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# Origin of species diversity in the catfish genus *Hypostomus* (Siluriformes: Loricariidae) inhabiting the Paraná river basin, with the description of a new species

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## Abstract

Within the Loricariidae, the genus *Hypostomus* is one of the most diversified freshwater catfish groups. Using new sequence data from the mitochondrial Control Region (D-loop) we examined the phylogeny of this genus. Our phylogenetic analyses suggest that, in the Paraná river basin, species diversity in the genus *Hypostomus* has been shaped by two processes: 1) by inter-basin diversification, generating groups of species that inhabit different basins, as a result of dispersal events; and 2) via intra-basin speciation as a result of basin fragmentation due to past marine transgressions, which produced groups of species within a basin. Using the D-loop as a molecular clock, each event of diversification was dated and linked with documented hydrological events or sea level changes. We also assessed the possible dispersal routes between the Paraná and Uruguay rivers, in addition to the obvious dispersal route via the Río de la Plata estuary. Finally, we describe a new species of *Hypostomus* inhabiting Middle Paraná river, *Hypostomus arecuta* n. sp. This species can be separated from all other *Hypostomus* by having light roundish dots on a darker background and by number of premaxillary/dentary teeth.

**Key words:** Armored catfish; Control Region; phylogeny; Paraná river.

## Introduction

In South America, the Loricariidae is the most species-rich endemic family of freshwater fishes. This family of suckermouth-armored catfishes comprises 818 species (Eschmeyer and Fricke, 2011) and new species are frequently discovered and described (e.g. Hollanda Carvalho *et al.*, 2010; Zawadzki *et al.*, 2010; Rodriguez *et al.*, 2011; Cardoso *et al.*, in preparation). Within the Loricariidae, the genus *Hypostomus* constitutes a rich assemblage of species, with approximately 130 recognized species (Weber, 2003; Ferraris 2007; Zawadzki *et al.*, 2010, Hollanda Carvalho *et al.*, 2010). Representatives of *Hypostomus* are bottom-dwelling fishes widely distributed throughout tropical and subtropical South America, occurring in a variety of freshwater ecosystems such as mountain streams and large lowland rivers and ponds. Species delineation and diagnosis in *Hypostomus* is difficult, in particular due to the diversity and widespread distribution of the genus, to elevated intra-specific morphological variability, and because some older descriptions are too short or incomplete.

Numerous species of *Hypostomus* inhabit the La Plata basin, which comprises the Paraguay, Paraná, and Uruguay rivers and the Río de la Plata (López and Miquelarena, 1991). Understanding the diversification history of *Hypostomus* as a "model" genus might allow the development of a comprehensive view of the processes that shaped the rich ichthyological diversity in the Paraná river basin.

According to the reconstruction of paleo basins in South America, from about 60 to10 million years ago (Ma), the paleo Amazon–Orinoco system was a large watershed with waters flowing northward toward the Caribbean Sea, while the La Plata basin was already oriented as present (Lundberg, 1998). This author suggested that at 12–10

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Ma, the boundary between the paleo Amazon–Orinoco and the La Plata basins underwent a final and important shift southward to its current location. This boundary displacement must have occasioned exchanges of water and fishes between the two main basins 12 to 10 Ma, as proposed by Montoya-Burgos (2003). However, the boundary displacement might have been more progressive, covering the last 10 Ma (Rasanen *et al.*, 1995; Lundberg *et al*., 1998), opening occasional dispersal routes between these two river systems.

During the Miocene (24-5 Ma), two main components of the La Plata basin, the upper plus middle Paraná river on one hand, and Uruguay river on the other, which were forming a single large river flowing southward into the Rio de la Plata estuary, disconnected from one another resulting in the modern configuration (Beurlen, 1970). According to Bonetto (1994), geological changes caused this disconnection by modifying the course of the middle Paraná river that subsequently reached the course of the Paraguay river. Today, the Paraná and Uruguay rivers are connected exclusively via the Río de la Plata estuary.

Furthermore, in the second half of the Miocene, 15-5 Ma, marine transgressions occurred at least twice along distinct paleogeographic corridors. The first maximum flooding event occurred between 15 and 13 Ma and the second between 10 and 5 Ma. The two marine transgressions covered most of the Paraná river basin (Hernández *et al.*, 2005). It is likely that the diversity of strictly freshwater organisms might have been seriously impacted by these marine transgressions. For example, the museum hypothesis of diversification (Nores, 1999) states that the Miocene marine incursions have been major diversifying events via the fragmentation of emerged land resulting in allopatric speciation.

The goals of the present work are: (1) to expand the phylogeny of the genus *Hypostomus* that was previously proposed by Montoya-Burgos (2003) using new sequence data from the mitochondrial Control Region, (2) to infer the origin of the diversity of *Hypostomus* species in the Paraná river basin, (3) to assess the possible dispersal routes between the Paraná and Uruguay river, in addition to the obvious dispersal route via the Río de la Plata estuary, and (4) to describe a new species of *Hypostomus* inhabiting the Middle Paraná river basin.

The use of genetic markers is a powerful tool to estimate the extent of hidden biodiversity. For example, the mitochondrial D-loop region is frequently used for answering a broad range of biological questions relative to population processes, phylogeography (e.g. Cardoso and Montoya-Burgos, 2009) and species identification (e.g. Cardoso *et al.*, 2011). Here we used this molecular tool in order to infer the phylogenetic relationships among *Hypostomus* species and to analyse the origin of species diversity in the río Paraná basin.

## Materials and methods

### Taxon sampling and morphological analyses

Fish specimens were collected in 15 different localities in the Paraná river basin, Argentina (Fig. 1). Most of them were sampled in the middle and lower section of the Paraná river basin. We also used available data from the upper section of this basin taken from GenBank. Fishes were caught using gill nets, cast nets, hand nets, and seine. Tissue samples for genetic studies were preserved in ethanol 96 % and frozen at -20 °C, the vouchers specimens were fixed in formalin 10 % for morphological studies and deposited at MHNG, IPLA, and MACN according to the institutional abbreviations are as listed in Ferraris (2007). Table I has more information about the specimens analysed.

All measurements were taken point to point with digital calipers to the nearest 0.01 mm, under a dissecting microscope when necessary. Measurements and counts of bilaterally symmetrical features were taken from the left side of the body whenever possible; if a feature was missing or broken on the left side, it was examined on the right side. Counts and measurements follow Boeseman (1968), Weber (1985), and Reis *et al.* (1990). Body plate counts and nomenclature follow Oyakawa *et al.* (2005). The oral disk width was measured at point of insertion of the maxillary barbels.

### DNA amplification and sequencing

The genomic DNA was extracted using the salt-extraction protocol (Aljanabi and Martinez, 1997). The PCR amplification of the Control Region (D-loop) of the mitochondrial DNA was performed using the following primers: DLA-III 5'-TATTTAAAGRCATAATCTCTTGAC-3' and HygDL-R 5'–WTGCKARTATGTGCCGYYTG–3'. The amplifications were performed in a total volume of 50 µl, containing 5 µl of 10x reaction buffer, 1 µl of deoxyribonucleoside triphosphate (dNTP) mix at 10 mM each, 1 µl of each primer at 10 µM, 0.2 µl of *Taq* DNA

Polymerase equivalent to 1 unit of Polymerase per tube, and 1 µl of DNA. Cycles of amplification were programmed as follows: (1) 3 min. at 94°C (initial denaturing), (2) 30 sec. at 94°C, (3) 30 sec. at 55–57°C, (4) 1 min. at 74°C, and (5) 5 min. at 74°C (final elongation). Steps 2 to 4 were repeated 42 times. The PCR products were purified and sequenced by the company MAGROGEN (Korea). Sequences were deposited in GenBank (Table I).

**TABLE I**. Details of the specimens used in the molecular phylogeny with GenBank accession numbers.

| Species | GenBank | Field number | Locality |
|---|---|---|---|
| *H. arecuta* | JF290442 | AG09-163 | Yahapé (27º22'12.1"S-57º39'14.6"W) |
| *H. arecuta* | JF290441 | AG09-181 | Ituzaingó (27º 29'32"S-56º 39'38"W) |
| *H. arecuta* | JF290445 | AG09-198 | Ituzaingó (27º 29'32"S-56º 39'38"W) |
| *H. arecuta* | JF290446 | PR-031 | Santa Fé, Santa Fé, Argentina |
| *H. arecuta* | JF290443 | TAE01 | Ituzaingó (27º 29'32"S-56º 39'38"W) |
| *H. arecuta* | JF290444 | TAE02 | Ituzaingó (27º 29'32"S-56º 39'38"W) |
| *H. derby* | JF290447 | YC10-316 | Uruzú (25°55'38.25"S-53°56,031'W) |
| *H. paranensis* | JF290449 | YC-025 | Suquia (31°24'11,9"S-64°12'11,4"W) |
| *H. paranensis* | JF290448 | YC-026 | Suquia (31°24'11,9"S-64°12'11,4"W) |
| *H. commersoni* | JF290450 | AG09-129 | Tabay (26º59'56.3"S-55º10'44.9"W) |
| *H. commersoni* | JF290451 | YC09-124 | Manucho (31°15'S- 60°53' W) |
| *H. commersoni* | JF290452 | YC-957 | El Bosque (34°54'37.55"S-57°56'15.65"W) |
| *H. commersoni* | JF290453 | YC-607 | Corrientes (29°48,574'S-59°23.600'W) |
| *H. commersoni* | JF290454 | 14802 | P. N. Pre-Delta (32º08'08.8"S- 60º37'26.2"W). |
| *H. commersoni* | JF290455 | AG09-013 | Ituzaingó (25º29'54.5"S- 56º42'47.0"W) |
| *H. commersoni* | JF290456 | Reg02 | Ensenada (34°50'23,96"S-57°55'13,04"W) |
| *H. commersoni* | JF290457 | AG09-077 | Garupa (27º29'10.2"S-55º44'23.1"W) |
| *H. commersoni* | JF290458 | YC-926 | El Pescado (34°57,790'S- 57°46,696'W) |
| *H. cochliodon* | JF290476 | AG09-016 | Ituzaingó (27º 29'32"S-56º 39'38"W) |
| *H.luteomaculatus* | JF290471 | YC-162 | Antequera (27°27'43.43" S-58°52'0.03" W) |
| *H. luteomaculatus* | JF290469 | UR004 | Pedra Fortaleza, Itapiranga, Brazil |
| *H. luteomaculatus* | JF290470 | UR002 | Pedra Fortaleza, Itapiranga, Brazil |
| *H. luteomaculatus* | JF290468 | AG09-157 | Ituzaingó (27º 29'32"S-56º 39'38"W) |
| *H. luteomaculatus* | JF290467 | AG09-200 | Ituzaingó (27º 29'32"S-56º 39'38"W) |
| *H. luteomaculatus* | JF290459 | AG09-012 | Ituzaingó (27º 29'32"S-56º 39'38"W) |
| *H. luteomaculatus* | JF290466 | CIA283 | Candelaria (27º26'92"S-55º44'50"W) |
| *H. microstomus* | JF290461 | AG09-015 | Ituzaingó (27º 29'32"S-56º 39'38"W) |
| *H. myersi* | JF290472 | YC10-256 | Deseado (25°47'1.30" S-54°2'21.40" W) |
| *H.myersi* | JF290474 | AG09-123 | Tabay (26º59'56.3"S-55º10'44.9"W) |
| *H. myersi* | JF290473 | AG09-124 | Tabay (26º59'56.3"S-55º10'44.9"W) |
| *H. myersi* | JF290475 | AG09-131 | Tabay (26º59'56.3"S-55º10'44.9"W) |
| *H. regani* | JF290460 | Reg.06 | Rio Mogi Guaçu, Brazil |
| *H. ternetzi* | JF290462 | YC-164 | Antequera (27°27'43.43" S-58°52'0.03" W) |
| *H. ternetzi* | JF290463 | AG09-160 | Yahapé (27º22'12.1"S-57º39'14.6"W) |
| *H. uruguayensis* | JF290464 | AG09-159 | Yahapé (27º22'12.1"S-57º39'14.6"W) |
| *H. uruguayensis* | JF290465 | 14731 | P. N. Pre-Delta (32º07'18.0"S- 60º40'12.0"W) |

**Sequence alignment, phylogenetic reconstruction, and molecular clock calibration**

The mitochondrial D-loop sequences were obtained for 36 individuals from Argentina (for more details see Fig.1 and Table I). We also used sequences of different species of *Hypostomus* deposited in GenBank and nine others species of the family Loricariidae as outgroups, as in Montoya-Burgos (2003). The editing of the new sequences and the alignment were performed using BioEdit 7.0.1 (Hall, 1999). Prior to phylogenetic reconstruction, appropriate substitution models were estimated with the Akaike information criterion (AIC) as implemented in MrAIC (Nylander, 2004). We used a total of 74 sequences of *Hypostomus* to reconstruct the phylogeny. Two phylogenetic reconstruction methods were used. First, maximum likelihood (ML) phylogenetic reconstruction was performed using TreeFinder (Jobb *et al.*, 2004). Confidence values for the edges of the ML tree were computed by bootstrapping (Felsenstein, 1985), with 1000 replications. Second, Bayesian Inference analysis (BI) was conducted using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Four chains were run simultaneously (three heated, one cold) for 20,000,000 generations, with tree space sampled every 100th generation. After a graphical analysis of the evolution of the likelihood scores, the first 300, 000 generations were discarded as burn-in. The remaining trees were used to calculate the final consensus tree.

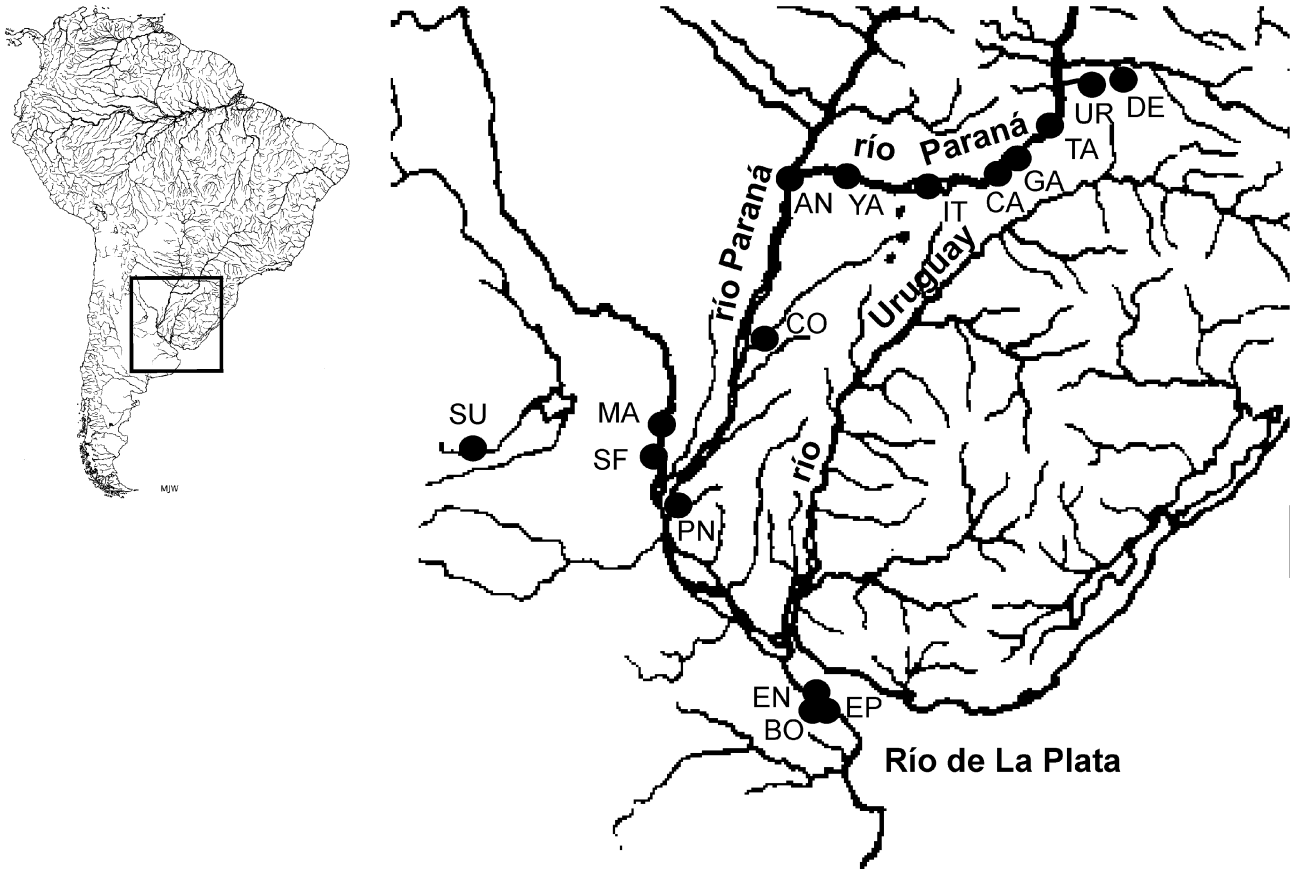


**FIGURE 1.** Map showing the sampling localities. The abbreviations mean: DE (arroyo Deseado, Iguazú river, Misiones); UR (arroyo Uruzú, Paraná river, Misiones); GA (arroyo Garupá, Paraná river, Misiones); TA (arroyo Tabay, Paraná river, Misiones); CA (Candelaria, Paraná river, Misiones); IT (Ituzaingó, Paraná river, Corrientes); YA (Yahapé, Paraná river, Corrientes); CO (Corrientes river, Corrientes); AN (Antequera, Paraná river, Chaco); SU (Suquia river, Cordoba); MA (Manucho river, Paraná river, Santa Fé); SF (Paraná river, Santa Fé); PN (Parque Nacional Pre-Delta, Paraná river, Entre Ríos); EN (Ensenada, Río de La Plata, Buenos Aires); EP (arroyo El Pescado, Río de la Plata, Buenos Aires) and BO (lago del Paseo del Bosque, La Plata).

Additionally, we performed molecular clock tests with HyPhy (Kosakovsky Pond *et al.*, 2005) using the HKY85 model. The null hypothesis of constant molecular clock was tested for the ingroup taxa using the log likelihood ratio test (Huelsenbeck and Crandall, 1997). Prior to these analyses, the data set was pruned to include only one representative of each species. In addition, the sequence corresponding to *H. fonchii* and *H.* sp. Tib1 – used in Montoya-Burgos (2003) – were discarded because it showed a particularly long branch in the phylogenetic tree.

**TABLE II**. Morphometric data and counts of holotype and 23 paratypes of *Hypostomus arecuta* n. sp.

| | **Holotype** | **range** | **Mean / SD** |
|---|---|---|---|
| **Standard length (mm)** | 185.5 | 127.0–268.1 | |
| | | Percents of SL | |
| Predorsal length | 38.6 | 37.4–44.0 | 39.1 ± 1.57 |
| Head length | 32.1 | 29.5–34.4 | 31.3 ± 1.32 |
| Cleithral width | 32.3 | 29.2–32.7 | 30.9 ± 0.89 |
| Head depth | 19.5 | 17.2–19.9 | 18.9 ± 0.70 |
| Dorsal-fin spine length | 33.1 | 26.8–34.5 | 31.6 ± 2.28 |
| Dorsal-fin base length | 27.2 | 25.1–30.0 | 27.2 ± 1.20 |
| Dorsal-adipose distance | 16.3 | 15.1–16.9 | 16.1 ± 0.48 |
| Thoracic length | 24.0 | 19.8–26.2 | 23.3 ± 1.56 |
| Pectoral-fin spine length | 33.4 | 29.7–35.6 | 32.0 ± 1.23 |
| Abdominal length | 25.6 | 22.1–25.6 | 23.9 ± 0.84 |
| Pelvic-fin spine length | 24.9 | 22.3–25.8 | 24.1 ± 0.95 |
| Caudal-peduncle length | 27.4 | 27.4–33.3 | 30.6 ± 1.52 |
| Caudal-peduncle depth | 11.7 | 10.4–12.0 | 11.3 ± 0.45 |
| Adipose-fin spine length | 10.3 | 8.0–10.9 | 9.6 ± 0.86 |
| Anal width | 12.6 | 10.0–13.5 | 11.7 ± 0.79 |
| Upper caudal-fin ray length | 33.0 | 27.4–35.3 | 30.7 ± 2.06 |
| Lower caudal-fin ray length | 36.2 | 28.1–36.3 | 31.9 ± 2.44 |
| | | Percents of head length | |
| Head depth | 60.8 | 57.7–63.6 | 60.7 ± 1.91 |
| Snout length | 61.3 | 61.2–66.8 | 63.0 ± 1.75 |
| Orbital diameter | 18.9 | 16.0–19.0 | 18.1 ± 0.94 |
| Interorbital with | 38.4 | 33.6–38.9 | 37.3 ± 1.47 |
| Maxillary barbel length | 12.2 | 9.3–15.2 | 12.1 ± 1.66 |
| Mandibulary ramus length | 24.5 | 21.7–25.4 | 23.3 ± 1.23 |
| | | | |
| Counts | | | mode |
| Median plates series | 27/27 | 26/28 | 27 |
| Predorsal plates | 3 | 3–3 | 3 |
| Dorsal plates below dorsal-fin base | 9 | 9–10 | 9 |
| Plates between dorsal and adipose fin | 6 | 5–6 | 6 |
| Plates between adipose and caudal fin | 4 | 4–5 | 5 |
| Plates between anal and caudal fin | 14 | 12–14 | 14 |
| Premaxillary teeth | 74/79 | 66–85 | 77 |
| Dentary teeth | 71/72 | 63–84 | 80 |

In order to evaluate the temporal diversification of species in the genus *Hypostomus* in the Paraná river basin, the rate of evolution of the D-loop region needed to be calibrated. To do so, we used the same calibration point as in Montoya-Burgos (2003), which is based on the following reasoning: the phylogeny of *Hypostomus* shows that *H. hondae*, distributed only in the Lago Maracaibo and Magdalena basins, is the closest relative to *H. plecostomoides*, which is known only from the Orinoco basin and some localities of upper Amazon. Because these distribution patterns match the vicariant episode that separated Lago Maracaibo system from Amazon and Orinoco basins 8 Ma (Hoorn, 1993), it is reasonable to attribute this age to the speciation event that gave rise to *H. hondae* and *H. plecostomoides*. This geological event has also been used for calibrating other Neotropical fish phylogenies (e.g. Lovejoy *et al.*, 2000, Sivasundar *et al.*, 2001).

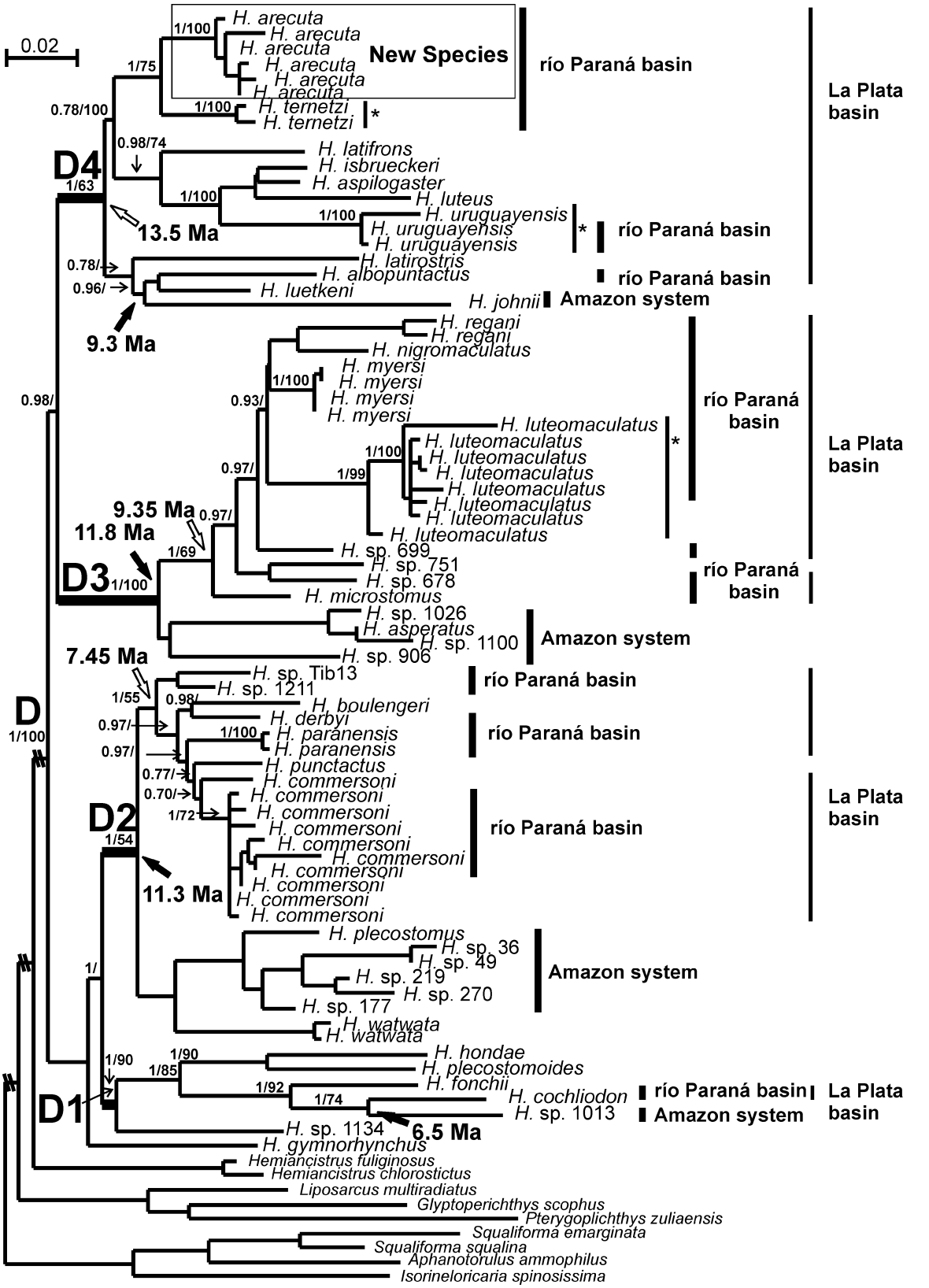


**FIGURE 2.** Maximum likelihood *Hypostomus* phylogenetic tree based on D-loop haplotypes (-lnL = 5192.34998). The ML tree was derived using the HKY + G model of sequence evolution. Numbers next to branches are Bayesian posterior probabilities followed by bootstrap values when these are above 50%, respectively. These support values are showed only for the relevant relationships of this work. Bold letters are abbreviations used for naming clades (see text). The specimens from Paraná river basin and Amazon system (Amazon basin, French Guyana and Northeastern South America coastal rivers) are indicated. The three species marked with * inhabit the Paraná and Uruguay rivers, but not on the Río de La Plata. Also, we show the estimated ages of dispersal events between basins (black arrow) and for vicariance events inside the La Plata basin (white arrow).

## Results

### Phylogenetic analyses

A molecular phylogenetic approach was used to investigate the diversity of *Hypostomus* species from the Paraná river basin. The sequence alignment comprised 592 positions, from which 179 were variable within the ingroup. Base composition and structural characteristics of *Hypostomus* D-loop sequences are described elsewhere (Montoya-Burgos *et al.*, 2002). The model of sequence evolution that fit the best our sequence data set is HKY + gamma, according to MrAIC (Nylander, 2004). The ML and Bayesian phylogenetic trees obtained have similar topologies. The ML tree is shown in Fig. 2.

The evolutionary relationships of the outgroup species is the same as found in Montoya-Burgos (2003). All *Hypostomus* species form a monophyletic clade named Clade D (Fig. 2). This clade can be organized into four monophyletic groups, D1, D2, D3, and D4. Clade D1 clusters together with D2 and Clade D3 with D4. The relationships among these clades are supported by high Bayesian posterior probabilities but relatively low bootstrap values. *Hypostomus gymnorhynchus* is placed as the sister species to clades D1 and D2 and thus forms a distinct monospecific lineage. The Clade D1 (Fig. 2) forms the *H. cochliodon* group including *H. cochliodon* from the Paraná river basin, *H. plecostomoides* from Orinoco basin, *H. hondae* from Lago Maracaibo and Magdalena basins, *H. fonchii* and *Hypostomus* sp. 1013 from the Amazon basin (see Montoya-Burgos (2003) for the non described species metioned in this work).

Clade D2 is subdivided into two monophyletic groups: the first contains species from French Guyana (*H. watwata* and *H. plecostomus*), Amazon basin (represented with *Hypostomus* spp.: 36, 49), and Northeastern South America coastal rivers (*Hypostomus* spp.: 177, 219, and 270 from Gurupí, Itapicurú, and Parnaíba rivers, respectively). The second clade includes species from Eastern South America coastal rivers (*H. puntactus* from Ubatiba), and the La Plata basin (*H. commersoni, H. derbyi, H. paranensis, H. boulengeri,* and two *Hypostomus* spp.: Tib13 and. 1211).

Clade D3 is also subdivided into two groups, one clade including species from the Amazon basin (*H. asperatus* and three *Hypostomus* spp.: 906, 1100, and 1026). The other clade contains species from La Plata basin and São Francisco river (*H. regani; H. luteomaculatus*; *H. microstomus*; *H. myersi*, *H. nigromaculatus*, and three *Hypostomus* spp.: 678, 699, and 751).

Finally, clade D4 includes species inhabiting the La Plata basin: *Hypostomus arecuta* n. sp. (described below), *H. ternetzi*, *H. uruguayensis*, *H. aspilogaster*, *H. luteus*, *H. isbrueckeri*, *H. latifrons*, *H. latirostris*, and *H. albopunctactus.* This clade comprises also *H. luetkeni* from Eastern South America coastal rivers (Paraíba river) and *H. johnii* from an northeastern South America coastal river (Parnaíba river).

Our results show that at least one species inhabiting Paraná river basin is present in each of the four main *Hypostomus* clades (i.e. *H. cochliodon* in clade D1; *H. commersoni, H. derbyi, H. paranensis,* and *Hypostomus* spp: Tib13 and 1211 in clade D2; *H. luteomaculatus*; *H. microstomus, H. myersi, H. regani*, *H. nigromaculatus*, and *H.* sp 699 in clade D3; finally, the new species *Hypostomus arecuta* n. sp., *H. ternetzi, H. uruguayensis*, and *H. albopunctatus* in clade D4).

### Phylogenetic tree calibration.

When analysing the ingroup taxa, with the exclusion of *H. fonchi* which has a particularly long terminal branch (clade D in Fig. 2), the log-likelihood ratio test of homogeneous evolutionary rate showed no significant differences between the likelihood scores obtained when enforcing or not the molecular clock ($X^2 = 56.03$; d.f. = 43; $P = 0.087$). This result indicates that the sequences of the ingroup representatives are evolving at a homogeneous rate. With the aim of evaluate the temporal diversification of the genus *Hypostomus* in the Paraná river basin, we calibrated the D-loop region. We found that the splitting between the *Hypostomus* from the Amazon system (comprising the Amazon basin, French Guyana and North-eastern South America coastal rivers) and La Plata basin is estimated to 6.5 Ma in clade D1, 11.3 Ma in clade D2, 11.8 Ma in clade D3 and 9.3 Ma in clade D4. Moreover, the origin of temporal diversification among the lineages inhabiting the La Plata basin is dated to 7.45 Ma in clade D2, 9.35 Ma in clade D3 and 13.5 Ma in clade D4.

**FIGURE 3.** *Hypostomus arecuta* n. sp., Holotype. MACN-ict 9677 (198), 185.5 mm SL. Dorsal, lateral, and ventral views. Photos by Yamila P. Cardoso.

***Hypostomus arecuta*** n. sp.
Fig. 3.

Here, we describe a new species that inhabit the Paraná river basin and which contributes to the understanding of the origin of the species diversity in this basin (see discussion).

**Holotype:** MACN-Ict 9677 (198), 185.5 mm SL, Argentina, Corrientes province, Ituzaingó, Paraná River (27°29'54.5"S - 56°42'47.0"W). Col: Gonzalez *et al.*, November, 2009.

**Paratypes:** MACN-ict 9678 (163, 166), 2 ex., 174.0–243.3 mm SL, Argentina, Corrientes province, Yahapé, Paraná river, (27°22'12.1"S-57°39'14.6"W). Col: Gonzalez *et al.*, November, 2009. MACN-ict 9679 (181, 191, 192, 193, 195, 196, 197, 199), 9 ex., 156.5–268.1 mm SL, same data as holotype. MACN-ict 9680 (CIA 284–285), 2 ex., 127.0–134.7 mm SL, Argentina, Misiones province, Candelaria city, Paraná river (27º26'92''S-55º44'50''W). Col: Aichino and Capli , November, 2009.

**Diagnosis**

*Hypostomus arecuta* n. sp. is distinguished from its congeners by the following combination of characters: dorsum of head and body and all fins dark grey covered by numerous rounded cream dots. Ventral surface of head and belly a plain cream color. This color pattern distinguishes *H. arecuta* n. sp. from *Hypostomus* species that have dark roundish dots on a lighter background (such: *H. ancistroides*, *H. brevis*, *H. commersoni*, *H. fluviatilis*, *H. hermanni*, *H. iheringii*, *H. nigromaculatus*, *H. paulinus*, *H. topavae*, *H. isbruekeri*, *H. aspilogaster*, *H. uruguayensis*, *H. latifrons*, and *H. latirostris*). Among the species that have light roundish dots or irregular light marks on a darker background, *H. arecuta* n. sp. is distinguished by number of premaxillary/dentary teeth (66–85/63–84) as compared to *H. albopuctatus* (26–32/22–26), *H. luteus* (22–38/26–40), *H. regani* (63–107/63–104), *H. luetkeni* (30–69/38–68), *H. strigaticeps* (about 60), *H. multidens* (115–260/122–267) and *H. microstomus* (7–11/7–13). *Hypostomus arecuta* n. sp. is distinguished from its sister species *H. ternetzi* by the colour pattern of dorsum of head and body, and all fins dark grey covered by numerous rounded cream dots vs. dorsum homogeneously dark, and greater number of scutes at dorsal-fin base (9–10 vs. 8). Also, some morphometric characters differentiate *H. arecuta* n. sp. from *H. ternetzi*: cleithral width (3.0–3.4 vs. 2.8–2.9 in SL), abdominal length (3.9–4.5 vs. 4.6–5.4 in SL), eye diameter (5.2–6.2 vs. 6.2–6.9 in HL), pelvic fin-spine length (3.9–4.5 vs. 3.1–3.8 in SL), caudal penduncle depth (8.3–9.6 vs. 7.5–8.1 in SL), upper caudal-ray length (2.8–3.6 vs. 2.0–2.3 in SL), lower caudal-ray length (2.4–3.5 vs. 1.8–2.0 in SL), and right mandibular ramus (3.9–4.6 vs. 4.8–5.6 in HL). Besides, *H. arecuta* n. sp. differs from *H. luteus* by short dorsal spine length (mean 31.6 % vs. 34.4 % of SL), the length of right mandibular ramus (3.9–4.6 vs. 4.8–6.1 in HL), abdominal length (3.9–4.5 vs. 4.4–5.0 in SL), head depth (1.6–1.7 vs. 1.7–1.9 in HL), and interorbital width (2.6–3.0 vs. 2.9–3.6 in HL). Also some ratios distinguish *H. arecuta* n. sp. from *H. luetkeni*: predorsal length (2.3–2.6 vs. 2.5–3.0 in SL), cleithral width (3.0–3.4 vs. 3.3–4.0 in SL), pectoral-fin spine length (2.8–3.3 vs. 3.1–3.8 in SL), and caudal peduncle length (3.0–3.6 vs. 2.8–3.1). Finally, short dorsal spine length separates *H. arecuta* n. sp. from *H. luteomaculatus* (mean 31.6 % vs. 40% of SL).

*Hypostomus arecuta* can be differentiated from *H. boulengeri*, *H. commersoni* and *H. cochliodon* by the colour pattern. Also, *H. commersoni* has strong lateral keels which are absent in *H. arecuta*, *H. cohliodon* bears fewer premaxillary and dentary teeth than *H. arecuta* n. sp (8/9 vs. 66–85/63–84, respectively). *Hypostomus arecuta* shares with *H. luetomaculatus* and *H. microstomus* a similar dorsal colour pattern, however *H. luteomaculatus* and *H. microstomus* have dark ventral surface of head and body with light vermiculated dots vs. head and belly plain cream in *H. arecuta*. Some counts distinguish *H. arecuta* from *H. luteomaculatus*: scutes along lateral line 26–28 (mode 27) vs 28–29 (mode 29), scutes between end of dorsal fin to adipose fin 5–6 (mode 6) vs 6–7 (mode 7); scutes from adipose to caudal fins 3–5 (mode 5) vs. 5–8 (mode 6), and scutes from anal to caudal fins 12–14 (mode 14) vs. 14–16 (mode 16), respectively. Finally *H. arecuta* n. sp. has a greater number of teeth than *H. microstomus* (66–85/63–84 vs. 7–11/7–13).

**Description**

Meristic and morphometric data are presented in Table I. Dorsal profile slightly convex from snout tip to anterior margin of eyes, straight at interorbital area, convex from interorbital area to dorsal-fin origin, and almost straight from dorsal-fin origin to end of adipose fin. Body width at cleithral region larger than head depth. Head broad and shallow dorsally covered with plates, except for a quadrangular naked area on snout tip.

Supraoccipital bone with a shallow median ridge, and with a relatively well developed posterior process bordered by a wide nuchal plate. A shallow ridge originating laterally to the nares, passing through supraorbital, and extending to median portion of pterotic-supracleithrum. Opercle small, with odontodes more developed distally.

Oral disk ovoid, lower lip covered with numerous papillae decreasing in size posteriorly. Maxillary barbels moderately developed, about as long as orbital diameter. Sixty-six to 85 (holotype 74) teeth in premaxilla, 63 to 84 (holotype 72) in dentary. Teeth bicuspid, curved inward distally, mesial cusp two or three times longer than lateral cusp, distal margin of mesial cusp rounded in replacement teeth and straight in functional ones. Body covered with five rows of moderately spinulose scutes. Tip of snout mostly naked even in large specimens, bearing two lateral vertical patches of odontodes.

Ventral surface of head naked, with small or large patch of platelets before branchial opening. Abdomen covered with minute platelets, with exception of small area around pectoral fin and small or large area around pelvic-fin insertions, and small area at urogenital opening. Preanal plate absent. Caudal peduncle laterally compressed, rather ovoid in cross section.

Twenty-one to 23 (mode 22) dorsal plates, 25–26 (mode 24) mid-dorsal plates, 24–25 (mode 25) median plates, 26–28 (mode 27) mid-ventral plates, 21–22 (mode 22) ventral plates. Three predorsal plates, 9–10 (mode 9) plates below dorsal fin, 5–6 (mode 6) preadipose plates, 4–5 (mode 5) plates between adipose fin and caudal fin, 12–14 (mode 14) plates between anal fin and caudal fin.

Dorsal-fin II,7, its origin placed at vertical closer to pelvic-fin origin than pectoral-fin origin. Dorsal-fin margin straight. Adipose-fin spine compressed and curved backward. Pectoral fin I,6, its posterior border straight. Pectoral-fin spine slightly curved inward, covered with weakly developed odontodes, slightly more developed on its distal portion in larger specimens. Tip of pectoral fin reaching one-third pelvic-fin spine length. Pelvic-fin I,5, its posterior border slightly roundish. Pelvic-fin spine surpassing anal-fin origin. Anal fin I,4, its tip reaching the 6th plate after its origin, 2$^{ed}$ and 3$^{rd}$ branched rays longer. Caudal-fin margin concave, I,14,I, with inferior lobe longer than superior one.

**Phylogenetic position of *Hypostomus arecuta***

The new species described above, *Hypostomus arecuta*, is distinguished from others species of the genus by a combination of morphological and molecular features. *Hypostomus arecuta* is apparently endemic to the Paraná river in Argentina. According to our phylogenetic tree, *H. arecuta*, together with *H. ternetzi*, *H. isbruekeri*, *H. aspilogaster*, *H. uruguayensis*, *H. latifrons*, *H. latirostris*, *H. luteus*, *H. albopuntactus*, *H. johnii* and *H. luetkeni* form the clade D4 (see Fig. 2). Although our results show that the node that clusters clades D4 and D3 shows low statistical support in the ML analysis, other data support this relationship: following Muller and Weber (1992), the *Hypostomus* species of clade D4 shares with species of clade D3 the presence of white spots on the body, wide mandible, and long-crowned teeth (defining the so called *Hypostomus regani* group). Species belonging to other clades (D1 and D2, Fig. 2) display black widespread spots on the body, medium-sized mandible, and short-crowned teeth (forming the so called *Hypostomus plecostomus* group). Moreover, karyological studies show that some species of the *Hypostomus regani* group have a fundamental chromosome number of near 72 and some species of the *Hypostomus plecostomus* group have a fundamental number near 68 (Zawadzki *et al.*, 2004). Therefore, these morphological, colour pattern and karyological data support our phylogenetic analyses showing a division of *Hypostomus* species into two principal clades, clade D1+D2 (*Hypostomus plecostomus* group) and clade D3+D4 (*Hypostomus regani* group). Thus, the new species *H. arecuta*. is considered as a member of the *Hypostomus regani* group.

**Colour in alcohol**

Overall ground colour of body and fins dark grey. Overall ground color of ventral area a plain, lighter, cream color in some specimens. Dorsal surface of head, body, and fins entirely covered by numerous rounded cream dots, smaller and closer on head. Dorsal, pectoral, and pelvic- fins with dots regularly or irregularly arranged in rows along their rays. Adipose fin with rounded, cream dots. Caudal fin with scattered, rounded cream dots on rays and membranes.

**Distribution**

*Hypostomus arecuta* is known from the Paraná river at Yahapé and Ituzaingó (Corrientes province), Candelaria (Misiones province), and Santa Fé (Santa Fé province), Argentina. *Hypostomus arecuta* is sympatric with *H. commersoni, H. cochliodon, H. uruguayensis, H. latifrons, H. ternetzi*, *H. luteomaculatus, H. microstomus,* and *H. boulengeri*.

**Etymology**

The specific epithet *arecuta* is a Guaraní word *arecutá* that means loricariid fish.

**Habitat**

The specimens of *Hypostomus arecuta* were collected in coastal areas of the Paraná river main channel. The bottom was composed mostly by large boulders of sandstone with patches of sand and pebbles. The species was found in well oxygenated waters having moderate current speed, about 0.60 m $s^{-1}$. Water transparency was within the most frequent range registered in the river (1.50–2.40 m). Conductivity was generally low and typical for the river (50.9–59.6 µS $cm^{-1}$). The pH was slightly acidic to neutral (6.8–7.1).

**Discussion**

**The origin of species diversity of *Hypostomus* in the Paraná river basin**

According to the results presented here and to Montoya-Burgos (2002, 2003), the phylogenetic tree of the genus *Hypostomus* can be divided into four principal clades (Fig. 2). Since each clade includes at least one species from the Paraná river basin and at least one from the large Amazon system, it can be deduced that old inter-basin allopatric speciation has participated in the diversification of *Hypostomus* in the Paraná river basin. In addition, lineages with multiple species inhabiting the Paraná river basin are found in clades D2, D3 and D4. This indicates that speciation within the basin also shaped the diversity of *Hypostomus* there.

The biogeographic analysis of the inter-basin relationships in clade D1 shows that *H. cochliodon,* from the Paraná river, clusters with *Hypostomus* sp. 1013, from the Amazon basin (Fig. 2), and our calibrations indicate that the splitting event can be dated to 6.5 Ma. To explain this age, we would have to invoke temporal connections between the upper Paraguay and Southern tributaries of the Amazon posterior to the inferred age of the boundary displacements and water interchange between the Northern paleo Amazon-Orinoco basin and La Plata basin (11.8–10 Ma) (Lundberg *et al.*, 1998). When these temporal interconnections ceased, the isolation of populations in both basins would have resulted in the allopatric speciation that gave rise to *H. cochliodon* and *H.* sp. 1013.

The clade D2 shows that species inhabiting the Amazon system cluster with species from La Plata basin + Eastern South America coastal rivers (Fig. 2). According to the D-loop molecular clock, the splitting event between these two groups can be dated to 11.3 Ma. This result is in accordance with the estimated date for this clade in Montoya-Burgos (2003). This inferred age matches with the documented boundary displacements between the Northern paleo Amazon-Orinoco system and the La Plata basin that occurred at about 11.8–10 Ma (Lundberg *et al.*, 1998). Once the headwater exchanges due to the boundary displacement were finished, the isolation of populations in both basins would have resulted in speciation, giving rise to the two lineages of clade D2.

Within clade D3, the species inhabiting the Amazon basin and those from La Plata basin + São Francisco river (see Fig.2) form two distinct lineages that originated about 11.8 Ma according to the D-loop data. In Montoya-Burgos (2003), this event had a slightly more recent date (10.2–10.1 Ma.). However, both estimations correspond well with the date estimated for the last major water interchange between the paleo Amazon-Orinoco and La Plata basin reported above for clade D2.

In clade D4, the splitting between species inhabiting North-eastern South America coastal rivers (being part of the Amazon system), represented by *H. johnii,* and species from the Paraná river + Eastern South America coastal rivers, represented by *H. albopunctatus + H. luetkeni*, was dated to 9.3 Ma. Differing from what was proposed in Montoya-Burgos (2003), this estimated age does not correspond to the boundary displacements between the Northern paleo Amazon-Orinoco system and La Plata basin (11.8–10 Ma). This difference can be explained by the exclusion of the sequence of *Hypostomus* sp. (Tib 1), used in Montoya-Burgos (2003), from our analysis and also because in this work we used a smaller segment of D-loop marker than in Montoya-Burgos (2003). According to our results, the diversification episode within clade D4 would be another case of more recent water interchange via a temporal connection between the two main basins, as was already the case for clade D1. In addition, the

evolutionary position of *H. johnii* within the clade D4 (fig. 2) would allow us to suggest that the direction of the dispersal event was from the La Plata basin towards the Amazon system.

In summary, our results suggest that at least four independent allopatric speciation episodes occurred between the Amazon system and the Paraná basin river + São Francisco + Eastern South America coastal rivers. In clade D2 and D3, these allopatric speciations may be explained by the boundary displacement between the Northern paleo Amazon–Orinoco and the Southern La Plata basin which occurred between 11.8–10 Ma (Lundberg *et al.*, 1998). As indicated in clades D1 and D4, two more recent allopatric speciations (6.5 Ma for clade D1 and 9.3 Ma for clade D4) occurred by headwater exchanges and subsequent isolation between the northern and southern river systems, involving probably the upper Paraguay and Southern tributaries of the Amazon (Lundberg *et al.*, 1998). Accordingly, more recent population splitting between the Amazon and Paraná river basins has been reported (between 2.3 and 4.1 Ma) for the migratory fish *Prochilodus* (Sivasundar *et al.*, 2001). Moreover, other recent dispersal events between these two basins are further exemplified by the distribution ranges of *Pygocentrus nattereri* (Hubert *et al.*, 2007) and *Pseudotylosurus augusticeps* (Lovejoy and De Araújo, 2000). These data and our results suggest that the temporary connections between the Amazon and Paraná river basins would be more frequent than previously thought.

The second origin of the diversity of *Hypostomus* species in the Paraná river basin is shaped by intra-basin speciation and occurs within the La Plata basin. In clade D2, the node including all species present in La Plata basin (seven species) was estimated to 7.45 Ma according to our molecular clock. This date coincides with the second reported event of maximum flooding of marine transgression during the Miocene (10-5 Ma) (Hernández *et al.*, 2005). The extensive marine incursion onto the Paraná river basin could have isolated the tributaries of this basin, generating several allopatric speciations in different and strictly freshwater organisms that habited the La Plata basin. Once the sea retreated, the newly formed species would have dispersed throughout the current La Plata basin, enriching its biodiversity. It is important to note that *H. puntactus* from Eastern South American coastal rivers emerges within the group inhabiting the La Plata basin. This fact was explained by Montoya-Burgos (2003) as a dispersal event from the La Plata basin towards the Eastern South American coastal rivers.

In clade D3, the calculated age for the origin of the species occupying the La Plata basin is 9.35 Ma. (eight species). This estimated age also coincides with the event of the marine transgression that occurred 10-5 Ma (Hernández *et al.*, 2005). Also, within this clade, there are species of the São Francisco river, which reveal, according to Montoya-Burgos (2003), a colonization event of the São Francisco river from the La Plata basin.

In clade D4, the node that contains the species inhabiting the La Plata basin is dated to 13.5 Ma; since then at least 10 species were formed. This estimated age is in accordance with the first event of maximum flooding of the marine transgression that occurred 15-13 Ma (Hernández *et al.*, 2005). As previously mentioned, within this clade emerges *H. johnii* from Northern South America coastal rivers and *H. luetkeni* from Eastern South America coastal rivers. This result demonstrates two dispersal events; both are posterior to the origin of the species diversity of the La Plata basin.

It is important to note that intra-basin diversification increases with the age of the origin of the lineage. In clade D2 the intra-basin diversification started 7.45 Ma and generated seven species; in clade D3 it started 9.35 Ma and gave rise to eight species; in clade D4 it started 13.5 Ma and resulted in ten species. These species numbers are underestimates as new species might be discovered and others might have become extinct. In this respect, the new species described here, *H. arecuta*, contributes importantly to our understanding that clade D4 is the most ancient and diverse *Hypostomus* lineage inhabiting almost exclusively the La Plata basin. This high diversity is the result of intra-basin speciations.

Therefore, we see that *Hypostomus* species diversity in the Paraná river, and in consequence in the La Plata basin, is moulded by two processes. One is the inter-basin diversification, which generated groups of species inhabiting different basins as result of dispersal events, as proposed by the hydrogeological hypothesis (Montoya-Burgos, 2003). In this context, paleo hydrogeological changes during the Miocene have promoted vicariance and dispersal routes yielding a high degree of diversification of species of fishes in the Neotropical region. The other process that shaped species diversity of *Hypostomus* is intra-basin speciation, which produced groups of species inside a basin due to habitat fragmentation. We see that the origin of species diversity inside the La Plata basin temporally matches with the marine transgression in the Paraná river basin. As proposed by the museum hypothesis (Nores, 1999), this marine incursion could have fragmented the La Plata basin resulting in several allopatric speciation events. The historical biogeography of *Hypostomus* argues that several documented hydrological and sea level changes deeply influenced the cladogenetic events observed in the phylogeny of this genus.

**Possible connection between the Paraná and Uruguay rivers**

The hydrographical patterns of the Paraná and Uruguay rivers indicate that they can be considered as belonging to the same basin (La Plata basin) as the Lower Paraná is connected to the Lower Uruguay via the Río de la Plata estuary. This configuration has been maintained almost unchanged for the last 10 Ma (Lundberg *et al.*, 1998). Sivasundar *et al.* (2001) mentioned that several conspecific populations are currently distributed along the Paraná and Uruguay rivers, dispersing probably through the Río de la Plata estuary. This dispersal route, which allows gene flow between the two rivers, may explain why representatives of *H. commersoni* are genetically similar in those two rivers as well as in the Río de la Plata estuary. However, according to the distribution range of some *Hypostomus* species in the La Plata basin, we can propose other possible ancient dispersal routes between the Paraná and Uruguay rivers. The examined material in this work and the bibliography about *Hypostomus luteomaculatus*, *H. uruguayensis*, and *H. ternetzi* show that these species are distributed in the Paraná and Uruguay rivers. Contrary to *H. commersoni*, these species have never been reported from the Río de la Plata estuary. These three species could have either gone extinct or have left the Río de la Plata estuary. Alternatively, they might have dispersed through temporal connections between Northern tributaries of the Paraná and Uruguay rivers.

During the Miocene (24-5 Ma), the Paraná and Uruguay rivers became disconnected from one another resulting in the present configuration (Beurlen, 1970). However, the topology and proximity between some tributaries of these two rivers allows us to hypothesize that water pathways between the Paraná and Uruguay rivers could have existed during flood periods. Weber (1987) suggested that the Aguapey river can be a connection between the Paraná and Uruguay rivers. Later on, Casciotta *et al.* (2005) mentioned that it is probable that at present ichthyofaunal exchanges can take place between the Laguna Iberá (Paraná river basin) and the Miriñay river (Uruguay river basin) during flood periods. An exhaustive population genetic analysis could be useful to understand the dispersal routes used by some *Hypostomus* species to maintain gene flow between populations inhabiting Paraná and Uruguay rivers basin.

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**Additional specimens examined**

Argentina. *Hypostomus boulengeri*, MACN-ict 9644, 1 ex., 121.8 mm, Corrientes Province, Paraná river at Ituzaingó. *Hypostomus commersoni*: ILPLA1907, 8ex, El Pescado, La Plata, Buenos Aires. *Hypostomus paranensis*: ILPLA1914, 2 ex, Córdoba, Córdoba Capital, Suquia river. *Hypostomus ternetzi*, MACN-ict 9645, 1 ex. 150 mm Corrientes Province, Paraná river at Yahape. *Hypostomus uruguayensis*, MACN-ict 9651, 1 ex., 159.0 mm, Corrientes Province, Paraná river at Yahape. Brazil. *Hypostomus luteus*: MCP19991, 1ex., Santa Catalina, Uruguay river basin, Uruguai river, proximo a pedra da Fortaleza. MCP20751, 1ex., Santa Catalina, Uruguay river basin, Uruguai river, proximo a pedra da Fortaleza. *Hypostomus regani*: MCP19989, 1 ex., Santa Catalina, Uruguay river basin, Uruguai river, proximo a pedra da Fortaleza. MCP28628, 1 ex., Rio Grande do Sul, Uruguay river basin, Uruguai river, no Remanso da Timbaúva, cerca de 1500m do início do Salto do Yucuma. MHNG 2547.017, 3ex. , 141, 86–162,09 mm, Sao Pablo, Mogui Guazu Cach.

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