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Phylogenetic relationships between Hypostominae and Ancistrinae (Siluroidei: Loricariidae): first results from mitochondrial 12S and 16S rRNA gene sequences[1]

Juan-Ignacio MONTOYA-BURGOS*, Sonia MULLER*, Claude WEBER* & Jan PAWLOWSKI**

Phylogenetic relationships between Hypostominae and Ancistrinae (Siluroidei: Loricariidae): first results from mitochondrial 12S and 16S rRNA gene sequences. - Partial 12S and 16S mitochondrial rRNA gene sequences were obtained from 16 species of South American catfishes belonging to the subfamilies Hypostominae, Ancistrinae, and Loricariinae sensu Isbrücker (Siluroidei: Loricariidae). The analysis of these sequences indicates that within the clade of Hypostominae + Ancistrinae, Chaetostoma + Ancistrus form the sister group of all other analysed ancistrines and hypostomines. The Ancistrinae as presently defined, include all analysed Hypostominae and therefore are paraphyletic. The monophyly of *Ancistrus*, including the recently described *A. ranunculus*, is strongly supported.

Key-words: Catfish - Ancistrinae - Hypostominae - Molecular phylogeny - Mitochondrial rRNA genes.

INTRODUCTION

According to the most recent classification the catfish family Loricariidae includes more than 600 species grouped in 70 genera and 6 subfamilies: the Lithogeneinae, the Neoplecostominae, the Hypostominae, the Ancistrinae, the Hypoptopomatinae, and the Loricariinae (ISBRÜCKER 1980). Loricariids are externally characterised by a sucker-like mouth located ventrally and by bony plates or scutes covering the body. The family is representative of the dramatic diversity of many teleost groups in Neotropical freshwaters (SCHAEFER 1986). New species, often placed in new genera, are regularly described (e.g. ISBRÜCKER & NIJSSEN 1989; REIS *et al.* 1990; WEBER 1991; REIS & SCHAEFER 1992; MULLER & ISBRÜCKER 1993). The systematics of the Loricariidae are still incompletely resolved.

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* Muséum d'histoire naturelle, Case postale 6434, CH-1211 Genève 6, Switzerland.

** Station de Zoologie, Université de Genève, 154 route de Malagnou,1224 Chêne-Bougeries, Genève, Switzerland.

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One of the major taxonomic problems concerns the phylogenetic relationships and the taxonomic status of two groups of species presently referred to the Ancistrinae and Hypostominae. Long considered as the single taxon Hypostominae (formerly Plecostominae) (EIGENMANN & EIGENMANN 1890; REGAN 1904; GOSLINE 1948), the two subfamilies were recognised by ISBRÜCKER (1980) who based his decision on a character shared by all Ancistrinae: the presence of evertible interopercular odontodes. This character was already used by KNER (1853, 1854) for the distinction of two subgroups, and also by GOSLINE (1947) who stated, however, that the two subgroups were not totally discontinuous. In a study mostly based on cranial myology, HOWES (1983) considers the Ancistrinae as polyphyletic and places their representatives within the Hypostominae and the Chaetostominae (fig. 1A). However, in the last and the most extensive phylogenetic analysis of loricariid subfamilies, SCHAEFER (1986, 1987) found uniquely derived osteological characters supporting the group Hypostominae + Ancistrinae as well as the monophyly of the Ancistrinae but found no evidence suggesting the monophyly of the Hypostominae (fig. 1B). This author also recognised the Loricariinae as the monophyletic sister group of Ancistrinae plus Hypostominae.

The present study is a first attempt to resolve the phylogenetic relationships between Hypostominae and Ancistrinae species using molecular data. In comparison with morphological techniques, molecular methods have the great advantage of avoiding the problems of phenotypic variabilities (AVISE *et al.* 1987). The high rate of mutational changes and the matrilineal mode of inheritance make mitochondrial DNA a particularly appropriate and powerful tool for this kind of investigations (MORITZ *et al.* 1987; MEYER 1993). HILLIS & DIXON (1991) suggested that mitochondrial rRNA genes are especially useful for investigating relationships among groups that diverged less than 65 million years ago. The paleontological data allow no conclusions about loricariids evolution since the scarce fossil records of this group belong to the Miocene and are very close to modern species (ARRATIA & CIONE 1996, LUNDBERG 1996). The mitochondrial rRNA genes were used for inferring the phylogeny of some antarctic Notothenioidei (BARGELLONI *et al.* 1994), the phylogeny of Gymnotoidei (ALVES-GOMES *et al.* 1995) as well as the one of piranhas and Characiformes (ORTI *et al.* 1996, ORTI & MEYER 1997).

Here we present phylogenetic relationships of 10 species of Ancistrinae, four species of Hypostominae and two species of Loricariinae, based on partial 12S and 16S mitochondrial rRNA gene sequences.

MATERIAL AND METHODS

FISH SPECIMENS AND DNA SEQUENCING

Sixteen specimens were used in this study: 10 specimens of Ancistrinae representing six genera, four specimens of Hypostominae representing three genera and two specimens of Loricariinae representing two genera. The specimens are deposited in the Natural History Museum of Geneva (MHNG). Species, origins, museum collection numbers (MHNG) and EMBL/GenBank accession numbers are given in Appendix 1.

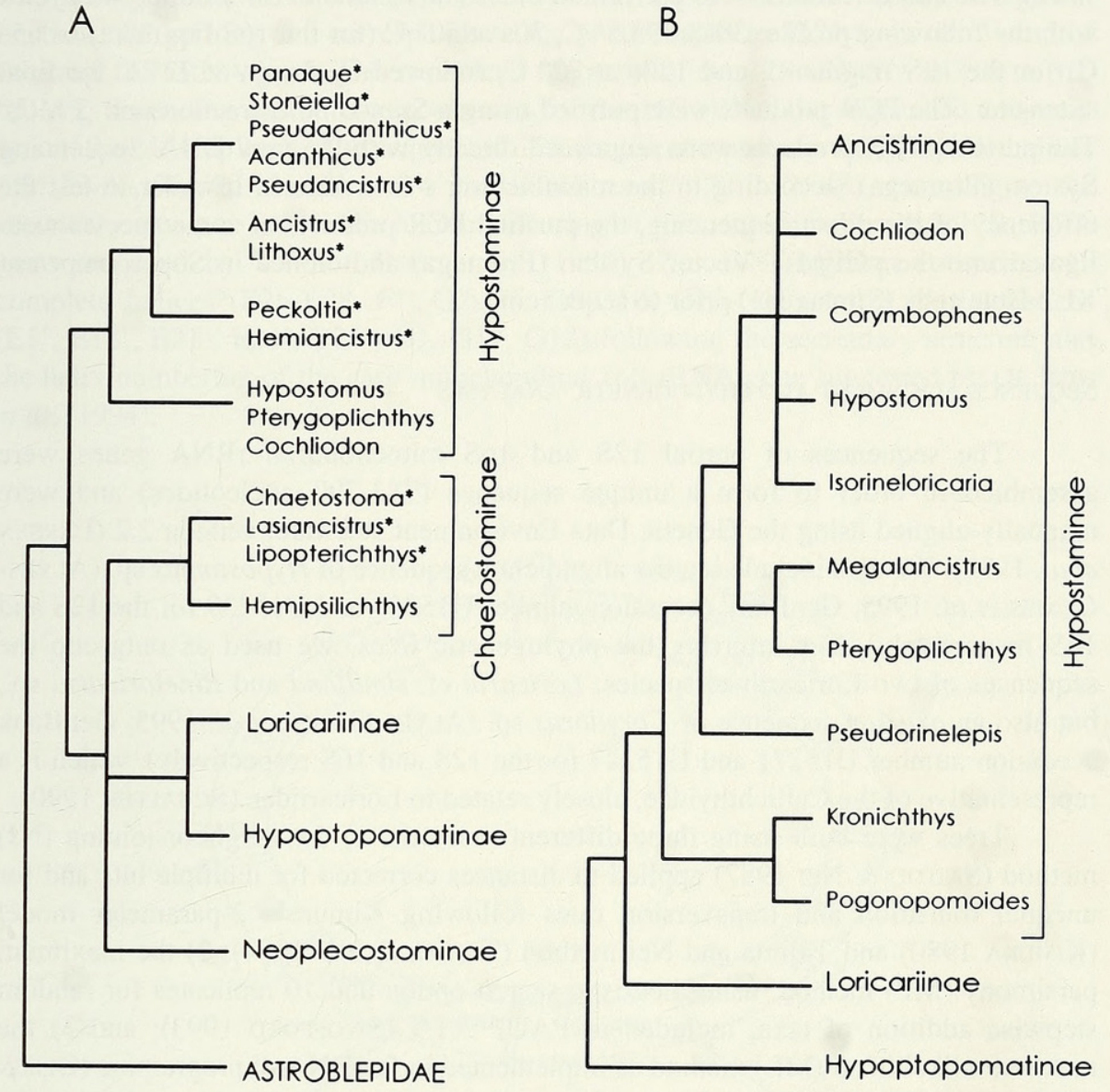

FIG. 1

Previous hypotheses of phylogenetic relationships of present Ancistrinae and Hypostominae species. A: HOWES (1983) hypothesis based on myological and osteological characters. Genera included in the Ancistrinae *sensu* SCHAEFER are indicated with an asterisk. B: SCHAEFER (1986, 1987) hypothesis based on osteological characters.

Total DNA was extracted from fresh, frozen or ethanol preserved muscle tissue samples using a rapid one-step extraction method (STEINER *et al.* 1995) or the standard DNA extraction protocol, using SDS-based extraction buffer and Proteinase K digestion (KOCHER *et al.* 1989; ALVES-GOMES *et al.* 1995).

Partial 12S rRNA and 16S rRNA mitochondrial genes were amplified by the polymerase chain reaction (PCR) with the following primers: L1091 and H1478 for the 12S gene, L2510 and H3059 for the 16S gene (as given by ALVES-GOMES *et al.*

1995). The amplifications were performed in a total volume of 50 γ during 40 cycles with the following profile: 30s at 93,5° C, 30s at 50° C (for the 16S fragment) or 65° C (for the 12S fragment), and 120s at 72° C, followed by 5 min at 72° C for final extension. The PCR products were purified using a Spin-Bind extraction unit (FMC). The purified PCR products were sequenced directly with the *fmol* DNA Sequencing System (Promega), according to the manufacturer's instructions. In order to test the efficiency of the direct sequencing, the purified PCR products of some species were ligated into the pGEM-T Vector System (Promega) and cloned in Supercompetent XL2-Blue cells (Stratagene) prior to sequencing.

Sequence alignment and phylogenetic analyses

The sequences of partial 12S and 16S mitochondrial rRNA genes were assembled in order to form a unique sequence (783-790 nucleotides) and were manually aligned using the Genetic Data Environment software, version 2.2 (Larsen *et al.* 1993). We also included in the alignment a sequence of *Hypostomus* sp. (Alves-Gomes *et al.* 1995, GenBank accession number U15263 and U15239 for the 12S and 16S respectively). For inferring the phylogenetic trees, we used as outgroup the sequences of two Loricariinae species, *Loricaria* cf. *simillima* and *Rineloricaria* sp., but also an existing sequence of *Corydoras* sp. (Alves-Gomes *et al.* 1995, GenBank accession number U15271 and U15247 for the 12S and 16S respectively), which is a representative of the Callichthyidae, closely related to Loricariidae (Schaefer 1990).

Trees were built using three different methods: 1) the neighbor-joining (NJ) method (Saitou & Nei 1987) applied to distances corrected for multiple hits and for unequal transition and transversion rates following Kimura's 2-parameter model (Kimura 1980) and Tajima and Nei method (Tajima & Nei 1984); 2) the maximum parsimony (MP) method, using heuristic search option and 10 replicates for random stepwise addition of taxa, included in PAUP 3.1.1 (Swofford 1993); and 3) the maximum likelihood (ML) method as implemented in fastDNAml programme (Olsen *et al.* 1994). The reliability of internal branches of the trees was assessed using the bootstrap method (Felsenstein 1988); 1000, 500 and 200 bootstrap replications were performed for the NJ, MP and ML methods respectively. The Phylo-win programme (N. Galtier & M. Gouy, unpublished) was used for distance computations, NJ and ML trees building and bootstrapping. Phylogenetic trees were plotted using Njplot programme (M. Gouy, unpublished). The Kishino & Hasegawa (1989) test implemented in the Dnaml programme of Phylip (version 3.5, Felsenstein 1993) was used to compare our molecular tree with the competing morphological hypotheses.

RESULTS

Sequence analysis

The position of the mitochondrial rRNA genes and of the amplified fragments is shown in figure 2. The amplified fragment of the 12S rRNA is about 435 base pairs (bp) long and corresponds to the position 419 to 854 starting from the 5' end of the

carp mitochondrial 12S rRNA gene (CHANG *et al.* 1994). The 16S amplified fragment is about 623 bp long and corresponds to the position 898 to 1521 from the 5' end of the carp 16S rRNA gene. The sequenced fragment of the 12S rRNA was 314 to 316 bp long depending on the taxon, whereas for the 16S rRNA the sequenced fragment was 469 to 474 bp long. The sequenced 12S fragment comprises 12 complete helices (31, 33-36, 38-42, 45, 47) and 4 partial helices (2', 22', 32, 48) according to the secondary structure and to the helix numbering of the carp mitochondrial 12S rRNA gene proposed by VAN DE PEER *et al.* (1994). The 16S fragment comprises 15 complete helices (E26-E28, F1, G2-G3, G6-G10, G13-G16) and 8 partial helices (E1', E18', E21', E24', E25', G1, G17, G18) following the secondary structure and the helix numbering of the carp mitochondrial 16S rRNA gene suggested by DE RIJK *et al.* (1994).

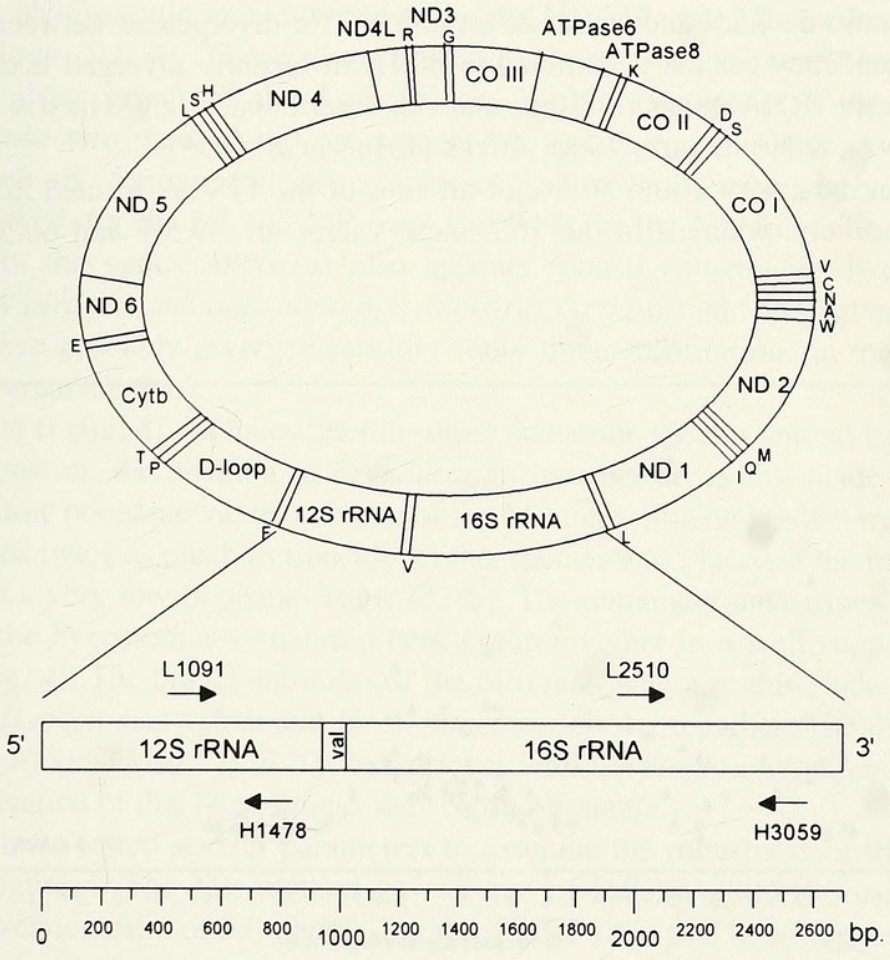

FIG. 2

General organization of the genes in fish mitochondrial DNA (CHANG *et al.* 1994; ZARDOYA *et al.* 1995). The tRNA genes are given by the one-letter amino acid code. Enlarged are the two rRNA genes and the position of the primers we used.

A total of 315 and 466 sites were aligned in the 12S and 16S genes respectively. Seven sites located within the 16S rRNA could not be aligned unambiguously and were discarded from further analysis. The two partial rRNA gene sequences were joined together and analysed as a single sequence. Invariant positions represented 77.5% of the 12S fragment and 74.7% of the 16S fragment. From 68 informative sites in the ingroup (Ancistrinae + Hypostominae), 34 were located in each of the fragments. In the ingroup, the mean proportion of transitions (TS) for all pairwise comparisons is 4.51% of total sites in the 12S and 2.94% in the 16S fragment whereas for transversions (TV) these values are 0.76% of total sites in the 12S and 0.69% in the 16S. In the combined data set, sequence divergence among species within a genus ranged from 0.3% to 3.5%, whereas the genera of the ingroup showed sequence divergences ranged from 0.9% to 7.3%. In the combined dataset, the TS/TV ratio plotted against sequence divergences for all pairwise comparisons (fig. 3) increases from a mean value of 3.2 for divergences between 0.5% and 1.5% to a mean value of 8 for divergences between 3.5% and 4.5%. As the divergence among taxa increases, the TS/TV ratio declines and stabilises around 1.6 for divergences between 10% and 13%. The tendency for the accumulation of TS in recently diverged taxa has been observed in mt rRNA genes of fishes and other vertebrates (e.g. HIXSON & BROWN 1986; MINDELL & HONEYCUTT 1990; ALVES-GOMES *et al.* 1995).

In our data, 49.4% and 50.6% of all sites of the 12S are located in stems and loops respectively, whereas in the 16S, these values are 39.5% and 60.5% respec-

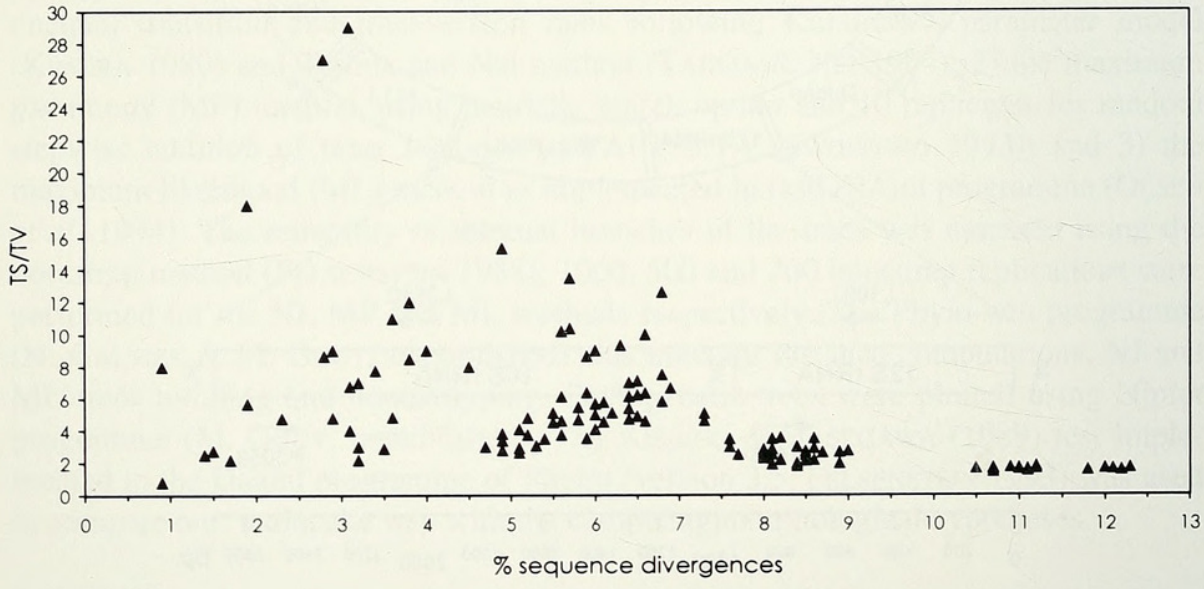

FIG. 3

Transition (TS) / Transversion (TV) ratio plotted against % sequence divergences for all pairwise comparisons of taxa.

tively. The sites located in loops are 1.68 and 2.96 times more variable than the ones in stems in the 12S and 16S respectively. Moreover, almost all transversion events occured in unpaired regions (80% and 92% of the sites showing transversions were located in loops in the 12S and 16S respectively).

Base composition showed no important differences among all examined taxa. Mean GC content in the 12S and 16S rRNA is about 49.9% and 46.5% respectively. In stems, mean GC content is about 59% in both segments whereas in loops this value is 41% in the 12S and 38% in the 16S. The low GC content found in loops may be a consequence of an overrepresentation of adenosine nucleotides (41.7% and 42.3% of all four nucleotides for the 12S and 16S respectively). The base composition bias between stems and loops observed in this study is congruent with those observed in mitochondrial rRNA genes of other fishes (ORTI *et al.* 1996).

PHYLOGENETIC RECONSTRUCTIONS

The phylogenetic trees inferred using the NJ, MP, and ML methods have the same topology (fig. 4). The well supported clade of Ancistrinae + Hypostominae forms the sister group of the Loricariinae. Within the group of Ancistrinae + Hypostominae two main clades are present (A and B, fig 4). Clade A consists of *Chaetostoma* aff. *fischeri* and five species of *Ancistrus*. Its monophyly is strongly supported (97% for the NJ and MP trees and 94% for the ML tree). The branching order within the genus *Ancistrus* also appears robust: *ranunculus* diverging first followed by *pirareta* and *dolichopterus*. *Ancistrus cirrhosus* and *multispinis* are sister taxa and have probably diverged recently (only three substitutions in the combined data set separate the two).

Clade B (fig. 4) includes the four other ancistrine species and all hypostomine species. However, the position of *Pseudacanthicus spinosus* in this clade is not well supported (low bootstrap values). In one of the MP trees obtained (when transversions were counted twice as much as transitions) this species was placed at the root of clade A, but with a very low bootstrap value (37%). The remainder ancistrines of clade B and all of the hypostomines analysed here group together in a well supported clade (clade C, fig. 4). The branching order of the different genera in this clade is not well supported. *Hypostomus affinis* and *H.* cf. *punctatus* cluster together. The third (*Hypostomus* sp., ALVES-GOMES *et al.* 1995) branches with *Glyptoperichthys joselimaianus*. The identification of this *Hypostomus* may be questionable.

We have tested several parameters to examine the robustness of the tree presented in figure 4. The ML method gave a tree of ln(L)= -2809.186 using a transition/transversion ratio of 2.0. Increasing the TS/TV ratio to 5.0, as suggested by the above analysis of sequences, does not change the topology. When all characters were uniformly weighted using the MP method, a single shortest tree was found of length=325 (CI = 0,56 excluding uninformative characters) the topology of which is identical to the NJ and ML trees. When transversions were counted twice as much as transitions, two shortest trees were found (length=380): the first has the same topology as the previous one, the second differs from the general tree only in the

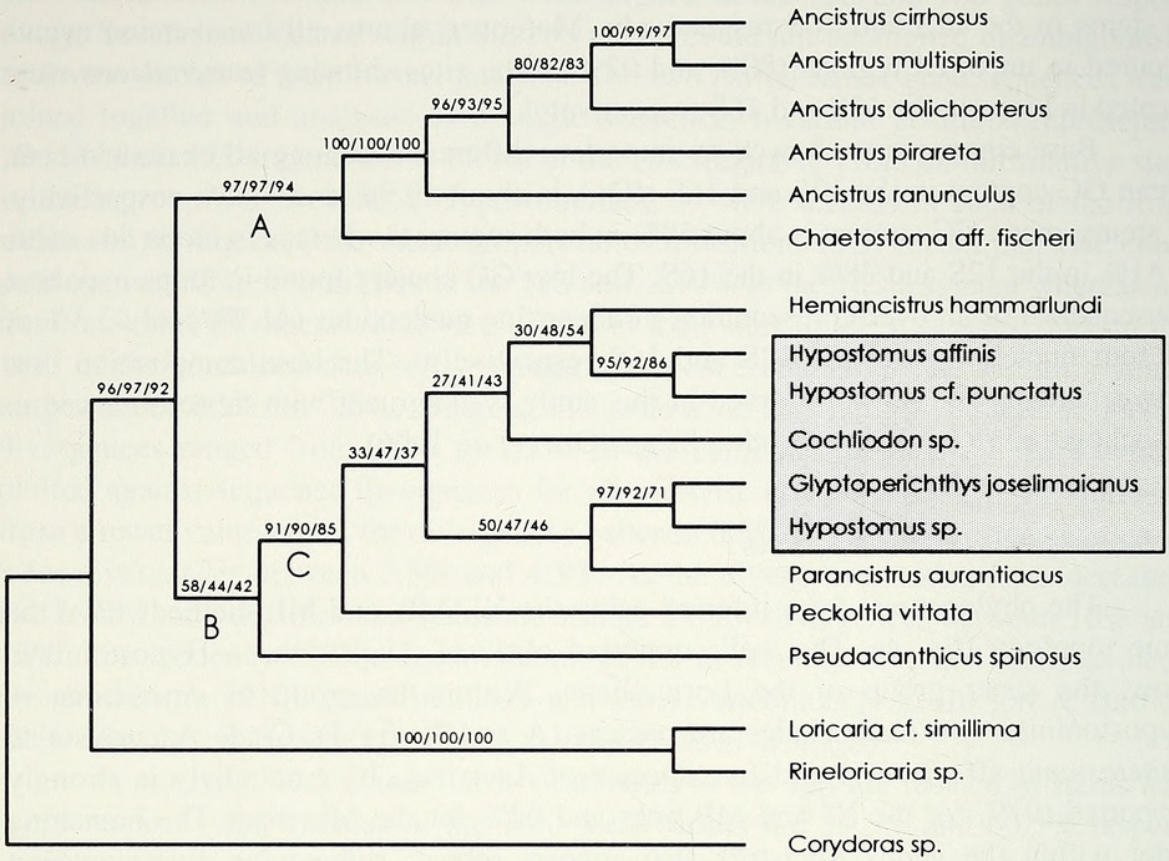

Fig. 4

Maximum parsimony unweighted phylogenetic tree (lengh = 325, CI = 0,56 excluding uninformative characters). Neighbor-joining and maximum likelihood methods gave the same topology. Numbers above each branch represent bootstrap values for neighbor-joining / maximum parsimony / maximum likelihood respectively. A, B, and C are the names of the clades (see text). The shadowed box presents the Hypostominae species analysed in this study.

position of *Pseudacanthicus spinosus* which is shown as the basal genus of the A clade (fig. 4). In order to check if any topological artefact was due to the overrepresentation of the genus *Ancistrus*, we discarded all but one of its species and tested with the NJ and ML methods. The general topology was conserved in both cases.

The KISHINO & HASEGAWA (1989) test showed that the proposal of HOWES (1983) as well as of SCHAEFER (1986, 1987) have a significantly lower log-likelihood than our molecular hypothesis, i.e. less significant ($\Delta l = 43.14 \pm 20.78$ and $\Delta l = 51.42 \pm 24.35$ respectively).

DISCUSSION

Our preliminary results do not contradict the hypothesis of SCHAEFER (1986, 1987) supporting the monophyly of Ancistrinae + Hypostominae. The monophyly of the Ancistrinae suggested by ISBRÜCKER (1980) and SCHAEFER (1986), however, is not

supported by the analysis of the mitochondrial rRNA genes. Instead, our data suggest a paraphyly of the Ancistrinae as it includes all the Hypostominae we have examined. Using the MP method we can enforce the monophyly of the ancistrines but the shortest tree obtained was nine steps longer than the one without topological assumptions. This test reinforces the suspected paraphyly of the Ancistrinae. Thus, there is a contradiction between the molecular data and the uniquely derived osteological characters found by SCHAEFER (1986, 1987) supporting the monophyly of Ancistrinae. Nevertheless, our results do not contradict the hypothesis that the mecanism of evertibility of the interopercular tuft of odontodes has evolved only once in the history of Ancistrinae (ISBRÜCKER 1980). If they are comfirmed by further investigations that would imply that this mecanism of evertibility and its related structures have disappeared in the lineage leading to present Hypostominae.

Our molecular hypothesis is also in contradiction with HOWES (1983) proposal, which includes a polyphyletic view of ancistrines, because it discards *Chaetostoma* from the clade including *Ancistrus* and it places the Loricariinae within the clade of Hypostominae + Ancistrinae as presently defined.

The tree in figure 4 shares one point with the consensus tree of ancistrine relationships given by SCHAEFER (1986) (fig. 5): *Ancistrus* and *Chaetostoma* are members of a same lineage (the higher ancistrines of SCHAEFER). However, in our tree

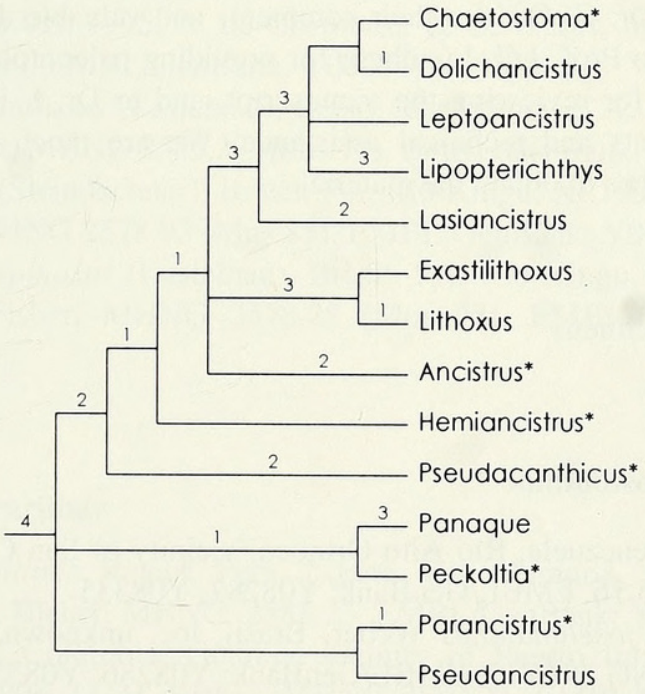

FIG. 5

Phylogenetic hypothesis of relationships among Ancistrinae species proposed by SCHAEFER (1986), based on osteological characters. Numbers above each branch represent the number of uniquely derived characters found by SCHAEFER. Taxa included in this study are indicated with an asterisk.

this last clade forms the sister group of the hypostomines plus the lower ancistrines *sensu* SCHAEFER analysed here. All the *Ancistrus* species included in our study cluster together in a well-supported clade including the type species, *A. cirrhosus* (Valenciennes). There is strong evidence that *A. ranunculus* belongs to this genus, as proposed by MULLER *et al.* (1994).

The phylogenetic relationships among the genera of clade C (fig. 4), and especially the three genera of hypostomines, could not be established with our sequence data. This is probably due to an insufficient sampling size of the numerous hypostomine group rather than a lack of sequence variability because the well resolved *Chaestostoma* + *Ancistrus* clade showed lower sequence variability than the clade C. It is also possible that these taxa have undergone an explosive radiation which could explain the difficulty in resolving the internal branches. In consequence, no indication about the monophyly of the Hypostominae is given.

Our high level taxonomy analysis of the relationships between some Ancistrinae and Hypostominae revealed the presence of two clades which do not correspond to the present definition of Hypostominae and Ancistrinae, the later being paraphyletic. The analysis of new species sequences could clear up the phylogeny of this group.

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APPENDIX 1: Specimens

INGROUP TAXA:

Loricariidae, Hypostominae

- *Cochliodon* sp.,Venezuela, Rio Alto Orinoco, vicinity of San Carlos, lg H. Bleher, MHNG 2585.16. EMBL/GenBank: Y08287; Y08335.
- *Glyptoperichthys joselimaianus* Weber, Brasil, loc. unknown, 1995, commercial source, MHNG 2585.17. EMBL/GenBank: Y08286; Y08334.
- *Hypostomus affinis* (Steindachner), Brasil, RJ, Rio Paraiba do Sul, 3 km W. of Sapucaia, 10.XII. 1990, lg R. Mazzoni, W. Costa & C. Weber, MHNG 2543.65 (Br 153). EMBL/GenBank: Y08288; Y08336.
- *Hypostomus* cf. *punctatus* Valenciennes, Brasil, RJ, Ubatiba, Marica, 4.XII.1990, lg. R. Mazzoni & C. Weber, MHNG 2543.27 (Br 148). EMBL/GenBank: Y08289; Y08337.

Ancistrinae

- *Ancistrus cirrhosus* (Valenciennes), Argentina, Buenos Aires, La Choza, Rio Lujan drainage, 15.IX.1995, lg. O. Fernandez-Santos & S. Körber, MHNG 2583.37 (Mus 97). EMBL/GenBank: Y08277; Y08325.
- *Ancistrus dolichopterus* Kner, aquarium F1 of: Brasil, AM, Alto Rio Negro, close to the mouth of Rio Demini, 1989, lg. W. Römer (26.VII.1990, K. Holota), MHNG 2585.13 (Mus -). EMBL/GenBank: Y08276; Y08324.
- *Ancistrus multispinis* (Regan), Brasil, RJ, Cachoeira de Macacu, 14.XII.1994, lg. C. Bizerril, P. Perez-Neto & C. Weber, MHNG 2572.3 (Br 94-2). EMBL/GenBank: Y08279; Y08327.
- *Ancistrus pirareta* Muller, Paraguay, Cordillera, Salto Pirareta, Rio Tebicuary-mi drainage, 15-16.XI.1990, lg. C. Dlouhy, V. Mahnert & S. Muller, MHNG 2542.84 (Pira 8+12). EMBL/GenBank: Y08278; Y08326.
- *Ancistrus ranunculus* Muller, Rapp Py-Daniel & Zuanon, Brasil, PA, Rio Xingu, IV.1995, aquarium import D. Fisher, MHNG 2583.38 (Mus 105). EMBL/GenBank: Y08280; Y08328.
- *Chaetostoma* aff. *fischeri* Steindachner, Ecuador, Manabi, Solanillo, Rio Daule drainage, 16.VIII.1995, A. de Chambrier & C. Weber, MHNG 2575.45 (EC 95-6). EMBL/ GenBank: Y08281; Y08329.
- *Hemiancistrus hammarlundi* Rendahl, Ecuador, Manabi, Solanillo, Rio Daule drainage, 16.VIII.1995, A. de Chambrier & C. Weber, MHNG 2575.46 (EC 95-5+ unnum.). EMBL/GenBank: Y08284; Y08332.
- *Parancistrus aurantiacus* (Castelnau); Brasil, loc. unknown, X. 1994, aquarium import D. Fisher, MHNG 2583.39 (Mus 73). EMBL/GenBank: Y08282; Y08330.
- *Peckoltia vittata* (Steindachner), Brasil, PA, Rio Xingu, XI.1995, aquarium import D. Fisher, MHNG 2578.93 (Mus 85). EMBL/GenBank: Y08285; Y08333.
- *Pseudacanthicus spinosus* (Castelnau), Brasil, PA, Rio Xingu, XI.1995, aquarium import D. Fisher, MHNG 2578.28 (Mus 77). EMBL/GenBank: Y08283; Y08331.

OUTGROUP TAXA:

Loricariidae, Loricariinae

- *Loricaria* cf. *simillima* Regan, Peru, Loreto, Rio Maranon, vicinity of Iquitos, I.1996, lg. H. Bleher, MHNG 2583.23. EMBL/GenBank: Y08290; Y08338.
- *Rineloricaria* sp., Colombia, Guainia, vicinity of Puerto Inirida, Rio Guaviare drainage, I.1996, lg. H. Bleher, MHNG 2583.24. EMBL/GenBank: Y08291; Y08339.

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