

1 **Connection and isolation of watersheds affecting the diversification and**
2 **distribution of genetic variability of coastal freshwater fishes.**

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19 fishes

20 **Abstract:** The Atlantic coast of South America is characterized by a great
21 diversity and endemism of fishes. Past eustatic changes that promoted cycles
22 of isolation, expansion, and connection of coastal watersheds are considered
23 putative drivers of genetic differentiation and phylogenetic diversity. Recent
24 eustatic movements should have left signs of impact on the demographic
25 history and local distribution patterns of freshwater fishes. This study addressed
26 the phylogeography and demographic history of two siluriform (*Scleromystax*
27 *barbatus*, *Rineloricaria* sp.) and one characiform (*Mimagoniates microlepis*) fish
28 species from the coastal plain of Paraná State, around the Paranaguá Bay,
29 Brazil. Nucleotide sequence data of > 800 bp of the mitochondrial cytochrome b
30 gene supports the genetic differentiation between populations of the three
31 species among all studied watersheds. Haplotype networks indicate different
32 histories with scenarios of secondary contact, population expansion, and
33 isolation of the studied populations. Neutrality tests and the reconstructed
34 patterns of demographic history in mismatch distributions were also consistent
35 with secondary contact in northern basins and population expansion mainly to
36 western populations. The results are consistent with the reconstructed
37 paleodrainages in the region and the hypothesis that recurrent reconnection
38 and isolation of streams associated with eustatic changes strongly influences
39 the current pattern of diversity and reflect the distribution of freshwater fishes in
40 this coastal hydrographic system.

41

42 Key words: drainage systems, glaciation, Late Pleistocene, seal-level changes.

43 Introduction

44

45 Roughly 40% of all fish species occur in freshwater (Dudgeon et al.
46 2006) and, on a global scale, the neotropical freshwater ichthyofauna is
47 considered the most diverse (Schaefer, 1998; Reis *et al.*, 2003; Albert & Reis,
48 2011). Approximately 70% of neotropical freshwater fish species occur
49 exclusively in the streams of the Atlantic forest in the Brazilian coast (Abilhoa et
50 al. 2011). In particular, the drainage systems of eastern South America
51 represent regions with high level of endemism of fish species (e.g. Vari 1988;
52 Bizerril 1994; Schaefer 1997). Communities that are confined in drainage
53 systems provide ideal conditions for studies that may reflect the past traits in
54 their populations due to the environmental changes (Lévêque *et al.*, 2008).

55 The physiography of the Atlantic coast in the Paraná State, Brazil, is an
56 ideal model to investigate diversification processes triggered by geological
57 history and paleoclimatology. The region is part of South-East Brazilian
58 ichthyofaunal province (Gery 1969) and the Southeastern Mata Atlantica
59 ecoregion (Abell et al. 2008). These coastal plains are surrounded by the
60 foothills of the Serra do Mar mountain range formed by uplifts during the
61 Cenozoic (Almeida 1976), and all streams and rivers are running West-East and
62 drain into the Atlantic Ocean. The coastal plains of Paraná are recognized as a
63 distinct area in terms of their ichthyofauna with a predominance of small-sized
64 fish species such as tetras (Characidae) and armored catfish (Callichthyidae
65 and Loricariidae) (Abilhoa et al. 2011).

66 Erosive processes were decisive in determining the composition of fish
67 communities in the coastal watersheds of eastern of Brazil (Ribeiro 2006).
68 However, extensive sea-level changes, responsible by marine transgressions
69 and regressions since the Pleistocene, strongly influenced the exchange and
70 diversification in the region (Suguio et al. 1985; Martin et al. 1996). The sea-
71 level exceeded the current level about 123 kya and 5.1 kya (Suguio et al. 1985;
72 Martin et al. 1996; Angulo & Lessa 1997; Martin 2003) but was roughly 130 m
73 below the present sea-level some 18 kya (Kowsmann & Costa 1974; Correa
74 1996). Such cyclic eustatic movements are presently accepted as drivers of
75 genetic diversity, demographic history, and regional distribution patterns of
76 freshwater fishes worldwide (e.g. Roxo et al. 2012; April et al. 2013; Chakona et

77 al. 2013a; Pereira et al. 2013; Roxo et al. 2014; Bruno et al. 2015; Tschá et al.
78 unpublished).

79 Weitzman et al. (1988) was the first to propose the diversification of
80 fishes in the presently isolated watersheds of the South American coast
81 associated to glaciation cycles and sea-level changes during the Late
82 Pleistocene. Subsequently, studies documented that eustatic changes and
83 geographic barriers influenced the radiation of *Odontesthes* species, the
84 silverside fish, in southern lakes of Brazil (Beheregaray et al. 2002) and affected
85 the patterns of dispersion and vicariance of *Hoplias malabaricus*, a widely
86 distributed species in the coastal lakes of Brazil (Pereira et al. 2013). Marine
87 retreats during glacial periods of the Pleistocene provided opportunities for
88 dispersal of freshwater fishes among currently isolated watersheds along the
89 Brazilian coast and acted as a major factor structuring the recent divergence of
90 *Hollandichthys multifasciatus* (Eigenmann & Norris, 1900), an endemic fish of
91 drainages in southeastern coast of Brazil (Thomaz et al. 2015). Additionally,
92 Baggio et al. (unpublished) revealed distinct patterns of genetic variation of
93 estuarine fishes (*Atherinella brasiliensis* (Quoy & Gaimard, 1825) influenced by
94 eustatic movements that matched features of continental shelf along the
95 Brazilian coast. Finally, a recent study of Tschá et al. (unpublished)
96 documented three different kinds of demographic history signals when studying
97 eleven fish species of the coastal lowlands and highlands plateaus in Paraná,
98 Brazil. These authors tied a unique and common pattern observed in fish
99 populations in the coastal lowlands to sea-level changes. In general, the
100 species from lowland watersheds present clear demographic expansion
101 correspond that overlapped with periods of sea-level increase of the sea-level
102 by more than 100 meters.

103 Under this putative scenario, cycles of marine regression and
104 transgression should allow connection (with expansion) and isolation of fish
105 populations from local watersheds. Expansion should result in the mixture of
106 genetic profiles evolved in allopatry during the preceding transgression among
107 neighboring watersheds. In the special case of the watersheds surrounding the
108 Paranaguá Bay transgressions with associated reduction in habitat should
109 result in bottleneck events, that should have been imprinted in the genome. This
110 cyclic pattern of expansion and isolation, with the associated consequences,

111 should be observed at several levels of biological complexity, including in
112 population genetics, as in the present study. However, under the present
113 scenario, this model of genetic diversification and distribution can only be
114 observed if the marine and estuarine environments truly represent a barrier for
115 extensive dispersion of the species. If saltwater indeed acted as a barrier to
116 gene flow among freshwater fish species of the coastal river basins in Southern
117 Brazil, it is expected that even species in geographically close watersheds
118 should have evolved unique genetic structure, while still showing signatures of a
119 common gene-pool. Among the consequences of the last eustatic movement, a
120 demographic signal of bottleneck should precede a populational increase
121 overlapping the period of marine transgression. In order to test these
122 predictions and the model of genetic diversification, this study focuses on three
123 freshwater fish species distributed in watersheds in the coastal plain of Paraná
124 State, Brazil, and correlates sea-level changes and historical conditions of
125 paleodrainages to the genetic signals of expansion and isolation in populations.

126 Thus, there are two major objectives to evaluate: 1) The significance of
127 salt and brackish water as a barrier for the freshwater fish species, and 2) the
128 existence of common genetic signals in populations of this fish assemblage
129 linked to the last cycle of regression/transgression.

130

131

132 **Materials and Methods**

133

134 *Sampling and molecular procedures*

135 Three fish species were targeted in this study, representing distinct
136 biology and ecology. *Mimagoniates microlepis* (Steindachner, 1877)
137 (Characiformes: Characidae), *Scleromystax barbatus* (Quoy & Gaimard, 1824)
138 (Siluriformes: Callichthyidae), and an unidentified species of *Rineloricaria*
139 Bleeker, 1862 (Siluriformes: Loricariidae) were caught by cast nets or electro-
140 fishing under license number 10007 (Instituto Chico Mendes de Conservação
141 da Biodiversidade - ICMBio, Brazil). These species inhabit the same clearwater
142 coastal streams but differ substantially in their life history. *Mimagoniates*
143 *microlepis* is widely distributed (Weitzman et al., 1988), but prefers waters with
144 vegetal covering (Mazzoni & Iglesias, 2002). Furthermore, it has a higher

145 vagility and a rather low salinity tolerance (Freire et al. 2008). The catfish
146 *Scleromystax barbatus* prefers lentic sites. A diurnal foraging pattern in
147 backwaters of shallow streams is characteristic for this species (Aranha et al.,
148 1998; Golçalves & Cestari, 2013). In contrast, *Rineloricaria* species occur in
149 various habitats, including streams or larger rivers with strong water rapids but
150 also lakes with shallow water and sandy soil (Reis & Cardoso, 2001). However,
151 little is known specifically about the biology and taxonomy of the target species
152 of *Rineloricaria* in the target area.

153 Samples were collected from six river sub-basins around the bay of
154 Antonina and bay of Laranjeiras, part of the complex of the Paranaguá bay,
155 Paraná, Brazil. The river sub-basins were: Sagrado River, Nhundiaquara River,
156 Cacatu River, Tagaçaba River, Serra Negra River, and Guaraqueçaba River
157 (Fig. 1). As there are no obvious migration barriers within each sub-basin, all
158 specimens of a species within each sub-basin were considered as belonging to
159 the same panmictic population. Accordingly, the fish from different sampling
160 sites within each sub-basin were pooled in the analyses. Three of the selected
161 sub-basins are located around the Antonina bay (western sites 1, 2, and 3) and
162 another three around the Laranjeiras bay (northern sites 4, 5, and 6). Details of
163 the sampling sites are illustrated in Figure 1, and the respective geographic
164 coordinates are provided in Table S1.

165 Muscle tissue samples were fixed in hypersaturated saline DMSO
166 solution (Seutin et al., 1991), while the remaining parts of the sampled fish were
167 preserved in 95% ethanol, and subsequently registered into the collection of
168 Museu de História Natural do Capão da Imbuia (access number xxxx to xxxx).
169 Total genomic DNA was extracted using the EZ-DNA kit (Applied Biosystems)
170 and the DNA iPrep™ (Invitrogen) robot with the standard protocol of the iPrep™
171 ChargeSwitch gDNA Tissue kit™ (Invitrogen). A fragment of > 800 bp of the
172 mitochondrial cytochrome b gene (CytB) was amplified for all specimens using
173 the primers GLUDG.L (Palumbi, 1996) and H16460 (Perdices & Doadrio, 2001).
174 PCR amplifications were performed in 25 µL containing 1 ng/µl genomic DNA
175 template, 1x buffer, 3 mM MgCl₂, 2.5 U Taq polymerase, 0.4 mM of each dNTP,
176 and 2 nM of each primer. The PCR protocol consisted of an initial denaturation
177 step of 4 min at 95 °C; followed by 35 cycles of 45 s at 94 °C, 45 s at 45 °C, 45
178 s at 72 °C; and a final extension of 5 min at 72 °C. PCR success was controlled

179 by agarose gel electrophoresis, and the positive PCR products were purified
180 with PEG 8000 (Amresco). DNA sequencing of the PCR products was carried
181 out in 10 µl reaction mixes consisting of: 5 ng/µl template DNA, 0.5 µl Big Dye™
182 (Applied Biosystems), 0.2 µM of primer, and 0.1 x reaction buffer. The
183 sequencing reactions were purified using Sephadex™ G-50™ (GE Healthcare
184 Bio-Sciences AB). Nucleotide sequences obtained for both strands were edited
185 using Staden 1.6.0 (Staden 1996), and subsequently aligned using ClustalW
186 (Thompson et al., 1994) as implemented in BioEdit v.7.0.5.2 (Hall, 1999).
187 Nucleotide sequences were deposited in GenBank with the accession numbers
188 KR423944-KR423986, KR424002-KR424087, and XXXXXXX-XXXXXX.

189

190 *Genetic analysis*

191 The estimation of genetic diversity indices as well as the analysis of
192 molecular variance (AMOVA) were done using Arlequin v.3.5 (Excoffier &
193 Lischer, 2010). Overall and pairwise F_{ST} values were calculated for all sub-
194 basins as well as for the pooled western (1-3) and northern (4-6) sub-basins
195 using a permutation of 1,000 iterations. Significance levels for multiple pairwise
196 comparisons were corrected with the Bonferroni method (Rice, 1989). Mantel
197 tests (Mantel, 1967), to test isolation by distance, were also performed in
198 Arlequin v.3.5 (Excoffier & Lischer, 2010), using F_{ST} values. For this purpose,
199 geographic distance was measured as straight-lines between the estuaries of
200 each watershed using Google Earth™.

201 To reconstruct the demographic history for each sub-basin, haplotype
202 networks were constructed, mismatch distribution (Rogers & Harpending, 1992)
203 were plotted, and neutrality tests according to Tajima (1989) and Fu (1997)
204 were calculated. Tajima's D and Fu's F_s statistics and mismatch distribution
205 were produced in Arlequin v.3.5 (Excoffier & Lischer, 2010). Haplotype
206 networks were derived in TCS v.1.21 (Clement et al., 2000) using the
207 parsimony criterion. Demographic histories were also reconstructed by
208 Bayesian Skyline Plots using BEAST v.1.7.5 (Drummond et al., 2012) and
209 Tracer v.1.5 (Rambaut & Drummond, 2009). The best fitting nucleotide
210 substitution model was determined using jModelTest v.0.1 (Posada, 2008)
211 using the corrected Akaike Information Criterion (AICc). Bayesian Inference
212 analyses were conducted in four independent MCMC runs for 100 million

213 generations sampled at each 1,000 trees. The first 10% of trees were discarded
214 as burn-in. To provide an approximate time frame for coalescent reconstruction
215 we used the commonly accepted substitution rates of 0.0076
216 substitutions/site/million years for CytB mtDNA of freshwater fishes (Zardoya &
217 Doadrio 1999).

218 Haplotype accumulation curves were performed to evaluate sampling
219 effort and to compare the haplotype diversity among populations from northern
220 and western sub-basins. I was conducted in the statistical program R v.3.1.2 (R
221 Development Core Team) using the package spider (Brown et al. 2012). This
222 process calculated the mean accumulation of haplotypes and its standard
223 deviation through 1000 random permutations, similar to the method that
224 generate rarefaction curves (Gotelli and Colwell 2001).

225

226 *Paleodrainages reconstruction*

227 The current underwater topographical relief through bathymetric data
228 was used to infer connections from ancient rivers in the coast of Paraná State
229 and to predict the past landscape exposed above water due to marine
230 regressions. Ancient rivers (paleodrainagens) that existed during low sea-levels
231 were modeled by the digital elevation model (DEM) GEBCO_08 at 30 arc-
232 second resolution and based on bathymetric and topographical data (Available
233 at <http://www.gebco.net/>). Paleodrainages were revealed in ArcGIS v.10.3.1
234 with surface and hydrological tools. Raster imperfections were corrected
235 through the fill option and sink option to identify the gaps in DEM. From this
236 corrected image we generated the flow direction and flow accumulation of
237 raster. Thus, the drainage system was reconstructed with the map algebra
238 operator with a 100 pixels threshold and contour lines were added to show the
239 relief based on nowadays depth. This inference of paleodrainages followed the
240 methodology applied in recent studies for wider coastal areas (e.g. Dias et al.,
241 2014; Thomaz et al., 2015).

242

243

244 **Results**

245

246 For all three targeted fish species, a substantial number of mitochondrial
247 haplotypes were detected. Haplotype diversity (h) and nucleotide diversity (π)
248 were in the same order of magnitude for *M. microlepis* ($n = 173$; 43 haplotypes,
249 $h = 0.904 \pm 0.016$; $\pi = 0.003 \pm 0.002$), *S. barbatus* ($n = 181$; 47 haplotypes; $h =$
250 0.875 ± 0.0145 ; $\pi = 0.006 \pm 0.003$), and *Rineloricaria* sp. ($n = 205$; 41
251 haplotypes, $h = 0.806 \pm 0.023$; $\pi = 0.004 \pm 0.002$). Most haplotypes were only
252 found in one locality, but in *M. microlepis* four, *S. barbatus* three, and
253 *Rineloricaria* sp. five haplotypes were found in more than one sampling site.
254 Details on the haplotypes and their distribution are listed in Table S2.

255 The haplotype networks for the three species illustrate the low number of
256 more frequent and widespread haplotypes, and the few instances of haplotypes
257 shared between the sampled sub-basins (Fig. 2). In *S. barbatus* there are two
258 well-defined haplogroups; the first one restricted to the western sub-basins and
259 the second comprising haplotypes only detected in the northern sub-basins. In
260 *Rineloricaria* sp. and *M. microlepis* there are also haplogroups found at the
261 northern sites; however, the respective haplotypes occur also in some
262 watersheds in the west.

263 The AMOVA revealed higher genetic variation within than between
264 populations in the six watersheds for *M. microlepis* and *Rineloricaria* sp.
265 (60.4%, $p < 0.01$ and 48.0%, $p < 0.01$, respectively). For both species just a
266 relatively low fraction of 18.6% ($p < 0.01$) for *M. microlepis* and 24.3% ($p < 0.01$)
267 for *Rineloricaria* sp. of variation related to northern vs western groups. However,
268 a somewhat different result was obtained for *S. barbatus*, in which 59.0% ($p <$
269 0.01) of the variation was between the western and northern groups, and only
270 23.3% ($p < 0.01$) was due to within population variation. Furthermore, variation
271 between populations in the six watersheds was higher than within populations.
272 Pairwise F_{ST} values indicated significant genetic differentiation between almost
273 all populations for the three species, except for the Tagaçaba sub-basin and
274 Serra Negra sub-basin populations of *S. barbatus* (Table 1). Mantel tests
275 provided statistical support only for isolation by distance in *S. barbatus* (79.5%,
276 $p = 0.01$), but these were not significant for *M. microlepis* (27.5%, $p = 0.14$) and
277 *Rineloricaria* sp. (8.6%, $p = 0.32$).

278 The mismatch distribution analyses revealed unimodal curves in all fish
279 populations from the Tagaçaba sub-basin to the Sagrado sub-basin. Although

280 few pairwise differences were observed to *S. barbatus* in Tagaçaba sub-basin.
281 The historical demographic reconstruction for *Rineloricaria* sp. and *S. barbatus*
282 demonstrate populations from the Guaraqueçaba and Serra Negra sub-basins
283 with bimodal curves. In these sub-basins *M. microlepis* present characteristic of
284 unimodal curves (Fig. 3).

285 The Bayesian Skyline Plots indicate for all three species recent
286 significant increases of N_e for the populations in the western sub-basins. For *M.*
287 *microlepis* and *Rineloricaria* sp. a similar pattern was observed for the
288 Tagaçaba and Guaraqueçaba sub-basins. In the other northern sub-basins,
289 however, changes in N_e were less pronounced showing either a slight increase
290 or even a decrease in N_e (Fig. 4). Tajima's D and Fu's FS were negative for all
291 species in all sampled sub-basins, but there was largely no statistical support
292 for these estimates (Table S2).

293 Haplotype accumulation curves revealed that both groups of watersheds
294 showed distinct levels of diversity at given sampling efforts (Fig. 5). It was clear
295 that there are significant differences in their contributions to overall diversity
296 estimates. By far, the western group of watersheds captured the largest
297 diversity in terms of haplotypes in all species, although, it was revealed that the
298 CytB diversity have not been fully sampled in the western group, as indicated by
299 the steep slopes of lines.

300 Reconstruction of paleodrainages from topographic and bathymetric data
301 predicted ancient rivers that probably were connected in the past during the last
302 lowest sea-levels (Fig. 6). There is an obvious relationship between the
303 connectivity of submerged ancient rivers and the current distance of
304 watersheds. Nearby watersheds are previously connected in a downstream
305 direction, however, we observe a pattern of western connection linked to a
306 northern channel that are connected before the Mel Island (see details in Figure
307 1) in an area with no more than 10 m deep.

308

309

310 Discussion

311

312 The physiography of the coastal plain of the Paraná State, Brazil, especially
313 in the region around the Paranaguá Bay, represents an area that was

314 particularly affected by extensive sea-level changes caused by marine
315 transgressions and regressions during the Pleistocene. This area of the coastal
316 plain is rich in small watersheds that are presently isolated by the branches of
317 the Paranaguá Bay and the foothills of the Serra do Mar. Under this geological
318 scenario, eustatic changes promoted cycles of isolation and reconnection of
319 these watersheds (Suguió et al. 1985; Martin et al. 1996) and consequently, of
320 their freshwater fauna. We suggest that these recurrent cycles promoted
321 diversification and may have influenced the regional richness of the freshwater
322 fish fauna (e.g. Vari 1988; Bizerril 1994; Schaefer 1997). However, our results
323 also suggest that intrinsic characteristics of the species studied and regional
324 episodic events promoted genetic exchange between watersheds and resulted
325 in a complex molecular signal.

326 The marine environment indeed represents a significant barrier for the
327 species studied is evidenced by the strong genetic differentiation among
328 populations of the characiform, *M. microlepis*, and the siluriforms, *S. barbatus*
329 and *Rineloricaria* sp., inhabiting distinct watersheds draining into the Antonina
330 Bay and Laranjeiras Bay. Data available on historical sea-levels, strongly
331 indicate that the fragmentation and isolation of these populations were
332 maximum at the sea-level maximum, at 8 ± 2 m and 4.8 ± 0.5 m above present
333 level about 123,000 and 5,100 years ago (Martin et al., 1996; Martin, 2003).
334 Thus, the observed genetic differentiation may be interpreted as the result of
335 the interaction between the geologic features of the region and the most recent
336 transgression of sea-level associated with the end of the last glaciation period.

337 Indeed, recurrent changes in sea-level have been suggested to shape the
338 evolution and diversification, and putatively affected the level of endemism of
339 coastal fish populations (e.g. Chakona et al. 2013a; Pereira et al. 2013, Roxo et
340 al. 2014; Baggio et al., unpublished). Low sea-levels resulted in the confluence
341 of rivers within geomorphological sections of the Eastern Brazilian coast, while
342 during the current sea-level or periods with higher sea-levels the watersheds
343 gradually isolated from each other by the rising sea-level with saltwater posing a
344 geographic barrier for local freshwater fishes. We should, thus, expect to detect
345 genetic signals associated with the last cycle of expansion (low sea-level) and
346 isolation (high sea-level). With expansion, mixture of older haplotypes should be
347 detectable while more recent haplotypes should present higher endemism

348 since they likely originated in allopatry due to the fragmentation of the collective
349 older watershed. The proximity of the mountain range to the Paranaguá Bay
350 during high sea-levels should result in signals of multiple bottlenecks (for
351 distinct watersheds) followed by expansion, as a result of allopatric
352 diversification within the fragments.

353 The topologies of the overall haplotype networks of the three target fish
354 species (Fig. 2) are consistent with the scenario of bottleneck (single most
355 abundant and central haplotypes) followed by secondary expansion (star-
356 shaped pattern), as it should be expected with subsequent population isolation
357 and reduction of the area of distribution. This pattern is especially conspicuous
358 in the regions of the haplotype networks associated with watersheds located in
359 the southwest margins of the Paranaguá Bay (Sagrado, Nhundiaquara, and
360 Cacatu sub-basins). Further, especially for *Rineloricaria* sp. and *S. barbatus*,
361 the existence of multiple central haplotypes with their respective satellite
362 haplotypes is consistent with fragmentation into multiple watersheds and
363 genetic drift followed by allopatric diversification, as indicated by the many
364 endemic satellite haplotypes.

365 Population expansions, a consequence of allopatric diversification of
366 haplotypes, are suggested also by the reconstructed patterns of demographic
367 history inferred by the unimodal distribution in mismatch analyses, especially in
368 western populations, (Fig. 3). Mismatch plots suggest secondary contact for
369 populations of *S. barbatus* and *Rineloricaria* sp. from Guaraqueçaba and Serra
370 Negra sub-basins (Fig. 3). The Bayesian Skyline Plot reconstructions also
371 support population expansion more pronounced in the western populations (Fig.
372 4). Values of Tajima's D and Fu's FS were not significant and thus, are
373 inconclusive (Table S2).

374 The results above clearly point to differences in the demographic
375 histories of populations from the western and northern watersheds of the
376 Paranguá Bay. While the signals of recent isolations of previously large
377 populations linked to bottleneck followed by subsequent expansion is clear for
378 western populations, the same pattern is not observed in the northern
379 watersheds. There are two possible general explanations for these differences.
380 1) That there are fundamental differences in the pattern generated for these two
381 groups watersheds, likely associated to the geomorphology of the areas that

382 hindered the establishment of the pattern of diversification predicted from the
383 oscillation in sea-level; or 2) That the same expected diversification pattern
384 occurred equally in both regions but the populations from the northern
385 watersheds have lost the genetic signal of these events.

386 Haplotype accumulation curves (Fig. 5) indicate that the haplotype
387 richness of northern populations are indeed smaller for all species studied
388 herein, independent of sampling density. Haplogroups for the populations of
389 Siluriformes (*Rineloricaria* sp. and *S. barbatus*) of the northern watersheds (Fig.
390 2) are composed almost exclusively by unique haplotypes, do not depict a clear
391 star-like shape, and shows many indications of absent or extinct (missing
392 intermediate) haplotypes. A unique haplogroup is also observed for the northern
393 populations of *M. microlepis*. Further, general mismatch analyses (Fig. 3)
394 indicate expansion for most northern population, as in the case of the western
395 populations but many watersheds depict signals of secondary contact (for both
396 species of Siluriformes). However, in Bayesian Skyline plots (Fig. 4), expansion
397 is not as evident in the studied species from the northern watersheds.

398 However, there is no reasonable explanation to ascertain that the pattern
399 of diversification expected from sea-level variations should be absent from
400 northern watersheds in the Paranaguá Bay. It is, thus, proposed that differences
401 in the diversification patterns likely reflect more recent processes, which could
402 mask the genetic signals corresponding to the influence of sea-levels by
403 promoting, as suggested by the haplotrees and mismatch analysis, local
404 extinction and secondary dispersion of haplotypes among the northern
405 watersheds. In some way, this hypothesis incorporates both alternative
406 hypotheses propose to provide explanation to the differences encountered
407 between the northern and the western watersheds.

408 Once again, the haplotrees provide some insights on the events that
409 could have taken place and determined the differences observed between the
410 northern and western watersheds. In all watersheds, the coexistence of
411 haplotypes in populations genetically structured might be related to two
412 process. First, sharing of more recent halotypes could be associated to current
413 processes of dispersal and occasional weather conditions, such as the heavy
414 precipitation events. Second, sharing of older (central) haplotypes appear to be

415 related to the spatial relationships of paleodrainages linked with the expansions
416 and connection of presently isolated watersheds during periods of low sea-level.

417 Years with strong El Niño show extreme rainfalls, far above the historical
418 mean in the Paraná Coast (Vanhoni & Mendonça, 2008; Pscheidt & Grimm,
419 2009), and may cause catastrophic and sporadic flooding in the region (see
420 Abilhoa et al. 2011). In such years, large pluviometric volumes promote flooding
421 that connect freshwater bodies. The large river discharge generates flushes into
422 the bay freshwater species and creates unusually extensive freshwater plumes
423 in the river mouth, allowing dispersion of species between neighboring river
424 systems. For the studied fish species such a scenario might seem unlikely
425 since, in general, Characiformes and Siluriformes are primary freshwater fish
426 species with apparent weak tolerance to even slight salinity levels. However,
427 there are evidences that *M. microlepis* can sustain periods of exposition to
428 marine salinity, maintaining their weight muscle stable for more than one hour
429 (Freire et al. 2008) and, thus, should present greater ability to disperse between
430 neighboring watersheds. This is likely associated with differences observed
431 between the siluriform species and *M. microlepis*. *Mimagoniates microlepis* is
432 also benthopelagic - as opposed to demersal as *Rineloricaria* sp. and *S.*
433 *barbatus* (see Froese & Pauli, 2015) which likely favours dispersal between
434 neighboring watersheds, in the less dense freshwater plumes. Survival of the
435 siluriform species are, for the same reasons above, expected to be smaller
436 during periods of high river flow, something that is consistent with the signals of
437 haplotype extinctions for these species.

438 Marine regression records and bathymetric data are clues about the past
439 connection of watersheds in the region. Expansion of regional watersheds
440 formed a wide freshwater drainage system thousands of years ago. The
441 connection facilitated contact between fish stocks of originally isolated
442 drainages and expansion of these populations into adjacent drainages. This
443 process may have an important significant role in the diversification of fishes in
444 coastal streams (e.g. Montoya-Burgos 2003; Ribeiro 2006; Roxo et al. 2012,
445 Dias et al. 2014; Roxo et al. 2014; Bruno et al. 2015; Thomaz et al. 2015).
446 Bathymetric data of Paraná coast (for details see Angulo et al. 2006, Lamour et
447 al. 2007) suggest two past regions of confluence of rivers draining into the
448 Paranaguá bay. There is one drainage channel from the bay of Antonina and

449 another one from the bay of Laranjeiras that come together west of the Mel
450 Island (Fig. 6). This morphological paleochannel system indicated by the
451 paleodrainage reconstruction is congruent to the genetic pattern found in the
452 coastal plain. The pattern of haplogroups differentiated between western and
453 northern populations, principally in *S. barbatus*, reflects the bifurcated
454 geography in the bay and is related to the connectivity of ancient rivers.
455 Haplogroups from western populations are related to the Antonina Bay and
456 rivers that likely ran together in this region, while haplogroups related to
457 northern populations are associated to the river branches on Laranjeiras Bay.

458 The largest continental shelf in South America is on the Paraná coast,
459 with a distance of roughly 230 km from the shoreline to the continental slope
460 (Mahiques et al. 2010). Therefore, periods of low sea-level may have been ideal
461 for the confluence of river basins in deltas or estuaries in regions of the
462 continental shelf (e.g. Suguio et al. 1985). The current hydrographic systems
463 that are isolated by the saltwater formed a distinct scenario in the past. A
464 connected system of river channels and valleys, today submerge, were
465 connected in during low sea-level and allowed freshwater fish migrations and
466 gene flow on the entire Coastal Plain.

467 Our results are consistent with signatures of isolation with eventual
468 secondary contact, and population expansion. Therefore, we propose a model
469 of genetic diversification of freshwater fish species by oscillatory episodes. The
470 cyclic pattern observed in this study, with population expansion and contact
471 associated with subsequent retraction and isolation strongly resembles the
472 evolutionary dynamic known as taxon pulse hypothesis. Pulses of diversification
473 were described by Erwin (1979, 1981, 1985) as repeated periods of contraction
474 and biotic dispersion of taxa beginning in a central area of origin. According to
475 this hypothesis, species generated by allopatric speciation could become
476 widespread after overcoming barriers or after the breakdown of these barriers in
477 repetitive episodes. Marine water acted as a barrier that isolated freshwater
478 populations while sea-level regression, thousands of years later, allowed the
479 genetic mixture among populations through the expansion of fish species
480 previously isolated. This process is clearly a phenomenon of interest to
481 biologists. Studies of historical biogeography in the region may reveal genetic
482 diversity patterns of fish populations which, we predict, will comply with the

483 assumptions of taxon pulse. Molecular dating is essential to infer if the mixing of
484 lineages is associated with periods of sea retreat and genetic divergences with
485 periods of transgression.

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