces de Diplozoidae : *Eudiplozoon nipponicum* et *Diplozoon homoion* que nous avons intégré dans une phylogénie moléculaire avec les séquences déjà disponibles. L'étude de la relation entre Diplozoidae et Octomacridae semble montrer qu'ils ne sont pas monophylétiques, ce qui sous-entend que la colonisation des poissons d'eaux douces primaires par ces deux familles pourrait avoir eu lieu indépendamment.

**MOTS CLÉS :** Diplozoidae, Octomacridae, Cyprinidae, Catostomidae, poissons d'eau douce, phylogénie moléculaire.

Polyopisthocotyleans reach their greatest diversification mainly on marine teleost fishes. Nevertheless, in this subclass, the Diplozoidae and Octomacridae are the only families parasitising primary freshwater teleosts. The Diplozoidae with more of 50 species actually described are really diversified on Cyprinidae and Characidae, the Octomacridae with only five species described on Catostomidae and Cyprinidae are less diversified (Khothenovsky, 1985). These two parasite families along with the Discocotylidae constitute the suborder Discocotylina (Boeger & Kritsky, 1993; Lebedev, 1995; Boeger & Kritsky, 1997; Boeger & Kritsky, 2001). The few morphological characters in favour of the grouping of these three families in this suborder are not very strong and this decision is debatable. Boeger & Kritsky (1993) claimed that two morphological characters support the monophyly

of the Discocotylina, namely the absence of spines on the male copulatory organ and the absence of anchors at all stages of development. But a phylogenetic analysis based on partial D2 sequences of 28S rDNA showed that monophyly of the Discocotylina is questionable because the Discocotylidae appeared to be only distantly related to the Diplozoidae (Jovelin & Justine, 2001). A divergence of the Discocotylidae is likely since they are essentially parasites of Salmonidae. This fish family contains many anadromous fishes and is not a primary freshwater family, unlike the Cyprinidae, Characidae and Catostomidae. On this basis, Khothenovsky (1985) has proposed the association of Diplozoidae and Octomacridae in a suborder, the Octomacrinea. Since Diplozoidae and Octomacridae are the only polyopisthocotyleans diversified on continental freshwater fishes, it is possible that they have diverged from a recent common ancestor already present on freshwater teleosts.

The present-day diversity is thus the result of co-speciation and switch between fishes and parasites, the freshwater habitat being a simple shared phylogenetic character (synapomorphy). So, a detailed examination

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of their phylogenetic relationship could reveal whether the colonisation of primary freshwater teleosts by polyopisthocotyleans took place once by their common ancestor or several times in independent events of colonisation.

The exploration of the phylogenetic relationship between these two families could also provide us with the opportunity to address another intriguing question: the Diplozoidae exhibits one of the most striking modes of reproduction (Lambert *et al.*, 1987). Indeed, the hermaphrodite adults develop and reach sexual maturity only after the permanent fusion of two larvae. If a family close to the Diplozoidae could be found, it may shed light on the origin of this intriguing sexual graft. The Octomacridae seems to be the most likely candidate, but very few morphological arguments support this hypothesis. The genitalia are absent in the Diplozoidae due to the sexual graft. So, the only morphological character supporting this phylogenetic proximity, is the presence of only one testis in the hermaphrodite individuals of the two families. A study of the spermatozoid ultrastructure (Hathaway *et al.*, 1995) undertaken to explore this phylogenetic relationship failed to show any common characters between the two families. But this result is due to the aflagellate structure of the spermatozoid of the Diplozoidae, a characteristic and specific trait of this family (Justine *et al.*, 1985; Justine, 1991).

A molecular investigation was necessary to overcome this lack of phylogenetically informative morphological characters and to elucidate the colonisation pattern and the evolution of the reproductive system.

## MATERIALS AND METHODS

We sequenced 640 pb of the 3' end of the 18S rDNA and 250 pb of the 5' end of the 28S rDNA region D1 of two Diplozoidae: *Diplozoon homoion* from *Rutilus rutilus* and *Eudiplozoon nipponicum* from *Cyprinus carpio* collected in southern France. Those two partial sequences correspond to the molecular information already available for the Octomacridae and other Polyopisthocotylea (Littlewood *et al.*, 1998; Littlewood *et al.*, 1999; Mollaret *et al.*, 1997; Mollaret *et al.*, 2000). This molecular information is available in Genbank data base for: *Octomacrum lanceatum* (Octomacridae) parasitising *Catostomus catostomus*, *O. mexicanum* (Octomacridae) parasitising *Catostomus sp.*, *Neopolystoma spratti* (Polystomatidae) parasitising *Chelodina longicollis*; *Zeuxapta seriola* (Axinidae) parasitising *Seriola hippos*; *Bivagina pagrosomi* (Microcotylidae) parasitising *Chrysophrys aurata*; *Kubnia scombrus* (Mazocraeidae) parasitising *Scomber scombrus*. The tree was rooted with Polystomatidae

that are tetrapod parasites. Mazocraeidae divergent from other teleost's parasites (Mollaret *et al.*, 2000) were chosen in order to evaluate the phylogenetic distance between the others families: Axinidae, Microcotylidae, Diplozoidae and Octomacridae that are closed in the cladistic morphological analysis (Boeger & Kritsky, 1993).

The DNA of members of the Diplozoidae was obtained with a CTAB buffer and amplified as previously described by Sicard *et al.* (2001). The 28S rDNA was amplified with the primers cer58S2249: 5'GCTCACGTGACGATGAAGAG<sup>3</sup> and cer28S3116: 5'TCGCTATCGGACTCGTGCC<sup>3</sup>; the 18S rDNA with the primer cer18S386: 5'AACGGCTACCACATCCAAGG<sup>3</sup> and reverse primer cer18S1585: 5'GCAGG-GACGTATTGAGCACA<sup>3</sup> (the numbers in the name of the primer refer to the number position in the *Coenorhabditis elegans* sequences). PCR products were purified with the GeneClean kit (Bio 101) and sequenced with the same primers as for PCR with the ThermoSequenase kit (Apbiotech). The electrophoresis was performed in an ALFred (Apbiotech) automatic sequencer. The sequences were first aligned automatically on the Multalin server (Corpet, 1988) ([www.toulouse.inra.fr/multalin](http://www.toulouse.inra.fr/multalin)) and manually revised using the Software Genedoc ([www.psc.edu/biomed/genedoc](http://www.psc.edu/biomed/genedoc)). The partial sequence of 18S rDNA and partial sequence of 28S rDNA (full domain C1, full D1 and partial D2) were used. The phylogenetic analyses were performed by Phylowin (Galtier *et al.*, 1996). Trees were constructed with the bio-Neighbour-joining (bioNJ), the maximum likelihood (ML) and the maximum parsimony (MP) methods. Bootstrap values were calculated for bioNJ, ML and MP with 500 replicates and likelihood of the topologies was tested with Phylowin.

## RESULTS AND DISCUSSION

When we compiled the 18S rDNA and the 28S rDNA in the same phylogenetic analysis, we obtain the most strongly supported topology (Fig. 1). The relative rate of evolution of internal branches was estimated with RRTree (Robinson *et al.*, 1998) to detect a potential "long-branch" effect. In our analysis of the tree, only the Mazocraeidae shows a significantly faster evolutionary rate compared to the other polyopisthocotyleans using *Neopolystoma* as reference. This probably explains why the values of the bootstrap of the branch between *Kubnia* and the others are so low.

The phylogenetic tree obtained modulates the idea of close phylogenetic relationship between Diplozoidae and Octomacridae, and at the same time the monophyly of the Discocotylinae obtained from morpholo-

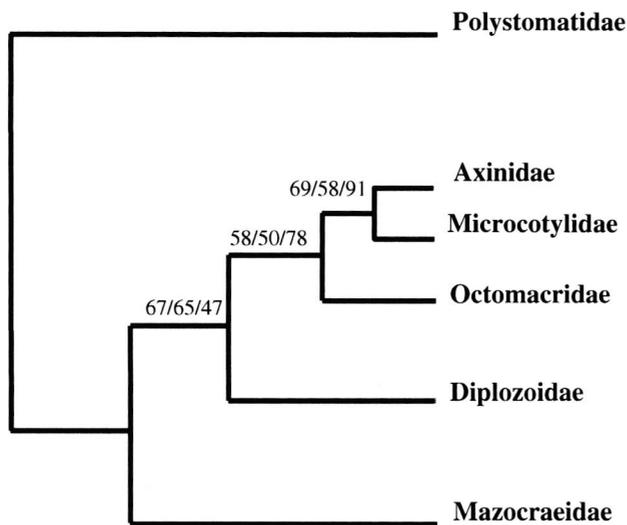


Fig. 1. – Molecular Phylogenetic relationship between Diplozoidea, Octomacridae and other teleost's Polyopisthocotylea inferred from partial 18S rDNA sequences and partial 28S rDNA sequences.

We have compiled the 18S rDNA and the 28S rDNA sequences in the same phylogenetic analysis and we obtained a most strongly supported topology than the separate analysis. Numbers on branching are the bootstrap proportion calculated with 500 iterations respectively with bio neighbour-joining method, MP and ML. The likelihood for other imposed topology was tested with Phylowin and this tree still the best one. The relative rate of evolution of internal branches was estimated with RRTree to detect a potential "long-branch" effect. Only the Mazocraeidae show a significantly faster evolutionary rate compared to the other Polyopisthocotyleans using *Neopolystoma* as reference.

gical analysis (Boeger & Kritsky, 1993; Lebedev, 1995; Boeger & Kritsky, 1997; Boeger & Kritsky, 2001). In fact, the Diplozoidea seems to be the sister group of a clade including Microcotylidae, Axinidae (Microcotylinae) and Octomacridae. Thus, this phylogenetic tree suggests that there is no recent common ancestor for Diplozoidea and Octomacridae. On the contrary, the topology as well as the relative branch length suggests the existence of other taxa that might branch somewhere between Mazocraeidae and Diplozoidea. In conclusion, it seems that Octomacridae is not the right candidate for studying the origin of the special traits of the Diplozoidea. Concerning the colonisation of the primary freshwater teleosts, the paraphyly of Diplozoidea and Octomacridae suggests that it could result of two different events.

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