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FILOGENIA E FILOGEOGRAFIA DO GÊNERO *Hollandichthys* EIGENMANN 1909 (TELEOSTEI: CHARACIDAE)

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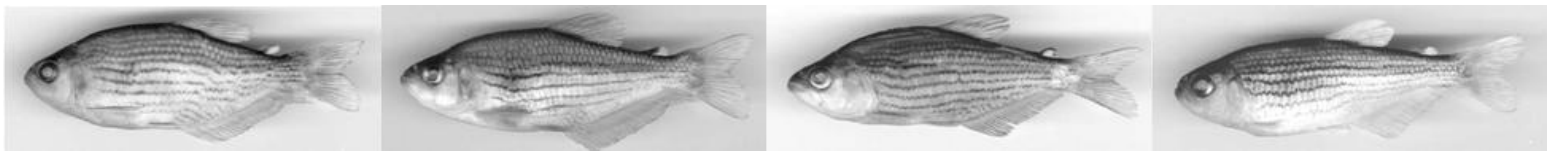

Filogenia e Filogeografia do Gênero *Hollandichthys*

Eigenmann 1909 (Teleostei: Characidae)

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DEDICATÓRIA

"Je hais les voyages et les explorateurs"

"Odeio as viagens e os exploradores"

(Claude Lévi-Strauss, 1908-2009)

Aos meus queridos pais:

Ana e Jamba.

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Aos genômicos pela alta diversidade haplotípica, possibilitando uma grande troca de conhecimentos, opiniões e boas risadas.

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SUMÁRIO

RESUMO

Hollandichthys é um gênero de caracídeos inseminadores cujas relações de parentesco e diversidade ainda estão por serem descobertas. Suas relações ainda são incertas, tendo sido considerado como *incertae sedis* dentro de Characidae. Algumas hipóteses recentes propuseram *Hollandichthys* como relacionado alternativamente a dois gêneros distintos, *Pseudochalceus* e *Rachoviscus.* É considerado como um gênero monotípico habitando pequenos riachos associado à Mata Atlântica (uma das regiões mais endêmicas do mundo), mas esconde uma grande diversidade por trás desta única espécie válida – *H. multifasciatus.* Para propor as relações filogenéticas de *Hollandichthys* dentro da família, nós analisamos genes mitocondriais e nucleares representando 41 espécies de Characidae. Para inferir a história evolutiva do gênero, nós sequenciamos 201 espécimes pertencentes a 20 populações de toda sua área de distribuição. Nós propomos aqui o gênero *Rachoviscus* como o grupo-irmão de *Hollandichthys*. Além disso, os resultados suportam a evidência de que a inseminação evoluiu independentemente pelo menos três vezes em Characidae. Nas análises filogeográficas, nós inferimos uma separação clara, datada de 1.9 milhões de anos, em dois grupos distintos (Norte e Sul) isolados pelo estuário de Paranaguá. As diversas populações agrupam-se consistentemente em cinco grupos que melhor representam a diversidade molecular e geográfica dos nossos dados. De modo geral, a história evolutiva inferida para esse gênero é estritamente correlacionada com as mudanças climáticas que causaram impacto na área da Floresta Atlântica. Um evento de *bottleneck* teria ocorrido durante o ultimo máximo glacial, seguido de um crescimento populacional que coincide com a expansão da floresta – de pequenas e isoladas áreas para uma distribuição contínua.

ABSTRACT

Hollandichthys is a genus of inseminating characid fishes whose relationships and diversity are still to be discovered. Its relationships are uncertain, having been considered as *incertae sedis* in Characidae. Some recent hypothesis set *Hollandichthys* alternatively related to two different genera, *Pseudochalceus* and *Rachoviscus*. It has been long considered a monotypic genus living in small creeks associated with the Atlantic Forest (one of the most endemic regions in the world), but it hides a great diversity behind a single valid species name - *H. multifasciatus*. To access the phylogenetic relationships of *Hollandichthys* we have analyzed mtDNA and nuclear genes representing 41 species of Characidae. To access the evolutionary history of the genus, we have sequenced 201 specimens from at least 20 populations from all distributional range. We found *Rachoviscus* as sister-group of *Hollandichthys*. Furthermore, the results support the evidence that insemination evolved at least three times inside this family. In the phylogeographic approach, we found a clear separation in two different groups (North and South) in the area of Paranaguá estuary in the Brazilian Coast, dating from 1.9 Mya, and the several populations consistently arranged into five groups that better fits to the diversity of our molecular and geographic dataset. In a general manner, the evolutionary history inferred for this genus is strictly correlated with the climatic changes that caused impact in the Atlantic Forest area. A bottleneck would have happened during the last maximum glacial, followed by a population growth that coincides with the expansion of the forest - from small isolated areas to a large continuum.

APRESENTAÇÃO

1. A REGIÃO COSTEIRA E SUA ICTIOFAUNA

A costa brasileira apresenta cerca de 9.200km de extensão e, além dos importantes ecossistemas que a constituem, é a região em que se distribui a maior parte da população humana do país. Os diferentes condicionamentos geológicos e climáticos ao longo de sua extensão são responsáveis por uma grande diversidade de aspectos morfológicos (planícies, falésias, costões rochosos...), tendo sua origem com os eventos que conduziram a abertura do Oceano Atlântico Sul, que foram iniciados no Jurássico (130 milhões de anos) e resultaram na ruptura do super continente Gondwana, a partir de um sistema de fraturas (Villwock *et al.*, 2005).

Devido ao deslocamento das placas tectônicas no sentido Leste ➡ Oeste, a costa do Oceano Atlântico da América do Sul está desenvolvida sobre uma margem continental passiva, o que se contrapõe à costa do Oceano Pacífico, construída sobre uma margem continental ativa. O cavalgamento da Placa Sul-Americana sobre a Placa de Nazca, na margem oeste da América do Sul, desenvolve relevos muito acidentados, como a Cordilheira dos Andes. Já a margem leste, é construída por regiões mais baixas em relação à costa oeste onde, em ambiente atividade tectônica menos ativa, se desenvolveram planícies costeiras com sistemas lagunares e ilhas-barreira que transicionam para extensas plataformas continentais (Villwock *et al.*, 2005).

Ao sul de São Paulo e, principalmente no Paraná e norte de Santa Catarina, as planícies costeiras abrigam extensos complexos estuarino-lagunares, como Paranaguá e Guaratuba (Villwock *et al.*, 2005). Na margem leste brasileira, desde o sul da foz do

rio São Francisco até o rio da Prata, ocorrem numerosos riachos isolados, com pequena área de drenagem. Esses riachos são especialmente curtos e de declive acentuado ao longo da porção sudeste da serra (Lundberg *et al.*, 1998).

A área de abrangência deste estudo, que compreende desde o litoral norte do Rio Grande do Sul até o sul do Rio de janeiro, é marcada por costas altas onde promontórios rochosos se alternam com pequenas planícies costeiras. O padrão de drenagens do escudo brasileiro é uma herança do período Jurássico-Cretáceo. Após a ruptura e abertura do oceano Atlântico os rios costeiros passaram a evoluir em consonância com processos tectônicos e erosivos locais. O soerguimento de blocos, que acabaram por formar a Serra do Mar, causaram reajustes localizados tais como capturas de cabeceiras que permitiram a inversão no sentido de alguns cursos de rios que passaram a correr para o interior do continente (Villwock *et al.*, 2005). Além disso, diversas invasões marinhas desde a abertura do oceano Atlântico tiveram um papel muito importante para a paisagem desta área. Devido a esses eventos geológicos ocorrentes nos últimos milhões de anos, a região costeira vem apresentando grandes oscilações climáticas e na disponibilidade de habitats, culminando em áreas altamente dinâmicas, com um elevado grau de endemismo em sua fauna (Ribeiro, 2006).

Bizerril (1994) calculou a taxa de endemismo para a região costeira brasileira e obteve 23,4% ao nível de gêneros e 95% com relação a espécies. Esse elevado grau de endemismo deve-se ao fato de que peixes de água doce dependem, para sua dispersão, de conexões diretas entre os sistemas de drenagens. Desta forma, devido a história de interconexões entre as bacias, a distribuição íctica ao longo da planície costeira reflete o desenvolvimento geológico implícito. Análises de biogeografia

histórica de peixes permitem criar fortes inferências sobre a biota e a evolução geológica da região analisada (Bermingham and Martin, 1998). Entretanto, informações históricas sobre as comunidades de peixes das drenagens da região costeira são escassas e fragmentadas, dificultando o entendimento dos processos históricos que determinaram a composição e distribuição da fauna de peixes de água doce ao longo da costa brasileira (Bizerril and Lima, 2000).

2. O GÊNERO *Hollandichthys*

O gênero *Hollandichthys* corresponde a um grupo neotropical de peixes de água doce popularmente chamados de lambari-listrado, sendo facilmente reconhecidos pela presença de listras pretas longitudinais. Habitam rios costeiros e ilhas marinhas, além da porção superior do rio Tietê na drenagem do rio Paraná (Langeani, 1989). Após a realização de recentes expedições, sua distribuição, anteriormente conhecida desde o norte do estado de Santa Catarina ao sul do Rio de Janeiro, foi ampliada à região norte do Rio Grande do Sul. Nesses locais, são encontrados em pequenos riachos ou poças laterais, com pouca água corrente e fundo lodoso; sempre associados à densa vegetação ripária de mata Atlântica, e apenas em porções preservadas desse bioma (Bertaco, 2003). Segundo Britski (1972), *H. multifasciatus* pode ser capturado em água salobra, podendo ser o caracídeo mais resistente a variações de salinidade.

Esse gênero é composto por uma espécie válida - *H. multifasciatus*. Entretanto, a partir de estudos morfológicos (Bertaco, 2003), foram identificadas oito morfoespécies no gênero, agrupadas com base em 20 sinapomorfias compartilhadas, sendo todas alopátricas e obedecendo a um padrão de distribuição geográfico. Duas

das oito morfoespécies de Bertaco corresponderiam as espécies nominais (*H. affinis* e *H. perstriatus*), atualmente sinônimos de *H. multifasciatus.* Essas morfoespécies distinguem-se principalmente pelo número de raios procorrentes dorsais e ventrais presentes na nadadeira caudal, número de escamas na série longitudinal, diâmetro orbital e colorido padrão (Bertaco, 2003). Entretanto, estas propostas ainda não foram publicadas, e por isso o gênero *Hollandichthys* permanece como gênero monotípico.

Hollandichthys já foi considerado sinônimo de *Pseudochalceus* (Schultz, 1966), sendo mais tarde revalidado como gênero (Weitzman *et al.*, 1986). No estudo filogenético de Bertaco (2003), baseado em caracteres morfológicos, o gênero *Pseudochalceus,* ocorrente na porção transandina do Equador e Colômbia, foi proposto como grupo-irmão de *Hollandichthys*. Em outro estudo (Quevedo, 2006), propôs o gênero *Rachoviscus* como grupo irmão. Mais recentemente, Mirande (2009) propôs *Rachoviscus* e *Hollandichthys* como pertencentes a grupos distintos na família Characidae, enquanto Javonillo *et al.* (2010) propuseram os dois gêneros como grupos irmãos.

3. ESTUDOS FILOGEOGRÁFICOS

A biogeografia da ictiofauna Neotropical é pouco conhecida (Ribeiro, 2006). Uma maneira para ampliar esse conhecimento seria através do estudo de pequenos grupos monofiléticos que se presuma que sejam relativamente recentes filogeneticamente e que estejam confinados em áreas restritas (Weitzman et al., 1988). Isso facilitaria a identificação de prováveis extinções, estudos de relações entre as áreas de endemismo e avaliação das hipóteses propostas sobre os mecanismos de evolução para esses organismos, o que proporcionaria ricas informações filogenéticas,

de fundamental importância para o entendimento de questões relativas à biogeografia histórica numa escala menor que a continental (Vari and Weitzman, 1990). A filogeografia, como uma subdisciplina da biogeografia, enfatiza aspectos históricos que levaram a distribuição contemporânea das linhagens gênicas (Avise *et al.*, 1998). Dessa forma, o entendimento de tais processos torna possível a inferência de modelos hierárquicos de diferenciação genética e de estrutura filogeográfica, que refletem, assim, a história geográfica da região (Beheregaray *et al.*, 2002).

A análise de diferentes grupos de peixes de água doce em uma determinada região frequentemente demonstra esses modelos hierárquicos de diferenciação genética e de estrutura filogeográfica, necessários para compreender a geografia histórica da região (Avise, 2000). Entretanto, a formulação e o teste de hipóteses gerais sobre a diversidade e diversificação desses peixes neotropicais requer conhecimento sobre sua filogenia e distribuição no tempo e no espaço (Lundberg, 1998).

Buscando respostas para essas questões, marcadores moleculares tem colaborado para um maior entendimento da ictiofauna, podendo corroborar hipóteses e auxiliar no esclarecimento de problemas evolutivos ainda não resolvidos. Para isso, a utilização de DNA mitocondrial (mtDNA) nos oferece grandes contribuições para obter um maior entendimento dos padrões geográficos de distribuição. É esperado que ele nos informe os níveis mais profundos de divergência filogenética (Sunnucks *et al.*, 2000) principalmente por apresentar uma evolução rápida nas populações da maioria dos animais e ser transmitido, raras exceções, pela linhagem materna, sem recombinação intermolecular, sendo por isso uma ferramenta muita utilizada em

estudos filogenéticos a nível de populações (Avise *et al.*, 1998). Além disso, as taxas de substituições de nucleotídeos são relativamente homogêneas dentro dos táxons, tornando possível estimar a cronologia da colonização e os eventos de diversificação (Bermingham and Martin, 1998).

Por outro lado, a utilização de genes nucleares tem aumentado nos últimos anos nesses tipos de estudos, entretanto, por sua evolução mais lenta são muito bons para estudos filogenéticos entre grupos mais antigos. A utilização dos íntrons nucleares ajudou os estudos filogeográficos a inferirem de forma mais completa a história evolutiva dos organismos (Avise, 2009).

4. OBJETIVOS

Este trabalho tem por objetivo (i) formular hipóteses de relações entre as diversas populações de *Hollandichthys*, (ii) testar a concordância entre os dados genéticos e as morfoespécies propostas e (iii) apresentar uma maior resolução das relações filogenéticas de *Hollandichthys* e os táxons supostamente inferidos como filogeneticamente aparentados. Para tanto esperamos:

a. Construir uma filogenia baseada em dados moleculares que auxilie no entendimento das relações de *Hollandichthys* dentro da família Characidae;

b. Compreender a distribuição espacial e temporal, verificando a estruturação haplotípica e eventos de expansão e contração populacional;

c. Avaliar a diversidade genética dentro do grupo foco do estudo;

d. Formular hipóteses que expliquem a história evolutiva da região, a qual culminou na distribuição atual do gênero *Hollandichthys*;

e. Ampliar o conhecimento sobre a ictiofauna da região que auxilie nos programas de conservação da mata Atlântica e principalmente dos corpos d'água desse bioma, a partir da diversidade genética estudada.

Capítulo 1

Relationships of *Hollandichthys* Eigenmann 1909 (Teleostei: Characidae): a Molecular Inference

(Manuscrito a ser submetido para o periódico *Neotropical Ichthyology*)

Relationships of *Hollandichthys* Eigenmann 1909 (Teleostei: Characidae): a Molecular Inference

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Key-word: phylogeny, *incertae sedis, "*clade C", *Rachoviscus, Pseudochalceus.*

Abstract

The phylogenetic relationships inside Characidae are complex with several genera remaining in an uncertain systematic position inside the family. The genus *Hollandichthys* is one of these problematic genera. It has been considered as *incertae sedis* inside this family, until two recent published phylogenies, one morphological and one molecular, have alternatively hypothesized *Hollandichthys* as related to two different genera, *Pseudochalceus* or *Rachoviscus*, respectively. In this paper, we infer the phylogenetic relations of these taxa based on arrangements of five genes sets (three mitochondrial - COI, ND2 and 16S; and two nuclear - Sia and Trop), totalling up to 2719 bp and 41 species in Characidae. Analyzed species include four *incertae sedis* characid taxa once hypothesized as related to *Hollandichthys*, but never analyzed in a single phylogeny (*Rachoviscus, Pseudochalceus, Nematocharax* and *Hyphessobrycon uruguayensis*). Here we propose the genus *Rachoviscus* as the sister-group of *Hollandichthys*, grouped in the same larger clade with the remaining *incertae sedis* taxa added to this study. In addition, we support the evidence that insemination evolved independently at least three times in Characidae.

Resumo

As relações filogenéticas dentro da família Characidae são complexas, com vários gêneros de posição incerta na sistemática da família. *Hollandichthys* é um desses gêneros problemáticos, tendo o status de *incertae sedis* dentro da família até recentemente, quando duas filogenias publicadas, uma morfológica e outra molecular, inferiram *Hollandichthys* como mais relacionado a dois gêneros distintos, *Pseudochalceus* ou *Rachoviscus,* respectivamente. Neste trabalho, foram feitas inferências filogenéticas baseadas em diversos arranjos com cinco genes (três mitocondriais - COI, ND2 e 16S; e dois nucleares - Sia e Trop), totalizando até 2719 pb e 41 espécies de Characidae. Este total inclui quatro táxons *incertae sedis* já referidos como supostamente relacionados a *Hollandichthys*, mas nunca analisados numa mesma filogenia (*Rachoviscus, Pseudochalceus, Nematocharax* and *Hyphessobrycon uruguayensis*). Nós propomos aqui o gênero *Rachoviscus* como o grupo-irmão de *Hollandichthys*, os quais, juntamente com os demais táxons *incertae sedis* adicionados nessa análise, ficaram posicionados em um mesmo clado mais abrangente. Além disso, os resultados suportam a evidência de que a inseminação evoluiu independentemente pelo menos três vezes em Characidae.

Introduction

The family Characidae is the most diverse of the order Characiformes and represents the fishes popularly known as “tetras”. Characids classification and taxonomy suffer constant changes due to the complex evolutionary history of the group and lack of well supported phylogenies (Mirande, 2009). Weitzman & Malabarba (1998) suggested the necessity to investigate the monophyly of several subfamilies in a tentative to clarify the resolution in Characidae. In view of the poorly known and incongruent relationships among groups inside this family, Lima *et al.* (2003) considered as valid only those subfamilies for which some evidence of monophyly was available. Thus, 88 genera were listed as *incertae sedis* in Characidae, including 620 species. Part of this total, corresponding to 53% or 47 genera, are monotypic, but probably representing several species complex (Lima *et al.*, 2003). Recently, a new phylogeny of Characidae, using morphological data, was suggested by Mirande (2009) in which he presents new combinations at the subfamily level.

The first molecular study proposing a phylogeny in Characidae was presented by Ortí & Meyer (1997), followed by Calcagnotto *et al.* (2005) and Javonillo *et al.* (2010). In this last one, the authors found three large monophyletic clades among characids (acting a supraorbital bone): the clade “A”, corroborating proposal of Malabarba & Weitzman (2003), with the subfamilies Glandulocaudinae and Stevardiinae and 19 *incertae sedis* genera; the clade “B” with the subfamilies Characinae, the monophyletic Cheirodontinae (Malabarba, 1998), Aphyocharacinae and Tetragonopterinae sensu strict (including only *Tetragonopteurs*); and clade “C” with the subfamily Stethaprioninae and several genera *incertae sedis*.

The genus *Hollandichthys* represents one of the many phylogenetic uncertainties regarding the family Characidae. This genus has the status of *incertae sedis* and is monotypic. It was first proposed by Eigenmann in 1909 based on the species *Tetragonopterus multifasciatus* Eigenmann & Norris, 1900. Two other species described in the genus *Pseudochalceus* (*P. affinis* Steindachner, 1908 and *P. perstriatus* Ribeiro, 1908) were considered as synonyms of *Hollandichthys multifasciatus* by Eigenmann (1910). The genus *Pseudochalceus* Kner, 1863, was always pointed as

being very similar to *Hollandichthys*, despite their disparate geographic distributions. *Hollandichthys* species lives in the Southeastern Atlantic coast of Brazil, while *Pseudochalceus* lives in coastal drainages of Ecuador and Colombia, in the South American Pacific coast. Even though, in 1966, Schultz referred *Hollandichthys* as a synonym of *Pseudochalceus* because he was unable to find any important anatomical difference.

Géry (1972) cited *Hollandichthys* as subgenus of *Pseudochalceus* and referred to the geographical disjunction of 4500km between the *Pseudochalceus* species as an interesting biogeographical problem. Finally, Weitzman *et al.* (1986) proposed to retain the name *Hollandichthys* until a cladistic phylogenetic study could demonstrate monophyly between *Hollandichthys* and *Pseudochalceus.* In this same paper they proposed the genera *Hollandichthys* and *Rachoviscus* Myers, 1926 as being possibly related with the genus *Nematocharax* Weitzman, Naércio & Menezes, 1986, based on the bear well-toothed maxillae and the relatively close geographic distribution

Only in 2003, Bertaco applied a morphological phylogenetic approach with *Hollandichthys*, proposing a sister-group relationship to the genus *Pseudochalceus* and these being closely related to a clade formed by the genus *Rachoviscus* + *Aphyocharax anisitsi* + *Nematobrycon palmeri*. On the other hand, Quevedo (2006) inferred phylogenetically the genus *Rachoviscus* as sister-group of *Hollandichthys,* both genera of inseminating fishes, and related to *Mimagoniates rheocharis* and *Diapoma speculiferum,* both species belonging to the clade "A". Globular expansions are found in the pectoral-fins of the species *Lophiobrycon weitzmani* Castro *et al.*, 2003 and in both species of *Rachoviscus* – *R. crassiceps* and *R. graciliceps* (Quevedo, 2006). In a sperm ultra structure analyses Quevedo (2006) found shared elongate spermatic nuclei in the spermatozoids of the genera *Rachoviscus, Hollandichthys, Lophiobrycon* and *Brittanichthys.*

Recently, two studies with different inference tools, positioned the genus *Hollandichthys* in different groups. The morphological study of Mirande (2009) proposed the genus *Hollandichthys* in

51 a group called "*Bramocharax"* formed by the genera *Hollandichthys* + *Pseudochalceus* +
52 *Bramocharax* + *Oligosarcus*. Conversely, molecular data was used to position *Hollandichthys* in a
53 clade with *Rachoviscus* + *Moenkhausia* + *Thayeria* (Javonillo *et al.*, 2010).

54 Attempting to test the relationships previously described between some characid genera,
55 we aim to propose a molecular phylogeny of *Hollandichthys* and putative related taxa, with the
56 objective to (1) infer the relationships of sister-group and more related taxa to the genus
57 *Hollandichthys* and (2) suggest a position to this genus inside the Characidae family.

58

59 **Material and Methods**

60 *Taxon sampling, DNA extraction and sequencing*

61 Tissue samples from 40 specimens, representing 14 *Hollandichthys* and 26 individuals of
62 other characiforms were obtained from fish collections and field work (Table 1). The ingroup
63 embraced all the *Hollandichthys* samples, the genera previously proposed as related to
64 *Hollandichthys*, such as *Pseudochalceus* (Bertaco, 2003)*, Rachoviscus* (Quevedo, 2006)*,*
65 *Nematocharax* (Weitzman *et al.*, 1986) and *Lophiobrycon* (Castro *et al.*, 2003), and species of
66 Characidae that share some morphological characters with *Hollandichthys*, as longitudinal black
67 stripes in *Hyphessobrycon uruguayensis. Mimagoniates microlepis* was included as a representative
68 of the Glandulocaudinae, which currently houses *Lophiobrycon. Aphyocharax anisitsi* was included
69 due to its relationships hypothesized to the genera listed above (Bertaco, 2003). We choose as
70 outgroup the genus *Bryconops* Kner, 1858, considered hypothetically basal species of the
71 Characidae (Weitzman & Malabarba, 1998).

72 DNA extraction from tissues maintained in 96% ethanol followed the modify salt-
73 precipitation protocol (Medrano *et al.*, 1990). We amplified five different genes, three as proposed
74 by Calcagnotto *et al.* (2005): one mitochondrial gene (16S), the nuclear gene seven in absentia (Sia)

and the intron 5 of the α-tropomyosin gene (Trop); and two more mitochondrial genes: cytochrome oxidase I (COI) and the NADH dehydrogenase 2 (ND2) (Table 2) by the PCR technique in 20µl reactions with the following concentrations: 10-50ng DNA, 0,2µM primer, 0,2mM of each dNTP, 1x Buffer, 1,5µM $MgCl_2$ and 1U *Taq* DNA polymerase *Platinum* (Invitrogen). In some cases 4% of Triton was added. PCR conditions are presented in Table 3. The PCR product were purified using EXOSAP (Exonuclease I and Shrimp Alkaline Phosphatase, GE Healthcare®) and the sequences reactions were performed using the Et Terminator cycle sequencing kit and run in both directions in the MegaBACE 1000 (GE Healthcare®).

Phylogenetic analyses

The forward and reverse chromatogram reads were assembled and visualized using the *Phred/Phrap/Consed* package (Ewing *et al.*, 1998; Gordon *et al.*, 1998). The consensus sequences were automatically aligned using the software Muscle 3.6 (Edgar, 2004) and manually checked with BioEdit 7 (Hall, 1999). The basic statistics, as nucleotide diversity (π) and nucleotide frequency, were calculated with the software DnaSP (Rozas *et al.*, 2003) and the pairwise distance among sequences was estimated with Mega 4 (Tamura *et al.*, 2007), with the Kimura-2-parameter method (Kimura, 1980). The mitochondrial coding genes COI and ND2 were concatenated because of the circular form of the mtDNA which makes them interconnected, under the same environmental pressures (Avise *et al.*, 1987). The gene 16S was not included in this concatenation because of the differences in the individuals sequenced.

Aligned sequences were analyzed with three different methods of phylogenetic inference: maximum parsimony, maximum likelihood and Bayesian analyses. The maximum parsimony was conducted in the software TNT (Goloboff, 2003), under the *New Technology Search* with Sectorial Searches, collapsing rules with minimum length=0, equal weighted and bootstrap values were estimated with 1000 replications. The maximum likelihood approach was inferred with the program RaxML 7 (Stamatakis, 2006) with a beginning evolutionary model of GTRGamma, one partition for

each gene and 1000 replications of bootstrap. The program Beast 1.5.3 (Drummond & Rambaut, 2007) was used to develop the Bayesian analyses with a partitioned dataset. The evolutionary models for each partition were selected in MrModelTest 2.3 (Nylander, 2004) with PAUP 4.0 (Swofford, 1998). Under the Akaike Information Criterion (AIC), we used the HKY+G model to the Sia gene partition, while we used the GTR+G model for the partitions containing Trop, 16S and the concatenated COI/ND2. We performed 100 millions of Markov Chain Monte Carlo (MCMC) sampled each 10000 generations, and 10% of the initial sampling trees were discarded as a burn-in. The nodal support was estimated based on its posterior probability.

To infer the position of *Hollandichthys* inside the family we used the sequences of Characidae available in GenBank (http://www.ncbi.nlm.nih.gov/) from the paper of Calcagnotto *et al.* (2005) for the genes Sia, Trop and 16S (Table 4) in addition of our sequences to the same three genes. The maximum parsimony and the maximum likelihood approaches followed the same steps as described above. For the Bayesian inference we re-estimated the evolutionary models as GTR+I+G for the genes 16S and Sia, and GTR+G for Trop, both estimated under AIC. We performed 200 millions of Markov Chain Monte Carlo (MCMC) sampled each 10000 generations, with 20% of the initial samples discarded as a burn-in. The remaining Bayesian parameters were implemented as described to the first set of analyses. All the threes inferred with the addition of Calcagnotto`s sequences were rooted in *Chalceus erythrurus* for being *Chalceus* the genus proposed as sister-group of all other so-called Characidae (Calcagnotto *et al.*, 2005).

One last analysis was performed with the sequences to all family to infer a species tree to Characidae using the method called *Beast under the multispecies coalescent model using a new algorithm proposed by Heled & Drummond (2009) and implemented in Beast 1.5.3 . It requires the combined information from multiple genes, adding the constrain that the time of common ancestry in each gene cannot be more recent than divergence time of the species (Heled & Drummond, 2009).

Results

Nucleotides

After the alignment, the Sia gene consisted of 400 base pairs (bp), the Trop with 192bp, 16S with 423bp and mitochondrial coding genes concatenated with 1704bp (COI = 639bp/ND2 = 1065bp). The first set of analyses comprised solely the sequences inferred by this study and we reached a total of 2719pb and 39 final taxa representing 10 species of characids. Of this total, 942bp were variable and 873 parsimony informative. The nucleotide diversity (π) varied from 0.07 to 0.01. The remaining basics statistics for each partition are accessible in Table 5.

For analyses regarding all the family Characidae a total of 1003bp were used with the genes Sia, Trop and 16S, with 205bp variable and 157bp parsimony informative sites. A total of 70 final taxa representing 41 species of the family were employed. In this case, the nucleotide diversity (π) varied from 0.05 to 0.03 and the mean distance between sequences was 0.036 (Table 5).

The Trop intron region varied in length from 300pb to 1200pb (Calcagnotto *et al.*, 2005) because of this great difference in the indels length and the difficulty in aligning them, this region was deleted of the sequences. In the 16S the loops corresponding to the secondary structure proposed by Ortí & Meyer (1997) were excluded from the analyses. With the nuclear genes, we used the IUPAC-IUB ambiguity code for the cases that single nucleotide polymorphisms were detected in some individuals.

Test of Putative Related Taxa

The three methods of phylogeny inference utilized in this set of analyses have shown similar tree topologies (Fig. 1-3). For some clades, however, low support values were inferred mainly in the shallow branches. The bootstrap values above 50 are shown in the nodes and the posterior probability over 0.7. Lower values were omitted. The maximum parsimony analysis found three trees with length= 1651, CI= 77 and RI=90.

149 The monophyly of the genus *Hollandichthys* was tested and, in all trees inferred, the
150 representatives of the genus have proved to share a common ancestral (Fig. 1-3). The genus
151 *Rachoviscus* was inferred to be the sister-group of *Hollandichthys* in all methods (Fig. 1-3) and these
152 two genera (*Hollandichthys + Rachoviscus*) form a clade with high support. A more inclusive clade
153 including (*Hollandichthys + Rachoviscus)* and other three taxa: *Pseudochalceus, Hyphessobrycon*
154 *uruguayensis* and *Nematocharax,* shows good support, proving they are more closely related than
155 other taxa tested. It is possible to infer *Nematocharax* and *Hyphessobrycon uruguayensis* as being
156 more closely related and *Pseudochalceus* position as still unclear inside the group.

157 The remaining taxa added to these analyses, as *Lophiobrycon, Mimagoniates* and
158 *Aphyocharax,* are distantly related to the *Hollandichthys´*s group. A clade with *Mimagoniates* and
159 *Lophiobrycon* are supported by high values of bootstrap/posterior probability, corresponding to the
160 Glandulocaudinae.

161 *Position in the Family Characidae*

162 Our maximum parsimony analysis (Fig. 4) has reached a similar topology as the Calcagnotto
163 *et al.* (2005), with small differences in the position of some taxa, probably due to the addition of
164 new species and the different root applied. The parsimony searches found 186 trees with a length
165 of 588, CI=45 and RI=74. The trees for each methodology are presented in figures 4 to 6. The
166 bootstrap values higher than 50 are shown in the nodes and the posterior probability higher than
167 0.7. Lower values were omitted.

168 In all trees it is possible to distinguish three major clades quite well supported. The first one
169 corresponds to the clade "A" (Malabarba & Weitzman, 2003), and the second and third clades
170 agree with clades "B" and "C" proposed by Javonillo *et al.* (2010). We follow this proposal (clades
171 "A", "B" and "C") since our results maintained the same pattern. The basal characids which are
172 always pointed as components of a single or multiple and successive sister-groups of the other

three clades (Malabarba & Weitzman, 2003; Calcagnotto *et al.*, 2005; Mirande, 2009) formed a unique clade in our analyses.

The relationships between clades “A”, “B” and “C” are very unclear and change according to the method. In the parsimony tree the clade “A” is sister-group of (“B”+“C”). To the likelihood, the clade “B” is sister-group of (“A”+“C”) and to the Bayesian analysis clade “C” is sister-group of (“A”+“B”). This incongruence show how contradictory the methods of molecular inference can be dealing with our data set at this level of analyses.

The genus *Hollandichthys* is positioned in the clade “C” along with *Rachoviscus.* The internal relationships of clade “C” are very plastic, depending on the method used in the tree inference. In the parsimony analysis (Fig. 4), this clade forms a big polytomy with only two clades well supported: *Rachoviscus + Hollandichthys* and *Astyanax scabripinnis + A. bimaculatus.* Because of this, the sister-group more related to *Hollandichthys + Rachoviscus* remains uncertain. The position of the genus *Pseudochalceus* is still unknown because of the incongruence inside this clade. In the parsimony tree (Fig. 4) this genus appears in a polytomy. In the likelihood tree (Fig. 5) it is positioned with *Hemigrammus bleheri* and some species of *Astyanax,* while in the Bayesian approach (Fig. 6) this genus is inferred to be the sister-group of the clade formed by *Hollandichthys* and *Rachoviscus*. Nevertheless, in the likelihood and Bayesian the low support keeps the position of *Pseudochalceus* still unclear.

Some groups can be defined by the likelihood (Fig. 5) and Bayesian (Fig. 6) trees with high support. In both trees *Nematocharax venustus* grouped with *Hemigrammus erythrozonus* and *Hyphessobrycon uruguayensis* with *Hemigrammus rodwayi*, these point both species as not closely related to *Hollandichthys* and the genus *Hemigrammus* as paraphyletic.

The genus *Lophiobrycon* is positioned inside clade “A” and just for the Bayesian approach it appears along with *Hemibrycon beni* with a relatively well value of posterior probability (0.8).

Species Tree

198 The topology proposed by this methodology (Fig. 7) is very similar to the gene tree
199 proposed by the Bayesian analysis (Fig. 6). The order between the major clades is the same (C +
200 (A+B)), but some differences are found. In this approach, the genus *Hollandichthys* is positioned as
201 sister-group of R*achoviscus graciliceps* making *Rachoviscus* paraphyletic*.* The species of the genus
202 *Astyanax* available in the data of Calcagnotto are positioned along with *Hemigrammus bleheri*
203 forming a group with (*Rachoviscus + Hollandichthys).* The genus *Lophiobrycon* is pointed as sister-
204 group of *Mimagoniates* clade. But the relationships that were not congruent among the gene trees
205 methodologies remain unclear by this methodology of inferring the species tree.

206

207 **Discussion**

208 Both analyses, the first performed with Sia, Trop, 16S and mitochondrial coding genes
209 including characid genera previously considered as possibly related to *Hollandichthys,* and the
210 second performed with genes Sia, Trop and 16S and including a larger number of characid
211 representatives, strongly support *Rachoviscus* as sister-group of *Hollandichthys*, corroborating the
212 morphological hypotheses of Quevedo (2006) and the molecular hypothesis of Javonillo *et al.*
213 (2010), and refuting other hypotheses presented above (Bertaco, 2003; Mirande, 2009). Quevedo
214 (2006) proposed a synapomorphy for these two genera: a body cavity encompassing the anus and
215 urogenital opening. Species of the two genera are inseminating and this cavity was inferred to play
216 some role in the spermatozoid transference from males to females.

217 The three large internal clades of Characidae previously proposed (Malabarba & Weitzman,
218 2003; Javonillo *et al.*, 2010) are clearly reconstructed in this study and seems to be valid taxonomic
219 units inside the family. Such a definition is useful in the clearance of the chaotic taxonomic status of
220 several intrafamilial taxa, and in furnishing guidelines in the choice of taxa in future phylogenetic
221 studies. The genera *Hollandichthys* and *Rachoviscus* are positioned in the clade "C" corroborating
222 the proposal by Javonillo *et al*. (2010). The addition of five *incertae sedis* (*Hollandichthys,*

Rachoviscus, Pseudochalceus, Nematocharax and *Hyphessobrycon uruguayensis*) inside the clade "C" is extremely helpful to clarify their relationships with other taxa inside this family (Weitzman & Malabarba, 1998).

Our results further corroborate the hypothesis that insemination evolved independently at least three times in Characidae (Malabarba, 1998), once in Clade C (*Hollandichthys + Rachoviscus* clade), once in Clade A (Stevardiinae and Glandulocaudinae - Menezes *et al.*, 2008), and once in clade B (in the tribe Compsurini of the Cheirodontinae, - Oliveira, 2007).

Although *Pseudochalceus, Nematocharax*, and *Hyphessobrycon uruguayensis* clustered together with *Hollandichthys* + *Rachoviscus* in the first analyses, they fail to group to these two genera in a more inclusive set of analyses including a larger number of characid taxa. Even though all genera belong to Clade C, their relationships to other genera are weakly supported, changing their position in tree topology according to the method of analyses. The low support values inside the clade "C" and the discordance of the position of some taxa inside this clade (Fig 4, 5, 6) was already noted in other molecular studies (Calcagnotto *et al.*, 2005; Javonillo *et al.*, 2010). This possibly occurs due to the large diversity inside this clade comparing to clades "A" and "B", and to the small number of representatives used so far in the phylogeny (Javonillo *et al.*, 2010).

Pseudochalceus is morphologically very similar to *Hollandichthys* in such a way they were once considered a single genus (Schultz, 1966) or sister taxa (Bertaco, 2003), but such similarities seems to represent convergence or the common sharing of plesiomorphic traits among them. A better resolved phylogeny is needed to support one of the hypotheses. Although not as morphologically similar to *Hollandichthys* as *Pseudochalceus*, the same is true for *Hyphessobrycon uruguayensis*, which has longitudinal black stripes along the body and this is the reason to doubts in the identification of this species with *Hollandichthys* and *Pseudochalceus.*

246 The tentative proposal of Mirande (2009) grouping *Rachoviscus* (not included in his
247 analysis) to the Aphyocharacinae is refuted, since *Rachoviscus* appears distant from *Aphyocharax*,
248 housed in Clade B.

249 Mirande (2009) found the genus *Nematocharax,* listed by him inside Rhoadsiinae, varied its
250 position in the cladogram in different analyses. Similarly, his clade formed by *Hollandichthys +*
251 *Pseudochalceus*, listed inside the *Bramocharax* clade, showed an unstable position between other
252 taxa. Although our analysis lacks representatives of *Bramocharax* or Rhoadsiinae, we always found
253 *Nematocharax*, *Pseudochalceus*, and *Hollandichthys* grouped to clade C taxa, showing a better
254 resolution than that of Mirande (2009). *Nematocharax* and *Pseudochalceus* were absent in Javonillo
255 *et al.* (2010) analyses.

256 *Lophiobrycon weitzmani* is here positioned in the clade known as clade "A" (Malabarba &
257 Weitzman, 2003) and the similar expansions in the pectoral fin rays found in this species and
258 *Rachoviscus* does not seems to be homologous just as inferred by Quevedo (2006).

259 Inside *Hollandichthys*, the small universe of sampling was enough to demonstrate the
260 diversity hidden behind a monotypic genus. The fourteen sequenced specimens resulted arranged
261 in two very well supported clades, suggesting that the several populations of *Hollandichthys* may
262 show a hierarchic pattern of relationships. It makes also possible that the two nominal species
263 available to the genus (*H. perstriatus* and *H. affinis*) and currently considered junior synonyms of *H.*
264 *multifasciatus*, become valid taxonomic units after a review of the *Hollandichthys multifasciatus*
265 complex (Bertaco, 2003). Relationships among several *Hollandichthys* populations are further
266 explored in Thomaz *et al*. (this volume).

267 The species tree approach is an inference that try to reduce potential sources of
268 discrepancy between gene trees and species tree as horizontal transfer, lineage sorting and gene
269 duplication/extinction (van der Niet & Peter Linder, 2008). Because of these events a gene history
270 can be different from the species phylogeny, like the *Rachoviscus* genus being paraphyletic in the

species tree. To the case of Characidae the inferred species tree (Fig. 7) reported a very similar to the Bayesian gene tree (Fig. 6). The small differences found are probably due to the complexity of the relationships inside the family and this approach is especially good for problematic with closely related species or species with large population sizes (Heled & Drummond, 2009). Others studies inferring species tree with a taxa group more related can better test this new methodology but, with no doubt, this multispecies coalescent method represents a step toward the unification of molecular systematics and phylogeography.

Our molecular tentative to position the genus *Hollandichthys* inside Characidae rises with the problematic relationships among the groups inside this family: the large and unknown diversity and the different levels of divergence along the time. Some recent studies are contributing for a better comprehension of this complex group (Calcagnotto *et al.*, 2005; Mirande, 2009; Javonillo *et al.*, 2010). Besides that, our results were efficient in determining *Rachoviscus* as the sister-group of *Hollandichthys* and inferring the position of some *incertae sedis* genera. With an increase of the number of studies dedicated to solve small phylogeny problems inside characids, like the presented here, it will be possible to better understand the relationships in higher levels.

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Table 1. Tissue samples, voucher specimens and sequences used to the phylogenetic inference. UFRGS = Universidade Federal do Rio Grande do Sul, MUSM = Museo de La Universidad de San Marcos , MCP = Museu de Ciências e Tecnologia PUCRS, MHNCI = Museu de História Natural Capão da Imbuia, MNRJ = Museu Nacional Rio de Janeiro, STRI = Smithsonian Tropical Research Institute.

Taxon	Voucher	Sequence				
		Sia	Trop	16S	COI	ND2
Aphyocharax anisitsi	UFRGS 9360 (TEC43)	✓	✓	✓	✓	×
Aphyocharax anisitsi	UFRGS 9361 (TEC44)	✓	✓	✓	✓	×
Astyanacinus multidens	MUSM 35742 A	×	×	✓	×	×
Bryconops sp.	MCP 35087	✓	×	✓	✓	×
Bryconops sp.	MCP 35087	×	×	✓	×	×
Bryconops sp.	MCP 35087	×	×	✓	✓	×
Hollandichthys multifasciatus	UFRGS 11793 (TEC842 E)	✓	✓	✓	✓	✓
Hollandichthys multifasciatus	UFRGS 11792 (TEC841 B)	✓	✓	✓	✓	✓
Hollandichthys multifasciatus	MCP 38333 (C)	✓	✓	✓	✓	✓
Hollandichthys multifasciatus	UFRGS 10579 (TEC 105)	✓	✓	✓	✓	✓
Hollandichthys multifasciatus	MHNCI	✓	✓	✓	✓	✓
Hollandichthys multifasciatus	MCP 30557	✓	✓	✓	✓	✓
Hollandichthys multifasciatus	UFRGS 11782 (TEC 739 A)	✓	×	✓	✓	✓
Hollandichthys multifasciatus	UFRGS 11784 (TEC 722 H)	✓	✓	✓	✓	✓
Hollandichthys multifasciatus	UFRGS 11785 (TEC 820 E)	✓	✓	✓	✓	✓
Hollandichthys multifasciatus	UFRGS 11787 (TEC 827 C)	×	✓	✓	✓	✓
Hollandichthys multifasciatus	UFRGS 11771 (TEC 888 A)	×	×	✓	✓	✓
Hollandichthys multifasciatus	UFRGS 11788 (TEC 859 A)	✓	✓	✓	✓	✓
Hollandichthys multifasciatus	UFRGS 11773 (TEC 897 C)	✓	×	✓	✓	✓
Hollandichthys multifasciatus	UFRGS 11776 (TEC 909 D)	✓	✓	✓	✓	✓
Hyphessobrycon uruguayensis	UFRGS 10564 (TEC 194 A)	✓	✓	✓	×	×
Hyphessobrycon uruguayensis	UFRGS 10564 (TEC 194 B)	✓	✓	✓	✓	×
Hyphessobrycon uruguayensis	UFRGS 11129 (TEC 325 A)	✓	✓	✓	×	×
Hyphessobrycon uruguayensis	UFRGS 11129 (TEC 325 B)	✓	✓	✓	✓	×
Lophiobrycon weitzmani	MNRJ 31664	✓	✓	✓	×	✓
Mimagoniates microlepis	MCP 31800	✓	×	✓	×	×
Mimagoniates microlepis	MCP 31800	✓	×	✓	×	✓
Nematocharax venustus	UFRGS 11649 (TEC 1121 A)	✓	✓	✓	✓	×
Nematocharax venustus	UFRGS 11649 (TEC 1121 B)	✓	✓	✓	✓	×
Nematocharax venustus	UFRGS 11649 (TEC 1121 C)	✓	✓	✓	✓	×
Nematocharax venustus	UFRGS 11655 (TEC 1132 A)	✓	✓	✓	✓	×
Nematocharax venustus	UFRGS 11655 (TEC 1132 B)	✓	✓	✓	✓	×
Pseudochalceus kyburzi	STRI-9309	×	✓	✓	✓	✓
Pseudochalceus kyburzi	STRI-9310	✓	×	✓	×	✓
Pseudochalceus kyburzi	STRI-9311	✓	✓	✓	×	✓
Rachoviscus crassiceps	UFRGS 9356 (TEC 102)	✓	✓	✓	✓	✓
Rachoviscus crassiceps	UFRGS 9356 (TEC 103)	✓	✓	✓	✓	✓
Rachoviscus graciliceps	UFRGS 11116 (TEC 1117 A)	✓	✓	✓	✓	✓
Rachoviscus graciliceps	UFRGS 11116 (TEC 1117 B)	✓	✓	✓	✓	✓
Rachoviscus graciliceps	UFRGS 11116 (TEC 1117 C)	✓	✓	✓	✓	✓

Table 2. Primers used in this study, with their sequence and source.

Gene	Primer sequence (liste from 5' to 3')	Source
16S ar	ACG CCT GTT TAT CAA AAA CAT	Palumbi (1996)
16S br	CCG GTC TGA ACT CAG ATC ACG T	Palumbi (1996)
sia/T3b	ATT AAC CCT CAC TAA AGT CGA GTG CCC CGT GTG YTT YGA YTA	Calcagnotto *et al.*(2005)
sia/T7b	AAT ACG ACT CAC TAT AGG AAG TGG AAG CCG AAG CAG SWY TGC ATC AT	Calcagnotto *et al*. (2005)
TROP F	GAG TTG GAT CGG GCT CAG GA GCG	Friesen *et al.* (1999)
TROP R	CGG TCA GCC TCT TCA GCA ATG TGC TT	Friesen *et al.* (1999)
COI-H2198	TAA AcT TcA ggg TgA ccA AAA AAT cA	Herbert *et al.* (2003)
COI-L1490	ggT cAA cAA ATc ATA AAg ATA TTg g	Herbert *et al.* (2003)
ND2-L5216	GGC CCA TAC CCC GRA AAT G	Sorenson (2003)
ND2-H6313	ACT CTT RTT TAA GGC TTT GAA GGC	Sorenson (2003)

Table 3. PCR conditions.

Primer	Desnaturation	Cycles	Extension
16S	95°C/10'	35x 95°C/30", 48°C/30", 72°C/45"	72°C/7'
SIA	95°C/5'	35x 95°C/30", 60°C/30", 72°C/45"	72°C/7'
TROP	95°C/10'	35x 95°C/30", 60°C/30", 72°C/45"	72°C/7'
COI	96°C/1'	40x 94°C/30", 50°C/20", 48°C/5", 46°C/5", 44°C/5", 42°C/5", 40°C/20", 72°C/1'	72°C/3'
ND2	94°C/4'	9x 94°C/30", 57°C-1°C/cycle/1', 72°C/1'30", 40x 94°C/30", 47°C/1', 72°C/1'30"	72°C/5'

Table 4. Sequences of Calcagnotto *et al.* (2005) added to the analyses.

Taxon	GenBank Accession Number		
	16S	Sia	Trop
Acestrorhynchus sp.	AY787956	AY790014	AY817181
Acestrorhynchus cf. nasutus	-	AY790015	AY817182
Aphyocheirodon sp.	AY787966	AY790025	-
Astyanacinus sp.	AY787969	AY790028	AY817190
Astyanacinus sp.	AY787987	AY790046	AY817209
Astyanax bimaculatus	AY787955	AY790013	AY817180
Astyanax scabripinnis	AY787967	AY790026	AY817188
Brycon sp.	AY787982	AY790041	AY817204
Brycon hilarii	AY787976	AY790035	AY817198
Bryconamericus diaphanus.	AY787984	AY790043	AY817206
Bryconops sp.	AY787985	AY790044	AY817207
Chalceus macrolepidotus	AY787999	AY790058	AY817217
Chalceus erythrurus	AY787990	AY790049	AY817211
Cheirodon sp.	AY787995	AY790054	-
Cheirodontops sp.	AY787996	AY790055	-
Creagrutus sp.	AY788001	AY790060	AY817219
Exodon paradoxus	AY788013	AY790072	AY817227
Gephyrocharax sp.	AY788014	AY790073	AY817228
Hemibrycon cf. beni	AY788020	AY790079	AY817234
Hemigrammus bleheri	AY788017	AY790076	AY817231
Hemigrammus erythrozonus	AY788023	AY790082	AY817236
Hemigrammus rodwayi	AY788034	AY790092	AY817245
Hyphessobrycon eques	AY788022	AY790081	AY817235
Inpaichthys kerri	AY788039	AY790097	AY817248
Knodus sp.	AY788041	AY790099	AY817249
Moenkhausia sanctaefilomenae	AY788054	AY790112	AY817261
Mimagoniates lateralis	AY788051	AY790109	AY817259
Prodontocharax sp.	AY788064	AY790122	-
Roeboides sp.	AY787994	AY790053	AY817214
Salminus brasiliensis	AY788080	AY790137	AY817282
Triportheus angulatus	AY788082	AY790139	AY817283

Table 5. Nucleotide diversity to each dataset used in the analyses (*=COI + ND2, only samples withiut missing data; L= length; N= number of sequences; V= variable sites; S= segregating sites; P= parsimony informative sites; π= nucleotode diversity; k= average number of nucleotide differences; d= average of pairwise distance with kimura-2-parameter).

										Nucleotide frequency			
Approach	**Segments**	**L**	**N**	**V**	**S**	**P**	**π**	**k**	**d**	**T**	**C**	**A**	**G**
Related taxa	**Concatenated***	1704	36	819	319	763	0.07	75.820	0.079	0.31	0.24	0.27	0.18
	16S	423	39	61	60	61	0.039	16.621	0.042	0.24	0.23	0.28	0.25
	Sia	400	34	50	50	41	0.027	10.699	0.028	0.17	0.38	0.17	0.28
	Trop	192	30	12	10	8	0.011	2.0050	0.011	0.14	0.19	0.28	0.39
Family	**16S**	423	60	101	95	79	0.050	20.541	0.052	0.24	0.23	0.28	0.25
	Sia	400	58	80	61	61	0.027	9.5610	0.028	0.17	0.37	0.18	0.28
	Trop	180	50	24	19	17	0.027	3.5550	0.029	0.14	0.18	0.29	0.39

Figure legends:

Fig. 1. Maximum parsimony tree inferred with Sia, Trop, 16S, COI and ND2 genes within taxa hypothesized as related to *Hollandichthys* [gray square]. Bootstrap values up to 50 are shown in the nodes.

Fig. 2. Maximum likelihood inference inferred with Sia, Trop, 16S, COI and ND2 genes within taxa hypothesized as related to *Hollandichthys* [gray square]. Bootstrap values up to 50 are shown in the nodes.

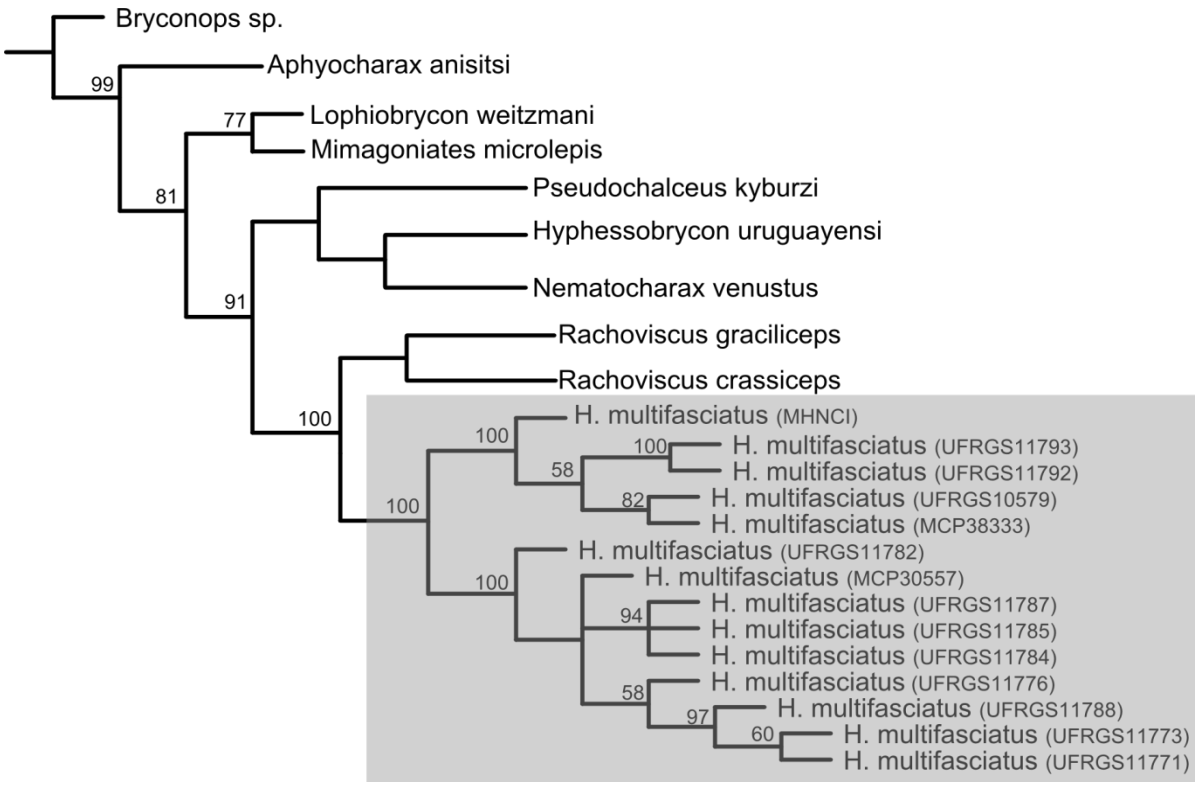
Fig. 3. Bayesian tree inferred with Sia, Trop, 16S, COI and ND2 genes to the taxa hypothesized as related to *Hollandichthys* [gray square]. Posterior probability values up to 0.7 are shown in the nodes.

Fig. 4. Phylogenetic inference to Characidae family with maximum parsimony analysis using the genes Sia, Trop and 16S. The number in the nodes represents the bootstrap values up to 50.

Fig. 5. Maximum likelihood tree with Sia, Trop and 16S genes to the family Characidae. The number in the nodes represents the bootstrap values up to 50.

Fig. 6. Bayesian tree with Sia, Trop and 16S genes to Characidae family with posterior probability up to 0.7 in the nodes.

Fig. 7. Species tree proposed to the family Characidae with the genes Sia, Trops and 16S. Posterior probability up to 0.7 in the nodes.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77
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Pseudochalceus kyburzi
Hyphessobrycon uruguayensi
Nematocharax venustus
91
Rachoviscus graciliceps
Rachoviscus crassiceps
100
H. multifasciatus (MHNCI)
100
100
H. multifasciatus (UFRGS11793)
H. multifasciatus (UFRGS11792)
58
82
H. multifasciatus (UFRGS10579)
H. multifasciatus (MCP38333)
100
H. multifasciatus (UFRGS11782)
100
H. multifasciatus (MCP30557)
H. multifasciatus (UFRGS11787)
94
H. multifasciatus (UFRGS11785)
H. multifasciatus (UFRGS11784)
H. multifasciatus (UFRGS11776)
58
97
H. multifasciatus (UFRGS11788)
60
H. multifasciatus (UFRGS11773)
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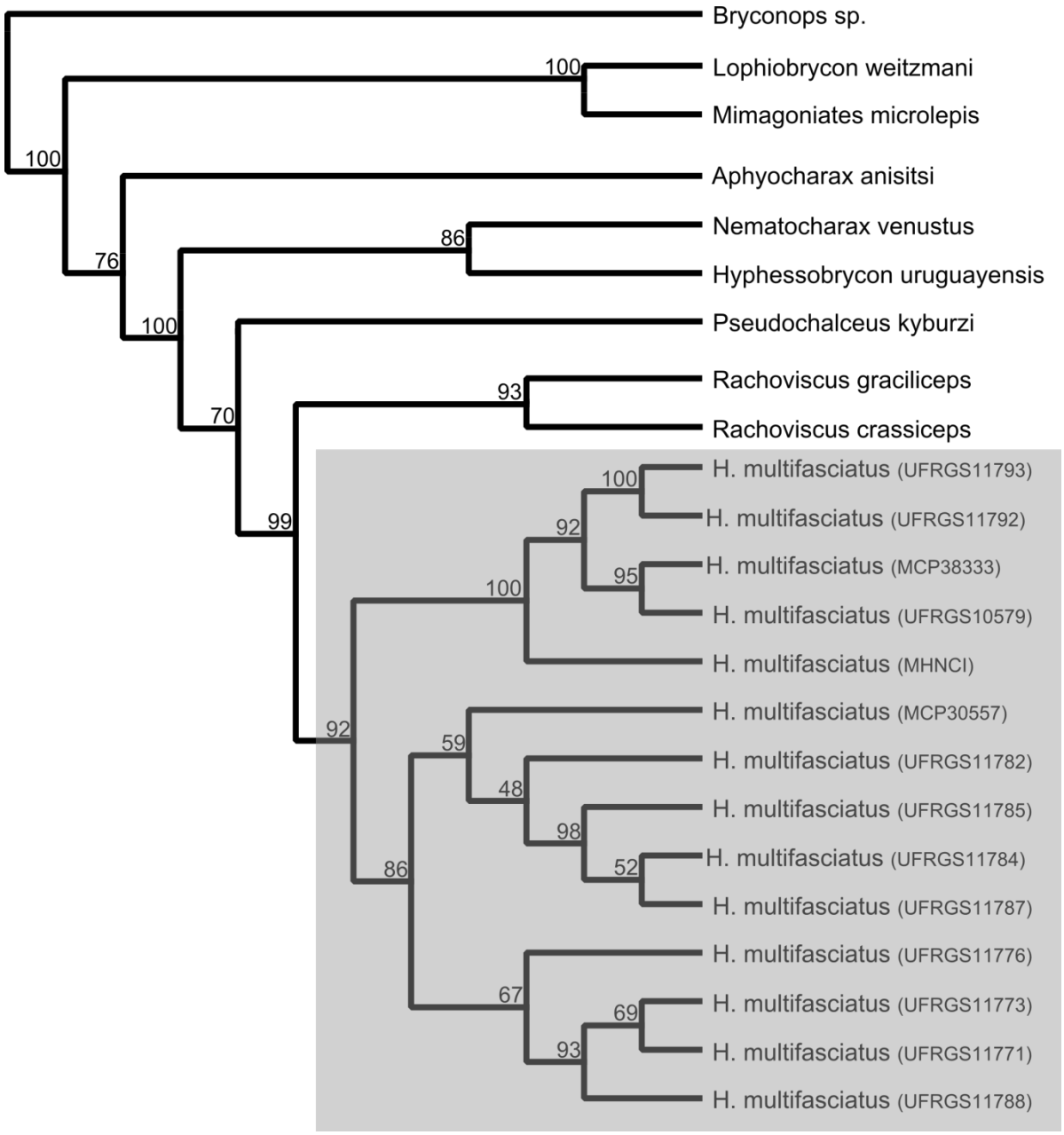
Fig. 1.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H. multifasciatus (UFRGS11793)
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H. multifasciatus (UFRGS11787)
H. multifasciatus (UFRGS11776)
H. multifasciatus (UFRGS11773)
H. multifasciatus (UFRGS11771)
H. multifasciatus (UFRGS11788)
100
100
100
86
76
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100
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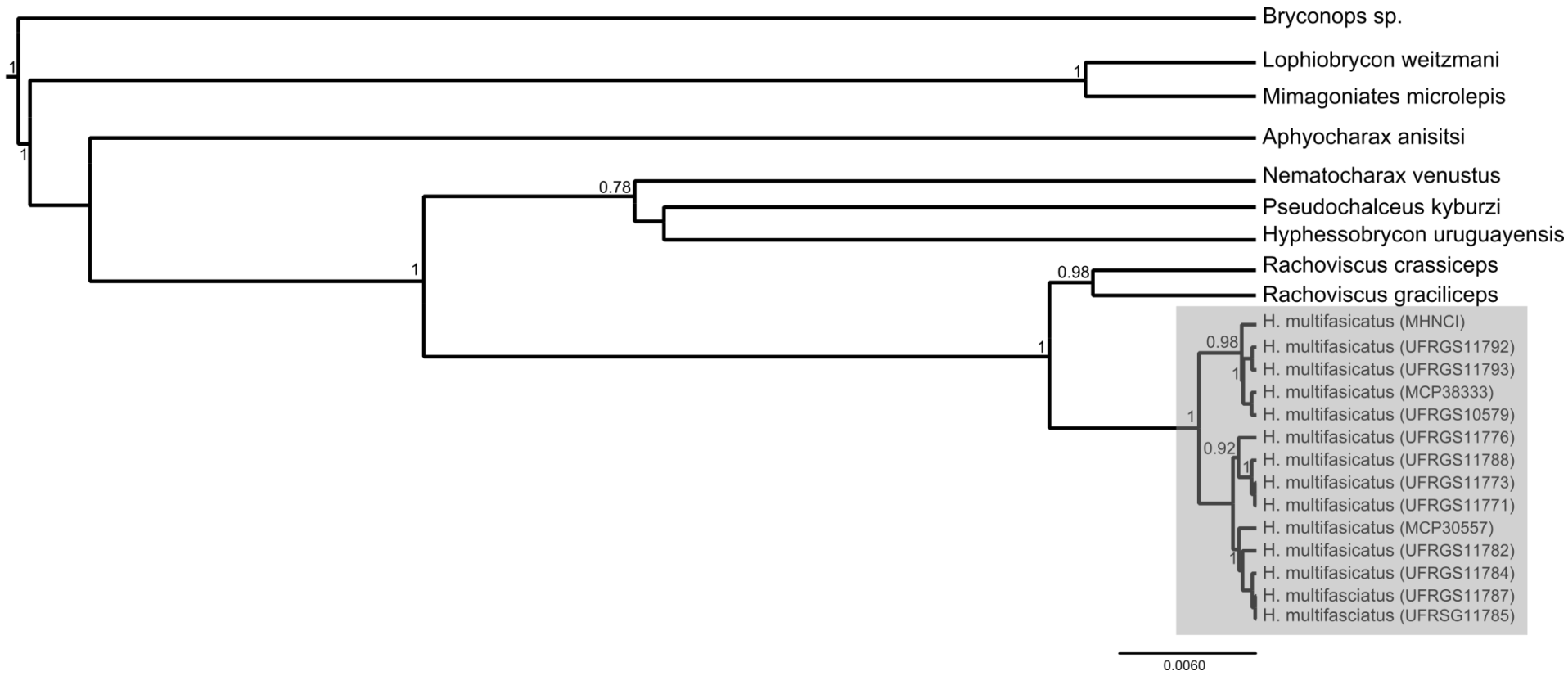
59
48
98
52
86
67
69
93

Fig. 2.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Rachoviscus graciliceps
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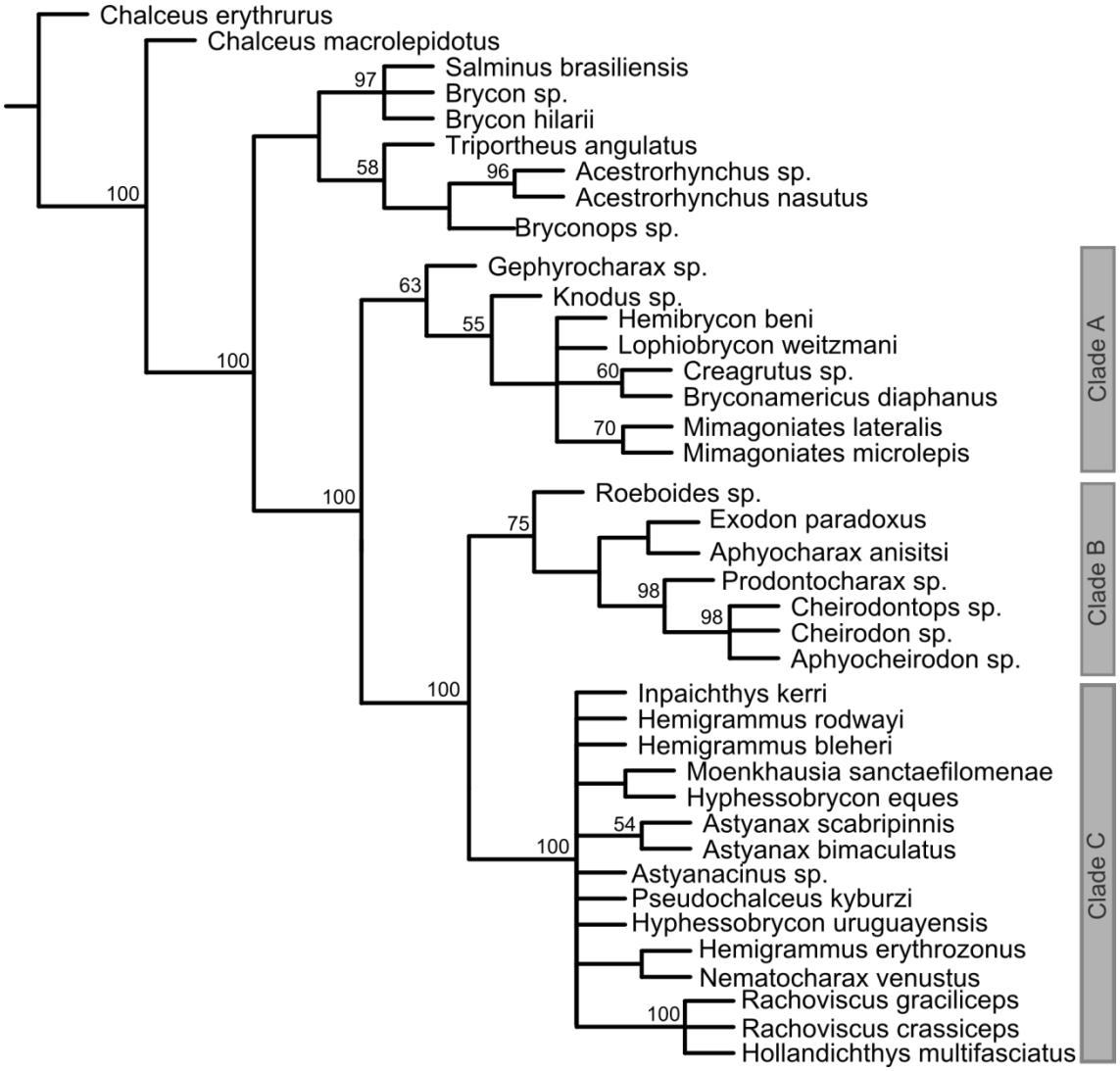
Fig. 3.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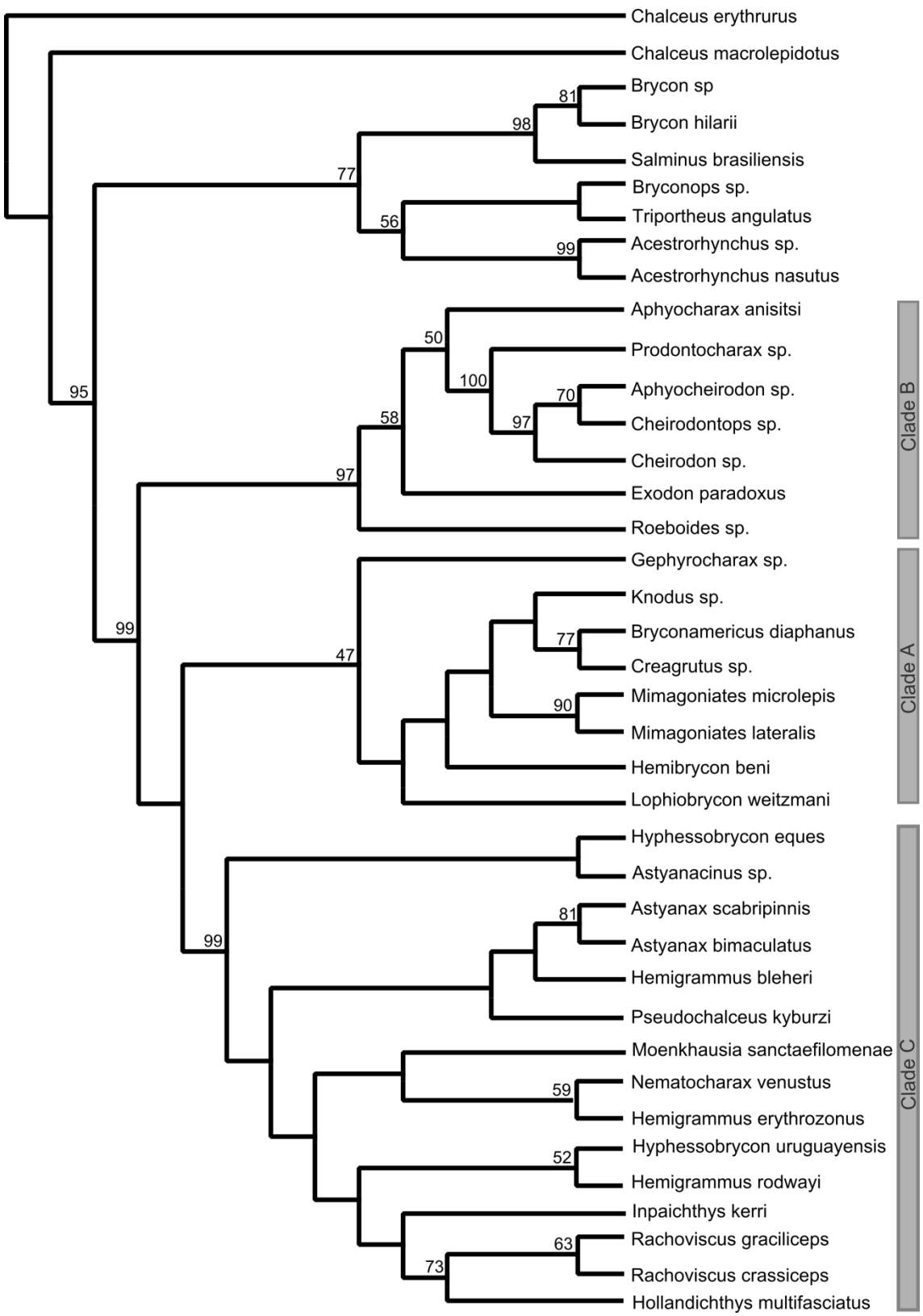
Bryconops sp.
Gephyrocharax sp.
Knodus sp.
Hemibrycon beni
Lophiobrycon weitzmani
Creagrutus sp.
Bryconamericus diaphanus
Mimagoniates lateralis
Mimagoniates microlepis
Roeboides sp.
Exodon paradoxus
Aphyocharax anisitsi
Prodontocharax sp.
Cheirodontops sp.
Cheirodon sp.
Aphyocheirodon sp.
Inpaichthys kerri
Hemigrammus rodwayi
Hemigrammus bleheri
Moenkhausia sanctaefilomenae
Hyphessobrycon eques
Astyanax scabripinnis
Astyanax bimaculatus
Astyanacinus sp.
Pseudochalceus kyburzi
Hyphessobrycon uruguayensis
Hemigrammus erythrozonus
Nematocharax venustus
Rachoviscus graciliceps
Rachoviscus crassiceps
Hollandichthys multifasciatus
97
58
96
100
100
63
55
60
70
100
75
98
98
100
54
100
100
Clade A
Clade B
Clade C

Fig. 4.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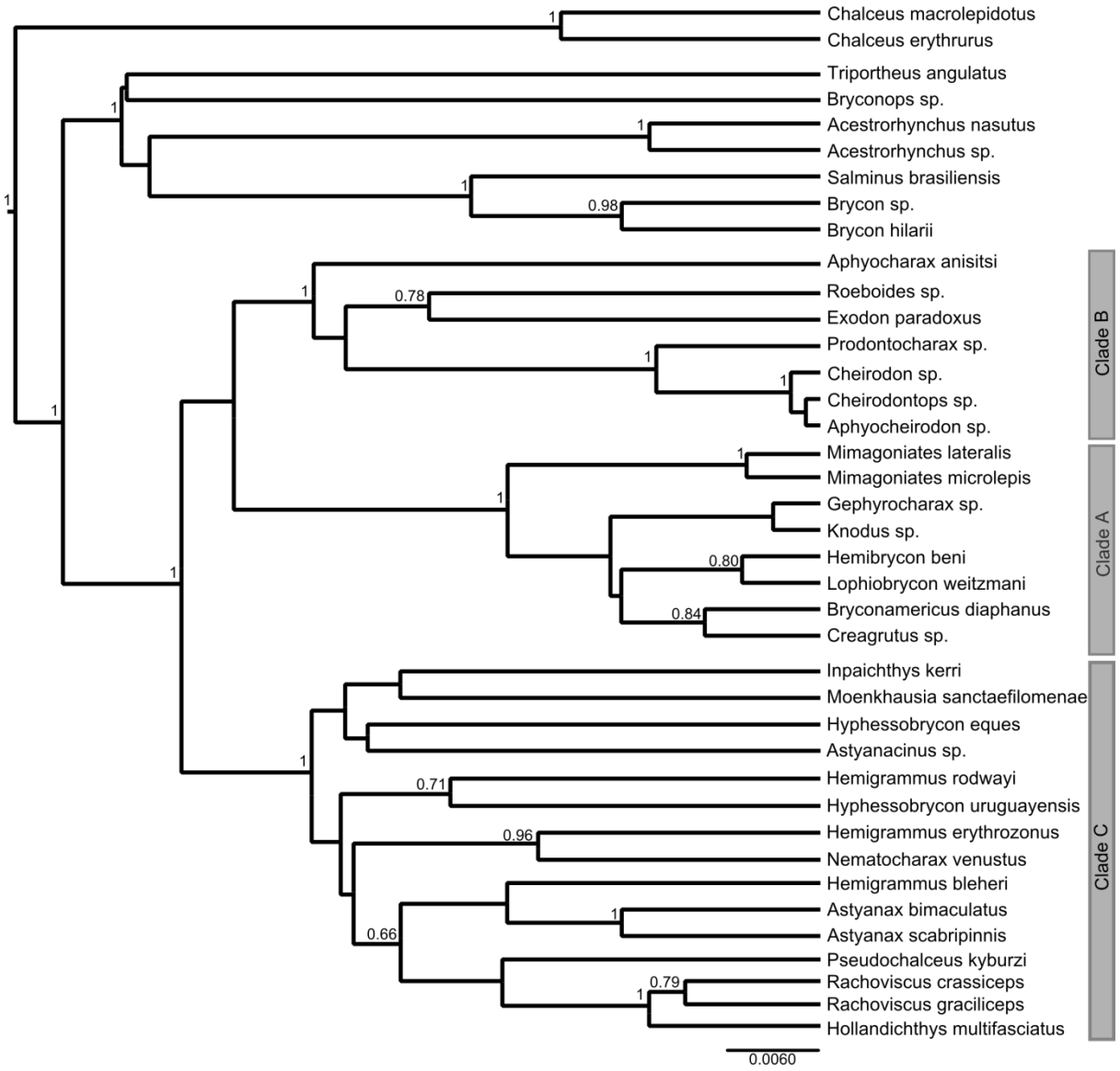
Hyphessobrycon uruguayensis
Hemigrammus rodwayi
Inpaichthys kerri
Rachoviscus graciliceps
Rachoviscus crassiceps
Hollandichthys multifasciatus
81
98
77
56
99
50
100
70
97
58
97
95
99
47
77
90
99
81
59
52
63
73
Clade B
Clade A
Clade C

Fig. 5.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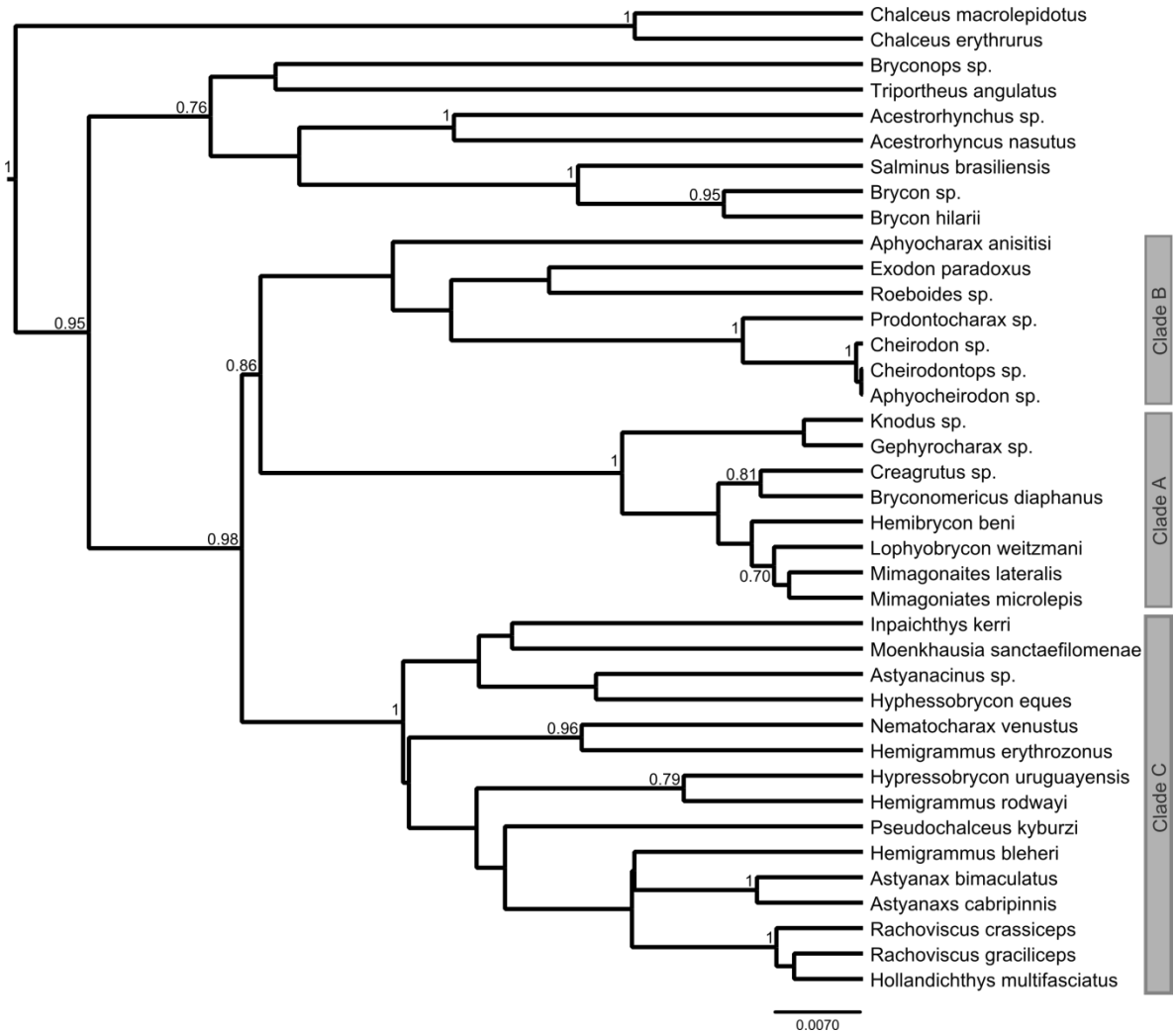
Astyanax scabripinnis
Pseudochalceus kyburzi
Rachoviscus crassiceps
Rachoviscus graciliceps
Hollandichthys multifasciatus
0.0060
Clade B
Clade A
Clade C

Fig. 6.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Hypressobrycon uruguayensis
Hemigrammus rodwayi
Pseudochalceus kyburzi
Hemigrammus bleheri
Astyanax bimaculatus
Astyanaxs cabripinnis
Rachoviscus crassiceps
Rachoviscus graciliceps
Hollandichthys multifasciatus
Clade B
Clade A
Clade C
0.0070

Fig. 7.

Capítulo 2

Phylogeography of the Species Complex

Hollandichthys multifasciatus Eigenmann & Norris, 1900 (Teleostei: Characidae)

In Brazilian Coastal Drainages

(Artigo a ser submetido ao periódico *Molecular Phylogenetics and Evolution*)

Phylogeography of the Species Complex *Hollandichthys multifasciatus* Eigenmann & Norris, 1900 (Teleostei: Characidae) in Brazilian Coastal Drainages

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Abstract

The Brazilian Atlantic coast corresponds to a system of isolated hydrographic basins, including important ecosystems as the Atlantic Forest. It is constantly under geological and climatic changes, especially because the glaciation and inter-glaciation periods. The ichthyofauna in this region suffered all these pressures, showing an extremely high endemism. *Hollandichthys multifasciatus* represents one of these cases and is known to have a great diversity behind this single name with eight morphotypes proposed. Results inferred with 201 samples of two mtDNA (COI and ND2) and more 40 sequences of three more conserved genes (16S, Trop, Sia) from 20 populations, proposed a consistent separation of two groups - North and South - in the Paranaguá estuary, dating from 1.8 Mya. The populations were consistently arranged into five groups that better fits to our molecular and geographic data set. In a general manner, the evolutionary history inferred for this genus is strictly correlated with the climatic changes that caused impact in the Atlantic Forest area. A bottleneck would have happened during the last maximum glacial, followed by a populational growth that coincides with the expansion of the forest - from small isolated areas to a large continuum.

Key-word: Atlantic Forest, Quaternary, endemism, speciation, morphotypes.

1. Introduction

The Brazilian Atlantic coast, with an extension of 9200km, contains one of the most important world ecosystems, the Atlantic Forest. The region is constantly under geological and climatic pressures, especially because the sea level fluctuations between the glaciation and inter-glaciation periods, and also processes linked with the global tectonic, like the uplift of crystalline shield resulting in the formation of Serra do Mar (Villwock et al., 2005). All these geological events started with the Gondwana break down. The rivers that constitute this region form a series of isolated hydrographic basins, known as Coastal Drainages of Eastern Brazil (Ribeiro, 2006). These smaller isolated basins are separated from the large inland drainages by the scarped, mountainous landscapes of the eastern margin of the Brazilian crystalline shield (Ribeiro, 2006).

The freshwater fishes are direct dependent of connections between river basins for their dispersal. The history of river drainages interconnections reflects the underlying geological development of landscapes (Bermingham and Martin, 1998). Since the Atlantic Coastal drainages correspond to a system of isolated creeks, it is expected that the living ichthyofauna in this region shares the same pattern, reflecting the geological history. Traditionally, this system of drainages has been recognized as a very distinct area in term of ichthyofauna (Ribeiro et al., 2006) because of the high number of endemic taxa. Bizerril (1994) calculated the endemicity rate of 285 species in 96 genera of fish in the coastal drainages. Of this total, 23.4% of the genera and 95% of the species were reported as endemic to this region. This extremely high level of endemism in species diversity is strictly related to a variety of continuing processes and unique events that created new freshwater habitats and fragmented the existing ones, providing opportunities for allopatric divergence. Lundberg (1998) proposed that the sea level fluctuations alternatively caused some coalescence of coastal rivers (during low stands) and truncations and isolation of rivers (during high stands) allowing isolation and temporary connections between ichthyofauna from different watersheds. Innumerous events of headwater capture are reported to this region, especially

between the Tietê and Paraíba do Sul rivers, which originally drained toward the Atlantic (Ab'Sáber, 1957; Malabarba, 1998). The ichthyofauna shared by these systems of drainages, like the genera *Hollandichthys* Eigenmann, 1909 and *Spintherobolus* Eigenmann, 1911, both present in the coastal drainages and adjacent uplands of the Tietê drainage (Langeani, 1989; Weitzman and Malabarba, 1999), agree with this theory of multiple headwaters captures (Ribeiro, 2006).

The genus *Hollandichthys* is a Neotropical freshwater fish, popularly known as "striped-tetras" ("lambari-listrado"). It constitutes one of the endemic genera in the Coastal Drainages of Brazil, ranging from the north portion of the Rio Grande do Sul state to the south of Rio de Janeiro state (Fig. 1). In this distributional range, the genus is found only in small creeks in the coastal drainages or in islands near the coast, with the exception of the upper part of the Rio Tietê basin, an upper tributary of the upper Rio Paraná (Langeani, 1989). *Hollandichthys* species lives in creeks always associated with the presence of preserved Atlantic Rainforest and its diet has pronounced dependence on the resources imported from the forest, like terrestrial insects and plants that fall in the stream, indicated by the surface picking behavior (Abilhoa et al., 2009).

Phylogeography is a field inside biogeography specially focused on the principles and processes that govern the distribution of genealogical lineages, within and among related species (Avise, 2000). *Hollandichthys*, a monotypic genus with a single valid species (*Hollandichthys multifasciatus*) has been morphologically diagnosed as a species complex with eight morphotypes (Bertaco, 2003). For this reason, along with its large distribution among several isolated coastal river drainages, it constitutes an interesting organism to be studied in a phylogeographic approach, because it has to deal with a great hidden diversity which probably suffered recent vicariant events during Quaternary (Ribeiro, 2006).

Moritz (1994) proposed the "Evolutionary Significant Units"(ESU) concept, which is the premise of reciprocal monophyly for mtDNA lineages. In others words, it is necessary sufficient complementary haplotypes extinction for a species to be recovered as monophyletic. On the

other hand, Rieppel (2009) argues that reciprocal monophyly means exclusivity of relationships and that the lineages discovered in the present cannot be stable over time. Gene trees mirror species history, but the ESU concept do not take in account the possibility of gene flow and adaptive diversity (Kizirian and Donnelly, 2004). The problem in assuming the reciprocal monophyly is that rapid events of speciation are not reflected in the mtDNA lineages coalescence (Maddison and Knowles, 2006). Phylogeography can be the field to provide a bridge between micro- and macroevolution, helping to understand the demographic process in the populations, as dispersal and genetic isolation, but how to determine a phylogeny that truly represents a phylogeographical history of the studied species will still be a problem (Diniz-Filho et al., 2008).

The aim of this study is to characterize phylogeographic patterns and demographic history of *H. multifasciatus* species complex, using mitochondrial genes and a large set of samples including all the geographic range of the genus. With these approaches, we aim to compare the patterns proposed for this genus with the geological events that contribute to the actual characteristics of the coastal drainages system. Besides that, we want to propose a molecular phylogeny to this genus using mitochondrial and nuclear genes, and compare it with the morphological study, testing if the eight morphotypes proposed agree with the organism evolutionary history proposed by the molecular data.

2. Material and Methods

2.1. Taxon sampling, DNA extraction and sequencin.

Tissues samples from 201 specimens of *Hollandichthys multifasciatus* from all distributional range were obtained from fish collections and field work (Table 1). We also included 5 individuals of the genus *Rachoviscus,* pointed as sister-group of *Hollandichthys* (Thomaz et al., this volume).

74 DNA extraction from tissues maintained in 96% ethanol followed the modified salt-
75 precipitation protocol (Medrano et al., 1990). We amplified five different genes, being three
76 mitochondrial: cytochrome oxidase I (COI), the NADH dehydrogenase 2 (ND2) and the ribosomal
77 16S; and two nuclear genes: seven in absentia (Sia) and the intron 5 of the α-tropomyosin (Trop)
78 (Table 2), by the PCR technique in 20µl reactions with the following concentrations: 10-50ng DNA,
79 0,2µM primer, 0,2mM of each dNTP, 1x Buffer, 1,5µM $MgCl_2$ and 1U *Taq* DNA polymerase
80 *Platinum* (Invitrogen. In some cases 4% of Triton was added to the mix. PCR conditions are
81 presented in Table 3. After this, PCR product was purified using EXOSAP (Exonuclease I and
82 Shrimp Alkaline Phosphatase GE Healthcare®) and the sequencing reactions were performed
83 using the Et Terminator cycle sequencing kit and read in both directions in the automated
84 sequencer MegaBACE 1000 (GE Healthcare®).

85 *2.2. Sequence alignment*

86 The forward and reverse chromatogram reads were assembled and visualized using the
87 *PHRED/PHRAP/CONSED* package (Ewing *et al.*, 1998; Gordon *et al.*, 1998). The consensus sequences
88 were automatically aligned using the software MUSCLE 3.6 (Edgar, 2004) and manually checked
89 with BIOEDIT 7 (Hall, 1999). The basic statistics, as nucleotide diversity (π) and nucleotide
90 frequency, were calculated with the software DNASP (Rozas et al., 2003) and the pairwise distance
91 among sequences was estimated with MEGA 4 (Tamura et al., 2007), with the Kimura-2-parameter
92 method (Kimura, 1980). The mitochondrial coding genes COI and ND2 were concatenated
93 because of the circular form of the mtDNA which makes them interconnected, under the same
94 environmental pressures (Avise et al., 1987).

95 *2.3. Phylogenetic inference*

96 The phylogenetic tree was inferred with all five genes and samples corresponding to 40 *H.*
97 *multifasciatus* specimens, one or two individuals for each sampling location, and five of the genus
98 *Rachoviscus* as outgroup of the analysis (Table 1). The Trop intron region varied in length, because

of this great difference in the indels length and the difficult aligning, this region was deleted of the sequences (Calcagnotto et al., 2005). In the 16S the loops corresponding to the secondary structure proposed by Ortí & Meyer (1997) were excluded from the analyzes. In the nuclear genes, we used the IUPAC-IUB ambiguity code for the cases that single nucleotide polymorphisms were detected in some individuals.

The evolutionary models were calculated in the program MRMODELTEST 2.3 (Nylander, 2004) with PAUP 4.0 (Swofford, 1998). The model proposed by the Akaike Information Criterion (AIC) to Sia, Trop and 16S genes partitions was HKY+G and to the concatenated COI+ND2 partition was GTR+I+G. The computational inference of the tree was developed under a Bayesian approach with the program BEAST 1.5.3 (Drummond and Rambaut, 2007) with partitioned genes dataset. We performed 200 millions of Markov Chain Monte Carlo (MCMC), sampling each 1000 generations, 10% of the initial sampling trees (20000) were discarded as a burn-in and the nodal support was estimated with posterior probability with the remaining trees.

2.4. Phylogeographic analyses

The sample of 201 *Hollandichthys* specimens was grouped according the watershed of the sampled creek in 20 different populations (Fig. 1/Table 1). To this set of analyses, only the genes COI and ND2 were used in the concatenated form. First, we performed an analysis of haplotype diversity (*Hd*) with the software DNASP (Rozas et al., 2003), following the construction of the haplotype network in the program NETWORK 4.5 (Bandelt et al., 1999), with the median-joining algorithm. Analyses of F statistics (Fst and ϕst) and Molecular Variance (AMOVA) (Excoffier et al., 1992) were carried in the program ARLEQUIN 3.11 with the haplotypic frequencies, with just one group to realize the variability among populations and with eight groups, to test the morphotypes proposed for this species complex (Bertaco, 2003). In populations with n=1, the specimen was excluded from these analyses (Garopaba and 3 Forquilhas). Because ϕst takes in account the mutational distances between the haplotypes and conventional Fst don`t (Holsinger and Weir,

2009), here we will analyze the values of ϕst because of the large mutational steps between the sequences in our data. In the tentative to infer the best geographic assemblage for the populations and possible barriers to gene flow, we used the spatial analysis of molecular variance (Dupanloup et al., 2002) with the program SAMOVA from K=2 until k=8 (http://web.unife.it/progetti/genetica/Isabelle/ samova.html).

To understand the dynamics of the population size fluctuation along the time, we used the Bayesian Skyline Plot method implemented in the program BEAST 1.5.3 (Drummond and Rambaut, 2007). Three different sets of data were constructed for this inference, one with all the samples and other two with the samples separated in two groups (North and South), defined according to previous analyses. All the sets were analyzed under a substitution rate of 0.01/mya (Avise et al., 1998; Bermingham et al., 1997; Ornelas-Garcia et al., 2008; Reeves and Bermingham, 2006) in a strict clock approach. For the analyses with the total set and also the North group, the evolutionary model GTR+I+G was selected by the AIC criterion in the program MRMODELTEST 2.3 (Nylander, 2004); each search was performed with 100 million MCMC, with sampling each 1000 generations. For the South group, the evolutionary model used was GTR+G; we employed 200 million MCMC with sampling every 1000 generations in a manner to stabilize the sampling in the universe of possibilities.

3. Results

3.1. Phylogeny

A total of 2719 base pairs (bp) were obtained from the five genes amplified; of this total, 355 bp were variable and the nucleotide diversity (π) varied from 0.035 to 0.003. The remaining basics statistics for each partition are accessible in Table 4.

147 In the Bayesian tree inferred for this dataset (Fig. 2), it is possible to distinguish two major
148 clades that correspond to *Hollandichthys* Northern and Southern geographic range. The major
149 clades proposed by this data set are well supported (posterior probability = 1). The resolution
150 inside the clades is better supported between the deepest clades and starts to be more complex
151 and weakly supported in the shallow groups. The eight morphotypes proposed for this genus
152 (Bertaco, 2003) do not fully agree with the molecular phylogeny inferred here. Only two
153 morphotypes (*H.* sp. A and *H.* sp. C) are monophyletic and supported under this molecular tree.

154 The coalescence of North and South clades are dated around 500 – 600 thousand years
155 and they seem to have been originated in the same period. The divergence between the clades,
156 which correspond to the coalescence for the genus, was estimated around 1.6 Mya.

157 *3.2. Phylogeographic pattern and population structure*

158 A total of 1704 base pairs (bp) were obtained with the mitochondrial genes COI and ND2,
159 from which 161 bp were variable. The nucleotide diversity (π) was 0.02 in the concatenated
160 dataset and a total of 65 haplotypes were found for *Hollandichthys* species complex (Table 4),
161 with the same two divergent clades found in the phylogeny inference, corresponding to the North
162 and South geographic range of *H. multifasciatus* and under an absolute allopatric model. The
163 South clade (S) is represented by 30 haplotypes and has a distribution from Rio Grande do Sul
164 state, in the locality of Maquiné, to the south of the Paranaguá estuary, in Paraná state. The North
165 clade (N), with 35 haplotypes, represents the specimens from Laranjeiras river (north of
166 Paranaguá estuary, Paraná state) to the north of the Paraty county, in Rio de Janeiro State, which
167 is the most northern population known in its distribution (Fig. 1).

168 The clades proposed in the phylogenetic inference (Fig. 2) with a wider set of genes are
169 coincident with the tree inferred just with the two mitochondrial partitions and an increased
170 number of individuals (Fig. 3). The largest difference was the coalescent dates inferred: with the
171 nuclear partitions, it was estimated around 500-600 thousand years ago to south and north

clades; with only the COI and ND2 genes, it was proposed to be between 700 and 900 thousand years and the genus coalescence in turn of 1.9 million years ago.

Within the two big clades, it is possible to recognize some groups inside them (Fig. 3). In the South clade, the more austral group (green) has high values of support and an origin estimated in 190 thousand years ago. In the network (Fig. 4), it is possible to visualize that the haplotypes S9 and S10, from the southern population of the Florianópolis island are more similar to the austral group than to the haplotypes of the north of this island. The clade formed by remaining specimens from Florianópolis, Joinville, S. Francisco and Guaratuba samples (S11 – S26) show a high level of similarity, with few mutational steps between the haplotypes in a wide distributional range between the continent and two islands. Florianópolis and São Francisco do Sul islands share haplotypes in the network to each gene partition (Fig. 5 and 6). However, Florianópolis population values of ϕst are significant for all pairwise comparisons (Table 5), but the lowest values are found for the comparison between Joinville, São Francisco and Guaratuba populations (0.37, 0.2 and 0.3, respectively). Comparing these three groups, all their ϕst values were not significant among them. The haplotype network for the group has a star format, suggesting a recent expansion. The Paranaguá population (S27 – S30) seems to be an isolated population for more than 200 thousand years and presents high support values (=1) (Fig. 3), which is corroborated by the ϕst values, ranging between 0.90 and 0.97 (Table 5).

The North clade is formed by four main subclades (Fig. 3). One is formed by the populations of Laranjeiras and Iguape, with a relatively low posterior probability (0.73); however, each population inside this clade is very well supported (=1), and do not share any haplotypes with others groups (Fig. 4) (N1-N4 and N26-N31). For both populations, the ϕst values are all significant (Table 5). A second and complex clade is formed by the isolated population of Peruíbe and more three populations: Santos + Alto Tietê + Bertioga populations. The clade as a whole is well supported. The haplotype from Peruíbe (N6) is isolated since 40 thousand years ago. The

197 remaining internal relationships are extremely complex, with haplotypes (N5) shared among
198 Santos + Alto Tietê + Bertioga populations (Fig. 4) and only the recent haplotypes of these
199 populations, with less than 30 thousand years (Fig. 3), follow a range agreeing with the
200 geographic distribution (N7-N9 and N10-N12). All ϕst values among these three populations were
201 very low and not significant (0.02, 0.08 and 0.12) (Table 5). Another well supported clade and
202 extremely isolated in the North group is the population of Paraty N (ϕst > 0.91 – Table 5). This
203 third clade is constituted by just one population and it seems to have been isolated since 0.7 Mya
204 and has 15 mutational steps (N32-N35), a large number in comparison to the other haplotypes
205 Finally, the last clade is constituted by the populations from S. Sebastião + Ilhabela Island +
206 Ubatuba (N and S) + Toca do Boi + one specimen from Bertioga. São Sebastião has five unique
207 haplotypes (N13-N17) and all ϕst values significant. These constitute the center of origin of the
208 haplotypes shared by Ubatuba N and Bertioga (N18), which is also the ancestral form to the
209 remaining populations: Ilhabela (N19-N20), Ubatuba N (N21-N22) and Toca do Boi (N23-N25), not
210 sharing haplotypes with other groups, presenting a recent monophyly, and without significant
211 values of ϕst (Table 5).

212 The Bayesian skyline plot scenario for the whole genus (Fig. 7a) showed a long period of
213 constant population size, followed by a discrete bottleneck event in Pleistocene, around 0.15
214 Mya, and a recent event of expansion starting around 0,1 Mya, with the population size increasing
215 consistently. When the two major clades are analyzed separately, the results corroborate this
216 recent expansion. In the North clade (Fig. 7b), the bottleneck event occurred around 0.03 Mya; on
217 the other hand, the South population (Fig. 7c) seems to have had an expansion around 0.1 Mya,
218 followed by a bottleneck (less than 0.03 Mya) and, after that, the same recent expansion
219 experienced by the North clade.

220 The AMOVA results showed high evidence of isolation among all the 20 populations. For
221 the conventional F-statistics, we found a FST value of 0.38, representing 62% of variation between

the populations, and using the Tamura-Nei method, the FST was even large (0.95), with both values being significant (p=0.000). Although, when comparing with the eight morphotypes proposed (Bertaco, 2003), the conventional F-statistics return a FCT value (between groups) extremely low (FCT = -0.00286, p= 0.5582±0.00528) and with Tamura-Nei correction, the FCT value was 0.68, which was significant but lower than the eight groups proposed furthermore by the SAMOVA test.

The results inferred by SAMOVA showed a better geographic/molecular conformation of groups than the morphotypes, with FCT = 0.89, maintaining the K=8. The groups proposed were (Maquiné, Mampituba, Araranguá)[*H.* sp. A] + (Florianópolis, S. Francisco, Joinville, Guaratuba)[*H.* sp. B, *H. affinis*] + (Paranaguá)[*H. affinis*] + (Laranjeiras)[*H.* sp. C] + (Iguape)[*H. perstriatus*] + (Peruíbe, Santos, Alto Tietê, Bertioga)[*H. perstriatus, H. multifasciatus, H.* sp. D] + (S. Sebastião, Ilhabela, Ubatuba N, Ubatuba S, Toca do Boi)[*H.* sp. D, *H.* sp. E] + (Paraty N)[*H.* sp. E]. Otherwise, in the moment that we test with smaller number of groups, it is possible to infer that the value of K that best fits to our data set is 5 (FCT=0.84). In this case, the FCT values seems to arrived in a plateau of stabilization (Fig. 8) and the groups proposed are: (Maquiné, Mampituba, Araranguá)[*H.* sp. A] + (Florianópolis, S. Francisco, Joinville, Guaratuba, Paranaguá)[*H.* sp. B, *H. affinis*] + (Laranjeiras, Iguape, Peruíbe, Santos, Alto Tietê, Bertioga)[*H.* sp. C, *H. perstriatus, H. multifasciatus, H.* sp. D] + (S. Sebastião, Ilhabela, Ubatuba N, Ubatuba S, Toca do Boi)[*H.* sp. D, *H.* sp. E] + (Paraty N)[*H.* sp. E].

4. Discussion

4.1. Phylogeographic patterns

The time of divergence estimated based on the tree with the nuclear and mitochondrial genes was 1.6 Mya, in contrast the tree with protein coding genes in mtDNA alone presented 1.9

Mya. This discrepancy of 200 thousand years between the results is probably due to the differences found in substitution rates: nuclear genes developed approximately four times slower than mitochondrial genes (Avise, 2009). This fact contributed to a probable deceleration in the general data rate for the substitution model with nuclear genes. Apart from this difference, both results reported similar coalescence dates for the genus *Hollandichthys*. In the last two millions years cyclic climatic events culminated in glacial and inter-glacial stages which were followed by significant changes on the sea level (Villwock et al., 2005). Since these events are congruent with the origin of the *Hollandichthys* lineages, it would to be expected for the geographic pattern of this fish to be adjusted by these climatic events. Analyzing the patterns inferred to *Hollandichthys* this does not seem to be the main rule. Some lineages are extremely old, like the Paranaguá population (0.9 Mya), refuting the theory of population link during the glacial stages when the sea-level was lower and the creeks were connected.

On the other hand, the formation of the shallow clades (Fig. 3) seems to coincide with the last maximum glacial (Wisconsin glaciation – 21000 years ago). It is possible to infer that this last glaciation was the one which affected more profoundly the neighbor populations in view of its great intensity – reported as the most severe glaciation during the Pleistocene (Schwarzbold and Schafer, 1984). Its consequences could have been the connection of creeks in a small distributional range or an expansion of the Atlantic Forest during Pleistocene sea-level changes (Carnaval and Moritz, 2008), which is crucial to the presence of this genus.

The populations proposed according to the hydrographic basins range turned out to be effective units in molecular approaches. Having in mind that watershed divides such as high mountains are often regarded as effective dispersal barriers for fish (Ingenito and Buckup, 2007) and the Brazilian coast is formed by an extensive system of micro-watersheds. The populations proposed demonstrated an incredible range of differentiation with an FST value of 0.95, indicating a high degree of isolation. It can be a reflection of the paleo-drainages distribution which has a big

correlation with the current position of the river mouths and the main paleo-valley flow lines. These results make reference to changes in current fluvial courses which used to be longer having to cross broad platforms, excavated during the periods of sea-level regression (Conti and Furtado, 2006).

The allopatry between the two major clades is complete (North and South), with their origin during the Pleistocene, around 0.5 to 0.9 Mya. The geographic barrier responsible for this breakdown corresponds to the Paranaguá region (Fig. 1), an extensive estuary delimited by the uplift of the Serra do Mar crystalline shield border. This estuary is mainly formed by the convergence of two rivers: the Paranaguá river in the southern portion of the estuary and the Laranjeiras river in the northern portion (Muehe, 2003), in between them we find the Ponta Grossa arch, a structure present in the area since the Cretaceous (Raposo, 1995), which seems to be an important barrier that only allows the convergence of those two rivers at a short distance from the ocean. By this point they comprise an estuary of 600km^2, primordially constituted of Pleistocene and Holocene beach ridges (Lessa et al., 2000). According to Ingenito & Buckup (2007) main river lowlands might act as an obstacle for fish dispersion, suggesting Paranaguá as a good breakdown area between the distributional range of *Hollandichthys.* To the Amazonian region a southern-northern shift in fish species distribution or genetic diversity can be attributed to the main channel of the Amazon river acting as a physical barrier to dispersal (Hubert and Renno, 2006) in a pattern similar of the found here.

Comparing the populations around this estuary (Paranaguá and Laranjeiras), each one in a separate clade, it is clear that there is a high degree of differentiation between them (38 mutational steps). As a result, this barrier can be seen as an old and effective separation, despite the tendency of the estuary-mouth migrating to the north during the last Pleistocene and Holocene (Lessa et al., 2000). Some other species of freshwater fishes suggest similar distribution with *Hollandichthys.* The genus *Spintherobolus* has one species in the northern part of

296 *Hollandichthys* distribution (*S. broccae)*, *S, leptoura* in the Ribeira de Iguape drainage, one in the
297 upper part of the Tietê (*S. papilliferus)* and *S. ankoseion* in the south of Paranaguá (Weitzman and
298 Malabarba, 1999), this last one working as an efficient and strong barrier. A North-South pattern
299 such as Paranaguá was found by Grazziotin et al. (2006) with the complex *Bothrops jararaca*
300 snakes.

301 *4.2. The South clade*

302 The South clade (S) is mainly formed by three groups: the southern clade (green) which
303 lives in an unusual habitat for *Hollandichthys*. While almost all *Hollandichthys* distribution is
304 characterized by high scarps where the boarder of the crystalline shield reaches a discrete coastal
305 plain; the referred southern portion has a large plain, formed by a chain of sand barriers and
306 lagoons. In these localities, the genus is found in lateral puddles of the rivers, while it is usually
307 found in small creeks associated with the Atlantic Forest. These differences are result of different
308 regional pressures in tectonic processes. Because of that, the clade with southern distribution
309 lives in an area where the large plain confines the Atlantic Forest between the scarps of the Serra
310 do Mar (Villwock et al., 2005).

311 The Florianópolis population is very diverse. The divergent haplotypes S9 and S10 (Fig. 4)
312 describe an ancient pattern more closely connected to the geographic demography – such as
313 populations separated by hills - than to the isolation of the island during the Holocene (Milne et
314 al., 2005). Those localities are in the south of the island while the other samples found in
315 Florianópolis are located in the north of the island separated by a series of scarps. On the other
316 hand, in the continent, Garopaba is geographically the nearest population to Florianópolis (S11-
317 Fig 4), which does not share haplotypes with the island. The third group is the isolate Paranaguá
318 population since 0.22 Mya, this probably occurs due to the position of the region between two
319 large bays: Paranaguá (already mentioned) and Guaratuba, in the south (Angulo and Lessa,
320 1997).The specimens here called Guaratuba population (Fig. 3, S19 and S20) were sampled in

creeks in the southern portion of this bay and, possibly because of this, they are genetically similar to Joinville, São Francisco and Florianópolis.

The fact that the islands of Florianópolis and São Francisco have so recently separated from the continent (Milne et al., 2005) can be a reason for both islands to still share same lineages (Fig 5-6, S4 and S6 respectively). The rises in the sea-level were responsible for the vicariant event of these islands formation and, consequently, for the isolation of the creeks in a small portion of land. The data assessed here still demonstrate to be suffering from incomplete lineage sorting (Maddison and Knowles, 2006). Different from the proposed by Fitzpatrick (2009) that the isolation time for a taxon of frogs on some islands near the Brazilian coast was enough to differentiate it from other species.

4.3. The North clade

The North group (N) shows complex phylogeographic patterns. The Laranjeiras and Ribeira de Iguape populations are isolated since 0.2 and 0.1 Mya, respectively. An extremely isolated population is Paraty N, in the northern portion. The coalescence between this group and other populations is estimated in 0.7 Mya, in an incredible divergent clade. This seems to be a result of very eventful coast line, with the crystalline shield boarder arriving until the ocean between this population and the remaining ones. The populations São Sebastião, Ubatuba N and S, Ilhabela and Toca do Boi share a same evolutionary history. Their shared haplotypes, or closed related ones, still permit to infer a history of connections between these hydrographic basins. The most divergent population in this group is São Sebastião - the presence of a deep valley between the continent (S. Sebastião) and the island (Ilhabela) in a southwestern direction, can be the reason for a great separation of two near locations as well as the isolation of S. Sebastião population around 0.13 Mya (Conti and Furtado, 2006). The ancient population in Ilhabela probably suffers from a founder effect because of the low level of diversity presented. It is interesting to observe that the Bertioga specimen shares the haplotype with Ubatuba population

346 (N18, Fig 3), which is possibly caused by a recent migration event, like, for instance, a flow that
347 would have permitted a temporary connection.

348 The multiple events of headwater captures between upper Tietê and the coastal
349 drainages have been the main explanation for the *Hollandichthys* presence in this drainage, in a
350 time when the upper Tietê drained in a composite drainage with the Paraíba do Sul river (Ingenito
351 and Buckup, 2007; Lundberg et al., 1998; Ribeiro, 2006; Weitzman et al., 1988). This hypothesis is
352 corroborated by two characids fossils from late Oligocene (35 to 23 Mya)*, Megacheirodon unicos*
353 and *Lignobrycon ligniticus,* closely related to the current fauna from coastal drainages
354 (Malabarba, 1998). These possible scenarios happened in a time before the *Hollandichthys*
355 coalescence (1.9 Mya). Our results, showing Alto Tietê samples sharing haplotypes with
356 populations on the coast at non-significant values of genetic divergence (Fig. 4/Tab. 5) do not
357 agree with these inferences, and we propose here an extremely recent single event of
358 colonization during the Holocene. According to Ingenito & Buckup (2007), species like the genus
359 *Astyanax,* tetras, are commonly used for artificial colonization of small dams. Therefore, the
360 presence of *Hollandichthys* in the Alto Tietê basin can be a result of artificial introduction by
361 humans, or a small scale and recent headwater transposal.

362 *4.4. Demographic dynamics*

363 Population expansion in both South and Norh clades can be linked with the end of the last
364 maximum glacial (21000) (Fig. 7). According to Carnaval and Moritz (2008) at 0.21 Mya the south
365 of Doce river - *Hollandichthys* distributional range - was not predicted to have retained a large,
366 stable habitat area for forest-dependent species, but rather small and medium-sized refuges only.
367 This finding corroborates to the bottleneck event, before the population growth. Our strong
368 phylogeographic structure in some populations can reflect on long-term population persistence in
369 isolated areas, like mid-sized refugium. In addition, the north group pattern of divergent clades
370 can infer the occurrence of several refugia in this area (Carnaval et al., 2009). Our late-Pleistocene

371 bottleneck followed by a population increase can be a reflection of the contraction and expansion
372 of the Atlantic forest. However, the poor predictive performance of the Carnaval & Moritz (2008)
373 model in the southeastern regions of the Atlantic forest emphasizes the need for phylogeographic
374 studies that focus on taxa with broader distributions in this region (Fitzpatrick et al., 2009).

375 The different sea levels do not appear to have been decisive in the formation of the
376 groups; their origins are older than the last maximum, 21 thousand year ago. The *Hollandichthys*
377 current distribution seems to be more related to the habitat where they live - creeks associated
378 with the Atlantic forest - and to the barriers, such as mountains. The geomorphological changes
379 that affected the river courses and the climatic changes during the Pleistocene caused allopatric
380 speciation in different ways. With our dataset some patterns can be proposed: the Paranaguá
381 break is congruent with the paleogeographic hypothesis, which predicts allopatric differentiation
382 among populations isolated throughout the species range distribution by the raising of the
383 paleoarches in the lowlands (Hubert et al., 2007). On the other hand, the southern population
384 (green) can be inferred as a hydrogeologic hypothesis predicting an increase in *Hollandichthys*
385 distribution range by dispersion, with dispersive populations being subsequently isolated (Hubert
386 et al., 2007; Montoya-Burgos, 2003).

387 *4.5. Phylogenetic patterns and morphotypes*

388 Only two morphotypes proposed (Bertaco, 2003) maintained the reciprocal monophyly in
389 the molecular approach, following the Moritz premises (1994). They are: *H.* sp. A and *H.* sp. C. The
390 process of finding groups that would maintain this pattern is extremely difficult, because of the
391 recent divergence between some clades: e.g. the populations on the islands and some possible
392 migrations as the specimen from Bertioga that shares haplotypes with a population in a northern
393 range. Following these criteria and keeping the morphospecies in monophyletic lineages, it would
394 only be possible to infer two genetic groups (South and North) in a species complex that is known

395 to be diverse and lives in a region under many external pressures such as climatic and geologic
396 ones.

397 When comparing the morphotypes with the best molecular and geographic groups
398 proposed by the Samova analyses (k=5), only the *H.* sp. A still remains as a genetic unit. When
399 comparing with the eight groups proposed by Samova analyses, just the two monophyletic
400 species (*H.* sp A and *H.* sp. C) are maintained. In all groups proposed by this methodology (K=2 to
401 8) the Paranaguá was always pointed as one of the geographic barriers and this seems to be
402 corroborated by the morphological study (Bertaco, 2003).

403 It is necessary to have in mind the extremely recent origin of this diversity and that
404 possible forces, as incomplete lineage sorting and recent migrations events, are still acting in the
405 molecular scale (Avise, 2000). Also, it is possible that the difference in the mode of speciation
406 (founder population versus fractioned species range) contributes to the differences in species
407 patterns found in nature (Mayr, 1954). Some mixtures in clades can be resulted of a former
408 widespread distribution followed by vicariant events and the still incomplete speciation cannot be
409 discarded (Hubert and Renno, 2006).

410 This discussion about what can be a species is still in the beginning and until the scientists
411 arrive in a consensus about what is a species, it is important, in the moment to take a decision, to
412 realize that the species proposed should mirror the hidden diversity in that moment, allowing it to
413 be accessible in an organized manner.

414 In the *Hollandichthys* case we found only two reciprocally monophyletic species based on
415 both molecular and morphological data (*H.* sp. A and C), but recognition of both species makes all
416 other six morphospecies paraphyletic regarding gene trees. If we recognize species based only in
417 the monophyly of gene trees, only two species are possible, corresponding to North and South
418 clades, but this would not allow the recognition of the *H.* sp. A separately from other populations
419 of the South clade, regardless the fact it is the most distinct, both morphologically and in habitat

use, among all *Hollandichthys* populations. Considering both evidences, we found molecular data extremely useful in accessing the evolutionary history among closely related populations of *Hollandichthys*, but it seems that events of speciation inside the south and north clades in *Hollandichthys* are recent and not fully reflected in the mtDNA lineages coalescence, as suggested for rapid speciation events by Maddison and Knowles (2006). Also there were no sufficient complementary haplotypes extinction for a species to be recovered as monophyletic, as proposed for "Evolutionary Significant Units" by Moritz (1994).

Species recognition based only in tree genes monophyly in this case seems problematic if we wish to reflect the *Hollandichthys* diversity at this moment. The non-recognition of *H.* sp. A population as a distinct species, for example, would hide an important evolutionary lineage of *Hollandichthys* recognizable in both morphological and habitat use aspects. We suggest morphological data to be re-examined along with gene tree information in accessing a better decision on *Hollandichthys* species level description.

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Table 1. Localities, vouchers, haplotypes and samples used to the phylogeny inference according to the morphotypes identification and the populational watershed separation.

Morphotypes	Population (Drainage)	Voucher	Locality	Lat.	Long.	Haplotype			Sequence		
						All	COI	ND2	Sia	Trop	16S
***H.* sp. A**	Maquiné	MCP 26969/38	arroio afluente do arroio do Ouro	-29.58	-50.28	S1	S1	S1			
		MCP 26969/39	arroio afluente do arroio do Ouro	-29.58	-50.28	S1	S1	S1			
		MCP 26969/40	arroio afluente do arroio do Ouro	-29.58	-50.28	S1	S1	S1			
		MCP 26969/41	arroio afluente do arroio do Ouro	-29.58	-50.28	S1	S1	S1			
		MCP 26969/42	arroio afluente do arroio do Ouro	-29.58	-50.28	S1	S1	S1			
		UFRGS 11793/TEC842A	riacho na localidade de Barra do Ouro	-29.59	-50.29			S1			
		UFRGS 11793/TEC842B	riacho na localidade de Barra do Ouro	-29.59	-50.29	S1	S1	S1			
		UFRGS 11793/TEC842C	riacho na localidade de Barra do Ouro	-29.59	-50.29	S1	S1	S1			
		UFRGS 11793/TEC842D	riacho na localidade de Barra do Ouro	-29.59	-50.29	S1	S1	S1	*	*	*
		UFRGS 11793/TEC842E	riacho na localidade de Barra do Ouro	-29.59	-50.29	S1	S1	S1	*	*	*
		UFRGS 11793/TEC842F	riacho na localidade de Barra do Ouro	-29.59	-50.29	S1	S1	S1			
		UFRGS 11793/TEC842G	riacho na localidade de Barra do Ouro	-29.59	-50.29	S1	S1	S1			
		UFRGS 11793/TEC842H	riacho na localidade de Barra do Ouro	-29.59	-50.29		S1				
	3 Forquilhas	MCP 29244/153	córrego lateral ao arroio da Barra	-29.43	-50.18	S2	S1	S2	*	*	*
	Mampituba	MCP 29241	arroio Molha Coco em Vila Rosa	-29.17	-49.99	S2	S1	S4			
		MCP 29242	arroio Molha Coco em Vila Rosa	-29.17	-50.00	S5	S1	S4			
		MCP 23625	arroio Molha Coco em Vila Rosa	-29.17	-50.00	S8	S1	S4			
		UFRGS 11790/TEC839	arroio Molha Coco	-29.17	-49.98	S5	S1	S4			
		UFRGS 11792/TEC841A	arroio Faxinalzinho	-28.79	-49.93	S6	S1	S4			
		UFRGS 11792/TEC841B	arroio Faxinalzinho	-28.79	-49.93	S5	S1	S4	*	*	*
		UFRGS 11792/TEC841C	arroio Faxinalzinho	-28.79	-49.93	S5	S1	S4			
		UFRGS 11792/TEC841D	arroio Faxinalzinho	-28.79	-49.93	S5	S1	S4			
		UFRGS 11792/TEC841E	arroio Faxinalzinho	-28.79	-49.93	S5	S1	S4			
		UFRGS 11792/TEC841F	arroio Faxinalzinho	-28.79	-49.93	S7	S1	S4			
		UFRGS 11792/TEC841G	arroio Faxinalzinho	-28.79	-49.93	S7	S1	S4	*	*	*
		UFRGS 11792/TEC841H	arroio Faxinalzinho	-28.79	-49.93	S5	S1	S4			
	Araranguá	UFRGS 11791/TEC840A	riacho afluente do rio Araranguá	-28.79	-49.93	S4	S2	S3	*	*	*
		UFRGS 11791/TEC840B	riacho afluente do rio Araranguá	-28.79	-49.93	S30	S1	S3			
		UFRGS 11791/TEC840C	riacho afluente do rio Araranguá	-28.79	-49.93	S30	S1	S3		*	*
		UFRGS 11791/TEC840D	riacho afluente do rio Araranguá	-28.79	-49.93	S30	S1	S3			
***H.* sp. B**	Garopaba	MCP 28734/108	riacho afluente do rio Araçatuba, drenagem rio Una	-28.07	-48.70	S11	S9	S7	*	*	*
	Florianópolis	MCP 28737/100	riacho na ilha de Florianópolis	-27.59	-48.48	S9	S3	S5			
		MCP 28737/101	riacho na ilha de Florianópolis	-27.59	-48.48	S10	S3	S5			
		MCP 28737/102	riacho na ilha de Florianópolis	-27.59	-48.48	S9	S3	S5			
		MCP 28747/104	riacho na ilha de Florianópolis	-27.51	-48.49	S15	S5	S6			
		MCP 28732/107	riacho na ilha de Florianópolis	-27.51	-48.49	S15	S5	S6			
		MCP 28732/125	riacho na ilha de Florianópolis	-27.51	-48.49	S17	S7	S6			
		MCP 37654/A	riacho na ilha de Florianópolis	-27.46	-48.42	S14	S6	S8			
		MCP 37654/B	riacho na ilha de Florianópolis	-27.46	-48.42	S18	S4	S10			
		MCP 38333/A	riacho afluente do rio Ratones na ilha	-27.52	-48.47	S15	S5	S6			
		MCP 38333/B	riacho afluente do rio Ratones na ilha	-27.52	-48.47	S15	S5	S6			
		MCP 38333/C	riacho afluente do rio Ratones na ilha	-27.52	-48.47	S15	S5	S6	*	*	*
		MCP 38333/D	riacho afluente do rio Ratones na ilha	-27.52	-48.47	S15	S5	S6	*	*	*
		MCP 38333/E	riacho afluente do rio Ratones na ilha	-27.52	-48.47	S16	S8	S6			
		MCP 38317/A	riacho na ilha de Florianópolis	-27.48	-48.44	S12	S4	S9			
		MCP 38317/B	riacho na ilha de Florianópolis	-27.48	-48.44	S12	S4	S9			
		MCP 38317/C	riacho na ilha de Florianópolis	-27.48	-48.44	S13	S4	S9			
		MCP 37635/A	riacho na ilha de Florianópolis	-27.48	-48.44	S14	S6	S8			

		MCP 37635/B	riacho na ilha de Florianópolis	-27.48	-48.44	S14	S6	S8			
		MCP 37635/C	riacho na ilha de Florianópolis	-27.48	-48.44		S6	S8			
		MCP 37635/D	riacho na ilha de Florianópolis	-27.48	-48.44	S18	S4	S10			
H. affinis	Joinville	MCP 30552/158	riacho na localidade de Araquari,SC	-26.38	-48.73	S23	S11	S11			
		MCP 30667/176	rio Cubatão do Norte	-26.19	-48.93	S21	S10	S11	*	*	*
		MCP 31486/177	rio Cubatão do Norte	-26.19	-48.93	S22	S10	S11	*	*	*
	S. Francisco	MCP 30553/159	arroio que deságua no rio Acaraí dentro da ilha	-26.33	-48.65	S24	S10	S11	*	*	*
		UFRGS 10579/TEC105	arroio que deságua no rio Acaraí dentro da ilha	-26.33	-48.65	S26	S4	S6	*	*	*
		UFRGS 9359/TEC345	arroio que deságua no rio Acaraí dentro da ilha	-26.33	-48.65	S25	S10	S12			
	Guaratuba	UFRGS 10578/TEC109	área alagada próxima a baía de Guaratuba	-25.88	-48.58	S20	S12	S6	*	*	*
		UFRGS 9358/TEC346	área alagada próxima a baía de Guaratuba	-25.88	-48.58	S19	S12	S6			
	Paranaguá	MCP 30556/161	riacho que converge para a baía de Paranaguá	-25.40	-48.87	S30	S13	S15	*	*	*
		MHNCI/1*	riacho que converge para a baía de Paranaguá	-25.43	-48.70	S29	S15	S13			
		MHNCI/2*	riacho que converge para a baía de Paranaguá	-25.43	-48.70			S13			
		MHNCI/3*	riacho que converge para a baía de Paranaguá	-25.43	-48.70	S28	S14	S14	*	*	*
		MHNCI/4*	riacho que converge para a baía de Paranaguá	-25.43	-48.70	S29	S15	S13			
		MHNCI/5*	riacho que converge para a baía de Paranaguá	-25.43	-48.70	S27	S14	S13			
		MHNCI/6*	riacho que converge para a baía de Paranaguá	-25.43	-48.70	S27	S14	S13	*	*	*
***H.* sp. C**	Laranjeiras	MCP 30557/162	riacho que desaguá na baía de Laranjeiras	-25.17	-48.42	N30	N5	N4	*	*	*
		MCP 30558/163	riacho que desaguá na baía de Laranjeiras	-25.21	-48.43	N27	N5	N4			
		UFRGS 11778/TEC755A	afluente do rio Tagaçaba	-25.22	-48.45	N31	N4	N2		*	*
		UFRGS 11778/TEC755B	afluente do rio Tagaçaba	-25.22	-48.45	N26	N6	N4			
		UFRGS 11778/TEC755C	afluente do rio Tagaçaba	-25.22	-48.45	N31	N4	N2			
		UFRGS 11778/TEC755D	afluente do rio Tagaçaba	-25.22	-48.45	N28	N5	N3		*	*
		UFRGS 11778/TEC755E	afluente do rio Tagaçaba	-25.22	-48.45	N28	N5	N3			
		UFRGS 11779/TEC763A	riacho na Enseada do Benito	-25.17	-48.42	N30	N5	N4			
		UFRGS 11779/TEC763B	riacho na Enseada do Benito	-25.17	-48.42	N27	N5	N4			
		UFRGS 11779/TEC763C	riacho na Enseada do Benito	-25.17	-48.42	N27	N5	N4		*	*
		UFRGS 11779/TEC763D	riacho na Enseada do Benito	-25.17	-48.42	N29	N5	N3			
H. perstriatus	Ribeira de Iguape	UFRGS 11780/TEC784	rio Pariquera-açú	-24.69	-47.89	N3	N2	N5			
		UFRGS 11781/TEC806A	riacho afluente do Ribeira de Iguape	-24.66	-47.49	N2	N1	N5			
		UFRGS 11781/TEC806B	riacho afluente do Ribeira de Iguape	-24.66	-47.49	N1	N1	N7			
		UFRGS 11781/TEC806C	riacho afluente do Ribeira de Iguape	-24.66	-47.49	N2	N1	N5			
		UFRGS 11781/TEC806D	riacho afluente do Ribeira de Iguape	-24.66	-47.49	N4	N3	N6	*	*	*
		UFRGS 11782/TEC739A	riacho afluente do Ribeira de Iguape	-24.65	-47.48	N2	N1	N5	*		*
		UFRGS 11782/TEC739B	riacho afluente do Ribeira de Iguape	-24.65	-47.48	N2	N1	N5			
	Peruíbe	MCP 30554/276	riacho que drena para o oceano	-24.36	-47.04	N6	N10	N8			
		MCP 30555/294	riacho que drena para o oceano	-24.37	-47.05	N6	N10	N8			
		MCP 30561/316	riacho que drena para o oceano	-24.36	-47.04	N6	N10	N8	*	*	*
		UFRGS 11783/TEC817A	riacho Cachoira da Anta na localidade de Guaraú	-24.36	-47.04			N8			
		UFRGS 11783/TEC817B	riacho Cachoira da Anta na localidade de Guaraú	-24.36	-47.04	N6	N10	N8			
		UFRGS 11783/TEC817C	riacho Cachoira da Anta na localidade de Guaraú	-24.36	-47.04	N6	N10	N8			
		UFRGS 11783/TEC817D	riacho Cachoira da Anta na localidade de Guaraú	-24.36	-47.04	N6	N10	N8			
		UFRGS 11783/TEC817E	riacho Cachoira da Anta na localidade de Guaraú	-24.36	-47.04	N6	N10	N8			
		UFRGS 11784/TEC 722A	riacho drenando para o mar na localidade de Guaraú	-24.37	-47.06	N6	N10	N8			
		UFRGS 11784/TEC 722B	riacho drenando para o mar na localidade de Guaraú	-24.37	-47.06	N6	N10	N8			
		UFRGS 11784/TEC 722C	riacho drenando para o mar na localidade de Guaraú	-24.37	-47.06	N6	N10	N8			
		UFRGS 11784/TEC 722D	riacho drenando para o mar na localidade de Guaraú	-24.37	-47.06	N6	N10	N8			
		UFRGS 11784/TEC 722E	riacho drenando para o mar na localidade de Guaraú	-24.37	-47.06	N6	N10	N8			
		UFRGS 11784/TEC 722F	riacho drenando para o mar na localidade de Guaraú	-24.37	-47.06		N10				
		UFRGS 11784/TEC 722G	riacho drenando para o mar na localidade de Guaraú	-24.37	-47.06	N6	N10	N8			

		UFRGS 11784/TEC 722H	riacho drenando para o mar na localidade de Guaraú	-24.37	-47.06	N6	N10	N8	*	*	*
H. multifasciatus	Santos	MCP 30560/290	arroio que deságua na baía de Santos	-23.84	-46.33	N12	N11	N8			
		MCP 30560/291	arroio que deságua na baía de Santos	-23.84	-46.33	N10	N9	N9			
		MCP 30559/341	arroio que deságua na baía de Santos	-23.86	-46.35	N10	N9	N9			
		MCP 30559/342	arroio que deságua na baía de Santos	-23.86	-46.35	N10	N9	N9			
		MCP 30559/343	arroio que deságua na baía de Santos	-23.86	-46.35	N10	N9	N9			
		MCP 30559/344	arroio que deságua na baía de Santos	-23.86	-46.35	N11	N9	N10	*	*	*
		UFRGS 11785/TEC820A	afluente do rio da Onça na estrada do Quilombo	-23.86	-46.35	N5	N9	N8			
		UFRGS 11785/TEC820B	afluente do rio da Onça na estrada do Quilombo	-23.86	-46.35		N9				
		UFRGS 11785/TEC820C	afluente do rio da Onça na estrada do Quilombo	-23.86	-46.35	N5	N9	N8			
		UFRGS 11785/TEC820D	afluente do rio da Onça na estrada do Quilombo	-23.86	-46.35		N9				
		UFRGS 11785/TEC820E	afluente do rio da Onça na estrada do Quilombo	-23.86	-46.35	N10	N9	N9	*	*	*
		UFRGS 11785/TEC820F	afluente do rio da Onça na estrada do Quilombo	-23.86	-46.35		N9				
		UFRGS 11785/TEC820G	afluente do rio da Onça na estrada do Quilombo	-23.86	-46.35	N10	N9	N9			
	Alto Tietê	UFRGS 11786/TEC826A	arroio na localidade de Paranapiacaba	-23.77	-46.33	N5	N9	N8		*	*
		UFRGS 11786/TEC826B	arroio na localidade de Paranapiacaba	-23.77	-46.33	N5	N9	N8			
		UFRGS 11786/TEC826C	arroio na localidade de Paranapiacaba	-23.77	-46.33	N5	N9	N8			
		UFRGS 11786/TEC826D	arroio na localidade de Paranapiacaba	-23.77	-46.33	N5	N9	N8			
		UFRGS 11786/TEC826E	arroio na localidade de Paranapiacaba	-23.77	-46.33	N5	N9	N8			
		UFRGS 11786/TEC826F	arroio na localidade de Paranapiacaba	-23.77	-46.33	N5	N9	N8			
		UFRGS 11786/TEC826G	arroio na localidade de Paranapiacaba	-23.77	-46.33	N5	N9	N8			
		UFRGS 11786/TEC826H	arroio na localidade de Paranapiacaba	-23.77	-46.33	N5	N9	N8			
		UFRGS 11787/TEC827A	arroio na localidade de Paranapiacaba	-23.77	-46.31	N5	N9	N8			
		UFRGS 11787/TEC827B	arroio na localidade de Paranapiacaba	-23.77	-46.31	N5	N9	N8			
		UFRGS 11787/TEC827C	arroio na localidade de Paranapiacaba	-23.77	-46.31	N5	N9	N8		*	*
		UFRGS 11787/TEC827D	arroio na localidade de Paranapiacaba	-23.77	-46.31		N9				
		UFRGS 11787/TEC827E	arroio na localidade de Paranapiacaba	-23.77	-46.31	N5	N9	N8			
		UFRGS 11787/TEC827F	arroio na localidade de Paranapiacaba	-23.77	-46.31	N5	N9	N8			
		UFRGS 11787/TEC827G	arroio na localidade de Paranapiacaba	-23.77	-46.31		N9				
		UFRGS 11787/TEC827H	arroio na localidade de Paranapiacaba	-23.77	-46.31	N5	N9	N8			
***H.* sp. D**	Bertioga	MZUSP 63130	arroio no município de Bertioga	-23.77	-45.98			N8			
		UFRGS 11771/TEC888A	tributário rio Vermelho, estrada Rio-Santos km 208	-23.78	-46.00	N18	N15	N11			*
		UFRGS 11771/TEC888B	tributário rio Vermelho, estrada Rio-Santos km 208	-23.78	-46.00	N5	N9	N8			
		UFRGS 11771/TEC888C	tributário rio Vermelho, estrada Rio-Santos km 208	-23.78	-46.00	N5	N9	N8			
		UFRGS 11771/TEC888D	tributário rio Vermelho, estrada Rio-Santos km 208	-23.78	-46.00	N5	N9	N8			
		UFRGS 11771/TEC888E	tributário rio Vermelho, estrada Rio-Santos km 208	-23.78	-46.00	N5	N9	N8			
		UFRGS 11771/TEC888F	tributário rio Vermelho, estrada Rio-Santos km 208	-23.78	-46.00	N5	N9	N8			
		UFRGS 11771/TEC888G	tributário rio Vermelho, estrada Rio-Santos km 208	-23.78	-46.00	N8	N9	N8			
		UFRGS 11771/TEC888H	tributário rio Vermelho, estrada Rio-Santos km 208	-23.78	-46.00	N5	N9	N8			
		UFRGS 11777/TEC980A	tributário rio Vermelho, estrada Rio-Santos km 208	-23.78	-46.00	N5	N9	N8			*
		UFRGS 11777/TEC980B	tributário rio Vermelho, estrada Rio-Santos km 208	-23.78	-46.00	N7	N9	N8			
		UFRGS 11777/TEC980C	tributário rio Vermelho, estrada Rio-Santos km 208	-23.78	-46.00	N5	N9	N8			
		UFRGS 11777/TEC980D	tributário rio Vermelho, estrada Rio-Santos km 208	-23.78	-46.00	N5	N9	N8			
		UFRGS 11777/TEC980E	tributário rio Vermelho, estrada Rio-Santos km 208	-23.78	-46.00	N5	N9	N8			
		UFRGS 11777/TEC980F	tributário rio Vermelho, estrada Rio-Santos km 208	-23.78	-46.00	N9	N12	N8			

		UFRGS 11777/TEC980G	tributário rio Vermelho, estrada Rio-Santos km 208	-23.78	-46.00	N5	N9	N8			
		UFRGS 11777/TEC980H	tributário rio Vermelho, estrada Rio-Santos km 208	-23.78	-46.00			N8			
	São Sebastião	MCP 30658/164	riacho no município de São Sebastião	-23.76	-45.71	N16	N14	N11			
		MCP 30660/166	riacho no município de São Sebastião	-23.79	-45.55	N14	N13	N11			
		UFRGS 11772/TEC891A	arroio Taquarubu	-23.76	-45.72	N16	N14	N11			
		UFRGS 11772/TEC891B	arroio Taquarubu	-23.76	-45.72	N16	N14	N11			
		UFRGS 11772/TEC891C	arroio Taquarubu	-23.76	-45.72	N16	N14	N11			
		UFRGS 11772/TEC891D	arroio Taquarubu	-23.76	-45.72		N14	N11			
		UFRGS 11772/TEC891E	arroio Taquarubu	-23.76	-45.72	N16	N14	N11			
		UFRGS 11772/TEC891F	arroio Taquarubu	-23.76	-45.72	N16	N14	N11			
		UFRGS 11772/TEC891G	arroio Taquarubu	-23.76	-45.72	N16	N14	N11			
		UFRGS 11772/TEC891H	arroio Taquarubu	-23.76	-45.72	N17	N14	N11			
		UFRGS 11795/TEC1229A	rio Boiçucanga na localidade de Boiçucanga	-23.77	-45.61	N16	N14	N11		*	*
		UFRGS 11795/TEC1229B	rio Boiçucanga na localidade de Boiçucanga	-23.77	-45.61	N15	N13	N1			
		UFRGS 11795/TEC1229C	rio Boiçucanga na localidade de Boiçucanga	-23.77	-45.61	N16	N14	N11			
		UFRGS 11795/TEC1229D	rio Boiçucanga na localidade de Boiçucanga	-23.77	-45.61	N13	N13	N1			
		UFRGS 11795/TEC1229E	rio Boiçucanga na localidade de Boiçucanga	-23.77	-45.61	N16	N14	N11			
		UFRGS 11795/TEC1229F	rio Boiçucanga na localidade de Boiçucanga	-23.77	-45.61		N14	N11			
		UFRGS 11795/TEC1229G	rio Boiçucanga na localidade de Boiçucanga	-23.77	-45.61	N15	N13	N1			
		UFRGS 11795/TEC1229H	rio Boiçucanga na localidade de Boiçucanga	-23.77	-45.61	N17	N14	N11			
		UFRGS 11788/TEC859A	rio Guaecá na estrada SP-55	-23.82	-45.45		N13	N11	*	*	*
		UFRGS 11788/TEC859B	rio Guaecá na estrada SP-56	-23.82	-45.45		N13				
***H.* sp. E**	Ilhabela	MCP 30661/358	riacho na ilha	-23.83	-45.36	N19	N15	N11			
		MCP 30661/359	riacho na ilha	-23.83	-45.36	N19	N15	N11	*	*	*
		MCP 30662/383	riacho na ilha	-23.82	-45.36	N19	N15	N11			
		MCP 30662/384	riacho na ilha	-23.82	-45.36	N19	N15	N11			
		UFRGS 11773/TEC897A	riacho da Cachoeira da Toca	-23.82	-45.35	N20	N15	N11			
		UFRGS 11773/TEC897B	riacho da Cachoeira da Toca	-23.82	-45.35		N15	N11			
		UFRGS 11773/TEC897C	riacho da Cachoeira da Toca	-23.82	-45.35	N19	N15	N11	*		*
		UFRGS 11773/TEC897D	riacho da Cachoeira da Toca	-23.82	-45.35	N19	N15	N11			
		UFRGS 11773/TEC897E	riacho da Cachoeira da Toca	-23.82	-45.35	N19	N15	N11			
	Ubatuba S	MCP 30663/170	arroio a oeste de Ubatuba	-23.43	-45.13	N22	N16	N12			
		UFRGS 11774/TEC900A	arroio na estrada Ubatuba-Taubaté no km 88	-23.41	-45.11		N16	N11			
		UFRGS 11774/TEC900B	arroio na estrada Ubatuba-Taubaté no km 88	-23.41	-45.11	N21	N16	N12	*	*	*
	Ubatuba N	UFRGS 11789/TEC866A	valo na beira da estrada do Cambucá	-23.35	-44.87		N15				
		UFRGS 11789/TEC866B	valo na beira da estrada do Cambucá	-23.35	-44.87	N18	N15	N11	*		*
	Toca do Boi	MCP 30664/387	córrego da Toca do Boi, próximo a praia Trindade	-23.33	-44.68	N23	N15	N11			
		MCP 30664/388	córrego da Toca do Boi, próximo a praia Trindade	-23.33	-44.68	N23	N15	N11			
		MCP 30664/389	córrego da Toca do Boi, próximo a praia Trindade	-23.33	-44.68	N24	N15	N11			
		UFRGS 11775/TEC908A	córrego da Toca do Boi, próximo a praia Trindade	-23.33	-44.68	N25	N15	N13	*	*	*
		UFRGS 11775/TEC908B	córrego da Toca do Boi, próximo a praia Trindade	-23.33	-44.68	N23	N15	N11			
		UFRGS 11775/TEC908C	córrego da Toca do Boi, próximo a praia Trindade	-23.33	-44.68	N25	N15	N13			
		UFRGS 11775/TEC908D	córrego da Toca do Boi, próximo a praia Trindade	-23.33	-44.68	N25	N15	N13			
		UFRGS 11775/TEC908E	córrego da Toca do Boi, próximo a praia Trindade	-23.33	-44.68	N23	N15	N11			
		UFRGS 11775/TEC908F	córrego da Toca do Boi, próximo a praia Trindade	-23.33	-44.68		N15				
		UFRGS 11775/TEC908G	córrego da Toca do Boi, próximo a praia Trindade	-23.33	-44.68		N15				
		UFRGS 11775/TEC908H	córrego da Toca do Boi, próximo a praia Trindade	-23.33	-44.68		N15				
	Paraty N	MCP 30665/174	córrego que drena para o oceano	-23.08	-44.70	N32	N7	N14			
		MCP 30666/175	córrego que drena para o oceano	-23.04	-44.69	N35	N8	N14			
		UFRGS 11776/TEC909A	afluente do rio Taquari	-23.04	-44.60	N34	N8	N15			
		UFRGS 11776/TEC909B	afluente do rio Taquari	-23.04	-44.60	N33	N8	N14			
		UFRGS 11776/TEC909C	afluente do rio Taquari	-23.04	-44.60	N33	N8	N14			

	UFRGS 11776/TEC909D	afluente do rio Taquari	-23.04	-44.60	N33	N8	N14	*	*	*
	UFRGS 11776/TEC909E	afluente do rio Taquari	-23.04	-44.60		N8				
	UFRGS 11776/TEC909F	afluente do rio Taquari	-23.04	-44.60	N33	N8	N14			
	UFRGS 11776/TEC909G	afluente do rio Taquari	-23.04	-44.60	N33	N8	N14			
	UFRGS 11776/TEC909H	afluente do rio Taquari	-23.04	-44.60			N14			
Rachoviscus crassiceps	UFRGS 9356/TEC102							*	*	*
Rachoviscus crassiceps	UFRGS 9356/TEC103							*	*	*
Rachoviscus graciliceps	UFRGS 11116/TEC1117A							*	*	*
Rachoviscus graciliceps	UFRGS 11116/TEC1117B							*	*	*
Rachoviscus graciliceps	UFRGS 11116/TEC1117C							*	*	*

Table 2. Genes used in this study.

Gene	Primer sequence (liste from 5' to 3')	Source
16S ar	ACG CCT GTT TAT CAA AAA CAT	Palumbi (1996))
16S br	CCG GTC TGA ACT CAG ATC ACG T	Palumbi (1996)
sia/T3b	ATT AAC CCT CAC TAA AGT CGA GTG CCC CGT GTG YTT YGA YTA	Calcagnotto (2005)
sia/T7b	AAT ACG ACT CAC TAT AGG AAG TGG AAG CCG AAG CAG SWY TGC ATC AT	Calcagnotto (2005)
TROP F	GAG TTG GAT CGG GCT CAG GA GCG	Friesen et al. (1999)
TROP R	CGG TCA GCC TCT TCA GCA ATG TGC TT	Friesen et al. (1999)
COI-H2198	TAA AcT TcA ggg TgA ccA AAA AAT cA	Herbert et al (2003)
COI-L1490	ggT cAA cAA ATc ATA AAg ATA TTg g	Herbert et al (2003)
ND2-L5216	GGC CCA TAC CCC GRA AAT G	Sorenson (2003)
ND2-H6313	ACT CTT RTT TAA GGC TTT GAA GGC	Sorenson (2003)

Table 3. PCR conditions applied to each gene.

Primer	Desnaturation	Cycles	Extension
16S	95°C/10'	35x 95°C/30", 48°C/30", 72°C/45"	72°C/7'
SIA	95°C/5'	35x 95°C/30", 60°C/30", 72°C/45"	72°C/7'
TROP	95°C/10'	35x 95°C/30", 60°C/30", 72°C/45"	72°C/7'
COI	96°C/1'	40x 94°C/30", 50°C/20", 48°C/5", 46°C/5", 44°C/5", 42°C/5", 40°C/20", 72°C/1'	72°C/3'
ND2	94°C/4'	9x 94°C/30", 57°C-1°C/cycle/1', 72°C/1'30", 40x 94°C/30", 47°C/1', 72°C/1'30"	72°C/5'

Table 4. DNA diversity estimates for *Hollandichthys multifasciatus* using all the studied segments.

												Nucleotide frequency			
Approach	**Segments**	**L**	**N**	**V**	**S**	**P**	**π**	**k**	**d**	**h**	***Hd***	**T**	**C**	**A**	**G**
Phylogeny	**Concatenated***	1704	45	331	179	314	0.035	36.28	0.037	31	0.981	0.31	0.24	0.28	0.17
	16S	423	45	9	9	9	0.006	2.406	0.006	6	0.757	0.24	9.23	0.27	0.25
	Sia	400	36	12	12	9	0.005	1.879	0.005	7	0.698	0.17	0.37	0.17	0.28
	Trop	192	40	3	3	3	0.003	0.046	0.003	4	0.424	0.14	0.19	0.28	0.39
Phylogeography	**Concatenated***	1704	177	161	160	127	0.020	33.29	0.020	65	0.957	0.32	0.25	0.29	0.15
	COI	639	195	49	44	42	0.016	9.974	0.017	31	0.906	0.33	0.25	0.23	0.19
	ND2	1065	188	112	39	85	0.019	8.132	0.019	30	0.889	0.28	0.23	0.34	0.15

* COI + ND2, only samples without missing data

L= lenght (bp)

N= number of sequences

h= number of haplotypes

V= variable sites

S= segregating sites

P= parsimony informative sites

π= nucleotode diversity

k= average number of nucleotide differences

Hd= Haplotype diversity

SD= standart deviation

d= average of pairwise distance with kimura-2-parameter

Table 5. Fst values calculated to the concatenated dataset. Fst values in the upward diagonal and ϕst values in the downward diagonal. Significant p values are presented in bold.

	Maq.	Mamp.	Arar.	Floria.	Joinv.	S.Franc.	Guarat.	Paran.	Laranj.	Iguape	Peruí.	Santos	A.Tietê	Bert.	S.Seb.	Ilhab.	Uba.S	Uba.N	Toca
Maq.		**0.64**	**0.65**	**0.39**	**0.62**	**0.53**	0.60	**0.50**	**0.42**	**0.54**	**0.73**	**0.47**	**0.74**	**0.55**	**0.45**	**0.65**	**0.53**	0.60	**0.47**
Mamp.	**0.91**		**0.54**	**0.33**	**0.50**	**0.40**	**0.46**	**0.40**	**0.34**	**0.45**	**0.67**	**0.40**	**0.68**	**0.48**	**0.38**	**0.57**	**0.40**	**0.46**	**0.39**
Arar.	0.21	**0.72**		**0.26**	0.43	0.28	0.34	**0.32**	**0.25**	**0.37**	**0.70**	**0.33**	**0.70**	**0.44**	**0.33**	**0.55**	0.28	0.34	**0.32**
Floria.	**0.70**	**0.75**	**0.64**		**0.20**	0.09	0.10	**0.16**	**0.12**	**0.20**	**0.43**	**0.19**	**0.43**	**0.27**	**0.19**	**0.32**	0.09	0.10	**0.17**
Joinv.	**0.98**	**0.96**	**0.90**	**0.37**		0.17	0.21	0.24	**0.18**	**0.30**	**0.68**	**0.27**	**0.68**	**0.39**	**0.27**	**0.51**	0.17	0.21	**0.25**
S.Franc.	**0.98**	**0.96**	**0.88**	**0.20**	0.00		0.00	0.12	0.07	0.18	**0.60**	0.16	**0.60**	**0.30**	0.17	**0.40**	0.00	0.00	0.14
Guarat.	**1.00**	**0.97**	0.93	**0.30**	0.81	0.74		0.14	0.08	0.21	**0.66**	0.19	**0.67**	0.33	0.19	0.46	0.00	0.00	0.16
Paran.	**0.90**	**0.90**	**0.81**	**0.63**	**0.75**	**0.72**	**0.74**		**0.15**	**0.24**	**0.55**	**0.22**	**0.56**	**0.32**	**0.22**	**0.40**	0.12	0.14	**0.21**
Laranj.	**0.99**	**0.98**	**0.97**	**0.92**	**0.97**	**0.97**	**0.98**	**0.94**		**0.19**	**0.47**	**0.18**	**0.47**	**0.27**	**0.18**	**0.33**	0.07	0.08	**0.16**
Iguape	**0.98**	**0.98**	**0.96**	**0.88**	**0.94**	**0.94**	**0.96**	**0.90**	**0.87**		**0.59**	**0.26**	**0.60**	**0.36**	**0.26**	**0.45**	0.18	0.21	**0.25**
Peruí.	**1.00**	**1.00**	**1.00**	**0.93**	**1.00**	**1.00**	**1.00**	**0.97**	**0.95**	**0.96**		**0.52**	**0.76**	**0.58**	**0.49**	0.69	0.60	0.66	**0.52**
Santos	**1.00**	**0.99**	**0.99**	**0.92**	**0.99**	**0.99**	**0.99**	**0.95**	**0.92**	**0.90**	**0.96**		**0.43**	**0.26**	**0.25**	**0.40**	0.16	0.19	**0.23**
A.Tietê	**1.00**	**1.00**	**1.00**	**0.93**	**0.99**	**0.99**	**1.00**	**0.97**	**0.95**	**0.93**	**1.00**	0.02		0.05	**0.49**	**0.70**	**0.60**	**0.67**	**0.53**
Bert.	**0.97**	**0.96**	**0.95**	**0.89**	**0.94**	**0.94**	**0.94**	**0.91**	**0.80**	**0.75**	**0.78**	0.08	0.12		**0.33**	**0.48**	**0.30**	0.31	**0.32**
S.Seb.	**0.99**	**0.99**	**0.98**	**0.94**	**0.98**	**0.98**	**0.98**	**0.96**	**0.95**	**0.95**	**0.97**	**0.96**	**0.97**	**0.90**		**0.38**	0.17	0.19	**0.23**
Ilhab.	**1.00**	**1.00**	**0.99**	**0.93**	**0.99**	**0.99**	**1.00**	**0.96**	**0.97**	**0.97**	**1.00**	**0.99**	**1.00**	**0.91**	**0.82**		**0.40**	0.46	**0.39**
Uba.S	**1.00**	**0.99**	**0.99**	**0.91**	0.99	0.99	1.00	**0.94**	**0.96**	**0.95**	**1.00**	**0.99**	**1.00**	**0.89**	**0.86**	**1.00**		0.00	0.14
Uba.N	**1.00**	**0.99**	0.98	**0.90**	0.98	0.98	1.00	**0.92**	**0.95**	**0.94**	**1.00**	**0.98**	**1.00**	**0.87**	**0.77**	0.00	1.00		0.03
Toca	**1.00**	**1.00**	**0.99**	**0.93**	**0.99**	**0.99**	**1.00**	**0.97**	**0.97**	**0.97**	**1.00**	**0.99**	**1.00**	**0.92**	**0.84**	0.00	**1.00**	0.00	
Par.N	**0.99**	**0.99**	**0.98**	**0.93**	**0.98**	**0.98**	**0.99**	**0.95**	**0.95**	**0.96**	**0.99**	**0.97**	**0.99**	**0.91**	**0.95**	**0.98**	**0.97**	**0.97**	**0.98**

Figure legends

Fig. 1. Geographical distribution of the *Hollandichthys multifasciatus* complex. Each point represents a sampled locality and each color represents a different population. This color pattern are follow in figures 2 to 6.

Fig. 2. Bayesian tree inferred with Sia, Trop, 16S, COI and ND2 genes with 40 *Hollandichthys* samples and out-groups. Colored branches indicate the morphotype definition. Numbers in red are the divergence times proposed and in black the posterior probability above 0.7.

Fig. 3. Bayesian tree inferred with COI and ND2 genes for 201 *Hollandichthys* samples. Colored branches indicate the sampled population. Numbers in red are the divergence times proposed and in black the posterior probability above 0.7.

Fig. 4. Median-joining network among haplotypes, inferred by the concatenated dataset (COI+ND2). Each circle represents a different haplotype with proportional size according to their frequencies. Each color represents a population as in Fig. 1. Crossed markers indicate the number of mutations in the branch when it is bigger than one. Between the two major clades North and South there are 38 nucleotide modifications.

Fig. 5. Median-joining network among haplotypes to COI partition, showing the two major clades proposed. Each circle represents a different haplotype with proportional size according to their frequencies. Each color represents a population as in Fig. 1. Crossed markers indicate the number of mutations in the branch when it is bigger than one.

Fig. 6. Median-joining network among haplotypes to ND2 partition, showing the two major clades proposed. Each circle represents a different haplotype with proportional size according to their frequencies. Each color represents a population as in Fig. 1. Crossed markers indicate the number of mutations in the branch when it is bigger than one.

Fig. 7. Bayesian skyline plot showing the effective population size fluctuations in the time **(a)** to all *Hollandichthys multifasciatus* species complex (solid line = median; grey area = confidence interval); **(b)** just to sequences in the North clade (solid line = median; grey area = confidence interval); **(c)** just to sequences in the South clade (solid line = median; grey area = confidence interval).

Fig. 8. FCT values according to the number of groups (k) proposed to *Hollandichthys multifasciatus* species complex.

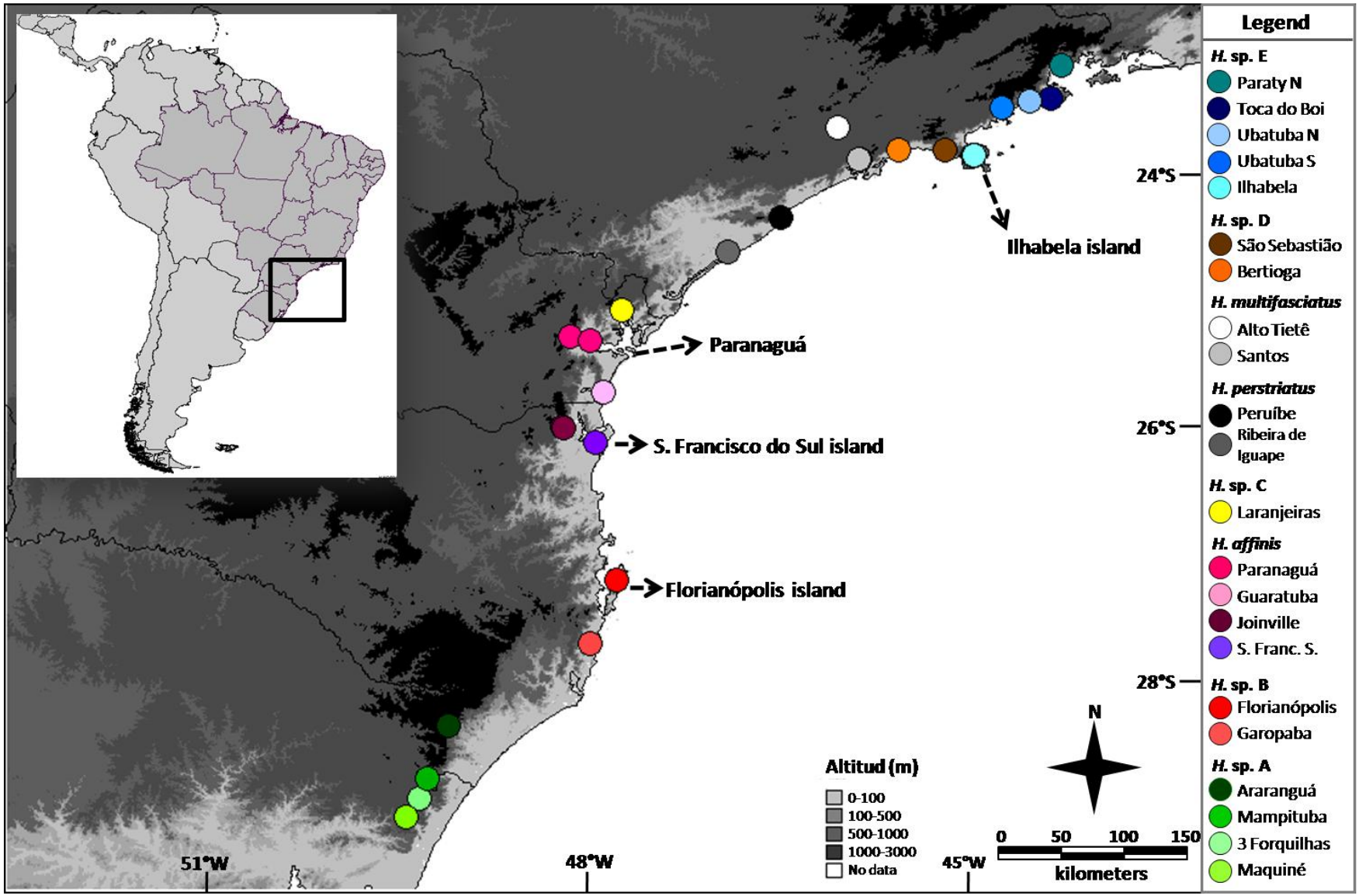
Legend
H. sp. E
Paraty N
Toca do Boi
Ubatuba N
Ubatuba S
Ilhabela
H. sp. D
São Sebastião
Bertioga
H. multifasciatus
Alto Tietê
Santos
H. perstriatus
Peruíbe
Ribeira de Iguape
H. sp. C
Laranjeiras
H. affinis
Paranaguá
Guaratuba
Joinville
S. Franc. S.
H. sp. B
Florianópolis
Garopaba
H. sp. A
Araranguá
Mampituba
3 Forquilhas
Maquiné
Ilhabela island
Paranaguá
S. Francisco do Sul island
Florianópolis island
24°S
26°S
28°S
51°W
48°W
45°W
Altitud (m)
0-100
100-500
500-1000
1000-3000
No data
N
0 50 100 150
kilometers

Fig. 1.

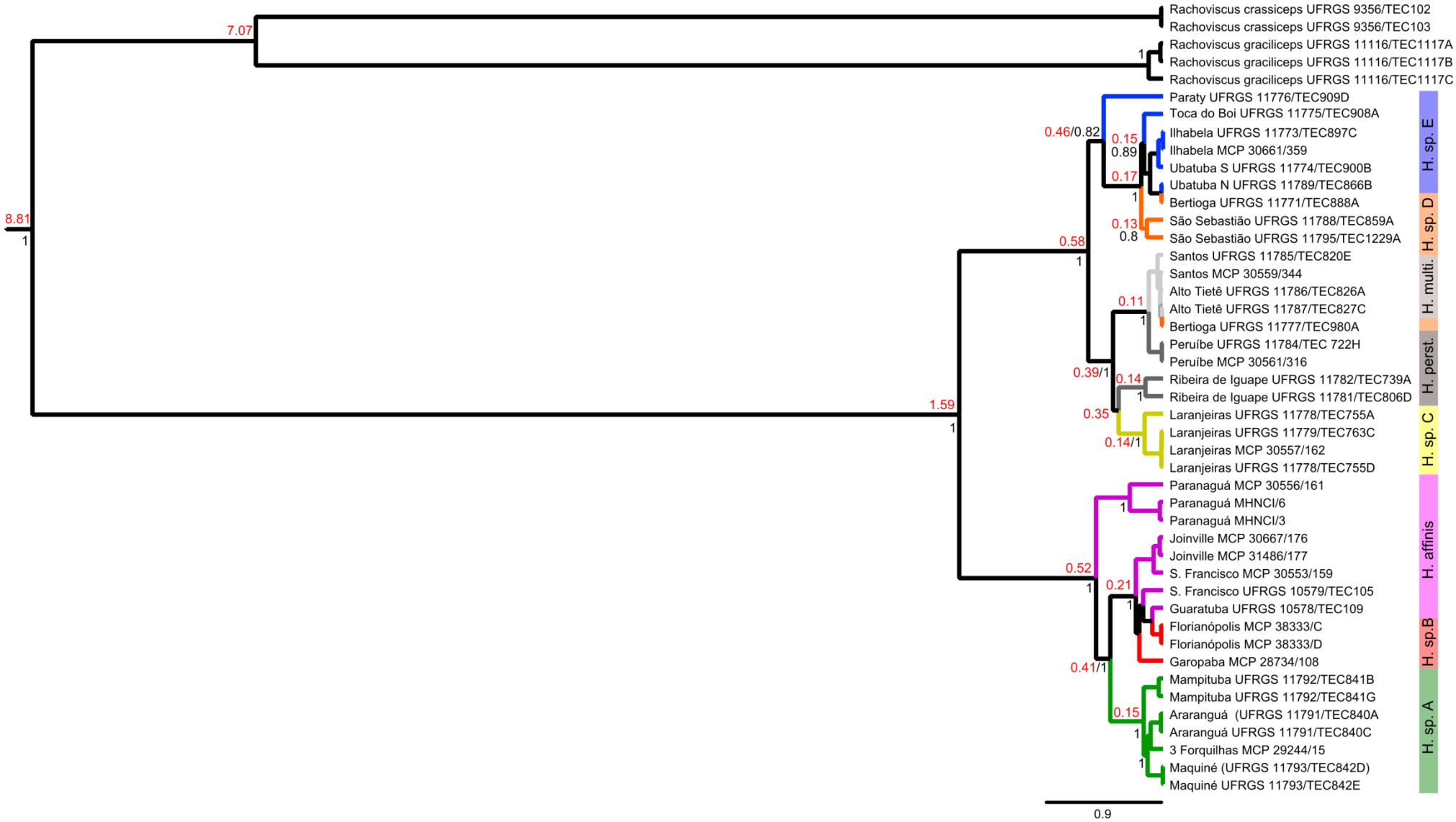
Rachoviscus crassiceps UFRGS 9356/TEC102
Rachoviscus crassiceps UFRGS 9356/TEC103
Rachoviscus graciliceps UFRGS 11116/TEC1117A
Rachoviscus graciliceps UFRGS 11116/TEC1117B
Rachoviscus graciliceps UFRGS 11116/TEC1117C
Paraty UFRGS 11776/TEC909D
Toca do Boi UFRGS 11775/TEC908A
Ilhabela UFRGS 11773/TEC897C
Ilhabela MCP 30661/359
Ubatuba S UFRGS 11774/TEC900B
Ubatuba N UFRGS 11789/TEC866B
Bertioga UFRGS 11771/TEC888A
São Sebastião UFRGS 11788/TEC859A
São Sebastião UFRGS 11795/TEC1229A
Santos UFRGS 11785/TEC820E
Santos MCP 30559/344
Alto Tietê UFRGS 11786/TEC826A
Alto Tietê UFRGS 11787/TEC827C
Bertioga UFRGS 11777/TEC980A
Peruíbe UFRGS 11784/TEC 722H
Peruíbe MCP 30561/316
Ribeira de Iguape UFRGS 11782/TEC739A
Ribeira de Iguape UFRGS 11781/TEC806D
Laranjeiras UFRGS 11778/TEC755A
Laranjeiras UFRGS 11779/TEC763C
Laranjeiras MCP 30557/162
Laranjeiras UFRGS 11778/TEC755D
Paranaguá MCP 30556/161
Paranaguá MHNCI/6
Paranaguá MHNCI/3
Joinville MCP 30667/176
Joinville MCP 31486/177
S. Francisco MCP 30553/159
S. Francisco UFRGS 10579/TEC105
Guaratuba UFRGS 10578/TEC109
Florianópolis MCP 38333/C
Florianópolis MCP 38333/D
Garopaba MCP 28734/108
Mampituba UFRGS 11792/TEC841B
Mampituba UFRGS 11792/TEC841G
Araranguá (UFRGS 11791/TEC840A
Araranguá UFRGS 11791/TEC840C
3 Forquilhas MCP 29244/15
Maquiné (UFRGS 11793/TEC842D)
Maquiné UFRGS 11793/TEC842E
H. sp. E
H. sp. D
H. multi.
H. perst.
H. sp. C
H. affinis
H. sp.B
H. sp. A
0.9

Fig. 2.

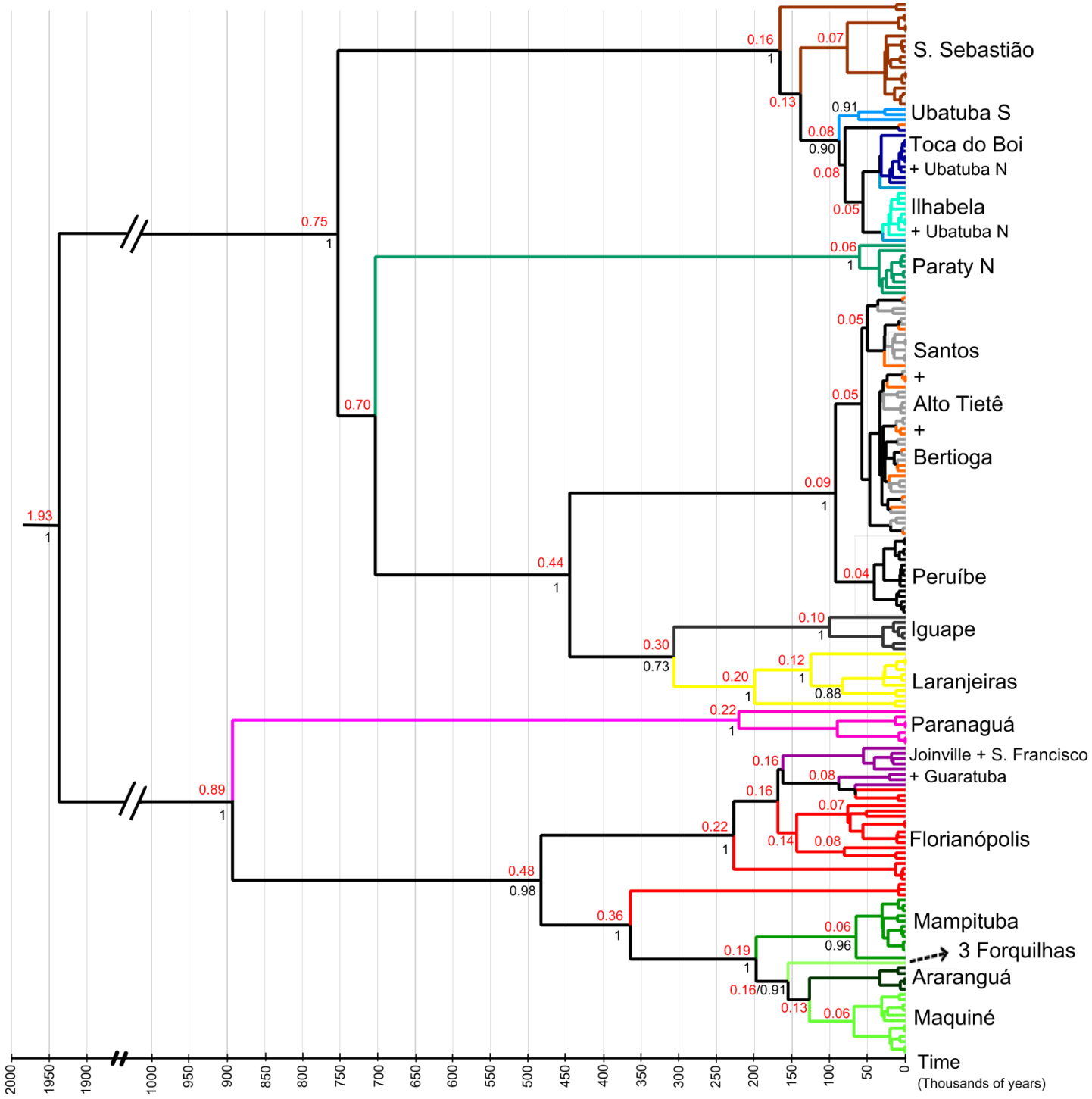
S. Sebastião
Ubatuba S
Toca do Boi
+ Ubatuba N
Ilhabela
+ Ubatuba N
Paraty N
Santos
+
Alto Tietê
+
Bertioga
Peruíbe
Iguape
Laranjeiras
Paranaguá
Joinville + S. Francisco
+ Guaratuba
Florianópolis
Mampituba
3 Forquilhas
Araranguá
Maquiné
Time
(Thousands of years)
2000
1950
1900
1000
950
900
850
800
750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0

Fig. 3.

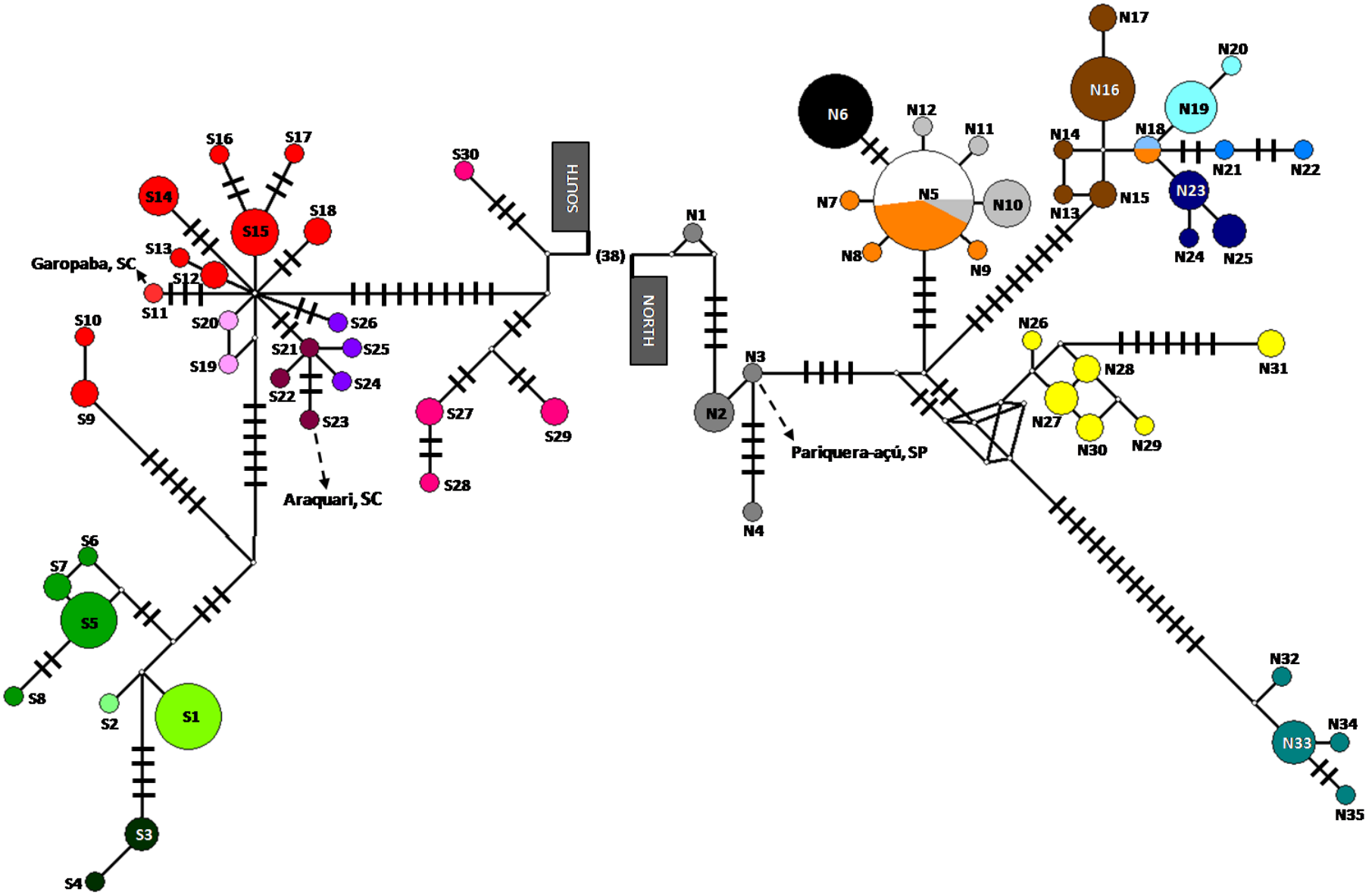
SOUTH
NORTH
(38)
Garopaba, SC
Araquari, SC
Pariquera-açú, SP
S1
S2
S3
S4
S5
S6
S7
S8
S9
S10
S11
S12
S13
S14
S15
S16
S17
S18
S19
S20
S21
S22
S23
S24
S25
S26
S27
S28
S29
S30
N1
N2
N3
N4
N5
N6
N7
N8
N9
N10
N11
N12
N13
N14
N15
N16
N17
N18
N19
N20
N21
N22
N23
N24
N25
N26
N27
N28
N29
N30
N31
N32
N33
N34
N35

Fig. 4.

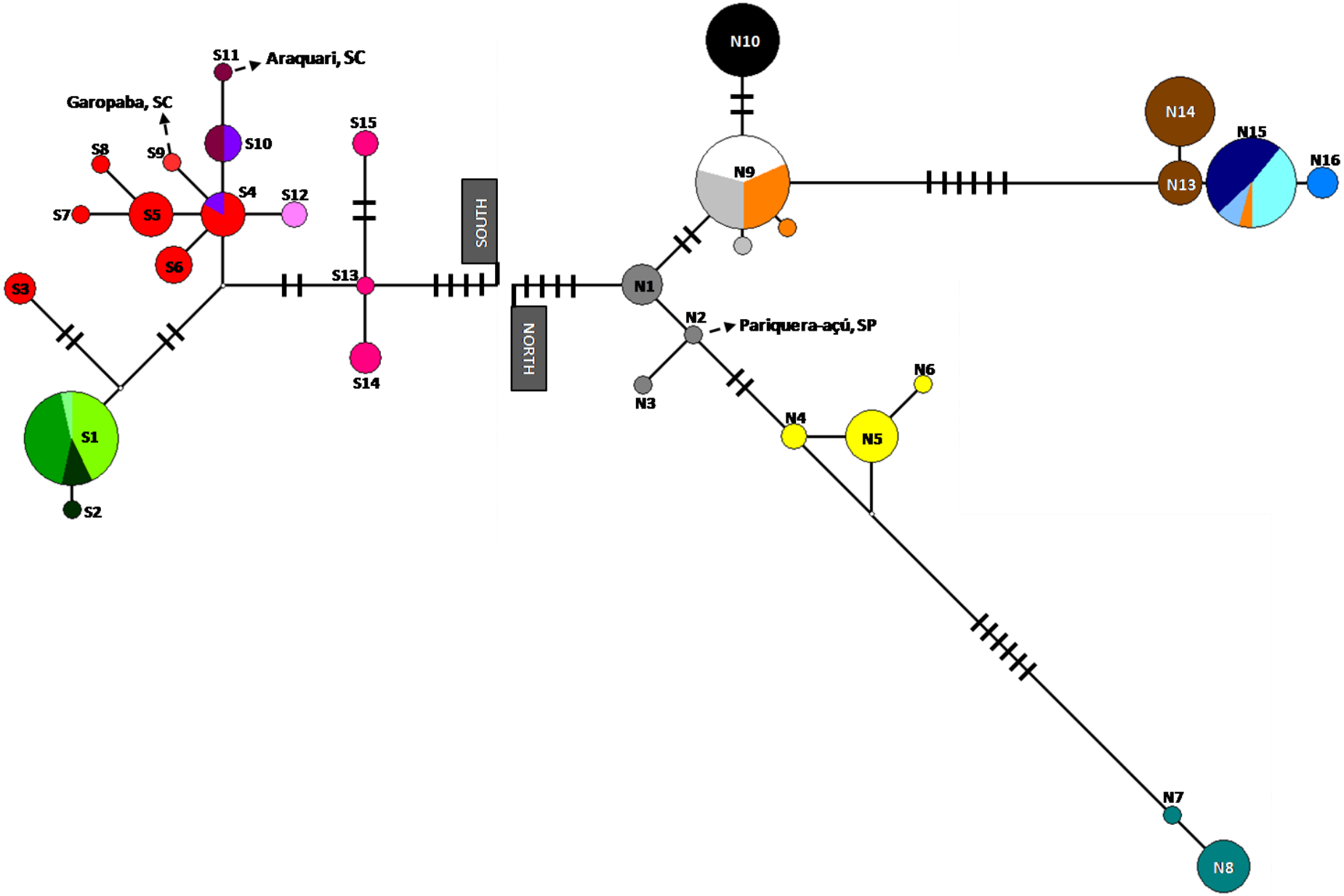
S11
Araquari, SC
Garopaba, SC
S10
S15
S8
S9
S4
S12
S7
S5
S6
S13
S3
S14
S1
S2
SOUTH
NORTH
N10
N14
N15
N16
N9
N13
N1
N2
Pariquera-açú, SP
N3
N6
N4
N5
N7
N8

Fig. 5.

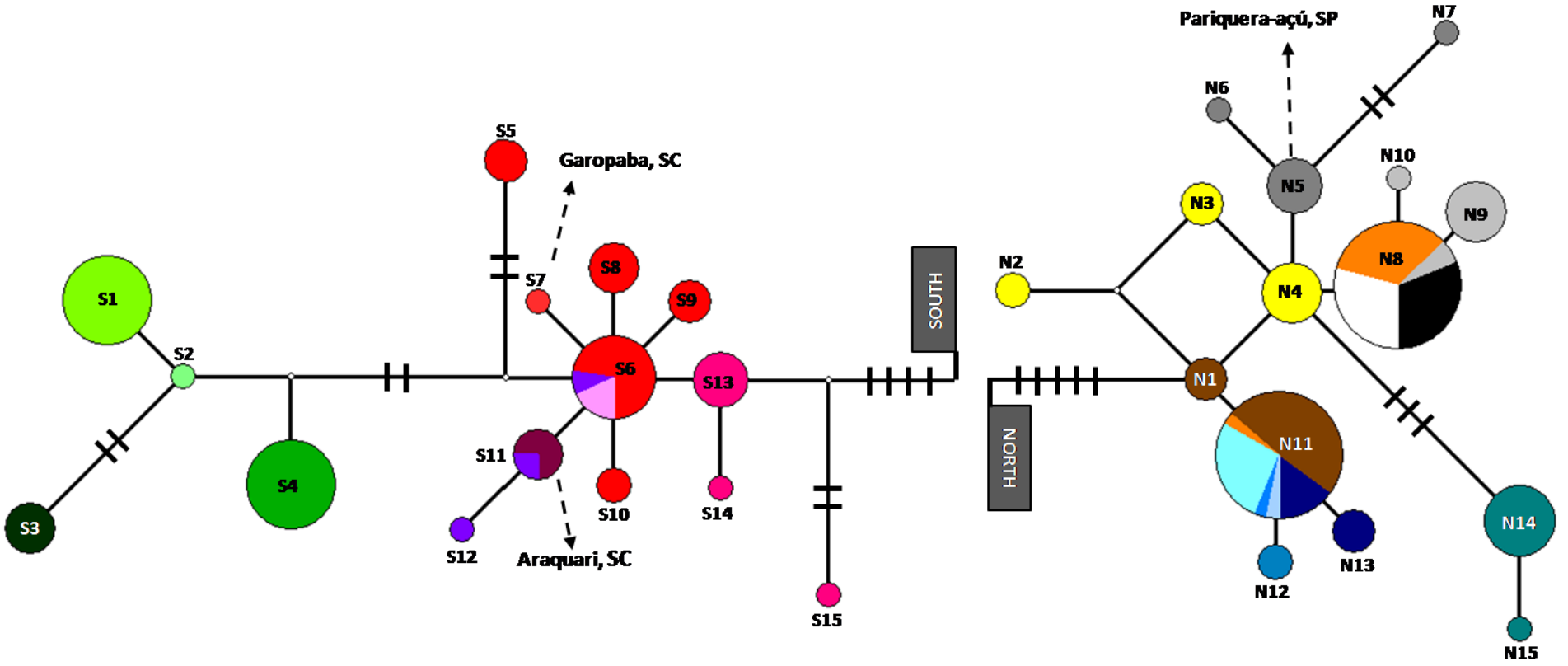
S1
S2
S3
S4
S5
S6
S7
S8
S9
S10
S11
S12
S13
S14
S15
Garopaba, SC
Araquari, SC
SOUTH
NORTH
Pariquera-açú, SP
N1
N2
N3
N4
N5
N6
N7
N8
N9
N10
N11
N12
N13
N14
N15

Fig. 6.

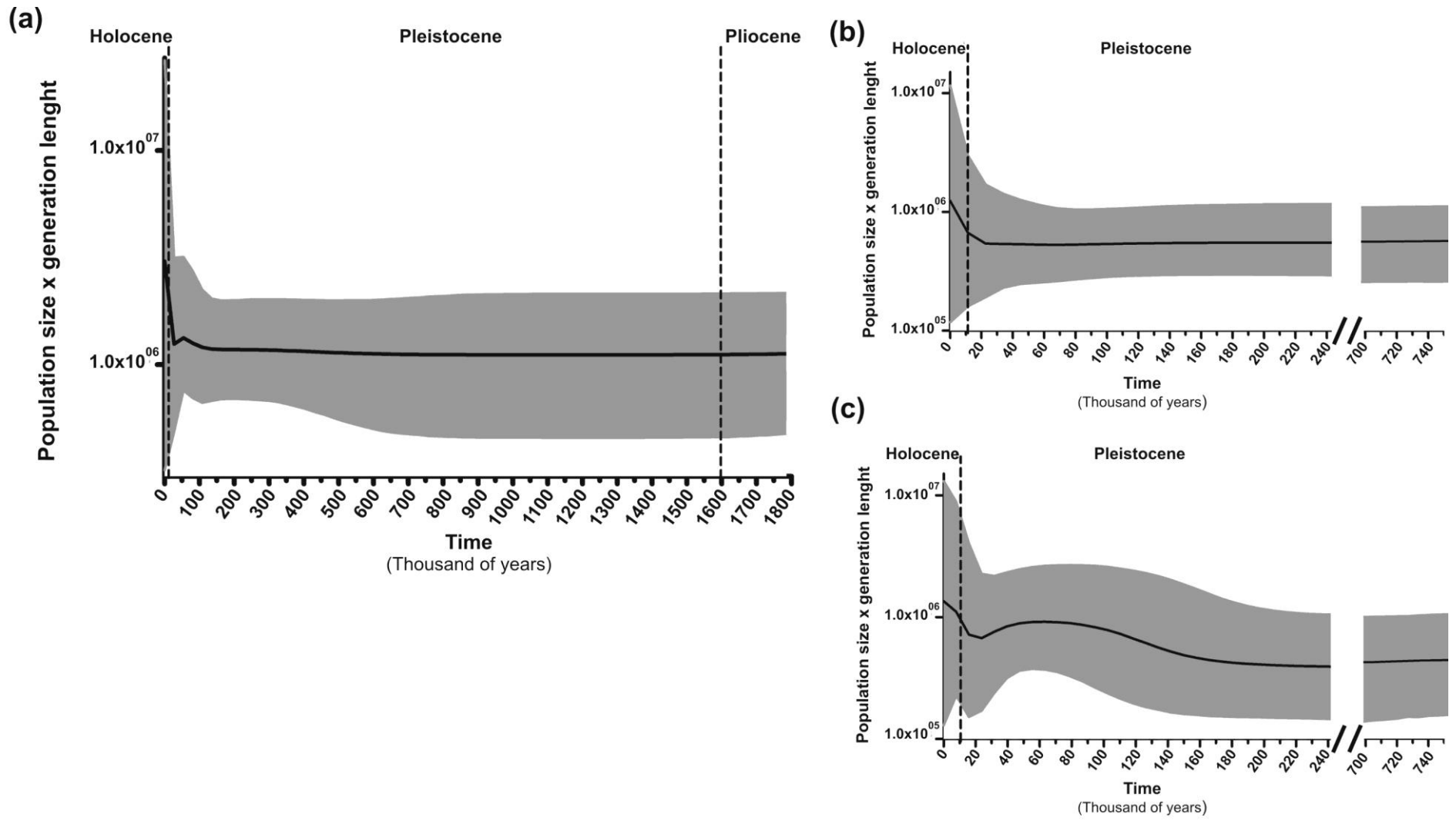
(a)
Holocene
Pleistocene
Pliocene
Population size x generation lenght
1.0x10^07
1.0x10^06
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
1700
1800
Time
(Thousand of years)
(b)
Holocene
Pleistocene
Population size x generation lenght
1.0x10^07
1.0x10^06
1.0x10^05
0
20
40
60
80
100
120
140
160
180
200
220
240
700
720
740
Time
(Thousand of years)
(c)
Holocene
Pleistocene
Population size x generation lenght
1.0x10^07
1.0x10^06
1.0x10^05
0
20
40
60
80
100
120
140
160
180
200
220
240
700
720
740
Time
(Thousand of years)

Fig. 7.

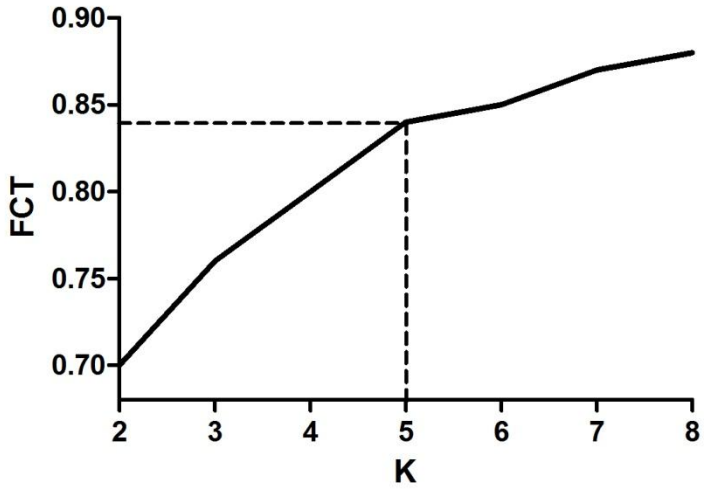

Fig. 8.

CONCLUSÕES

Hollandichthys multifasciatus é caracterizado como um complexo de espécies, ao invés de compor um gênero monotípico. Corrobora-se com forte suporte a indicação do gênero *Rachoviscus* como grupo-irmão de *Hollandichthys*, refutando outras hipóteses anteriores, e o posicionando no clado "C" (*sensu* Javonillo et al., 2010) dentro da família Characidae. Outros táxons *incertae sedis* em Characidae (*Pseudochalceus, Nematocharax* e *Hyphessobrycon uruguayensis)* são posicionados filogeneticamente e pela primeira vez no clado "C" dessa família. Embora não tenha sido obtida uma resolução satisfatória das relações internas do clado "C", este resultado possibilita a inferência de novas questões de relacionamentos entre estes táxons que antes não era possível devido aos seus posicionamentos duvidosos.

Dois grupos principais são claramente formados na filogenia do gênero. Um representa a distribuição ao norte e o outro ao sul da barreira inferida como o estuário de Paranaguá, sendo esta separação datada em torno de 1.9 Mya. Além disso, o estudo filogeográfico permitiu questionar a hipótese amplamente consolidada na literatura de que a presença de espécies em comum entre o alto Tietê e rios costeiros era relacionada a eventos de captura de cabeceira associados ao paleo rio Paraíba. Estes resultados suportam a presença extremamente recente de *Hollandichthys* no Alto Tietê, sendo possivelmente por introdução antrópica ou evento recente de captura de cabeceira em uma área geográfica bastante reduzida.

Os eventos de *bottleneck* e de aumento populacional observados estão mais correlacionados aos eventos climáticos e de expansão e retração da mata Atlântica, do que com os diversos eventos de subida e descida do mar, evidenciando a estrita relação desses peixes com o seu ambiente. O fato dos clados apresentarem datas relativamente antigas comprova essa relação e demonstra que as diversas transgressões e regressões marinhas

recentes não tiveram um papel fundamental para as relações e distribuição atual de *Hollandichhtys*. Algumas populações extremamente isoladas, por exemplo Paraty N, indica que o litoral bem recortado com muitas cadeias de montanhas são importantes barreiras para a dispersão desses peixes também. Já as ilhas, pelo seu curto tempo de isolamento, ainda apresentam uma relação próxima com algumas populações no continente.

Os resultados obtidos por este trabalho sugerem um padrão filogeográfico a ser testado para os demais componentes da ictiofauna vivente na região costeira do sudeste e sul do Brasil. A escassez de trabalhos filogenéticos e populacionais para peixes dessa região demonstra o pouco que se sabe sobre seus padrões de distribuição geográfica, refletindo na diversidade biológica que está ainda por ser descoberta. Além disso, essa região abriga a Mata Atlântica, um ecossistema que vem sofrendo grande ameça por mais de 500 anos tendo sua área reduzida em aproximadamente 80%. Neste trabalho verificamos uma grande importância desse ecossistema para a manutenção da diversidade existente dentro do gênero *Hollandichthys.*

Estes resultados evidenciam a necessidade de mais estudos dessa natureza com o objetivo de avaliar o real grau de endemismo dos peixes ali viventes. Estes organismos se desenvolveram de maneira única devido a diversidade de fatores geológicos e climáticos da região que resultaram na diversidade e conformação atuais. Além de possibilitar um maior entendimento desses processos, estes estudos podem auxiliar em tomadas de decisões mais eficazes para a manutenção dessa diversidade, já que mais organismos do que se imaginava devem ser dependentes diretos desse bioma.

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