Acta Scientiarum

http://www.uem.br/acta
ISSN printed: 1679-9283
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v35i2.11451

Molecular variants in populations of *Bryconamericus* aff. *iheringii* (Characiformes, Characidae) in the upper Paraná river basin

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ABSTRACT. There are evidences that *Bryconamericus* aff. *iheringii* represents a species complex. DNA molecular markers have been effective in studies on phylogeny, taxonomy, and identification of cryptic species. In this study, partial sequences of genes of *ATPase 6* and *8* were used to assess genetic diversity within and among populations of *B.* aff. *iheringii* of sub-basins of Tibagi, Pirapó and Ivaí rivers, belonging to the Upper Paraná river basin. The analysis of the sequences of genes pointed out high genetic diversity in *B.* aff. *iheringii* from the sub-basins studied with genetic distance values comparable to those found among different species. There was a division of the individuals into five groups. The comparison with other species of *Bryconamericus* that have sequences available in GenBank confirmed that the individuals studied have relevant values of genetic distance, found among different species. Nevertheless, with the available data it is not possible to refute the hypothesis that the populations correspond to a group resulting from hybridization or that there might have been introgression of mitochondrial DNA among different species.

Keywords: characiformes, *Bryconamericus*, mtDNA, genes of *ATPase* 6 and 8.

Variantes moleculares em populações de *Bryconamericus* aff. *iheringii* (Characiformes, Characidae) da bacia do alto rio Paraná

RESUMO. Há indícios de que *Bryconamericus* aff. *iheringii* represente um complexo de espécies. Os marcadores moleculares de DNA têm sido eficazes em estudos de filogenia, taxonomia e identificação de espécies crípticas. Neste estudo, as seqüências parciais de genes da *ATPase* 6 e 8 foram utilizados para avaliar a diversidade genética dentro e entre populações de *B.* aff. *iheringii* das sub-bacias dos rios Tibagi, Pirapó e Ivaí, pertencentes a bacia do Alto Rio Paraná. As análises das seqüências dos genes apresentaram alta diversidade genética em *B.* aff. *iheringii* das sub-bacias estudadas, com valores de distâncias genéticas semelhantes às encontradas entre espécies diferentes. Houve uma divisão dos indivíduos em cinco grupos. A comparação com outras espécies de *Bryconamericus* que têm seqüências disponíveis no GenBank confirmou que os indivíduos estudados possuem valores relevantes de distância genética encontrados entre espécies diferentes. No entanto, com os dados disponíveis não é possível descartar a hipótese de que as populações correspondem a um grupo resultante de hibridação, nem que houve introgressão de DNA mitocondrial entre espécies diferentes.

Palavras-chave: characiformes, *Bryconamericus*, mtDNA, genes da *ATPase* 6 e 8.

Introduction

One of the major groups of freshwater fish worldwide is formed by representatives of the Characiformes order (NELSON, 2006). Genetic and cytogenetic studies have confirmed the hypothesis shared by systematists that it is a non-monophyletic group (SAITOH et al., 2003). Many studies have indicated problems of classification from specific (CAPISTANO et al., 2008; PAINTNER-MARQUES et al., 2003; PRIOLI et al., 2004) to superorder levels (SAITOH et al., 2003). The family Characidae, included into Characiformes, has been divided into subfamilies by different authors, due to the large number of species and the similarities among groups of genera. Thus, the species with poorly known evolutionary relationships were considered Incertae sedis, including *Bryconamericus* genus (LIMA et al., 2005). The taxon *B. iheringii* is described as having its type-locality in São Lourenço City (Rio Grande do Sul State - Brazil), thus the species that occur in the Paraná river is probably new to science, so that it would be more appropriate to call them as *B.* aff. *iheringii* (GRAÇA; PAVANELLI, 2007)

Cytogenetic studies have been undertaken with individuals of *B.* aff. *iheringii*. In specimens of Água da Floresta river, at the sub-basin of Tibagi river, individuals showed a diploid number (2n) of 52 chromosomes distributed as 8M+22SM+10ST+12A, with a fundamental number (FN) of 92 (PAINTNER-MARQUES et al., 2003). The same 2n was found in a population of Keller stream, at the sub-basin of Ivaí river (PORTELA-CASTRO; JULIO-JUNIOR, 2002). However, three different cytotypes were detected in this population: I, with 12M+18SM+8ST+14A; II, with 10M+22SM+8ST+12A; and III, with 8M+28SM+6ST+10A. Capistano et al. (2008) examined specimens of *B.* aff. *iheringii* of three streams belonging to the Upper Paraná river basin (Keller stream, Maringá stream and Tatupeba stream) have registered 2n = 52, but three different karyotypes have been observed. As cytotype I (population of Maringá stream), the karyotype is composed of 12M+18SM+8ST+14A with FN of 90; the II (population of Keller stream) had 8M+28SM+10ST+6A, FN equal to 94 ; the cytotype III (species of Tatupeba stream) with 8M+20SM+8ST+16A with FN of 88. Although the 2n= 52 chromosomes is a characteristic of *Bryconamericus* sp., as described previously, the karyotypes for different species in this genus vary, suggesting that chromosomal rearrangements may be involved in the karyotypic evolution of this group of fish (PAINTNER-MARQUES et al., 2003). Portela-Castro and Julio-Junior (2002) suggests that these changes may have been the result of pericentric inversions and that perhaps these cytotypes correspond to different species of *Bryconamericus*.

The genetic variations can be analyzed by molecular markers, such as the sequences of mitochondrial DNA (mtDNA) (PRIOLI et al., 2002). The mitochondrial DNA molecule is highly conserved; however, the mitochondrial genes *ATPase 6* and *8* have the characteristic of accumulating nucleotide substitutions that can detect genetic variations among species or even among populations of the same species (BERMINGHAM; MARTIN, 1998; MACHORDON; DOADRIO, 2001; PERDICES; DOADRIO, 2001; WONG et al., 2004). Therefore, the molecular analysis, comparing different populations of *B.* aff. *iheringii,* can provide important information in elucidating the condition of these taxonomic species.

Material and methods

Specimens of *B.* aff. *iheringii* were collected in three localities of Paraná river Basin (Table 1 and Figure 1) and were deposited in the fish collection of Molecular Genetics laboratory at the Center for Research in Limnology, Ichthyology and Aquaculture (NUPÉLIA) of the Maringá State University. Total genomic DNA was obtained from tissue samples according to Monesi et al. (1998), with modifications.

Table 1. Geographical coordinates of the collection points of specimens of the *Bryconamericus* aff. *iheringii* in the upper Paraná river basin.

Sampling Localities	Coordinates	mtDNA samples
1. Tibagi river, Pitangui Stream, Ponta Grossa city, Paraná State	25° 01' S – 50° 04' W	12
2. Pirapó river, Maringá Stream, Maringá city, Paraná State	23° 20' S – 51° 51' W	5
3. Ivaí river, Keller Stream, Marialva city, Paraná State	23° 38' S – 51° 51' W	6

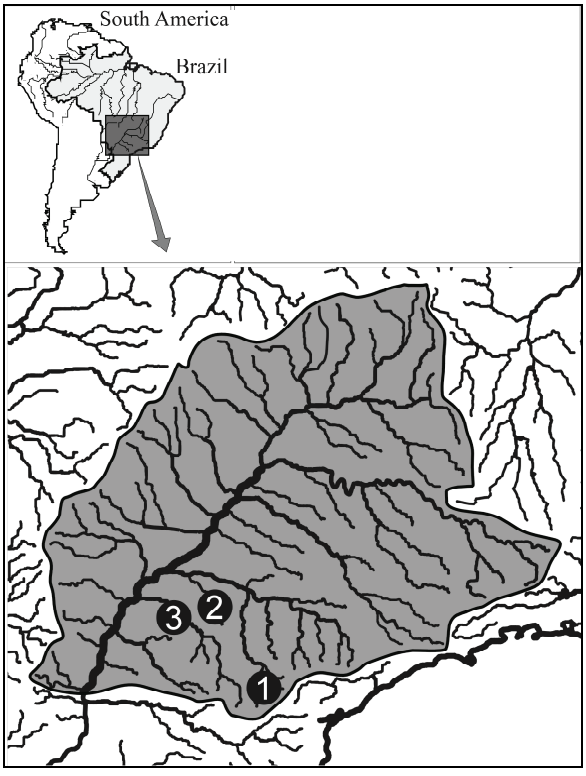

Figure 1. Collection areas of *Bryconamericus* aff. *iheringii* in the upper Paraná river basin. The numbers represent the collection points: 1 – Tibagi river, 2 – Pirapó river and 3 – Ivaí river.

A segment that corresponds to the complete sequence of the genes of *ATPase 6 and 8* and partial sequence of the genes $tRNA^{Lys}$ and *COIII* were amplified by PCR using primers H9236 (5´-GTTAGTGGTCAKGGGCTTGGRTC-3´) and L8331 (5´-AAAGCRTYRGCCTTTTAAGC-3´)

described by Lovette et al. (1998). Amplifications were carried out according to Prioli et al. (2002). The samples were purified after the amplification, according to Rosenthal et al. (1993).

The final product of PCR reaction was used in sequencing reactions of nucleotides in a MegaBace Automatic Sequencer (Amersham) following manufacturer's instructions.

The sequences amplified were aligned with the program Clustal W (THOMPSON et al., 1994) and edited in Bioedit (HALL, 1999). Procedures using the corrected Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC) of the program Modeltest 3.7 (POSADA et al., 1998) were determined by maximum likelihood. The differentiation among populations of different sub-basins was inferred from neighbor-joining and maximum likelihood trees, and Bayesian Inference using the model defined by the program Modeltest 3.7. The neighbor-joining tree, with 10,000 bootstrap samplings, was performed with the program MEGA 4.0 and the maximum likelihood tree, also using 10,000 bootstrap samplings, was obtained with the program PAUP4.0.b10b4 (SWOFFORD, 2002).

The Phylogeny of Bayesian inference, which calculated the posterior probability of genealogical relationships in a better model of development, was obtained by the Markov Chain Monte Carlo Simulation (MCMC) using the program MrBayes 3.0 (HUELSENBECK; RONQUIST, 2001). The tree was done with 300,000 generations, with the retention of 10 generations. The first 20,000 generations were discarded because the number was determined for the parameters converging to stability. Consequently, only 280,000 generations were used to calculate the *consensus* tree.

For the phylogenetic analysis, an individual of the species *B. scleroparius* was used as outgroup.

Scatter plots of the haplotypes were made in main coordination, with the program Statistica 6.0 (STATSOFT Inc., 2001).

GenBank sequences of other species of *Bryconamericus* (AF412573 - AF412627) were selected and analyzed to be compared to the sequences of *B.* aff. *iheringii* obtained in this study. In order to ensure greater reliability in the analysis, the sequences were aligned, and only those pairs of bases in the region of the genes of *ATPase 6* and *8*, were considered. The differentiation among populations of different species of *Bryconamericus* was inferred from neighbor-joining and maximum likelihood dendrograms, using the same model previously defined by the program Modeltest 3.7. The neighbor-joining clustering based on 10,000 bootstrap samplings was conducted with the program MEGA 4.0. The dendrogram of maximum likelihood was obtained with 500 bootstrap samplings of the program PAUP4.0.b10b4 (SWOFFORD, 2002).

Results

With the PCR amplification, it was possible to have a sequence of approximately 1,500 base-pairs (bp). However, a shorter sequence with 800 bp, covering the partial regions of the genes of *ATPase 6* and *8,* was selected for analysis due to its better quality sequencing after manual editing. The proportion of bases found in this sequence was A= 0.2910; C= 0.2857; G= 0.1074; T= 0.3159, with the transition/transversion rate ratios (Ti/Tv) of 4.099, the estimate of global genetic differentiation (Nst) equal to 6, with rate of invariant nucleotides (I) of 0.6693. Based on the characteristics found in the sequences studied, the evolutionary model that best applies to explain the genetic model was the Tamura Nei plus I (TrN+I) model. The alignment sequences analyzed showed nucleotide substitutions of 143 points, being 28 transversion and 125 transitions. There were a high number of substitutions associated with groups of individuals, indicating genetic similarity among them. In this way, individuals were classified into five groups. The neighbor-joining and maximum likelihood trees have confirmed the formation of five groups (Figure 2).

Table 2 lists the values of the average nucleotide diversity calculated with the TrN+I model among the five groups of *B.* aff. *iheringii* and the outgroup *B. scleroparius*. The values obtained were at least 0.04, between groups 1 and 3, and a maximum of 0.132, between groups 1 and 5. The scatter plot of haplotypes confirmed the formation of five distinct groups within the species (Figure 3).

The Bayesian Inference of Phylogeny tree has confirmed the groups trained in neighbor-joining and maximum likelihood trees (Figure 4). The high values of *a posteriori* probability indicate the trend of branches generated when it is assumed a large number of generations.

The neighbor-joining groups, with 10,000 bootstrap samplings, and maximum likelihood, with 500 bootstrap samplings, constructed with the TrN+I model among the five groups of *B.* aff. *iheringii* and the species *B. scopiferus*, *B. emperador*, *B. terrabensis*, *B. scleroparius*, *B. ricae* and *B. bayano* also supported the division of individuals of *B.* aff. *iheringii* into five groups (Figure 5). It was observed

that individuals of *B.* aff. *iheringii* analyzed in this study are genetically distant from the other species of *Bryconamericus,* whose sequences are available in GenBank.

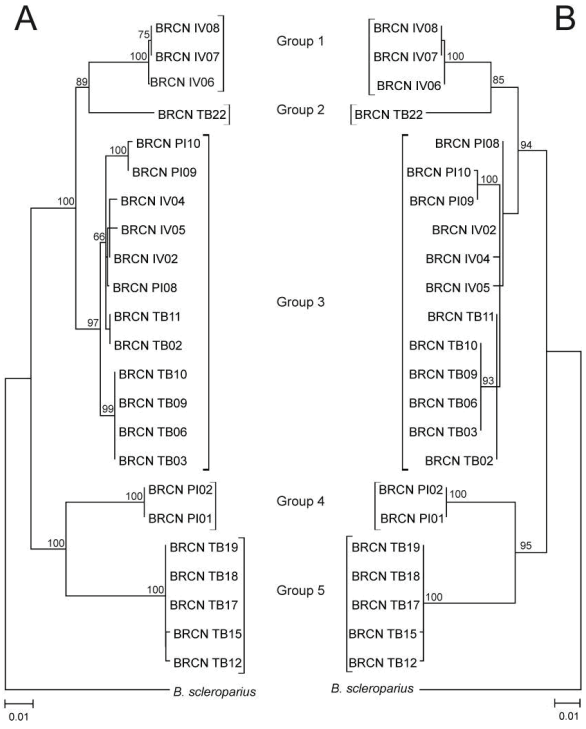

Figure 2. Neighbor-joining (A) and maximum likelihood trees (B) constructed with the TrN+I model, from partial sequences of the genes of ATPase 6 and 8 of individuals of B. aff. iheringii of the sub-basin of Tibagi river (BRCN TB), sub-basin of Pirapó river (BRCN PI) and sub-basin of Ivaí river (BRCN IV), located in the upper Paraná river basin. One individual of B. scleroparius was used in the analysis as an outgroup. The bootstrap analysis was based on 10,000 samplings, only values higher than 65 were represented.

Table 2. Average nucleotide diversity calculated with the TrN+I model among the five groups of *Bryconamericus.* aff. *iheringii* and the outgroup *Bryconamericus scleroparius*.

	Group 1	Group 2	Group 3	Group 4	Group 5
Group 1	0.001				
Group 2	0.045	NS*			
Group 3	0.040	0.043	0.008		
Group 4	0.124	0.120	0.110	0.000	
Group 5	0.132	0.128	0.119	0.064	0.001
B. scleroparius	0.199	0.212	0.185	0.205	0.201

NS* - non-significant.

Table 3 contains the values of average nucleotide diversity calculated with the TrN+I model among the five groups of *B.* aff. *iheringii* and the species of the genus *Bryconamericus* available in GenBank. The lowest values of genetic distances were observed among the five groups of *B.* aff. *iheringii* (0.04 to 0.13) and among the six species available in GenBank (0.03 to 0.093). The higher values of genetic distance were achieved when comparing the five groups of *B.* aff. *iheringii* with the species available in GenBank (0.18 to 0.217), evidencing the high differentiation of *B.* aff. *iheringii* in relation to the other species of *Bryconamericus.*

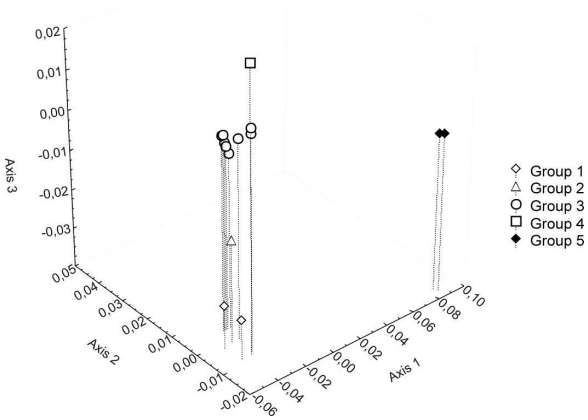

Figure 3. Scatter plot of haplotypes along the Principal Coordinates with three values of eigenvectors of the individuals of five groups of *Bryconamericus* aff. *iheringii* of Tibagi, Pirapó and Ivaí rivers.

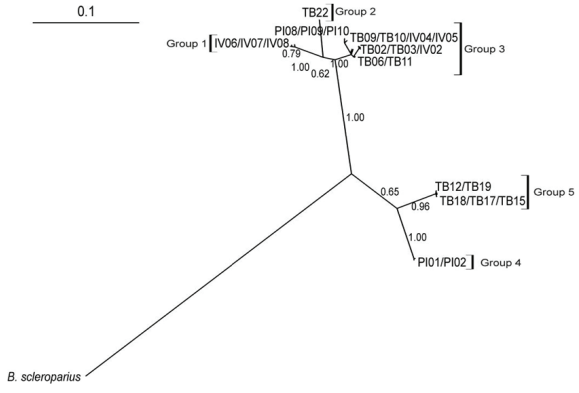

Figure 4. Tree of phylogeny of Bayesian inference calculated using the program Mr. Bayes 3.0 by the Markov Chain Monte Carlo Simulation (*MCMC*). The values in the branches are the percentage of posterior probability of the last 280,000 generations simulated using the model of nucleotide substitution TrN + I

Table 3. Average genetic distance between groups of individuals of *Bryconamericus* aff. *iheringii* of Tibagi, Pirapó and Ivaí rivers, and individuals of other species of the genus *Bryconamericus*, calculated with TrN + I from the 800 bp partial fragment of the genes ATPase 8 and 6.

	Gp 1	Gp 2	Gp 3	Gp 4	Gp 5	I	II	III	IV	V	VI
Group 1	*0.001*										
Group 2	0.046	NS*									
Group 3	0.041	0.044	*0.008*								
Group 4	0.123	0.119	0.109	*0.000*							
Group 5	0.130	0.126	0.116	0.062	*0.001*						
B. scopiferus (I)	0.207	0.217	0.200	0.216	0.209	*0.005*					
B. emperador (II)	0.202	0.213	0.194	0.214	0.208	0.054	*0.016*				
B. terrabensis (III)	0.193	0.202	0.183	0.201	0.201	0.082	0.086	*0.003*			
B. scleroparius (IV)	0.194	0.207	0.180	0.200	0.196	0.091	0.093	0.030	*0.000*		
B. ricae (V)	0.194	0.207	0.182	0.204	0.191	0.090	0.091	0.034	0.040	*0.007*	
B. bayano (VI)	0.205	0.217	0.191	0.215	0.211	0.091	0.091	0.032	0.042	0.042	*0.001*

NS* - non-significant.

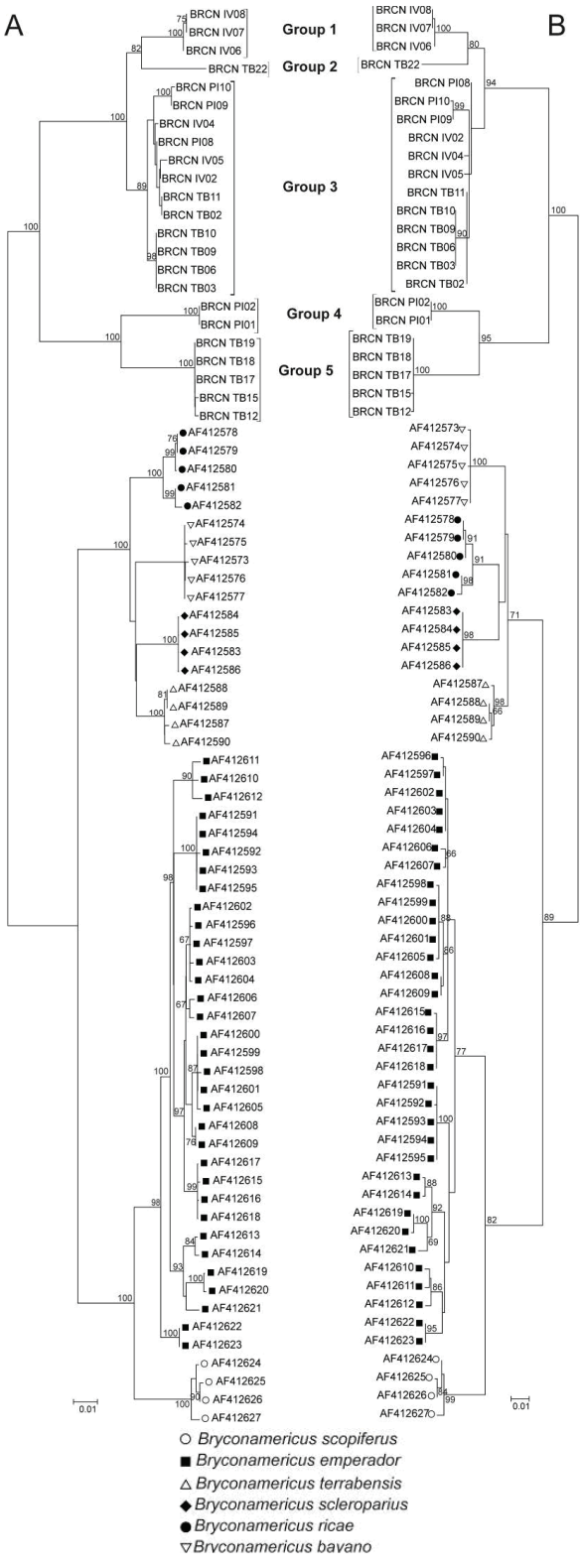

Figure 5. Neighbor-joining (A) and maximum likelihood (B) dendrograms constructed with the TRN + I model, based on partial nucleotide sequences of the genes for *ATPase 8* and *6* of *B.* aff. *iheringii*, *B. scopiferus*, *B. emperador*, *B. terrabensis*, *B. scleroparius*, *B. ricae* and *B. bayano*. Analyses were based on 500 bootstrap re-samplings, only values higher than 65 were represented.

Discussion

Several studies with different approaches have been performed using the genes of *ATPase 6* and *8* in fish. At lower taxonomic levels, with close phylogenetic relationships, such as populations of the same species and subspecies, the surveys have shown that the genetic distances, in absolute values, vary between 0.002 and 0.054 (FAULKS et al., 2008; KINZIGER et al., 2007; MACHORDOM; DOADRIO, 2001; PERDICES; DOADRIO, 2001; SIVASUNDAR et al., 2001). Among species of the same genus, the observed values are between 0.015 and 0.13 (FROUFE et al., 2005; MACHORDON; DOADRIO, 2001; PERDICES; DOADRIO, 2001; REID; WILSON, 2006; SIVASUNDAR et al., 2001). For higher taxonomic levels, the values of genetic diversity are also high, ranging from 0.062 to 0.16 (SIVASUNDAR et al., 2001; FROUFE et al., 2005).

In the analysis with the model of nucleotide substitution TrN+I, the distances obtained have showed five groups of *B.* aff. *iheringii*. The distances TrN+I between groups 1 and 3 (0.040) and groups 1 and 5 (0.132) are comparable with the distances TrN+I among *Bryconamericus* species with sequences available in GenBank, whose values are distributed from 0.030 to 0.093. In this distribution, the lowest values are compatible with intra-specific levels found in the literature. In contrast, the highest values are at levels found in congeneric species.

Considering that, in this study it was used only representatives of *B.* aff. *iheringii* from sub-basins located in the upper Paraná river, it was not expected high levels of molecular polymorphism. However, diversity could be expected at low levels in populations of *B.* aff. *iheringii* of the upper Paraná river, as found in previous studies, in some cytotypes, with the same diploid number, but with different karyotypic forms (PORTELA-CASTRO; JULIO-JUNIOR, 2002; CAPISTANO et al., 2008).

In cytogenetic studies, the individuals of Ivaí river had four different cytotypes, while in the molecular analysis, only two haplotypes. One of these haplotypes was also found in individuals of the Pirapó river, belonging to group 3, the only group that was distributed in all sub-basins studied. This fact can be justified due to the greater proximity between the collection points in the Ivaí and Pirapó rivers, with a greater possibility of connection between fish populations with a lower genetic diversity due to the increased gene flow.

Possibly this haplotype has arisen from populations in Tibagi river, which presents several

waterfalls that vary from 1.5 to 115 meters, along an altitude variation of 762 meters (FRANÇA, 2002). This condition favors the isolation of populations and the emergence of different haplotypes.

Until recently, there were no anthropogenic barriers that could have prevented the displacements source-mouth, thus it is plausible that regular migrants from group 3 have reached the basin of Paranapanema, Paraná and Ivaí rivers. Apparently, the haplotypes of groups 2 and 5 were restricted to Tibagi river basin (Figure 2). A probable explanation for the differences in the geographic distribution would be the greater aggressiveness and/or dispersal ability of the group 3. As a result of the wider geographic distribution, the average intra-group distance (d = 0.008) shows that the group 3 is more heterogeneous. Morphologically, it can be suggested that the populations of *B*. aff. *iheringii* belong to a single species. However, the neighbor-joining and maximum likelihood trees indicate the formation of two clades, because of the consistent values of genetic differences, which may indicate the presence of at least two ancestral species. The clade A with the groups 1, 2 and 3, and the clade B with the groups 4 and 5 (Figure 2). This configuration is confirmed by high bootstrap values in the trees. The scatter plots of haplotypes (Figure 3) corroborate the information provided by the group, and Bayesian inference (Figure 4) evidences a high value of a posteriori probability (1.00) for the branch between these two clades.

The distances found between clades A and B of *B*. aff. *iheringii* (0.11 to 0.132) (Table 2) are at levels equivalent to those found for different species of *Bryconamericus*, showing high genetic differentiation. Moreover, within each clade, the genetic distances among groups correspond to interspecific distances.

The genetic distances among groups and other species of *Bryconamericus* were higher, ranging from 0.18 to 0.217 (Table 3). Values are within a range of distances often found among species of the same family but of different genera. This result could be expected, since the sequences of *Bryconamericus* available in GenBank are of Central American species (REEVES; BERMINGHAM, 2006). Neighbor-joining and maximum likelihood groups (Figure 5) corroborate these results. Among the species in GenBank, the one that presents more genetic diversity is *B. emperador*, possibly because of the large number of individuals sampled. Nevertheless, the genetic distances among them are very low. Still, Reeves and Bermingham (2006) characterized *B. emperador* as the group "emperador". Thus, if low values are distant enough to reveal a species complex, then, the values determined in this study for the groups are pertinent to those found for different species. So, the analyses suggest that the levels of genetic differentiation of *B*. aff. *iheringii* of the upper Paraná river are consistent in indicating divergences compatible with a species complex, with up to five different species.

The nucleotide differences found among the haplotypes strongly indicate interspecific levels. However, with the data available it is not possible to discard the hypothesis that the populations may correspond to a group resulting from the hybridization of two or more species of *Bryconamericus*. Another possibility would be the introgression of mitochondrial DNA among different species. Regardless the explanation, it seems inevitable that there should be events of speciation. Nevertheless, for a greater understanding of the genetic overview of this group, further studies are required, using molecular markers more conserved than ATPase.

Conclusion

In this way, taking into account that *B.* aff. *iheringii* shows molecular evidences of formation of species complexes, it is likely that analysis of samples from other regions and other basins could reveal many other groups genetically differentiated at interspecific level. This analysis pointed out remarkable evidences of the diversity under the name *B*. aff. *iheringii*, but for now, only available in molecular analyses. Because of its magnitude, it is imperative that taxonomic studies, supported by molecular methods, promote the mapping of this diversity.

Acknowledgements

We would like to thank to COMCAP (Complexo de Centrais de Apoio à Pesquisa – UEM), for the sequencing of samples, to Dra. Carla Simone Pavanelli, for the taxonomic identification of fish, to Dra. Ana Maria Geahl for the collection of fish from the Tibagi river and, to the Post-Graduate Program in Comparative Biology of the State University of Maringá.

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Received on October 18, 2010.
Accepted on August 31, 2011.