Filogenia e Filogeografia gênero

Eurycheilichthys (Siluriformes: Loricariidae)

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Filogenia e Filogeografia gênero *Eurycheilichthys* (Siluriformes: Loricariidae)

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Sumário

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Resumo

A fauna de peixes de água doce da América do Sul é uma das mais ricas e, ao mesmo tempo, uma das menos conhecidas do mundo. O gênero *Eurycheilichthys* é endêmico do sul do Brasil, possuindo apenas duas espécies descritas na literatura. *Eurycheilichthys pantherinus* ocorre na Bacia do Uruguai, enquanto *E. limulus* ocorre somente na microbacia do alto Jacuí, na bacia do Guaíba. Atualmente, sete novas espécies estão sendo descritas com base na morfologia, as quais ocorrem na microbacia do Taquari-Antas, também na bacia do Guaíba. A área de ocorrência é restrita ao platô dos estados do Rio Grande do Sul e Santa Catarina, acima de 600 m de altitude. O presente trabalho estuda a filogenia e a filogeografia das espécies de *Eurycheilichthys* utilizando evidências moleculares. Foram seqüenciados os genes mitocondriais COI e ND2 de 126 indivíduos sendo os dados concatenados para as análises, totalizando 1208pb. A filogenia sugere estruturação entre as bacias hidrográficas, assim como para as microbacias. Das espécies novas, notadamente ficaram monofiléticas apenas aquelas três com distribuição restrita a pequenos rios, enquanto as espécies de ampla ocorrência ficaram não-monofiléticas. Quando estimado o tempo de divergência entre as espécies, utilizando duas taxas evolutivas (0,9% e 1,8% por milhão de anos), percebe-se que o processo de diferenciação das novas espécies foi recente (entre 0.5-1.0 Mya), sugerindo que a não-monofilia das espécies pode ser causada por compartilhamento de linhagens ancestrais, uma vez que é possível notar que existe uma expansão quase simultânea de todas espécies novas. A distribuição do gênero nessas bacias hidrográficas deve ter sido modulada por vicariância, com a formação da bacia do Uruguai, datada do Plioceno.

PHYLOGENY AND PHYLOGEOGRAPHY OF *Eurycheilichthys (*SILURIFORME: LORICARIIDAE)

Abstract

The Neotropical genus *Eurycheilichthys* is endemic to the southern Brazilian plateau, with two described species: *E. pantherinus,* which occurs in the upper Uruguay River basin, and *E. limulus,* that is found in the upper Jacuí River microbasin, which is part of the Guaíba Lake drainage. Seven new species are being described based on morphological characters and they are all endemic to the upper Taquari-Antas River microbasin, which is also part of Guaíba Lake drainage. We conducted the first molecular analysis concerning the phylogenetic relationships and phylogeography of the whole genus *Eurycheilichthys* using two mtDNA genes (COI and ND2). The phylogenetic trees and the network are structured by basin and microbasin and presented both described species as monophyletic. However, only three of the new species were monophyletic for these genes while some species showed several clades with restrict geographical distribution which could perhaps indicate other highly endemic cryptic species or the non-monophyly may be caused by incomplete lineage sorting. The origin of the Taquari-Antas clade was estimated around 0.5-1.0 million years ago while the divergence time among the species from this microbasin may have occurred around half a million years ago. Population analyses showed signals of a recent population growth for most of the species. The disjunct distribution among the river basins likely happened between the late Pliocene and the early Pleistocene, with the origin of the Uruguay basin. The high diversity and degree of endemicity of this group in the Taquari-Antas microbasin may be explained by its high declivity associated with the ecological restriction of the group.

KEYWORDS: molecular phylogeny, phylogeography, Loricariidae, *Eurycheilichthys*.

APRESENTAÇÃO

1. HISTÓRICO DO PROBLEMA

1.1 Introdução

A fauna de peixes de água doce da América do Sul é uma das mais ricas e, ao mesmo tempo, uma das menos conhecidas do mundo (Vari & Malabarba, 1998). Em 2003 foi publicado o livro *Check List of Freshwater Fishes of South and Central America* – CLOFFSCA, o qual listou as 4475 espécies e 1550 que estão por serem descritas, totalizando 6025 espécies, reunidas em 71 famílias (Reis et al., 2003). Esse levantamento permite perceber o quão desconhecida é a ictiofauna, podendo se inferir que no estado do Rio Grande do Sul existe uma grande quantidade de espécies a serem descritas.

O gênero *Eurycheilichthys* é endêmico do sul do Brasil, possuindo até o momento apenas duas espécies descritas na literatura (Reis & Schaefer, 1992; Reis & Schaefer, 1998). Porém, expedições realizadas no estado pela equipe do Laboratório de Ictiologia do Museu de Ciências e Tecnologia da PUCRS com o objetivo de enriquecer o conhecimento da ictiofauna do RS, apontam para a existência de ao menos sete novas espécies. A área de distribuição do gênero é bastante restrita, ocorrendo apenas no planalto do Rio Grande do Sul e parte de Santa Catarina, acima de 600 metros de altitude. Até o momento nenhum estudo enfocando a variabilidade genética e a história evolutiva do gênero foi realizada.

1.2. Sistemática do grupo

De acordo com Schaefer (1991) o gênero *Eurycheilichthys* encontra-se classificado conforme abaixo:

Classe: Actinopterygii
Ordem: Siluriformes
Família: Loricariidae
Sub-família: Hypoptopomatinae
Tribo: Otothyrini
Genêro: *Eurycheilichthys*

A classe Actinopterygii é composta pelos peixes de nadadeiras raiadas, e está dividida em 42 ordens. Os representantes da ordem Siluriformes ocorrem principalmente em ambientes de água doce, porém, existem duas famílias, Ariidae e Plotosidae, que possuem representantes em águas salinas. Os Siluriformes ocorrem em todos os continentes quando incluído os registros fósseis do Eoceno ou Oligoceno encontrados na Antártica. Algumas espécies são conhecidas por possuírem substâncias venenosas, produzidas em células epidérmicas do tecido que revestem os espinhos das nadadeiras. A maioria é passiva, utilizando o espinho para defesa contra predadores. A ordem Siluriformes é dividida em 34 famílias (Nelson, 1994).

Dentre estas, a família Loricariidae é a que contém o maior número de espécies de peixes na região Neotropical, e provavelmente no mundo. Atualmente é composta por 683 espécies, e a cada ano novas espécies são descritas. Esses peixes estão distribuídos desde o norte da Costa Rica até o sul da Argentina. A grande maioria das espécies encontra-se no lado leste da Cordilheira dos Andes, mas existem espécies que são restritas aos declives do lado oeste da mesma. Essa família apresenta-se dividida em seis subfamílias, sendo enfatizada a subfamília Hypoptopomatinae (Reis et al, 2006).

A subfamília Hypoptopomatinae é composta por 16 gêneros e 79 espécies. Estão distribuídos principalmente nas planícies cisandinas, desde a Venezuela até o norte da Argentina e ocorrem em pequenos e médios rios e córregos. Quando adultos são pequenos, variando entre 20mm e 75mm CP (comprimento padrão). Como outros membros dessa família, esses peixes são revestidos por placas ósseas. A boca é adaptada para sucção, sendo os lábios bastante carnudos. A alimentação é

predominantemente herbívora, e o hábito, diurno. A maioria é encontrada próxima à superfície da água, associada à vegetação das margens do rio (Reis & Schaefer, 2003). Entre as subfamílias de Loricariidae, a subfamília Hypoptopomatinae é a mais estudada e compreendida. As relações entre os gêneros foram quase totalmente resolvidas por Schaefer (1991), o qual posteriormente revisou alguns gêneros e dividiu essa subfamília em duas tribos monofiléticas: Hypoptopomatini e Otothyrini (Vari & Malabarba, 1998). Mais recentemente, no entanto, Lehmann (2006) não encontrou evidências para suporte da tribo Otothyrini.

1.4.1 O Gênero *Eurycheilichthys*

O gênero *Eurycheilichthys* (Fig. 1) é endêmico do estado do Rio Grande do Sul, possuindo somente duas espécies descritas na literatura: *E. pantherinus* (Reis & Schaefer, 1992) e *E. limulus* Reis & Schaefer, 1998. A área de ocorrência se restringe apenas ao planalto do estado, acima de 600 metros de altitude. A espécie *E. pantherinus* é encontrada apenas na bacia do Uruguai, enquanto *E. limulus* está presente na bacia do Guaíba (microbacia do alto rio Jacuí).

Expedições realizadas no estado pela equipe do Laboratório de Ictiologia do Museu de Ciências e Tecnologia da PUCRS, com o objetivo de enriquecer o conhecimento da ictiofauna do RS, possibilitaram verificar a existência de uma área mais ampla da distribuição do gênero *Eurycheilichthys,* tendo sido encontrado também na microbacia do Taquari-Antas, além das outras duas citadas acima (Fig. 2). A variação morfológica desses espécimes coletados na bacia do Taquari é muito grande, principalmente dos padrões da coloração. Esses indivíduos aparentemente são distintos das espécies já descritas, e possivelmente existem no mínimo sete novas espécies. Para todas as novas espécies, indivíduos foram coletados em diversos afluentes da bacia do Taquari, e em alguns locais foi possível verificar a ocorrência de mais de um táxon.

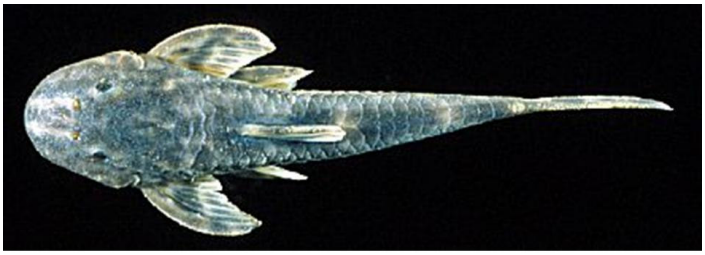

Figura 1: Espécime de *Eurycheilichthys*

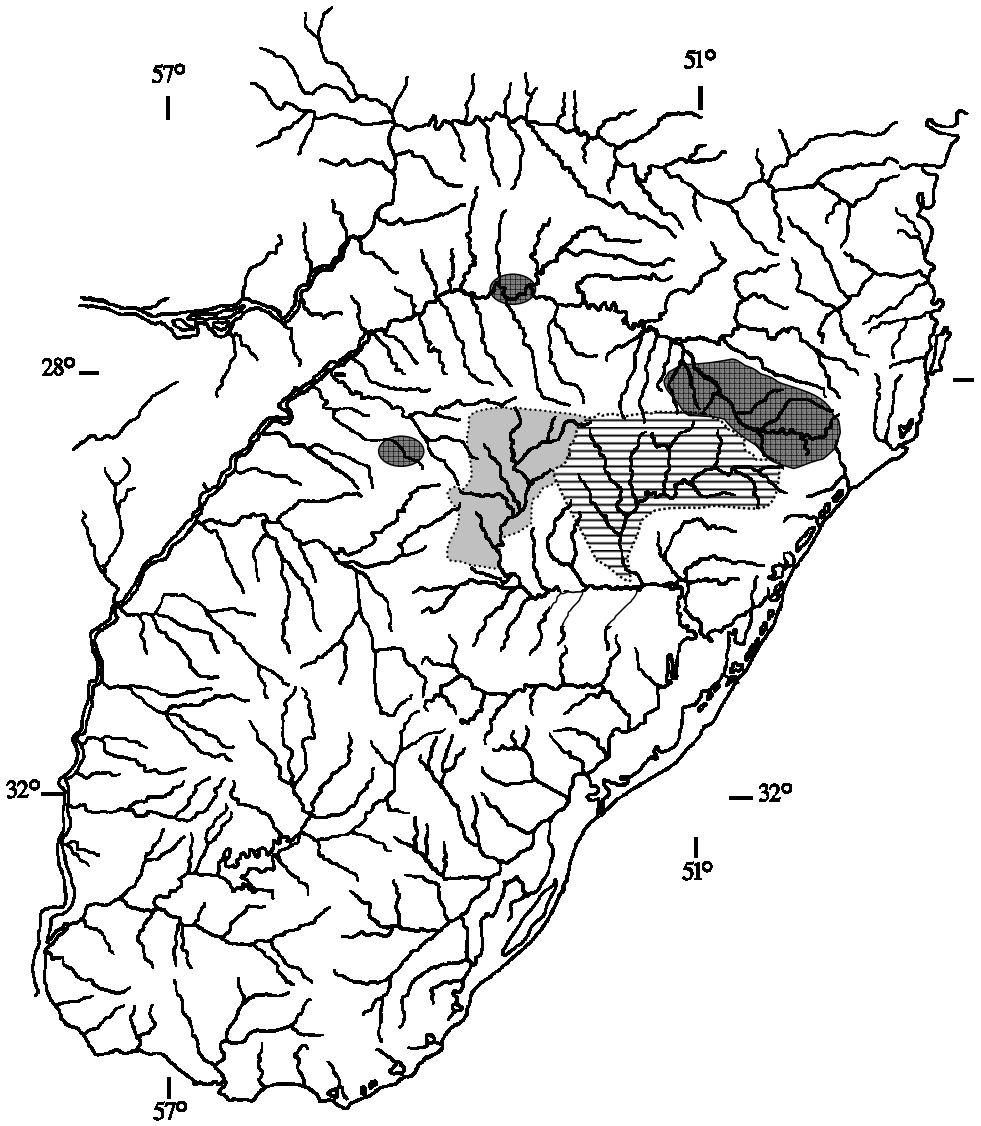

Figura 2: Área de distribuição de *Eurycheilichthys pantherinus* ■ ,

Eurycheilichthys limulus ▨ e espécies novas ▤

1.3 As Bacias Hidrográficas do Rio Grande do Sul

No Rio Grande do Sul é possível delimitar três bacias hidrográficas, reconhecidas pelas direções de escoamento dos rios. São elas: a bacia hidrográfica do Rio Uruguai, na parte norte e oeste, cujas águas desembocam no Rio da Prata; o sistema da Lagoa dos Patos, na região central, sul e leste, e a bacia do Rio Tramandaí, no litoral norte (Fontana et. al., 2003). É relevante detalhar as duas primeiras, a bacia do Rio Uruguai e o sistema da Lagoa dos Patos, pois é nessas regiões que se encontra a área de distribuição do gênero *Eurycheilichthys*.

A bacia do Rio Uruguai está subdividida em dez unidades, ou microbacias: Apuaê / Inhandava; Passo Fundo; Várzea; Turvo / Santa Rosa / Santo Cristo; Ijuí; Butuí / Piratinim / Icamaquã; Ibicuí; Quaraí; a bacia do Rio Santa Maria e a bacia do Rio Negro. A ocorrência do gênero *Eurycheilichthys* foi identificada em três microbacias: dos Rios Apuaê / Inhandava, Várzea e do Rio Ijuí. Provavelmente existe a ocorrência deste gênero nas demais microbacias, porém não há dados de coleta.

O sistema da Lagoa dos Patos é subdividido em bacias e o gênero *Eurycheilichthys* ocorre somente na Bacia do Guaíba. Essa bacia também foi subdividida, sendo formada por nove microbacias: Guaíba; Gravataí, Sinos, Caí e Baixo Jacuí; Alto Jacuí, Taquari-Antas, Pardo, Vacacaí / Vacacaí-Mirim. Duas são relevantes para o estudo, pois há registros de ocorrência de espécies do gênero *Eurycheilichthys*: a microbacia do Alto Jacuí, que corresponde ao segmento inicial do Rio Jacuí, e a microbacia do Taquari-Antas, que também desemboca no Rio Jacuí. Todas as bacias de interesse estão localizadas no Planalto Meridional.

A Bacia do Uruguai está contida na Bacia do Paraná, a qual distribui-se por quase toda porção meridional do Brasil, possuindo como leito as rochas formadas no derrame basáltico que originou o Planalto Meridional do Brasil. Essa formação basáltica provém de sucessivos eventos vulcânicos, entre 250 milhões de anos e 50 milhões de anos, tendo ocorrido o pico de atividade vulcânica em torno de 135 milhões de anos, no início do Cretáceo. Parte desse derrame basáltico está presente na costa da África, pois nessa época os continentes estavam unidos na Gondwana, cuja fragmentação começou cerca de 120 milhões de anos.

Após a separação dos continentes, houve a acomodação das placas tectônicas, que derivou no soerguimento da Serra Geral do Brasil. Esse soerguimento resultou na mudança na drenagem dos rios, escoando para o interior do continente até a confluência do Rio Uruguai com o Rio Negro, que deságuam no Rio da Prata (Potter, 1997)

1.4. Filogenia e Filogeografia

Dados moleculares são de grande importância para a inferência filogenética, devido à riqueza de informações. Esses dados são utilizados na sistemática como ferramentas para o acesso a questões evolutivas tradicionais. Diversos tipos de dados e de análises têm sido empregados, sendo que a abordagem mais direta é determinar a seqüência de nucleotídeos de um ou mais genes homólogos nas espécies em que se busca a filogenia.

Os dados de seqüências moleculares podem ser utilizados para estimar filogenias por diversas técnicas. Embora existam divergências sobre qual a melhor técnica a ser utilizada, alguns métodos como Máxima Verossimilhança, Máxima Parcimônia, Neighbor-Joining e Análise Bayesiana são constantemente aplicados e os resultados comparados (Holder & Lewis, 2003).

A filogeografia pode ser definida como estudo dos princípios e processos que governam a distribuição geográfica de linhagens genealógicas (Avise, 2000). As bases históricas desta ciência estão intimamente ligadas aos estudos empíricos com o DNA mitocondrial de animais, iniciados nos anos 70 e que têm sido usadas intensivamente para estudos filogenéticos, nos quais a distribuição dos grupos de haplótipos de mtDNA

através da área de uma espécie ou complexos de espécies é utilizado para inferir sobre a história das populações (Avise, 2000)

A filogeografia supre uma ponte empírica e conceitual entre a genética de populações e a biologia filogenética. E mesmo relações explicadas de forma estritamente ecológica, podem, através de uma interpretação filogeográfica, explicitar as bases filogenéticas para certas características da história natural de um grupo (ver Avise, 2000).

A utilização da variação entre seqüências do DNA mitocondrial tem sido a metodologia mais amplamente utilizada para inferir sobre os padrões filgeográficos das espécies (Avise, 2000). A evolução de algumas regiões mitocondriais é extremamente rápida comparada ao DNA nuclear (Nedbal & Flynn, 1998) o que habilita o mtDNA a ser utilizado como um marcador molecular para microevolução (Avise, 2000).

Análises de padrões filogeográficos intraespecíficos conduzem a observação de barreiras e estruturas geográficas (p.ex. Eizirik, 2001) e levam a um maior avanço em nosso conhecimento dos processos históricos biogeográficos.

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Artigo Científico

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Phylogeny and phylogeography of the endemic catfish genus *Eurycheilichthys* (Siluriformes: Loricariidae) from the southern Brazilian plateau

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Abstract

The Neotropical genus *Eurycheilichthys* is endemic to the southern Brazilian plateau, with two described species: *E. pantherinus*, which occurs in the upper Uruguay River basin, and *E. limulus*, that is found in the upper Jacuí River microbasin, which is part of the Guaíba Lake drainage. Seven new species are being described based on morphological characters and they are all endemic to the upper Taquari-Antas River microbasin, which is also part of Guaíba Lake drainage. We conducted the first molecular analysis concerning the phylogenetic relationships and phylogeography of the whole genus *Eurycheilichthys* using two mtDNA genes (COI and ND2). The phylogenetic trees and the network are structured by basin and microbasin and presented both already described species as monophyletic. However, only three out of the seven new species were monophyletic for these genes while some species showed several clades with restrict geographical distribution which could perhaps indicate other highly endemic cryptic species or the non-monophyly may be caused by incomplete lineage sorting. The origin of the Taquari-Antas clade was estimated around 0.5-1.0 million years ago while the divergence time among the species from this microbasin may have occurred around the half a million years ago. Population analyses showed signals of a recent population growth for most of the species of the genus. The disjunct distribution among the river basins likely happened between the late Pliocene and the early Pleistocene, with the origin of the Uruguay basin. The high diversity and degree of endemicity of this group in the Taquari-Antas microbasin may be explained by its high declivity associated with the ecological restriction of the group.

KEYWORDS: molecular phylogeny, phylogeography, Loricariidae, *Eurycheilichthys*.

1. Introduction

The Neotropical rivers of South and Central America host the most diverse freshwater fish fauna of the world, with almost 6,000 of the world's approximated 13,000 species (Reis et al., 2003). Within this huge diversity of species, the great majority belongs to the superorder Ostariophysi, in which Siluriformes, or catfishes, is the most diverse and widely distributed order (Nelson, 1994). The high diversity of habits and shape of the body within siluriformes is noteworthy, especially in the superfamily Loricarioidea, which contributes with more then two thirds of all catfish species found in the Neotropics.

The Neotropical *Eurycheilichthys* (Reis and Schaefer, 1992) include small-body species that belong to the family Loricariidae, subfamily Hypoptopomatinae and was included in the tribe Otothyrini by Schaefer (1991). *Eurycheilichthys* is endemic to southern Brazil, where it is restrictedly distributed throughout the plateaus of Rio Grande do Sul (RS) and Santa Catarina (SC) states (above 600 meters of altitude, Fig. 1). Currently, the genus has only two described species: *E. pantherinus* (Reis and Schaefer, 1992) and *E. limulus* Reis and Schaefer, 1998. *Eurycheilichthys pantherinus* is present only in the Uruguay River basin, which drains into the La Plata River; on the other hand *E. limulus* is found only in the upper Jacuí River microbasin, which is part of the Guaíba Lake drainage (Fig. 1). However, recent extensive collection in these areas have found at least seven additional new species for the genus (RER unpublished results) based on morphological characters. The new species also belong to Guaíba Lake drainage, but differently from *E. limulus* they are all endemic to the Taquari-Antas microbasin (Fig. 1). This microbasin has a high average declivity with little riparian forest left. It is geographically divided by altitude and declivity in three regions: The first is in the highest portions of the microbasin (at almost 1,200m above sea level), where the main river channel drains from east to west, and is deeply escavated with a declivity of 4.8m/km. In the second region the river has a moderated declivity with only 1.6 m/km and a direction from northeast to southeast. In the last portion the river flows out from the plateaus to a central depression in Rio Grande do Sul state, changing its feature to a plain river (Fig. 1). Fishes of the genus *Eurycheilichthys* inhabit mostly head streams with no more than 50 centimeters deep, in the first and second regions of the microbasin. They stay under rocks, protecting themselves from the watercourse, feeding the vegetation incrusted in the rock surfaces (Reis and Schaefer, 1992).

These new species are being described mainly based upon differences in morphology and color patterns, and there are two principal distributional patterns among them: some species are spread across several relatively distant streams (*E. sp. nov. 1*, *E. sp. nov. 2*, *E. sp. nov. 5*; Fig. 1) while others are limited to only one or a few near streams (*E. sp. nov. 3*, *E. sp. nov. 4*, *E. sp. nov. 6*, *E. sp. nov. 7*; Fig. 1). No genetic diversity study has been done to date in the genus or in any other member of the subfamily Hypoptopomatinae.

Fishes that present an endemic and speciously pattern have been helping us to better understand questions about the evolutionary process of speciation, from the classical works of D. E. Rosen with the fish fauna of the karst region in Guatemala (reviewed in Rosen, 1979) and A. Meyer (Meyer et al., 1990) with cichlids from the African lakes, to others works regarding similar patterns around the world (Robalo et al., 2006; Cunha et al., 2004; Alves et al., 1997) and the new approaches to these same classical problems (Won et al., 2006). These studies frequently presented complex results, sometimes contrasting species delimitation based on morphology with relationships based on gene trees (Funk et al., 2003; Pollard et al., 2006). This conflict is know to be caused by many distinct speciation scenarios as described by different phylogeographic patterns (e.g. Avise 2000).

In this present phylogeographic study of *Eurycheilichthys* we tried to better understand the evolutionary processes that gave rise to a group with a so unusual endemic diversity. We also explored the demography and biogeography of this group in a restricted area of the southern Brazilian plateau.

2. Material and Methods

2.1 Sampling procedures

A total of 126 specimens of *Eurycheilichthys* were used in this study, of which 123 specimens were collected by us in 22 sampling points, and three samples were supplied by FZB/RS (MCN in Table 1). We collected, when possible, around ten specimens in each locality (Fig. 1). They were preserved in alcohol 90%, deposited in MCP fish collection, and recorded under the catalog numbers as shown in Table 1. As outgroups, we used samples of two related genera (same tribe according Schaefer, 1991): *Epactionotus itaimbezinho* and *Hisonotus* sp., supplied by MCP.

2.2 DNA amplification and sequencing

Muscular tissue was isolated and total DNA was prepared according to the protocol of Ammonia Acetate (Sambrook et al., 1989). Fragments of NADH subunit 2 (ND2) and cytochromo oxidase I (COI) mitochondrial DNA genes were amplified using the polymerase chain reaction (PCR) and sequenced using the following primers pairs: L-5216 and H-6313 (Sorenson et al, 1999) and L-1490 and H-2198 (Herbert et al., 2003), respectively.

All PCR products were analyzed in 1% agarose gel, purified by standard Polietilenoglicol (PEG) 8000 precipitation and sequenced using the DYEnamic ET Dye Terminator Cycle Sequencing Kit (GE Healthcare) in a MegaBACE 1000 automated sequencer (GE Healthcare) following the manufacturer's protocols. The primers used for sequencing were the same as those used in the PCR for COI, but an internal primer was constructed to sequence the final portion of ND2 segment (FISH_ND2SEQ 5'-GCA CCA ATA CAC TTC TGA AT-3´). Chromatograms were visually checked with the CHROMAS 2.0 software (Technelysium) and sequences were aligned manually using BIOEDIT 6.0.7 (Hall, 1999).

2.3 Phylogenetic Analyses

Phylogenetic analyses were performed using the criteria of maximum likelihood (ML), maximum parsimony (MP), neighbor-joining (NJ), and Bayesian phylogenetic inference (BI). When appropriate, the model of nucleotide substitution was used as estimated by Modeltest 3.07 (Posada and Crandall, 1998) using the minimum theoretical information criterion (AIC).

Maximum likelihood, MP and NJ trees were estimated using PAUP* 4.0b10 (Swofford, 2003). We inferred ML trees with heuristic searches with the tree-bisection-reconnection (TBR) option and a NJ starting tree; statistical confidence was estimated with 100 replications of bootstrap using the same search strategy. MP was performed with heuristic searches (TBR) with starting tree produced by 100 replication of random stepwise addition. To assess the MP statistical confidence, 1000 bootstrap replicates were conducted using simple stepwise addition. The NJ analysis used the ML distance under the evolutionary model selected by Modeltest and to access the statistical support 1000 bootstrap replicates were conducted.

The BI was performed using MrBayes 3.1 (Huelsenbeck and Ronquist, 2001) with 2,000.000 generations for the Markov chain Monte Carlo (MCMC) algorithm using flat priors. Tracer 1.3 (http://evolve.zoo.ox.ac.uk/software/tracer/) was used to determine the burn-in and a consensus tree was estimated from the remaining trees using PAUP*. We consider bootstrap support (BS) higher than 70% and Bayesian posterior probability (PP) higher than 95% as a significant support for a clade (Felsenstein, 1985; Hillis and Bull, 1993; Huelsenbeck and Ronquist, 2001; Wilcox et al., 2002).

The Kishino-Hasegawa test (Kishino and Hasegawa, 1993), calculated using PAUP*, was used to test hypothesis of monophyly of the species as well as the hypothesis of an alopatric pattern concerning the genetic structure in the basins (i.e., the monophyly of the basins). A median-joining network (Bandelt et al., 1999) was constructed using the program Network 4.2 (www.fluxusengineering.com) to better investigate the relationship among the closely related haplotypes from the Guaíba Basin.

To estimate a time frame for the results, we first tested the molecular clock assumption comparing the likelihood of the best ML tree and the clock enforced tree using Shimodaira-Hasegawa test (Shimodaira and Hasegawa, 2001) performed in PAUP*. As there is neither a suitable calibration point for this group nor any substitution rate for the mtDNA of any close related taxa, we used two extreme substitution rates found in literature for fishes: 0.9%/My for COI described for goodeines by Webb et al. (2004) and 1.8%/My for *Centropomus* proposed by Kocher and Carleton (1997), based in the cichlid's ND2 gene.

2.4 Phylogeographic Analyses

We used the Mantel test to try to distinguish between the influence of the geographic distance and the species delimitation in the genetic diversity structure of the new species from the Guaíba Lake basin. For this we used Arlequin 3.01 (Schneider et al., 2000) to calculate the correlation between genetic distances matrix and two other matrices: geographical distances along the rivers and a discrete matrix in which one or zero were set to represent different or equal species, respectively. We also carried out an analysis of molecular variance (AMOVA Excoffier et al., 1992) with these two approaches using Arlequin 3.01. We set the populations using two approaches: first,

each species was assumed to be a population; and second, all specimens collected in the same or in close streams were considered a population.

Genetic summary statistics as nucleotide and haplotype diversity, Tajima's D (Tajima, 1983) and Fu's Fs (Fu, 1997) neutrality tests were estimated for each species. We also computed a mismatch distribution analysis for all species and for each microbasin to find out signals of population expansion. The summary statistics and mismatch distribution were performed in Arlequin 3.01. To estimate the dynamics of the population size along the time, we used the Bayesian Skyline Plot method implemented in the program BEAST 1.4 (Drummond and Rambaut, 2003). This Bayesian approach incorporates the uncertainty in the genealogy by using MCMC integration under a coalescent model, where the timing of divergence dates provides information about effective population sizes through time. It was used the evolutionary model suggested by Modeltest, a length chain of 10,000,000 and the substitution rates described above.

3. Results

3.1. Sequence analyses and alignments

Our alignments result in two matrices of 601bp and 607bp for the ND2 and COI fragments, respectively. Both genes concatenated produced a matrix of 1208bp of which 204 sites were variable and 119 sites were parsimony informative. We could not amplify the COI gene from the three specimens of *Eurycheilichthys pantherinus*, so we coded these sequences as missing data for COI in the concatenated matrix. This matrix resulted in 59 haplotypes with no shared haploype between species. Although it was not possible to obtain homogeneous sample sizes across the species, as some species are very rare, we compared summary statistics among those with more than 10 individuals. The nucleotide diversity ranged for 0.01% to 1.14% and the haplotype diversity ranged for 0.66 to 0.95 among the species (Table 2), with no correlation with the sample size. It is remarkable the high diversity found in *E.* sp. nov. 1 and *E.* sp. nov. 5, the latter presenting nucleotide diversity higher than the whole Taquari-Antas Microbasin. The sequences obtained in this study were deposited in the GenBank (sequences not yet submitted).

3.2. Phylogenetic analyses

For the whole data set the evolutionary model selected by Modeltest was GTR+I+G, with a proportion of invariable sites of 0.6723 and an alpha parameter of 3.0062. All phylogenetic approaches produced a similar pattern (Fig. 2 A): the topology presented two major monophyletic clades, one representing the Uruguay River basin where only *Eurycheilichthys pantherinus* occurs and the other is formed by all species of the Guaíba Lake basin, this latter clade presenting two subclades structured by microbasin: one consisting of *E. limulus* from the upper Jacuí microbasin and the other the seven new species from the Taquari-Antas microbasin. The Taquari-Antas microbasin monophyly occurred in the MP, NJ and BI trees but with low support, whereas in the ML trees two haplotypes from *E.* sp. nov. 5 (sampling point 18, Fig. 1) grouped with haplotypes of *E. limulus* from the upper Jacuí microbasin (not showed). However, the values of BS and PP support for this latter clade were very low and in the Shimodaira-Hasegawa test comparing this best ML tree with a ML tree estimated with a constrain where all haplotypes from the Taquari-Antas microbasin stayed monophyletic, showed they are non-significantly different. Besides, the median-joining network also supports the monophyly of the species from the Taquari-Antas microbasin (not shown).

The phylogenetic analyses highly support the monophyly of the two species described by Reis and Schaefer (1992, 1998), *Eurycheilichthys pantherinus* and *E. limulus*. On the other hand, only three of the new species of the Taquari-Antas microbasin are monophyletic by the mitochondrial lineages (*E.* sp. nov. 3, *E.* sp. nov. 4, and *E.* sp. nov. 7). To test the support for this result, we compared the best ML tree against the best tree were all species were constrained to be monophyletic using the Kishino-Hasegawa test. The monophyletic constrained trees found were significantly worst than the ML tree. The relationship among the more closely related haplotypes from the Guaíba Lake basin estimated using the median-joining network (Fig. 3) is similar to the phylogenetic trees, supporting the non-monophyletic tree and showing seven mutational steps between *E. limulus* and the other species of Taquari-Antas microbasin, also in agreement with the phylogenetic tree (Fig.2).

Within the Taquari-Antas microbasin species we could distinguish two common and two idiosyncratic phylogeographic patterns. First, there are the monophyletic species with a very restrict area of distribution (*E.* sp. nov. 3, *E.* sp. nov. 4 and *E.* sp. nov. 7); second there are non-monophyletic, geographically widespread species with non-monophyletic sample points (*E.* sp. nov. 1 and *E.* sp. nov. 2). In this latter pattern there is one haplotype of *E.* sp. nov. 1 shared by samples from two different and apart

locations: the sampling point 3 and 6 (Fig. 1). For all other species that have the same haplotype in individuals from different locations they are, in fact, in the same watercourse. There are also *E.* sp. nov. 6, that is also highly endemic but non-monophyletic and *E.* sp. nov. 5, that is geographically widespread and non-monophyletic but with the individuals from the same collecting point being monophyletic.

As the Shimodaira-Hasegawa test did not reject the molecular clock for the data, we used the slow (0.9%) and the fast (1.8%) rates of substitution to estimate divergence times along the tree (Fig. 4). The clade comprising *E. pantherinus* from the Uruguay River basin diverged from the clade found in the Guaíba Lake basin about 1.2 to 2.4 Mya (million years ago), while the divergence time between *E. limulus* from the upper Jacuí microbasin and the clade of species from the Taquari-Antas microbasin was estimated around 0.5 to 1.0 Mya. All internal clades of Taquari-Antas microbasin showed a recent radiation around 167,000 – 80,000 years ago. This radiation is visible in Fig.2 B, where all specimens are represented in a NJ tree.

3.3 Phylogeographic analyses

As the phylogenies showed that some species are separated in different clades which represent haplotypes found in the same river while others represent the whole species, we tried a simple test to see which variable (geographic distance along the rivers or the species) present a better correlation with the genetic distance among the haplotypes. The Mantel tests resulted in a correlation of 54% between genetic distance and geographical distance matrices but of only 7% between the genetic distance and the discrete matrix of species similarity (all statistics were significant at $P = 0.01$). However, in the analysis of molecular variance (Table 3) both approaches for grouping the haplotypes, by the morphospecies or by the river where they were found, resulted in similar partitions of the variation.

The neutrality tests and the mismatch distributions were estimated only for the species with sample size larger than 10 (Table 2). Fu's F_s, known to be more sensitive to demographic changes, presented significantly negative values for all monophyletic species calculated and for the whole clade from the Taquari-Antas Microbasin. As expected, Tajima's D being more conservative, was significantly negative for *E.* sp. nov. 3 only. Furthermore, the two non-monophyletic species tested (*E.* sp. nov. 1 and *E.*

sp. nov. 5) showed non-significant values. The suggestions for recent population expansion indicated by the F_s test were corroborated by the single wave mismatch distributions (not shown), while the other species presented a clear ragged distribution (as expected in cases of the non-monophyletic species). Moreover, the distribution estimated using the Taquari-Antas microbasin clade also resulted in a single wave pattern that supports a bottleneck followed by a population expansion (Fig. 4) around 0.46 and 0.92 Mya (using the slow and fast rates on a tau of 10.1, respectively).

The demographic history of the whole Taquari-Antas microbasin species could be estimated with more details using a Bayesian skyline plot (Fig. 5). Using both rates (0.9% and 1.8%), the results suggest that this group was relatively stable since its origin around one Mya but had suffered a population expansion about 70,000 – 130,000 years ago preceded by a moderate bottleneck.

4. Discussion

Three major mitochondrial clades were found in *Eurycheilichthys* and these are hierarchically structured in the drainages by basin and microbasin. Two of these clades coincide with the two known species of the genus (*E. pantherinus* and *E. limulus*) while the third clade is endemic to the Taquari-Antas microbasin but is now considered to comprise seven undescribed species. While three of these seven new species are monophyletic for the mitochondrial lineages, the other four are non-monophyletic. One obvious question is the significance of these phylogenetic results to the reality of these four species both as evolutionary units and as valid taxa.

First, although some of these species are quite similar, all of them could be morphologically diagnosed by the standard taxonomic methods and parameters used for the group (RER unpublished results). Moreover, it is now widely known that the relationship between closely related species and mitochondrial phylogenies (or actually any single gene tree) is complex. The non-monophyly in single gene trees of undisputed valid species have been observed in a wide number of phylogeographical studies (e.g., Fry and Zink, 1998 Andolfatto et al., 2003; Gifford et al., 2004, Grazziotin et al., 2006; Chang, 2007). Besides, there are rich theoretical demonstrations of speciation scenarios that could result in true species that are non-monophyletic in single gene trees, such as recent peripatric speciation that display paraphyletic gene tree patterns or sharing of ancestral lineages caused by incomplete lineage sorting after recent allopatric divergence in large ancestral populations (reviewed in Avise, 2000). This latter seems a

likely scenario to the Taquari-Antas microbasin endemic *Eurycheilichthys* species, where most internal clades arise in a narrow and recent (<1 Mya) time frame (Fig. 4) resembling an adaptive radiation scenario. This scenario could explain the phylogeographic patterns we described for this group, such as the monophyletic microendemic species (*E.* sp. nov. 3, *E.* sp. nov. 4, and *E.* sp. nov. 7) or the non-monophyletic, geographically widespread species with clades with haplotypes from different collecting points (*E.* sp. nov. 1 and *E.* sp. nov. 2). One additional hypothesis is that some of the clades that are geographically restricted (clades A-G, Fig. 2) but are found inside some widespread species, may perhaps represent cryptic species, what would increase the already high microendemicity of this genus in this microbasin.

A radical alternative hypothesis is that the whole Taquari-Antas microbasin clade is actually a single species with at least some of the undescribed morphospecies and maternal lineages representing subspecific evolutionary units. However, as species definitions are related to species concepts, and this is an unresolved and somewhat subjective issue, we would not like to enter this debate. The phylogeographic results presented here shed some light in the evolutionary history of the genus irrespective the taxonomic status assigned to these evolutionary units.

Our results suggest that the divergence between the clades from the Uruguay River and Guaíba Lake basins occurred between the late Pliocene and early Pleistocene (1.2-2.4 Mya). However, as the geological and hydrological history of this particular area is poorly known, it is difficult to know accurately the time and mode of the evolution of this drainage system. Nevertheless, there are some evidences that the Uruguay drainage basin was formed in the Pliocene (Maack, 1968; Bossi, 1969; Souza *et al*., 2005). Therefore, using the upper and lower limits of the molecular and geological estimates, respectively, it could not be rejected the hypothesis that a vicariant event between basins was the process that shaped this first divergence within the genus. However, we think it is more likely that these two drainages were established before our estimated molecular divergence times suggesting an alternative scenario to simple basin vicariance (see below). Interestingly, there are several other sister-taxa pair of fish species between the Uruguay and the Jacuí basins, e.g. *Gymnogeophagus* (Reis and Malabarba, 1988; Wimberger et al., 1998), *Parapimelodus* (Lucena et al., 1992), *Cnesterodon* (Lucinda, 2005), and *Hypostomus* (Reis et al, 1990).

The divergence between the clades from the two Guaíba microbasins (upper Jacuí and Taquari-Antas) was estimated to have occurred around the middle Pleistocene

(1.0-0.5 Mya) and the initial divergence of several species from the Taquari-Antas microbasin started soon after that. Unfortunately, the absence of information about the formation of the microbasins of the Guaíba basin precludes us, at this time, to draw specific correlations of our evolutionary scenario with the geological history. However, it is not likely that these microbasins, especially the Taquari-Antas, being constituted by valleys deeply excavated in a huge volcanic plateau, were formed later than the Pliocene (< 1.8 Mya).

Considering that there are no strong geographic barriers between these basins and microbasins, that they are geographically very close at their headwaters, and that several species are known to occur at these very close headstreams, another, perhaps more likely scenario to explain the above results (at least the divergence between two Guaiba microbasins clades) is past headwater capture between the basins and the microbasins (Beurlen, 1970). Considering the monophyly of the three drainage systems studied here under the above headwater capture scenario, only two headwater capture events are necessary to parsimoniously explain the results. However, it was not possible to establish the polarities of the unidirectional gene flow that these putative river captures imply.

Interestingly, no haplotype was found to be shared between the seven species of the Taquari-Antas microbasin, suggesting the absence of very recent gene flow across the species barriers. On the other hand, clade E is very singular, as it comprises haplotypes from four different species and it is widely distributed. This last pattern may be explained by an ancestral widespread population or by some gene flow in a relatively recent past. This last process may also explain the presence of haplotype 16 in two relatively distant collecting sites (sites 3 and 6, Figs. 1 and 2).

We detected, using the Bayesian skyline plot, a signal of a moderate population bottleneck followed by a significant populations size increase in the whole Taquari-Antas microbasin clade around 70-130 thousand years ago, that were also detected separately in some of the species and clades. This demographic fluctuation may be related to the climatic changes that occurred in the late Pleistocene around this time (Ledruet al. 1996; Behling and Lichte 1997), in special the more severe effect of the dry period estimated to have occurred in the southern part of the Atlantic Forest. Interestingly, a similar demographic fluctuation was estimated for the jararaca snake (*Bothrops jararaca*) in this region (Grazziotin et al., 2006).

What could explain the unusually high degree of microendemicity in the Taquari-Antas microbasin in contrast with the single species found in the other drainage systems? One possible explanation is the natural features of the Taquari-Antas microbasin which is distinct from those found in the other drainage systems included in this study. In the Taquari-Antas microbasin the rivers present a high declivity and the shallow headwaters where most species inhabit appear to be isolated from each other by more slow-flowing, deeper sections of river. On the hand, the upper Uruguay basin and the upper Jacuí microbasin are located mostly on the top of the plateaus, without clear geographical barriers.

One final comment concerns a conservation issue related to the Taquari-Antas microbasin, where three hydroelectric dams are being implanted (http://www.ceran.com.br), and several others are planned. It is not clear how these dams would affect those geographical barriers and consequentially the persistence of these evolutionary units.

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Table 1: *Eurycheilichthys* species, number of sampling point, haplotype, name of drainage, coordinate points and voucher catalog number.

Species	Sampling point	Haplotype	Drainage	Latitude, Longitude	Voucher
E. pantherinus	22	Haplo 1	Uruguay Basin	28º38'42"S, 50º17'01"W	MCP 35042 A
	22	Haplo 1	Uruguay Basin	28º38'42"S, 50º17'01"W	MCP 35042 B
	22	Haplo 2	Uruguay Basin	28º38'42"S, 50º17'01"W	MCP 35042 C
	22	Haplo 3	Uruguay Basin	28º38'42"S, 50º17'01"W	MCP 35042 D
	22	Haplo 1	Uruguay Basin	28º38'42"S, 50º17'01"W	MCP 35042 E
E. limulus	12	Haplo 4	Alto-Jacuí Microbasin	28º22'14"S, 52º30'37"W	MCP 35120 A
	12	Haplo 5	Alto-Jacuí Microbasin	28º22'14"S, 52º30'37"W	MCP 35120 C
	12	Haplo 6	Alto-Jacuí Microbasin	28º22'14"S, 52º30'37"W	MCP 35120 D
	12	Haplo 8	Alto-Jacuí Microbasin	28º22'14"S, 52º30'37"W	MCP 35120 E
	12	Haplo 7	Alto-Jacuí Microbasin	28º22'14"S, 52º30'37"W	MCP 35120 F
	12	Haplo 8	Alto-Jacuí Microbasin	28º22'14"S, 52º30'37"W	MCP 35120 G
	12	Haplo 6	Alto-Jacuí Microbasin	28º22'14"S, 52º30'37"W	MCP 35120 H
	12	Haplo 9	Alto-Jacuí Microbasin	28º22'14"S, 52º30'37"W	MCP 35120 J
	11	Haplo 10	Alto-Jacuí Microbasin	28°18'24"S, 52º28'13"W	MCP 35118 A
	11	Haplo 10	Alto-Jacuí Microbasin	28°18'24"S, 52º28'13"W	MCP 35118 B
	11	Haplo 11	Alto-Jacuí Microbasin	28°18'24"S, 52º28'13"W	MCP 35118 C
	11	Haplo 12	Alto-Jacuí Microbasin	28°18'24"S, 52º28'13"W	MCP 35118 D
	11	Haplo 13	Alto-Jacuí Microbasin	28°18'24"S, 52º28'13"W	MCP 35118 E
	11	Haplo 8	Alto-Jacuí Microbasin	28°18'24"S, 52º28'13"W	MCP 35118 F
	11	Haplo 10	Alto-Jacuí Microbasin	28°18'24"S, 52º28'13"W	MCP 35118 G
	11	Haplo 14	Alto-Jacuí Microbasin	28°18'24"S, 52º28'13"W	MCP 35118 H
E. sp. nov. 1	3	Haplo 15	Taquari-Antas Microbasin	29º03'30"S, 52º30'58"W	MCP 35058 A
	3	Haplo 16	Taquari-Antas Microbasin	29º03'30"S, 52º30'58"W	MCP 35058 B
	3	Haplo 16	Taquari-Antas Microbasin	29º03'30"S, 52º30'58"W	MCP 35058 C
	3	Haplo 16	Taquari-Antas Microbasin	29º03'30"S, 52º30'58"W	MCP 35058 D
	3	Haplo 17	Taquari-Antas Microbasin	29º03'30"S, 52º30'58"W	MCP 35058 E
	3	Haplo 15	Taquari-Antas Microbasin	29º03'30"S, 52º30'58"W	MCP 35058 F
	3	Haplo 16	Taquari-Antas Microbasin	29º03'30"S, 52º30'58"W	MCP 35058 G
	3	Haplo 16	Taquari-Antas Microbasin	29º03'30"S, 52º30'58"W	MCP 35058 I
	3	Haplo 16	Taquari-Antas Microbasin	29º03'30"S, 52º30'58"W	MCP 35058 J
	4	Haplo 18	Taquari-Antas Microbasin	28º48'24"S, 52º18'14"W	MCP 35057 A
	4	Haplo 19	Taquari-Antas Microbasin	28º48'24"S, 52º18'14"W	MCP 35057 B
	4	Haplo 19	Taquari-Antas Microbasin	28º48'24"S, 52º18'14"W	MCP 35057 C
	4	Haplo 19	Taquari-Antas Microbasin	28º48'24"S, 52º18'14"W	MCP 35057 D
	4	Haplo 19	Taquari-Antas Microbasin	28º48'24"S, 52º18'14"W	MCP 35057 E
	4	Haplo 20	Taquari-Antas Microbasin	28º48'24"S, 52º18'14"W	MCP 35057 F
	4	Haplo 19	Taquari-Antas Microbasin	28º48'24"S, 52º18'14"W	MCP 35057 G
	4	Haplo 19	Taquari-Antas Microbasin	28º48'24"S, 52º18'14"W	MCP 35057 H
	4	Haplo 19	Taquari-Antas Microbasin	28º48'24"S, 52º18'14"W	MCP 35057 J
	5	Haplo 21	Taquari-Antas Microbasin	28º49'44"S, 52º14'35"W	MCP 35122 A
	5	Haplo 21	Taquari-Antas Microbasin	28º49'44"S, 52º14'35"W	MCP 35122 B
	5	Haplo 21	Taquari-Antas Microbasin	28º49'44"S, 52º14'35"W	MCP 35122 C
	5	Haplo 21	Taquari-Antas Microbasin	28º49'44"S, 52º14'35"W	MCP 35122 D
	6	Haplo 16	Taquari-Antas Microbasin	28º34'06"S, 51º50'35"W	MCP 35045 B
	6	Haplo 22	Taquari-Antas Microbasin	28º34'06"S, 51º50'35"W	MCP 35045 C
	6	Haplo 16	Taquari-Antas Microbasin	28º34'06"S, 51º50'35"W	MCP 35045 D
	6	Haplo 23	Taquari-Antas Microbasin	28º34'06"S, 51º50'35"W	MCP 35045 E
	6	Haplo 24	Taquari-Antas Microbasin	28º34'06"S, 51º50'35"W	MCP 35045 F
	6	Haplo 25	Taquari-Antas Microbasin	28º34'06"S, 51º50'35"W	MCP 35045 G
	6	Haplo 26	Taquari-Antas Microbasin	28º34'06"S, 51º50'35"W	MCP 35045 I
	6	Haplo 27	Taquari-Antas Microbasin	28º34'06"S, 51º50'35"W	MCP 35045 J
	7	Haplo 28	Taquari-Antas Microbasin	29º01'06"S, 51º31'37"W	MCP 36837 A
	7	Haplo 28	Taquari-Antas Microbasin	29º01'06"S, 51º31'37"W	MCP 36837 B
E. sp. nov. 2	17	Haplo 29	Taquari-Antas Microbasin	28º21'51"S, 51º17'53"W	MCP 35119 A

	17	Haplo 30	Taquari-Antas Microbasin	28º21’51”S, 51º17’53”W	MCP 22800 A
	17	Haplo 30	Taquari-Antas Microbasin	28º21’51”S, 51º17’53”W	MCP 22800 B
	21	Haplo 31	Taquari-Antas Microbasin	29º05’34”S, 50º37’30”W	MCP 22374 A
E. sp. nov. 3	8	Haplo 32	Taquari-Antas Microbasin	28º31’36”S, 52º08’37”W	MCP 35043 B
	8	Haplo 33	Taquari-Antas Microbasin	28º31’36”S, 52º08’37”W	MCP 35043 C
	8	Haplo 34	Taquari-Antas Microbasin	28º31’36”S, 52º08’37”W	MCP 35043 D
	8	Haplo 34	Taquari-Antas Microbasin	28º31’36”S, 52º08’37”W	MCP 35043 E
	8	Haplo 34	Taquari-Antas Microbasin	28º31’36”S, 52º08’37”W	MCP 35043 F
	8	Haplo 34	Taquari-Antas Microbasin	28º31’36”S, 52º08’37”W	MCP 35043 G
	8	Haplo 34	Taquari-Antas Microbasin	28º31’36”S, 52º08’37”W	MCP 35043 H
	9	Haplo 34	Taquari-Antas Microbasin	28º21’29”S, 52º15’51”W	MCP 35121 D
	9	Haplo 35	Taquari-Antas Microbasin	28º21’29”S, 52º15’51”W	MCP 35121 F
	9	Haplo 34	Taquari-Antas Microbasin	28º21’29”S, 52º15’51”W	MCP 35121 H
	9	Haplo 34	Taquari-Antas Microbasin	28º21’29”S, 52º15’51”W	MCP 35121 I
	9	Haplo 34	Taquari-Antas Microbasin	28º21’29”S, 52º15’51”W	MCP 35121 J
	10	Haplo 36	Taquari-Antas Microbasin	28º21’08”S, 52º15’56”W	MCP 35049 A
	10	Haplo 35	Taquari-Antas Microbasin	28º21’08”S, 52º15’56”W	MCP 35049 B
	10	Haplo 34	Taquari-Antas Microbasin	28º21’08”S, 52º15’56”W	MCP 35049 C
	10	Haplo 34	Taquari-Antas Microbasin	28º21’08”S, 52º15’56”W	MCP 35049 D
	10	Haplo 34	Taquari-Antas Microbasin	28º21’08”S, 52º15’56”W	MCP 35049 E
	10	Haplo 32	Taquari-Antas Microbasin	28º21’08”S, 52º15’56”W	MCP 35049 F
	10	Haplo 35	Taquari-Antas Microbasin	28º21’08”S, 52º15’56”W	MCP 35049 G
	10	Haplo 34	Taquari-Antas Microbasin	28º21’08”S, 52º15’56”W	MCP 35049 H
	10	Haplo 32	Taquari-Antas Microbasin	28º21’08”S, 52º15’56”W	MCP 35049 I
	10	Haplo 34	Taquari-Antas Microbasin	28º21’08”S, 52º15’56”W	MCP 35049 J
E. sp. nov. 4	14	Haplo 39	Taquari-Antas Microbasin	28º38’04”S, 51º36’53”W	MCP 35062 A
	14	Haplo 40	Taquari-Antas Microbasin	28º38’04”S, 51º36’53”W	MCP 35062 B
	14	Haplo 37	Taquari-Antas Microbasin	28º38’04”S, 51º36’53”W	MCP 35062 D
	14	Haplo 38	Taquari-Antas Microbasin	28º38’04”S, 51º36’53”W	MCP 35062 E
	14	Haplo 39	Taquari-Antas Microbasin	28º38’04”S, 51º36’53”W	MCP 35062 F
	14	Haplo 40	Taquari-Antas Microbasin	28º38’04”S, 51º36’53”W	MCP 35062 I
	14	Haplo 39	Taquari-Antas Microbasin	28º38’04”S, 51º36’53”W	MCP 35062 J
	15	Haplo 40	Taquari-Antas Microbasin	28º39’35”S, 51º37’05”W	MCP 35041 A
	15	Haplo 41	Taquari-Antas Microbasin	28º39’35”S, 51º37’05”W	MCP 35041 B
	15	Haplo 42	Taquari-Antas Microbasin	28º39’35”S, 51º37’05”W	MCP 35041 D
	15	Haplo 40	Taquari-Antas Microbasin	28º39’35”S, 51º37’05”W	MCP 35041 F
	15	Haplo 39	Taquari-Antas Microbasin	28º39’35”S, 51º37’05”W	MCP 35041 G
	15	Haplo 43	Taquari-Antas Microbasin	28º39’35”S, 51º37’05”W	MCP 35041 H
	15	Haplo 40	Taquari-Antas Microbasin	28º39’35”S, 51º37’05”W	MCP 35041 I
E. sp. nov. 5	13	Haplo 44	Taquari-Antas Microbasin	28º44’25”S, 51º41’15”W	MCP 35037 C
	13	Haplo 45	Taquari-Antas Microbasin	28º44’25”S, 51º41’15”W	MCP 35037 D
	13	Haplo 46	Taquari-Antas Microbasin	28º44’25”S, 51º41’15”W	MCP 35037 E
	13	Haplo 46	Taquari-Antas Microbasin	28º44’25”S, 51º41’15”W	MCP 35037 F
	13	Haplo 46	Taquari-Antas Microbasin	28º44’25”S, 51º41’15”W	MCP 35037 H
	13	Haplo 46	Taquari-Antas Microbasin	28º44’25”S, 51º41’15”W	MCP 35037 I
	13	Haplo 46	Taquari-Antas Microbasin	28º44’25”S, 51º41’15”W	MCP 35037 J
	16	Haplo 47	Taquari-Antas Microbasin	28º24’19”S, 51º29’25”W	MCP 35125 A
	16	Haplo 48	Taquari-Antas Microbasin	28º24’19”S, 51º29’25”W	MCP 35125 B
	16	Haplo 49	Taquari-Antas Microbasin	28º24’19”S, 51º29’25”W	MCP 35125 C
	16	Haplo 50	Taquari-Antas Microbasin	28º24’19”S, 51º29’25”W	MCP 35125 D
	16	Haplo 47	Taquari-Antas Microbasin	28º24’19”S, 51º29’25”W	MCP 35125 E
	16	Haplo 49	Taquari-Antas Microbasin	28º24’19”S, 51º29’25”W	MCP 35125 G
	16	Haplo 49	Taquari-Antas Microbasin	28º24’19”S, 51º29’25”W	MCP 35125 I
	18	Haplo 51	Taquari-Antas Microbasin	28º23’26”S, 51º03’22”W	MCP 35123 A
	18	Haplo 52	Taquari-Antas Microbasin	28º23’26”S, 51º03’22”W	MCP 35123 B
	18	Haplo 52	Taquari-Antas Microbasin	28º23’26”S, 51º03’22”W	MCP 35123 C
	18	Haplo 52	Taquari-Antas Microbasin	28º23’26”S, 51º03’22”W	MCP 35123 D
	18	Haplo 52	Taquari-Antas Microbasin	28º23’26”S, 51º03’22”W	MCP 35123 E
	18	Haplo 52	Taquari-Antas Microbasin	28º23’26”S, 51º03’22”W	MCP 35123 H
E. sp. nov. 6	19	Haplo 53	Taquari-Antas Microbasin	29º16’41”S, 50º14’42”W	MCP 35044 A

	19	Haplo 54	Taquari-Antas Microbasin	29º16’41”S, 50º14’42”W	MCP 35044 C
	19	Haplo 55	Taquari-Antas Microbasin	29º16’41”S, 50º14’42”W	MCP 35044 E
	20	Haplo 56	Taquari-Antas Microbasin	29º07’24”S, 50º21’29”W	MCN 18563 A
	20	Haplo 56	Taquari-Antas Microbasin	29º07’24”S, 50º21’29”W	MCN 18563 B
	20	Haplo 56	Taquari-Antas Microbasin	29º07’24”S, 50º21’29”W	MCN 18563 C
E. sp. nov. 7	1	Haplo 57	Taquari-Antas Microbasin	29º02’53”S, 52º33’19”W	MCP 35124 C
	2	Haplo 58	Taquari-Antas Microbasin	29º02’51”S, 52º34’06”W	MCP 35071 A
	2	Haplo 57	Taquari-Antas Microbasin	29º02’51”S, 52º34’06”W	MCP 35071 B
	2	Haplo 59	Taquari-Antas Microbasin	29º02’51”S, 52º34’06”W	MCP 35071 D
	2	Haplo 59	Taquari-Antas Microbasin	29º02’51”S, 52º34’06”W	MCP 35071 E
	2	Haplo 58	Taquari-Antas Microbasin	29º02’51”S, 52º34’06”W	MCP 35071 F
	2	Haplo 58	Taquari-Antas Microbasin	29º02’51”S, 52º34’06”W	MCP 35071 H

Table 2. Summary statistics for the species of *Eurycheilichthys.*

	n	*h*	H_d	π	*D*	F_s
E. pantherinus	5	3	0.700 (0.218)	0.09 (0.08)	NA	NA
E. limulus	16	11	0.958 (0.036)	0.20 (0.13)	-1.436	-8.256*
Whole Taquari-Antas	105	45	0.979 (0.004)	1.03 (0.52)	-0.869	-17.770*
E. sp. nov. 1	32	14	0.909 (0.030)	0.82 (0.43)	0.237	-0.066
E. sp. nov. 2	4	3	0.666 (0.314)	0.44 (0.36)	NA	NA
E. sp. nov. 3	22	5	0.878 (0.039)	0.02 (0.03)	-1.729*	-14.731*
E. sp. nov. 4	14	7	0.923 (0.050)	0.14 (0.10)	-0.724	-5.329*
E. sp. nov. *5*	20	9	0.873 (0.046)	1.14 (0.59)	2.077	3.403
E. sp. nov. 6	6	4	0.857 (0.137)	0.52 (0.32)	NA	NA
E. sp. nov. 7	7	3	0.809 (0.129)	0.01 (0.02)	NA	NA

n, number of sequences; h, number of haplotypes; Hd, Haplotype diversity; π, Nucleotide diversity; D, Tajima's D; Fs, Fu's Fs. *P < 0.05; values in parentheses are the confidence interval; NA, not applicable.

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Table 3. AMOVA considering two different approaches to group the data

	Species approach		River approach	
Source of variation	*d.f.*	% of variation	*d.f.*	% of variation
Among groups	8	49.27	9	46.2
Among populations within groups	13	39.24	13	42.21
Within populations	103	11.49	103	11.58

Fig. 1. Geographic distribution of the *Eurycheilichthys* species with sampling localities. Solid line separates the Uruguay (up and right) from the Guaíba basins, and dashed line separate upper Jacuí (left) from Taquari-Antas microbasins. Symbols represent each species as in the internal legend, and numbers identify the sample sites.

Fig. 2. A) Bayesian tree, with all haplotypes, with support values (BI, MP, NJ and ML, respectively). * for <50 and – for nodes not found in the respective tree. B) NJ tree with all specimens

Fig. 3. Median-joining network for the Guaíba Lake basin haplotypes. The straight dashed line at the left demarcate the two microbasins. Each circle represents a different haplotype with size proportional to its relative frequency. The different shading patterns indicate the species as indicated in the internal legend. The demarcation with the oval dashed lines indicate the number of the site were the haplotypes were collected (see map in Fig. 1), showing only those that are not monophyletic. The crossed marks are nucleotide substitutions inferred in that branch.

Fig. 4. ML tree with the molecular clock forced for estimating divergence times using both substitution rate above 1.8%, bellow 0.9%.

Fig. 5. Mismatch distribution for the haplotypes of Taquari-Antas species. The observed frequency is represented by the diamond and black line, and the expected frequency under the expansion model is depicted by gray line connected by squares.

Fig. 6. Bayesian skyline plot showing the effective population size fluctuation throughout time of the Taquari-Antas microbasin clade (heavy line, median estimation; thin lines, confidence interval); above and below the x axis and left and right y axis are the time estimated using the 1.8% and the 0.9% My rates, respectively.

Fig. 1

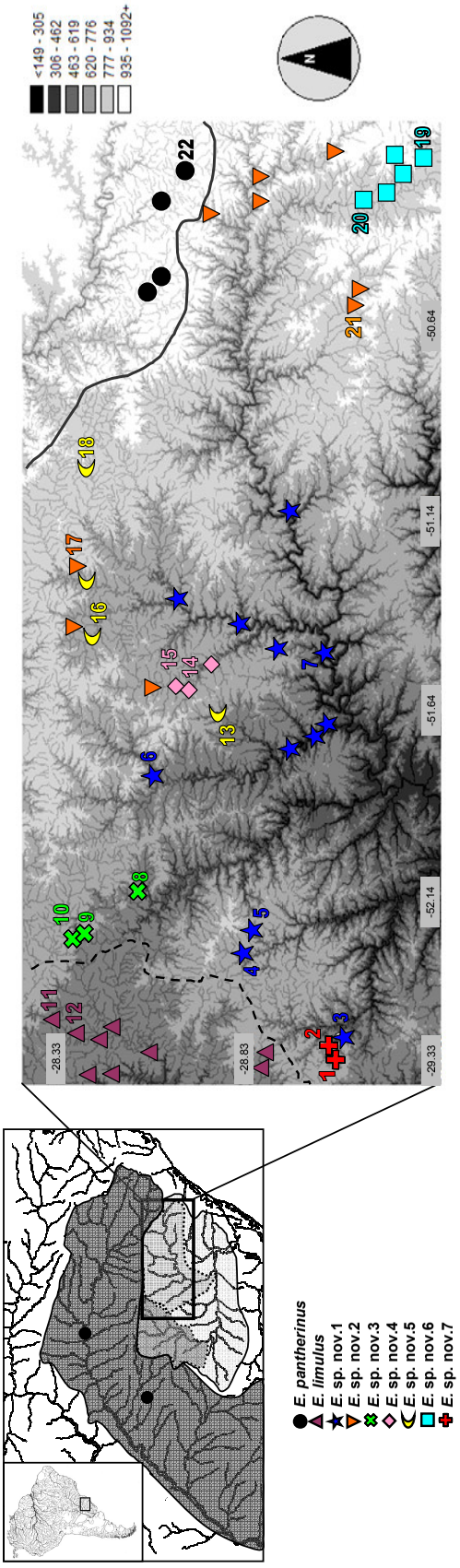
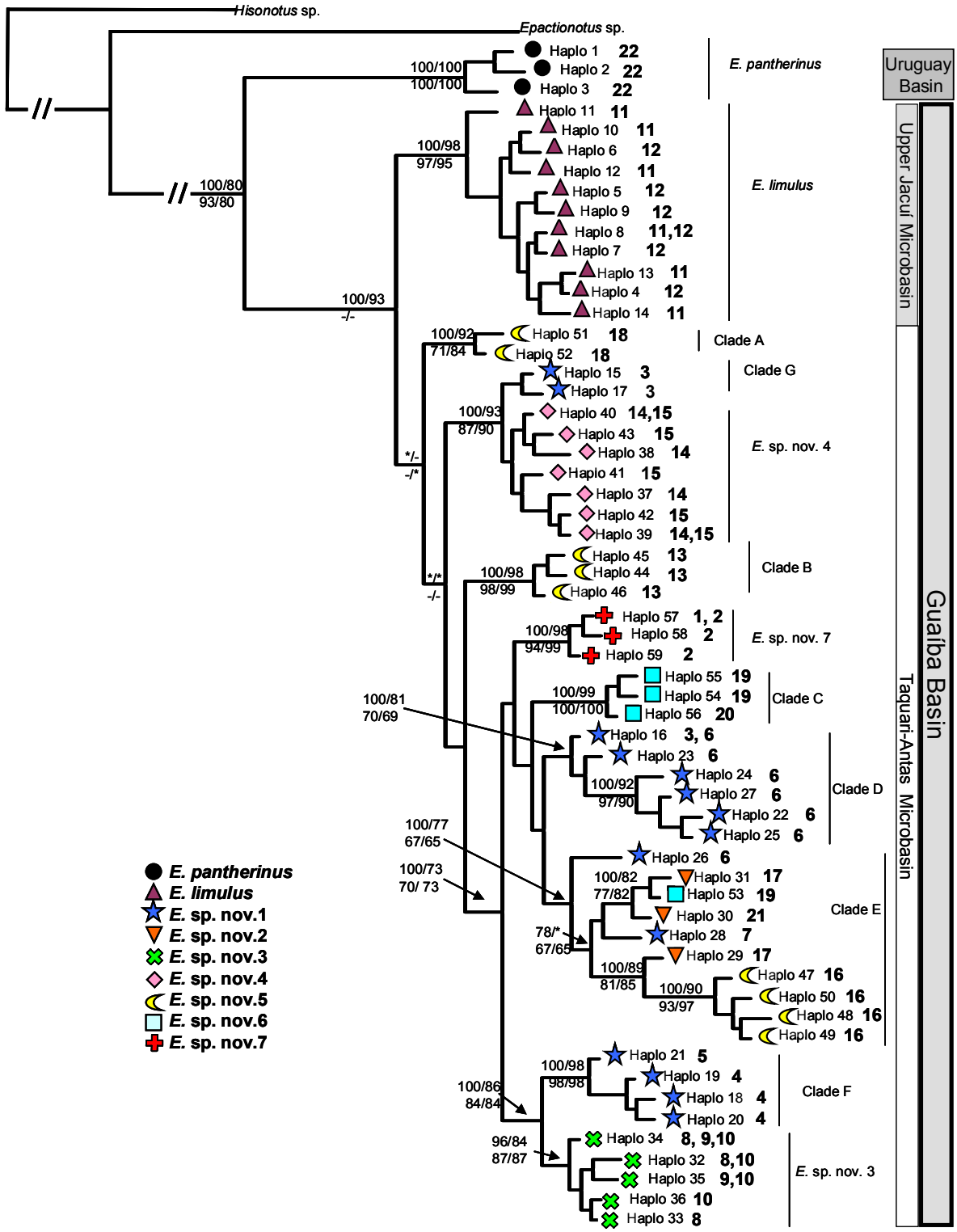

<149 - 305
306 - 462
463 - 619
620 - 776
777 - 934
935 - 1092+
N
-28.33
-28.83
-29.33
-52.14
-51.64
-51.14
-50.64
E. pantherinus
E. limulus
E. sp. nov.1
E. sp. nov.2
E. sp. nov.3
E. sp. nov.4
E. sp. nov.5
E. sp. nov.6
E. sp. nov.7

Fig. 2

A)


Hisonotus sp.
Epactionotus sp.
Haplo 1 22
Haplo 2 22
Haplo 3 22
E. pantherinus
Haplo 11 11
Haplo 10 11
Haplo 6 12
Haplo 12 11
Haplo 5 12
Haplo 9 12
Haplo 8 11,12
Haplo 7 12
Haplo 13 11
Haplo 4 12
Haplo 14 11
E. limulus
Haplo 51 18
Haplo 52 18
Clade A
Haplo 15 3
Haplo 17 3
Clade G
Haplo 40 14,15
Haplo 43 15
Haplo 38 14
Haplo 41 15
Haplo 37 14
Haplo 42 15
Haplo 39 14,15
E. sp. nov. 4
Haplo 45 13
Haplo 44 13
Haplo 46 13
Clade B
Haplo 57 1, 2
Haplo 58 2
Haplo 59 2
E. sp. nov. 7
Haplo 55 19
Haplo 54 19
Haplo 56 20
Clade C
Haplo 16 3, 6
Haplo 23 6
Haplo 24 6
Haplo 27 6
Haplo 22 6
Haplo 25 6
Clade D
Haplo 26 6
Haplo 31 17
Haplo 53 19
Haplo 30 21
Haplo 28 7
Haplo 29 17
Haplo 47 16
Haplo 50 16
Haplo 48 16
Haplo 49 16
Clade E
Haplo 21 5
Haplo 19 4
Haplo 18 4
Haplo 20 4
Clade F
Haplo 34 8, 9,10
Haplo 32 8,10
Haplo 35 9,10
Haplo 36 10
Haplo 33 8
E. sp. nov. 3
100/100
100/100
100/98
97/95
100/80
93/80
100/93
-/-
100/92
71/84
100/93
87/90
*/-
-/*
/
-/-
100/98
98/99
100/98
94/99
100/81
70/69
100/99
100/100
100/92
97/90
100/77
67/65
100/73
70/ 73
100/82
77/82
78/*
67/65
100/89
81/85
100/90
93/97
100/98
98/98
100/86
84/84
96/84
87/87
E. pantherinus
E. limulus
E. sp. nov.1
E. sp. nov.2
E. sp. nov.3
E. sp. nov.4
E. sp. nov.5
E. sp. nov.6
E. sp. nov.7
Uruguay Basin
Upper Jacuí Microbasin
Taquari-Antas Microbasin
Guaíba Basin

B)

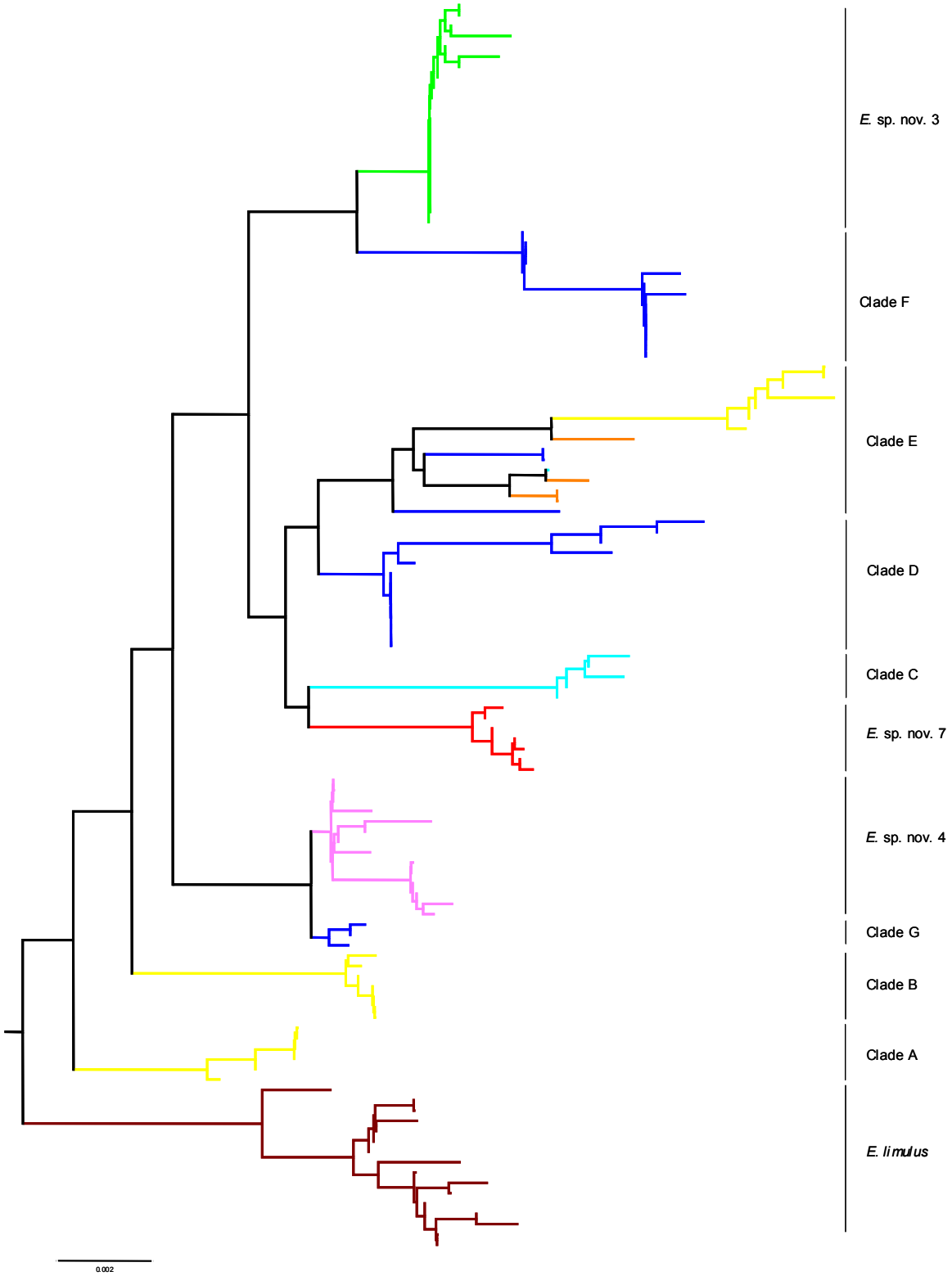

E. sp. nov. 3
Clade F
Clade E
Clade D
Clade C
E. sp. nov. 7
E. sp. nov. 4
Clade G
Clade B
Clade A
E. limulus
0.002

Fig. 3

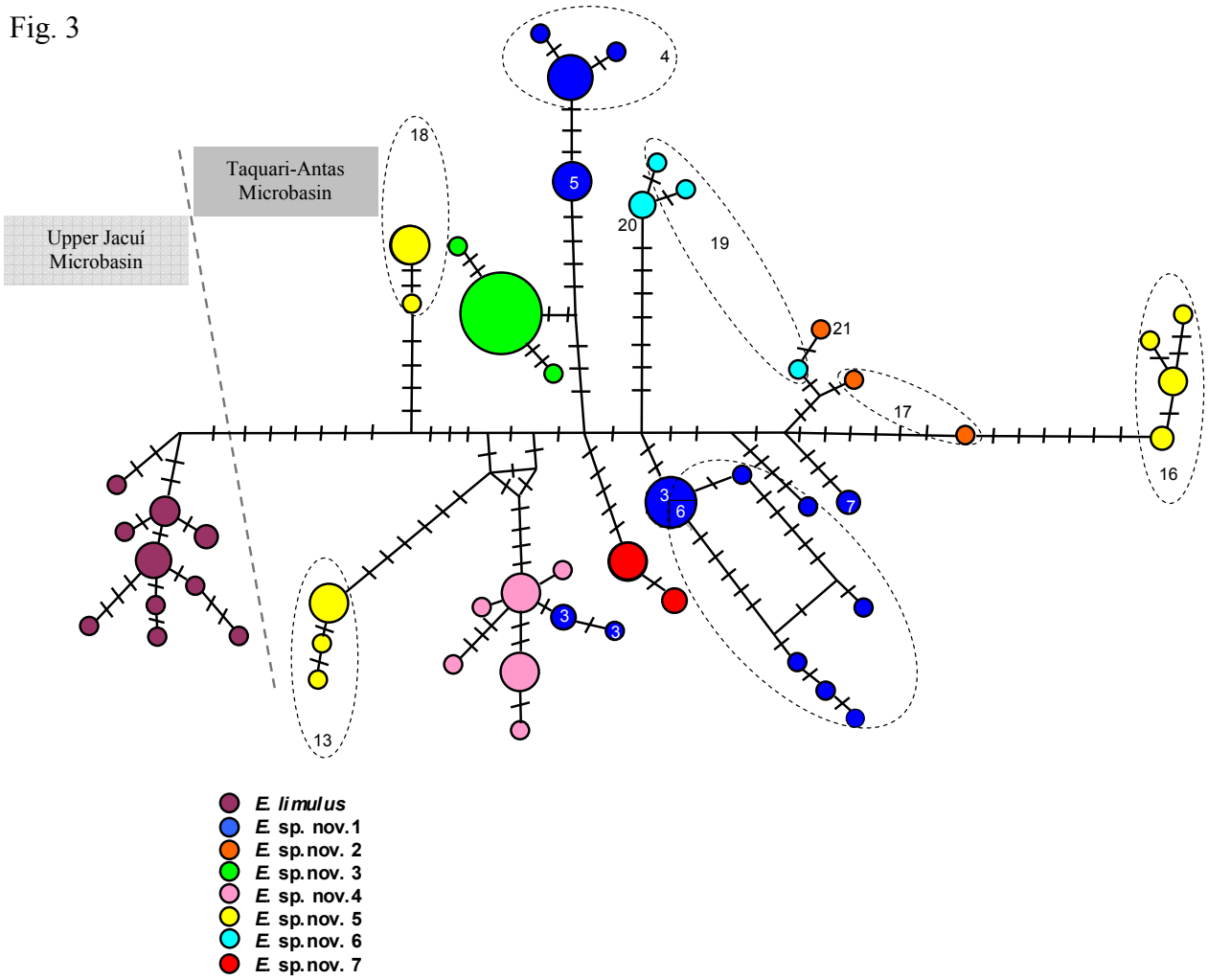

Taquari-Antas Microbasin
Upper Jacuí Microbasin
4
18
5
20
19
21
17
16
3
6
7
3
3
13
E. limulus
E. sp. nov. 1
E. sp. nov. 2
E. sp. nov. 3
E. sp. nov. 4
E. sp. nov. 5
E. sp. nov. 6
E. sp. nov. 7

Fig. 4

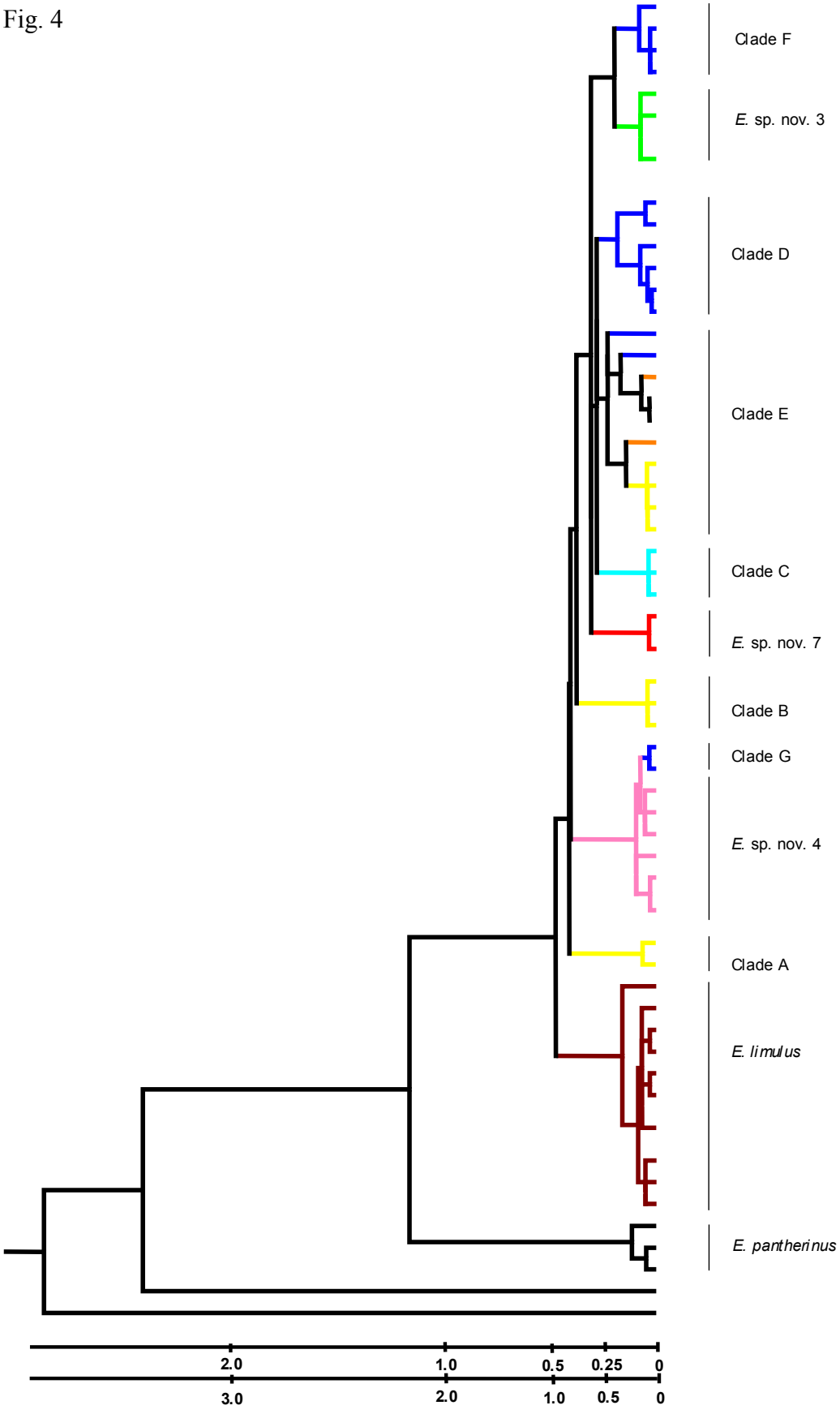

Clade F
E. sp. nov. 3
Clade D
Clade E
Clade C
E. sp. nov. 7
Clade B
Clade G
E. sp. nov. 4
Clade A
E. limulus
E. pantherinus
2.0
1.0
0.5
0.25
0
3.0
2.0
1.0
0.5
0

Fig. 5

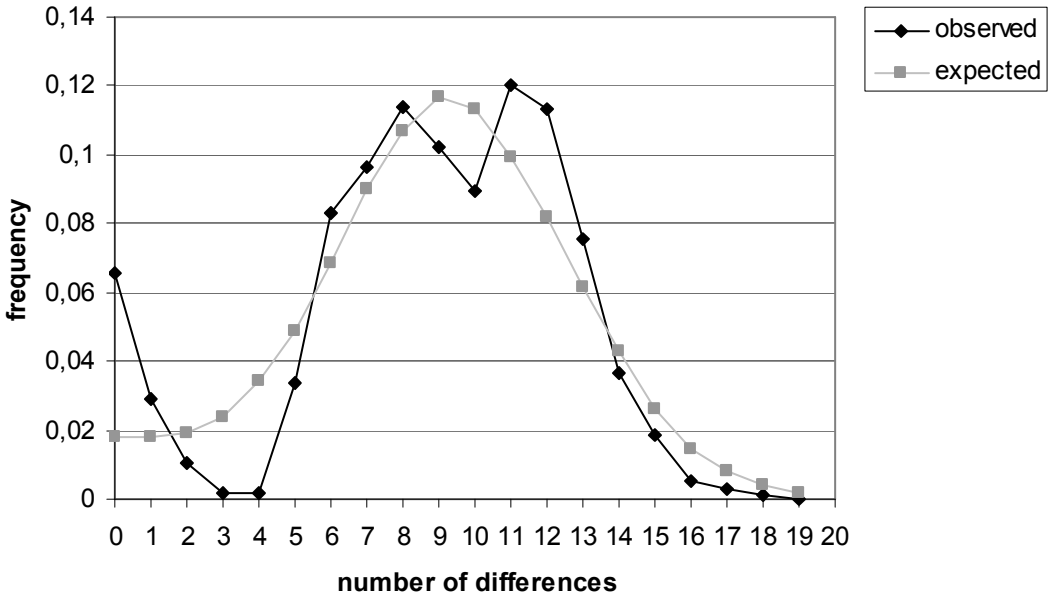
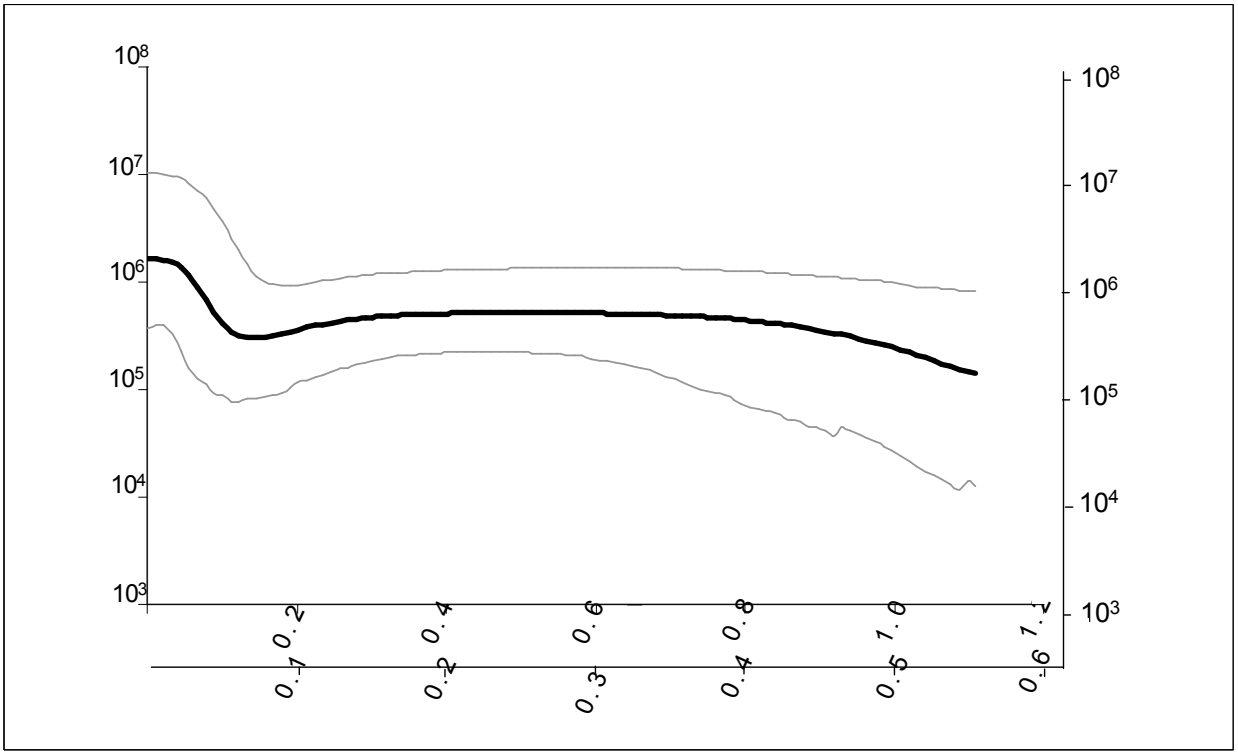

0,14
0,12
0,1
0,08
0,06
0,04
0,02
0
frequency
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
number of differences
observed
expected

Fig. 6


10^8
10^7
10^6
10^5
10^4
10^3
10^8
10^7
10^6
10^5
10^4
10^3
0.1
0.2
0.2
0.4
0.3
0.6
0.4
0.8
0.5
1.0
0.6
1.2