Phylogeography of *Hypostomus strigaticeps* (Siluriformes: Loricariidae) inferred by mitochondrial DNA reveals its distribution in the upper Paraná River basin

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In this study, phylogenetic and phylogeographic analyses of populations identified as *Hypostomus strigaticeps* from the upper Paraná River basin were conducted in order to test whether these different populations comprises cryptic species or structured populations and to assess their genetic variability. The sequences of the mitochondrial DNA ATP sintetase (subunits 6/8) of 27 specimens from 10 populations (one from Mogi-Guaçu River, five from Paranapanema River, three from Tietê River and one from Peixe River) were analyzed. The phylogeographic analysis showed the existence of eight haplotypes (A-H), and despite the ancestral haplotype includes only individuals from the Tietê River basin, the distribution of *H. strigaticeps* was not restricted to this basin. Haplotypes A, B and F were the most frequent. Haplotypes D, E, F, G, and H were present in the sub-basin of Paranapanema, two (A and B) were present in the sub-basin of the Tietê River, one (C) was exclusively distributed in the sub-basin of the Peixe River, and one (B) was also present in the sub-basin of the Grande River. The phylogenetic analysis showed that the populations of *H. strigaticeps* indeed form a monophyletic unit comprising two lineages: TG, with representatives from the Tietê, Mogi-Guaçu and Peixe Rivers; and PP, with specimens from the Paranapanema River. The observed degree of genetic divergence within the TG and PP lineages was 0.1% and 0.2%, respectively, whereas the genetic divergence between the two lineages themselves was approximately 1%. The results of the phylogenetic analysis do not support the hypothesis of existence of crypt species and the phylogeographic analysis confirm the presence of *H. strigaticeps* in other sub-basins of the upper Paraná River: Grande, Peixe, and Paranapanema sub-basins.

Neste estudo, foram conduzidas análises filogenéticas e filogeográficas de populações identificadas como *Hypostomus strigaticeps* na bacia do alto rio Paraná a fim de testar se essas populações compreendem espécies crípticas ou populações estruturadas e avaliar a variabilidade genética das mesmas. Foram analisadas sequências do DNA mitocondrial ATP sintetase (subunidades 6/8) de 27 espécimes de 10 populações (uma do rio Mogi-Guaçu, cinco do rio Paranapanema, três do rio Tietê e uma do rio do Peixe). A análise filogeográfica mostrou a existência de oito haplótipos (A-H), e apesar do haplótipo ancestral incluir apenas indivíduos da bacia do rio Tietê, a distribuição de *H. strigaticeps* não se restringe a esta bacia. Os haplótipos A, B e F foram os mais frequentes. D, E, F, G e H estão presentes na sub-bacia do rio Paranapanema, dois (A e B) estão presentes na sub-bacia do rio Tietê, um (C) está exclusivamente distribuído na sub-bacia do rio do Peixe, e um (B) também está presente na sub-bacia do rio Grande. A análise filogenética mostrou que as populações de *H. strigaticeps* realmente formam uma unidade monofilética que compreende duas linhagens: TG, com representantes do rio Tietê, rio Mogi-Guaçu e rio do Peixe, e PP, com espécimes do rio Paranapanema. O grau de divergência genética observada nas linhagens de TG e PP foram de 0,1% e 0,2%, respectivamente, enquanto que a divergência genética entre as duas linhagens foi de aproximadamente 1%. Os resultados da análise filogenética não suportam a hipótese da existência de espécies crípticas e a análise filogeográfica confirma a presença de *H. strigaticeps* em outras sub-bacias do alto rio Paraná: sub-bacias do rio Grande, rio do Peixe e rio Paranapanema.

Key words: ATPase, Bayesian Inference, Haplotype, Nested clade, Phylogeny.

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Introduction

The Neotropical freshwater fish fauna is extremely diverse, comprising approximately 71 families and 4,475 valid species of fish characterized by high endemicity (Reis *et al.*, 2003). The order Siluriformes represents a large and diverse group of bony fishes with 37 families and more than 2500 species identified (Burgess, 1989; Ferraris, 1995; Britto, 2003). One of the most species-rich families of siluriforms is the family Loricariidae whose members are known as suckermouth armored catfishes. The loricariids are characterized by a body completely covered by dermal plates, small bone-shaped structures (thorn odontodes) in the head and by a sucker-like mouth with fleshy papillae (Reis *et al.*, 2003).

The genus *Hypostomus* is the most diverse group within the family (Weber, 2003; Hollanda Carvalho *et al.*, 2010), with 126 species distributed from Central America to southern South America (Zawadzki *et al.*, 2010). The relative morphological polymorphism of the *Hypostomus* species make difficult to identify species-specific characters for the genus (Armbruster, 2004). This extensive morphological variation combined with the cytogenetic diversity observed, with diploid numbers ranging from 2n = 52 in *Hypostomus emarginatus* (Artoni & Bertollo, 2001) to 2n = 84 in *Hypostomus* sp. (Cereali *et al.*, 2008) evidence the great taxonomic diversity of group.

Studies related to phylogeography and genetic variability in the genus *Hypostomus*, are very scarce. A molecular study on the genus was conducted by Montoya-Burgos (2003) inferring about the biogeographic patterns of genus *Hypostomus* showed that in the upper Paraná River basin the genus represent a polyphyletic group. More recent studies involving the genetic characterization of *Hypostomus* populations used RAPD by Sofia *et al.* (2008) and Allozymes by Zawadzki *et al.* (2008) were conducted and these studies show a great genetic variability in some species, however not mentioned any phylogenetic relationships.

Hypostomus strigaticeps (Fig. 1) was described by Regan (1908) from specimens of the Piracicaba River a sub-basin of the Tietê River. According to Weber (2003) and Reis *et al.* (2003) it is restricted to the sub-basin of the Tietê River, in the upper Paraná River basin. This species was not mentioned in any other sub-basin of the upper Paraná River basin. Observed morphological variation in the *Hypostomus* populations along the sub-basins of the Paraná River (Paranapanema and Rio Grande sub-basins), raises the possibility that these morphotypes represent either polymorphic populations of *H. strigaticeps* or cryptic species morphologically similar to *H. strigaticeps*.

In order to test whether these different populations comprise cryptic species or structured populations, in this study we sequenced the mitochondrial ATPase 6/8 gene in different populations of *Hypostomus strigaticeps* from the Tietê river to assess their genetic variability. We also analyzed the genetic variability of *Hypostomus* specimens from three adjacent sub-basins of the Paraná River: the Paranapanema, Peixe and Grande rivers (Mogi-Guaçu River) sub-basins, to determine the current range of *H. strigaticeps* in the Paraná River, and to identify the historical process and dispersal patterns of this species.

Material and Methods

A total of twenty-seven *Hypostomus* specimens from four sub-basins of the Paraná River (Fig. 2) were examined. Specimens from 10 populations from four sub-basins were examined: three from the Tietê, five from Paranapanema, one from Grande (Mogi-Guaçu) and one from Peixe (Table 1). *Hypostomus ancistroides*, *H. paulinus*, and *H. iheringii* from the upper Paraná River basin were used as close outgroups, whereas *Rhamdia quelen* (Heptapteridae) was used as distant outgroup. The specimens were deposited in the collections of the Laboratório de Citogenética of Universidade Estadual Paulista (UNESP, Rio Claro, Brazil), and in the ichthyology collection of Nupelia (NUP) of Universidade Estadual de Maringá (UEM, Maringá, Brazil).

DNA was extracted with the phenol-chloroform-isoamyl alcohol technique (Sambrook & Russell, 2001). PCR amplification of the ATPase 6/8 gene region was obtained from a volume of 13.5 mL of a solution containing 6.25 mL of PCR Mix (Quiagen), 5.25 mL of Milli Q water, 0.5 µL primer F (10 µM), 0.5 mL of primer R (10 µM) and 1.0 mL of template DNA (200 ng). PCR was performed in a thermocycler (Eppendorf Mastercycler) and consisted of the following

Fig. 1. Dorsal, lateral and ventral views of *Hypostomus strigaticeps*. MZUSP 79646, Tietê River, São Paulo State, Brazil. 127.6 mm SL.

cycling conditions: an initial cycle of denaturation at 94 °C for 40 s, followed by 35 cycles at 94 °C for 30 s, annealing at 55 °C for 40 s, chain extension at 68°C. Check temperature extension for 2 min, and a final extension at 72°C for 5 min. The following set of primers was used: L8331 (5'-AA GCR TYR GCC TTT TAA GC-3') and H9236 (5'-GTT AGT GGT CAK GGG CTT GGR TC-3') (Perdices *et al.*, 2002), and resulted in an amplification of about 850 bp. The amplified DNA segments were run on 1% agarose gel stained with 2 mL SYBR Safe (10,000×) (Invitrogen®) in 1 mL of blue juice running buffer (10×) and visualized in ultraviolet light. The quantification of the amplified gene products was performed using the molecular weight marker Lambda (λ) DNA 50 ng and 30 ng. For the sequence analysis, the amplified DNA was purified with the EXOSAP enzyme and subsequently sequenced (MacroGen, Korea).

Model Test 3.06 (Posada & Crandall, 1998) was used to find the best fitting evolution model for our data. The model selected by the Akaike criterion was GTR+I+G (empirical base frequencies A=0.3338, C=0.3053, G=0.1023, T=0.2586; substitution rates: A-C=1.77, A-G=36.7617, A-T=0.5967, C-G=2.1775, C-T=17.2043, G-T=1.0). These parameters were used to estimate Maximum Likelihood (ML) distances in

Fig. 2. Map of the river system in Brazil, highlighting the upper rio Paraná basin and the collection points.

the Neighbor-Joining (NJ) analyses in PAUP*, with 1,000 bootstrap replicates. The maximum parsimony (MP) analysis was performed using heuristic searches with tree-bisection-reconnection (TBR) branch swapping and 10 random-taxon-addition replicates. Only minimal trees were retained and zero length branches were collapsed. A total of 1,000 bootstrap replicates were used to assess the relative robustness of the branches of the MP trees in PAUP*. The saturation analysis of data was performed in DAMBE 4.5.8 (Xia & Xie, 2001).

Bayesian inference (BI) phylogenies were inferred using MrBayes v. 3.1.2 (Huelsenbenck & Ronquist, 2001). Four simultaneous Monte Carlo Markov Chains (MCMC) were run for 2 million generations under the General Time Reversible (GTR) model of evolution, with sample frequency of 100 generations and chain temperature set to 0.2. Log-likelihood stability was reached after approximately 80,000 generations (excluding the first 80,000 trees). The remaining trees were used to compute a 50% majority-rule consensus tree. Posterior probability values were calculated to determine the level of support to the Bayesian topology.

We identified the haplotypes network (nested clade) to evaluate the association between haplotypes and their geographical distribution. This analysis was constructed using the TCS software package (Clement *et al.*, 2000), which employs the method of Templeton *et al.* (1992).

We used the mtDNA clock calibration of 1.30% sequence divergence per million years (calculate using uncorrected p-genetic distance) for marine fishes separated by the Pliocene closure of the Isthmus of Panama (Bermingham *et al.*, 1997) and previously applied for the genus *Rhamdia* from Central America (Perdices *et al.*, 2002).

Results

The alignment of the ATPase 6/8 gene sequences revealed 828 conserved and 14 variable base pairs, of which 11 were informative for the Maximum Parsimony analysis. Mean nucleotide base composition in the sequences was as follows: 28.7 (T), 29.5 (C), 30.1 (A) and 11.7 (G), the data obtained were not saturated.

All phylogenies (NJ, MP and BI) were congruent differing only in their branch support. The phylogenetic analysis showed the analyzed *Hypostomus* specimens from the upper Paraná River as a monophyletic group organized into two major mtDNA lineages: Lineage TG, with representatives from the Tietê, Grande (Mogi-guaçu) and Peixe River populations; and Lineage PP, with specimens from the Paranapanema River basin populations (Fig. 3) (Table 1). These lineages were highly supported by bootstrap values (>86%) and posterior probability values (>80%). The mean genetic distance between TG and PP lineages was 0.94% ± 0.20 whereas among individuals within the TG and PP lineages was 0.11% ± 0.08 and 0.27% ± 0.30, respectively. The genetic distance observed between *H. strigaticeps* and other species of the genus
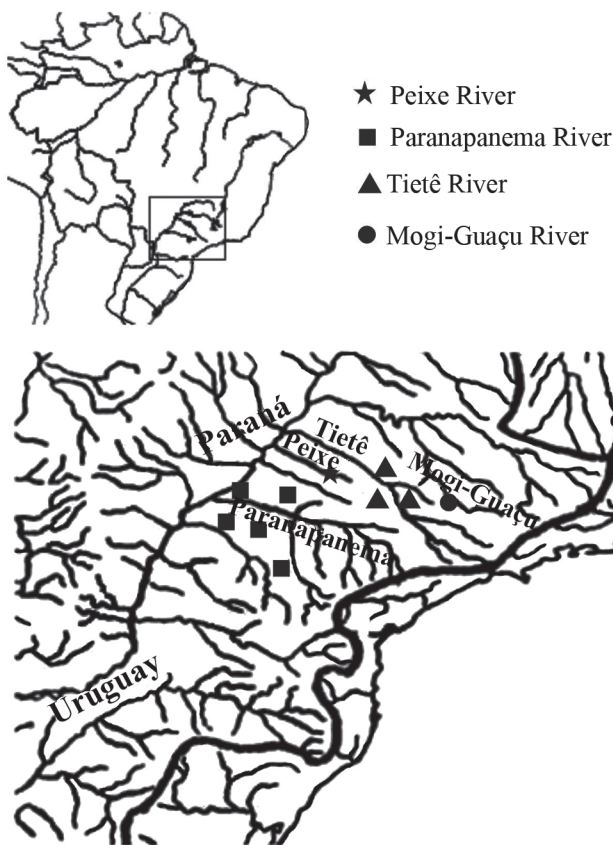
Table 1. Identification of *Hypostomus strigaticeps* specimens collected, respective collection points at the upper Paraná River basin and their haplotypes and mtDNA lineage.

Individual code	Basin	Sub-basin	Locality	Haplotypes	mtDNA lineage
LGP1092	upper Paraná	Paranapanema	Chavantes reservoir	D	PP
LGP1093	upper Paraná	Paranapanema	Chavantes reservoir	D	PP
LGP1098	upper Paraná	Paranapanema	Chavantes reservoir	D	PP
LGP1051	upper Paraná	Paranapanema	Pirapó River	E	PP
LGP1052	upper Paraná	Paranapanema	Pirapó River	E	PP
LGP14130	upper Paraná	Paranapanema	Hortelã stream	F	PP
LGP14133	upper Paraná	Paranapanema	Hortelã stream	F	PP
LGP14131	upper Paraná	Paranapanema	Hortelã stream	F	PP
LGP31518	upper Paraná	Paranapanema	Hortelã stream	F	PP
LGP31517	upper Paraná	Paranapanema	Hortelã stream	F	PP
LGP10982	upper Paraná	Paranapanema	Maringá stream	H	PP
LGP10983	upper Paraná	Paranapanema	Maringá stream	E	PP
LGP10984	upper Paraná	Paranapanema	Maringá stream	E	PP
LGP29800	upper Paraná	Paranapanema	Taquará River	G	PP
LGP10671	upper Paraná	Tietê	Araquá stream	A	TG
LGP10672	upper Paraná	Tietê	Araquá stream	A	TG
LGP10667	upper Paraná	Tietê	Araquá stream	A	TG
LGP10669	upper Paraná	Tietê	Araquá stream	A	TG
LGP10903	upper Paraná	Tietê	Corrumbatai River	B	TG
LGP10882	upper Paraná	Tietê	Corrumbatai River	B	TG
LGP19385	upper Paraná	Tietê	Conchas River	A	TG
LGP25930	upper Paraná	Mogi Guaçu	Água Boa stream	B	TG
LGP1060	upper Paraná	Peixe	Peixe River	C	TG
LGP1061	upper Paraná	Peixe	Peixe River	C	TG
LGP1062	upper Paraná	Peixe	Peixe River	C	TG
LGP1063	upper Paraná	Peixe	Peixe River	C	TG
LGP1064	upper Paraná	Peixe	Peixe River	C	TG
LGP1065 - *H. paulinus*	upper Paraná	Paranapanema	Pirapó River	-	-
LGP1066 - *H. iheringii*	upper Paraná	Peixe	Peixe River	-	-
LGP1067 - *H. ancistroides*	upper Paraná	Tietê	Claro stream	-	-
LGP1068 - *Rhamdia quelen*	upper Paraná	Tietê	Claro stream	-	-

analyzed, *H. iheringii*, *H. paulinus*, and *H. ancistroides* was $3.80\% \pm 0.09$, $4.06\% \pm 0.10$ and $5.82\% \pm 0.10$, respectively.

The phylogeographic analysis revealed eight haplotypes (A-H). Haplotype A was considered the ancestral haplotype, and it was present in two of the three populations analyzed from the Tietê River sub-basin (Araquá and Conchas Rivers). The individuals of the Corumbataí River (Tietê River sub-basin) showed haplotype B shared with individuals inhabiting Água Boa stream (Grande River) (Fig. 4). Haplotype C was exclusively found in the Peixe sub-basin whereas Haplotypes D, E, F, G, and H were exclusive of all rivers of the Paranapanema sub-basin (Table 1). Haplotypes A, C, and F had the highest frequency (18.51%).

Discussion

Due to the high degree of morphological similarity between the representatives of the genus *Hypostomus*, the use of molecular markers to identify and characterize its populations and species is extremely valuable. Our data supported the hypothesis that the different ingroup *Hypostomus* populations analyzed represented the same species, *Hypostomus strigaticeps*, as evidenced by the small genetic distances among the analyzed populations. The phylogenetic analysis showed that these populations form two major mtDNA lineages: TG, distributed in the sub-basins of the Tietê, Grande

(Mogi-Guaçu) and Peixe Rivers; and PP, represented in the sub-basin of the Paranapanema River. Similarly, the phylogeographic analysis also showed two clades: Tietê-Peixe-Grande and Paranapanema, corresponding to lineages TG and PP, respectively, in the phylogenetic analysis. Although all samples analyzed were *H. strigaticeps* individuals, our phylogenetic and phylogeographic results consistently showed a clear separation between the sub-basins. The phylogeographic analysis showed that the ancestral haplotype of *H. strigaticeps* was distributed in the Tietê River sub-basin. The observed network of haplotypes related haplotypes found in the Tietê River sub-basin and Grande River (Mogi-Guaçu) and Peixe River, and clearly separated haplotypes found in the Paranapanema sub-basin. Although further analysis with more populations and sub-basins of the Paraná River basin should be performed to have more consistent results, we found the ancestral haplotypes of *H. strigaticeps* distributed in the Tietê River sub-basin related to the adjacent sub-basins Peixe and Grande what suggests a recent gene flow.

Considering a rate of mutation of the ATPase 6/8 gene in fish of 1.3%/Ma (Bermingham *et al*., 1997; Perdices *et al*., 2002) and the observed genetic divergence of 0.94% between lineages TG and PP, we calculate the separation of both lineages as approximately about 723,000 years ago, during the Quaternary. Likely due to their bottom-dwelling body
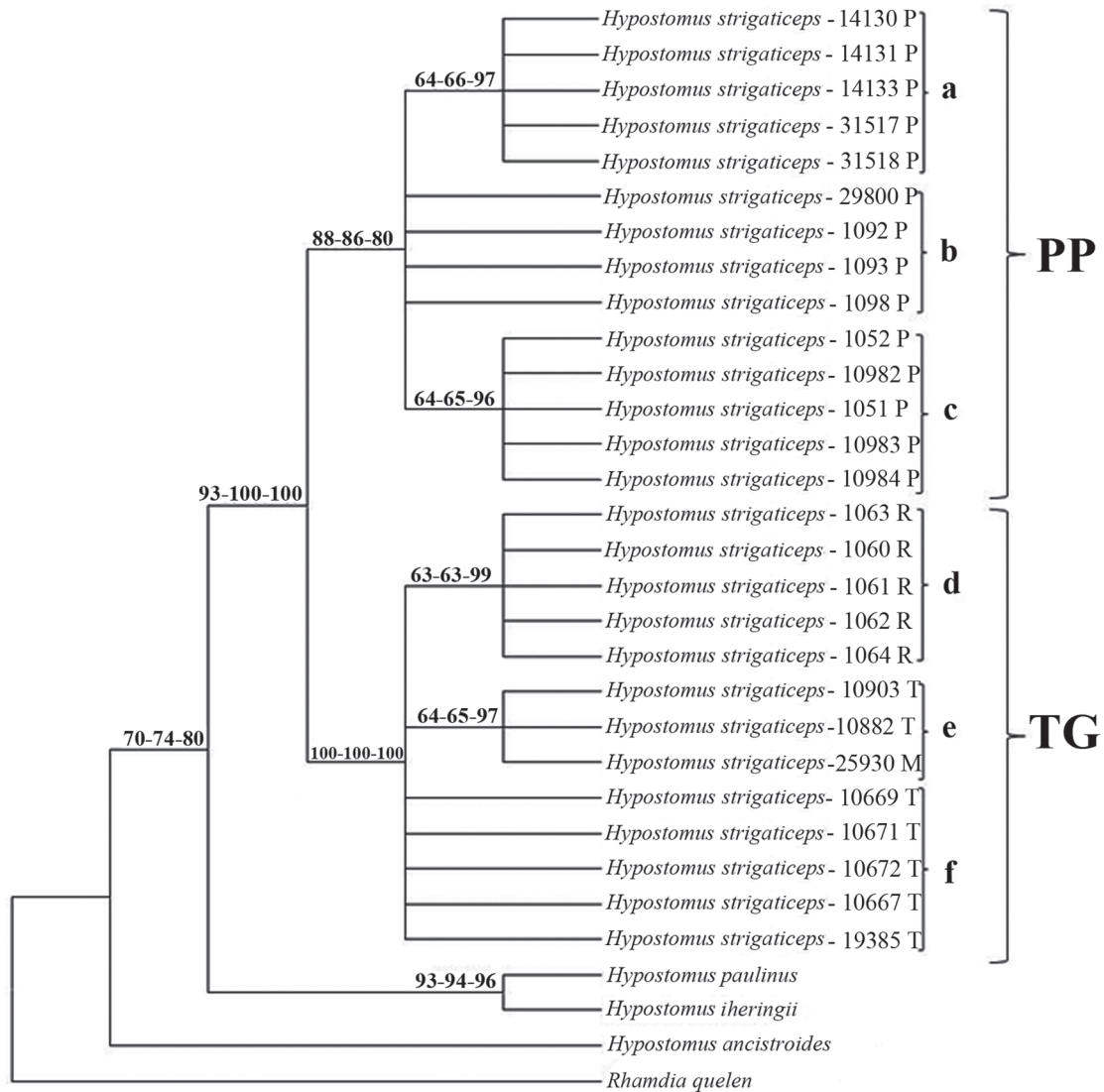
Fig. 3. Phylogenetic tree based on the ATPase 6/8 mitochondrial gene using NJ, MP and BI methods. Values on branches represent bootstrap values for NJ, MP and posterior probabilities for BI.

shape the *Hypostomus* species seems not a group of freshwater fishes prone to perform long river displacements (Weber, 2003). Genetic studies on other species of the genus *Hypostomus* as *H. ancistroides* (Sofia *et al.*, 2008; Endo *et al.*, 2012), *H. margaritifer* (Zawadzki *et al.*, 2002), and *H. regani* (Zawadzki *et al.*, 2008) have also found moderate structured populations as we found in the *H. strigaticeps* populations analyzed.

The hypothesis that *H. strigaticeps* is widely distributed in the Upper Parana River, is supported by the present data. Our phylogenetic results showed that all individuals analyzed from the four sub-basins investigated belong to *H. strigaticeps*. Genetic variability and the genetic distances found among individuals inhabiting all water systems analyzed indicated that all individuals belong to the same
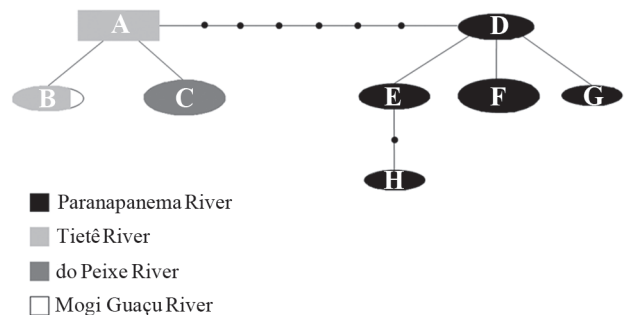

Fig. 4. Network of haplotypes generated by the TCS program.

species: *H. strigaticeps*. Moreover, all these individuals form a monophyletic group highly supported, that were not intimately related to any of the sympatric *Hypostomus* species analyzed that occur in these area. Our results of the phylogeographic analysis confirm the presence of *H. strigaticeps* in other sub-basins of the upper Paraná River: Grande, Peixe and Paranapanema sub-basins.

Acknowledgments

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