Short Communication

Population genetic structure of *Cichla pleiozona* (Perciformes: Cichlidae) in the Upper Madera basin (Bolivian Amazon): Sex-biased dispersal?

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1. Introduction

In the Upper Madera basin, the Tucunaré was recently identified as a distinct species than *Cichla monoculus*, its former name, on the basis of molecular data (Renno et al., 2006) and was ultimately described as *C. pleiozona* (Kullander and Ferreira, 2006). This new *Cichla* species inhabits preferably transparent waters in lakes and rivers draining the north territory of Bolivia and is particularly abundant in the Iténez River system, according to our sampling campaign. The clear and calm waters along and across the Iténez watershed provide favorable environmental conditions for the Tucunaré, which is widely spread in both connected and disconnected adjacent lakes and in the river channels (Muñoz et al., 2006). Nevertheless, the Tucunaré can also be observed, although in lesser abundance, in muddy – white water systems (originating mainly in the Andes) of the Bolivian Amazon, where it is found only in small clearer tributaries, creeks, and adjacent lakes with better water transparency than the main river channel.

As a medium-sized fish (40 cm standard length and approximately 2 kg in adult males), the Tucunaré is an important resource for local riverside communities, commercial fisheries and sport fishing (Carvajal et al., 2005; Winemiller, 2001). Threats on the Tucunaré, however, are increasing in the Upper Madera basin as local human communities are growing and intensifying their exploitation, mining activities increase, introduction of non-native species are promoted and habitat destruction intensified (Van Damme and Carvajal, 2005). Population structure analysis and patterns of molecular variation among groups of individuals separated geographically represent a cornerstone for management and conservation priorities at appropriate spatial scale. Renno et al. (2006) observed high mtDNA haplotype diversity with no shared haplotype among geographical populations from the Upper Iténez, Middle Iténez, Yata, Manuripi, Sécure and Ichilo rivers. They also recognized four significantly distinct phylogenetic clades in partial congruence with geography in relation with a demographic expansion. Precisely delimiting genetic populations, however, requires the utilization of nuclear DNA, which allows identifying reproductive units through panmixia analysis. Several studies have shown that introns are suitable neutral markers to describe population
structures (e.g. Berrebi et al., 2006; Hubert et al., 2007; Palumbi and Baker, 1994).

The present study aimed at studying the population structure of C. pleiozona in the Bolivian Amazon using EPIC-PCR analyses, at discussing ecological and geographic factors likely involved in the origin of the structure, and at comparing the results obtained from nDNA and mtDNA, using previously published sequences (Renno et al., 2006) and new sequences of the same sampling sites.

2. Material and methods

2.1. Location and sample collection

The sampling was designed to take into account (1) the variability in the geographic origins of the water in the Upper Madera basin (Andean Cordillera, Brazilian Shield or lowland plain), which influences its chemical and ecological parameters (Sioli, 1984), and (2) the geographic distance between the sampling localities, considering the extensive floodplain (Hamilton et al., 2004) as a possible way of dispersal for fish populations.

Sixty-seven specimens of C. pleiozona were sampled in the same localities of Renno et al. (2006): in the Iténez, Mamoré and Beni-Madre de Dios sub-basins in the Upper Madera basin (Fig. 1). In the Iténez clear water basin originating in the Precambrian Brazilian shield, two localities were collected: the San Martín River (11 specimens) in the middle part of the drainage, and the Paraguá River (11 specimens) in its upper part. Two localities were collected in the Mamoré basin: the Securé River (11 specimens) in the middle part and the Ichilo River (13 specimens) at the headwater. The Ichilo and Sécure rivers are directly influenced by muddy-white water from the Andes. The Yata River (11 specimens) is a lowland drainage of clear waters running through the forest between the Mamoré and Beni drainages. In the Madre de Dios-Beni system only the Manuripi River (10 specimens), in the lower portion, was sampled due to the low abundance of Tucunaré in this system. The Manuripi River is also a lowland drainage, although considered of mixed waters (clear and muddy) due to its seasonal change in water appearance. Indeed, during the dry season, the Manuripi River receives water from local small tributaries that run through the forest (clear water), while during the rainy season it tends to be muddy probably because of the increased erosion and possible contacts with the Madre de Dios River originating in Andes Cordillera (Navarro and Maldonado, 2002).

The fish were caught by hook and line and occasionally gillnets in rivers and lakes during the dry seasons of 2001–2003.
Approximately 1 cm³ of muscle tissue was removed and immediately preserved in 96% ethanol for subsequent DNA extraction.

2.2. mtDNA data analyses

Adding to the 41 sequences previously analyzed (Renno et al., 2006) and using the same methods, 75 new sequences (total 116) were used: 9 from the Manuripi, 4 from the Yata, 7 from the Sécure, 14 from the Ichilo, 32 from the San Martin, 9 from the Paraguá rivers. These new sequences were obtained from preserved tissues of the original fish sampling at the exact same localities, and each unique haplotype were deposited in DDBJ/EMBL/GenBank database (accession numbers between HQ267858 and HQ267932).

The genetic differences between each pair of geographical populations were established using the $\Phi_{ST}$ calculated with Arlequin version 3.5.1.2 (Excoffier and Lischer, 2010), considering the Tamura-Nei distance ($\chi^2 = 0.715$) selected by the Akaike Information Criterion value (AIC) in APE (Paradis et al., 2004). Gene flow was calculated as $N_m = 1 - \Phi_{ST} / 2 \Phi_{ST}$.

2.3. nDNA extraction, intron amplification and polymorphism assessment

Intron amplification (EPIC–PCR) and analyzes interpretations were carried out according to Hubert et al. (2006). EPIC-PCR enjoys several practical advantages: cross-species amplification is easier when primers are designed in coding sequences because exon sequences are more conserved across species, and PCR artefacts such as null alleles are expected to be less frequent for the same reason. A locus was identified when the combination of all their alleles followed a Mendelian inheritance. Four suitable pairs of available primers were used for: Aldolase B (Hassan et al., 2002); Mhc 1F/2R which amplifies the intron 1 of the Major histocompatibility complex class II antigen (Hassan et al., 2002); PmOpsi-F/-R designed from cDNA sequences of the opsin (Bierne et al., 2000); and PmAct1-F/-R designed from cDNA sequences of the actin (Bierne et al., 2000).

2.4. Intron length polymorphism analysis

Levels of genetic variation were based on classical parameters such as the allelic frequency per locus, levels of observed ($H_o$) and non-biased estimates of expected heterozygosities ($H_e$) (Nei, 1978). Inbreeding index ($F_{IS}$) (Wright, 1978), estimated by the $f$ estimator of Weir and Cokerham (1984), was calculated to evaluate the departures from panmixia. Significance was tested by 1000 permutations among the alleles. Overall differentiation at the population level was assessed by considering all loci using 1000 permutations among the alleles. Overall differentiation at the population level was assessed by considering all loci using 1000 permutations among the alleles. Evaluating the departure from panmixia. Significance was tested by the $\Phi_{ST}$ estimator test, for each of the six geographic samples, the hypothesis of panmixia cannot be rejected (Table 2).

3. Results

3.1. Population structure inferred from mtDNA

The observed haplotypic diversity was high with 85 haplotypes out of 116 sequences (Fig. 1f). The haplotypic diversity ranged from 0.738 ± 0.106 (Manuripi) to 1.000 ± 0.030 (Yata) and the nucleotide diversity from 0.003 ± 0.002 (Ichilo) to 0.026 ± 0.014 (Yata) (Table 1a). A single haplotype was shared by two geographical samples, the Manuripi and San Martin (Middle Iténez). All the other haplotypes were private of a particular locality. The Yata sample (11 specimens) was only composed of singleton haplotypes.

All the pairwise $\Phi_{ST}$ comparisons among geographical populations were significant (Table 1b). Despite obvious limited gene flow (significant $\Phi_{ST}$ values) between the Yata, Middle Iténez and Upper Iténez populations, the number of migrants per generation ($N_m$ calculated from $\Phi_{ST}$) were relatively high (3.6–7.0) compared to those between the other geographical populations (<1, Table 1c).

3.2. Population structure inferred from nDNA (EPICs)

Five polymorphic loci were identified with the four pair primers. For each amplified intron one locus was identified except for PmAct1-F/-R, which amplified two loci: PmAct1 and PmAct2. Two alleles were scored for Aldolase B, Mhc, PmOpsi and PmAct1, and three for PmAct2. Aldolase B and PmOpsi loci were polymorphic only in the Middle Iténez sample (Table 2, Fig. 1a–e).

According to the $f$ estimator test, for each of the six geographic samples, the hypothesis of panmixia cannot be rejected (Table 2).

The mean inter-sample $\theta$ was 0.44, with a range from 0.00 (Upper Iténez vs. Yata) to 0.96 (Manuripi vs Ichilo), rejecting the homogeneity of allele frequencies for all pairwise comparisons excepted between Yata-Middle Iténez ($\theta = 0.02$), Middle Iténez-Upper Iténez ($\theta = 0.03$), and Yata-Upper Iténez ($\theta = 0.00$), which showed the highest gene flow (Table 1c). The Manuripi, Ichilo and Sécure were clearly the most isolated geographical populations, differentiated from Yata, Middle and Upper Iténez (Table 1b), which grouped together and displayed a low $F_{IS}$ value when a single genetic unit was considered ($f = -0.08, P = 0.19$). All the pairwise $\theta$ comparisons among geographical populations were significant, excepted among the Yata, Middle and Upper Iténez (Table 1b). Contrarily to the mtDNA results, the higher number of migrants per generation observed among these three geographical populations (Table 1c) preventing significant differentiations, hence in stronger gene flow. Consequently, the consensus NJ radial tree based on Reynolds et al.’s genetic distance was built with four genetic populations showing a topology weakly supported by the bootstraps (Fig. 2).
The highest genetic variability, combining $H_{NB}$ and private alleles, was observed in the Yata-Iténez (Middle-Upper) population ($H_{NB}$ around 0.15, with three private alleles: Aldolase B (250), Mhc (300), PmOpsi (500)), whereas the Manuripi and Ichilo ($H_{NB}$ close to 0.00) populations showed the lowest. The Secure population had the same $H_{NB}$ that the Middle Iténez sample, but with only a single private allele in the locus Pm$\text{ActI}_1$ (730) (Table 2).

The AMOVA analysis revealed that the genetic differentiation among geographic population was not significantly related to the water origin ($\Phi_{CT} = 0.27, P = 0.11$), but significantly related to the geographical location and so to the distance separating them ($\Phi_{CT} = 0.40, P = 0.01$), with contributions to the variance of 26.96% and 39.79%, respectively (Table 3).

The correlation between genetic distance and the geographic distance following the sinuous pathway of the rivers courses was stronger than between genetic distance and geographic distance in straight line, $r = 0.38$ ($P = 0.0234, 13$ d.f.) and $r = 0.45$ ($P = 0.0458, 13$ d.f.), respectively.
The higher genetic variability was observed, with both nDNA and mtDNA, in the Iténez-Yata system (where we observed that Tucunaré are more abundant). This confirms previous results obtained with a lower number of sequences (Renno et al., 2006). The Iténez-Yata system might have acted as an aquatic refuge for some groups of fish (i.e. Hubert et al., 2007), including Tucunaré, during the last Pleistocene glaciation’s period and as a source for the subsequent colonization of the Bolivian Amazon. The low genetic variability observed in the Manuripi and Ichilo populations would then be the result of two independent founder events from the Yata-Iténez. Although the Refuge Theory (habitat contraction) (Haffer, 1982) has been questioned as a preponderant model on terrestrial Neotropical Biota diversification at the specific level (i.e. Colinvaux et al., 2000; Cortés-Ortiz et al., 2003), it explains coherently the results of previous intraspecific molecular analyses in different fish species in the Upper Madera basin: C. pleiozona, showing highest mtDNA haplotypic diversity in the Iténez River populations (Renno et al., 2006), Serrasalmus rhombeus with similar structure (EPIC-PCR and mtDNA, Hubert et al., 2007).

Spatial analysis of molecular variance did not show relationship between the nDNA geographical structure and the water origin of the watersheds. The same results were obtained with mtDNA, which confirms what was previously observed with fewer sequences (Renno et al., 2006). Correlation analysis between geographical and genetic distances suggested isolation by distance among populations. However discrepancies have been observed: two close geographic samples (Sécure and Ichilo, ~400 km by the main river course) are genetically differentiated populations, whereas more geographically distant samples (Yata and Upper Iténez, ~1000 km, by the main river course) represent a single population. Therefore, it cannot be ruled out that water origin at local scales might also play a role in Tucunaré dispersion in the Upper Madera.

As a whole, the results of both nDNA and mtDNA analyses concurred in indicating strong genetic differentiation among the geographical populations. AMOVA and correlation analyses indicated that this differentiation was mainly related to geographical isolation. Additionally, the higher genetic diversity in the Yata-Iténez system suggested it as the center of origin for the colonization of the Upper Madera by the Tucunaré, with probable founder effects in the Manuripi and Ichilo. Thus neutral genetic drift is likely to explain the major pattern of population differentiation observed by both nuclear and mitochondrial markers.

Some discrepancies, however, were observed between nDNA and mtDNA results, particularly in the Yata-Iténez system. Indeed, while no genetic differentiation is observed between the Yata, Middle and Upper Iténez samples using nDNA, these three localities are genetically distinct populations using mtDNA, with no haplotype sharing (see also Renno et al., 2006). In the same way, using nDNA, the Manuripi and Ichilo populations are strongly differentiated from the other populations although they do not hold private alleles, whereas neither of them did share mitochondrial haplotype with other populations, apart from a single one (shared between Manuripi and Middle Iténez). Several works have highlighted important differences about the population structure of a species when mtDNA and nDNA results are confronted (e.g. Larmuseau et al., 2010; Lemaire et al., 2005; Palumbi and Baker, 1994; Shaw et al., 2004). Discordances on spatial structure between nuclear and cytoplasmic markers can be the result of selection, drift, mutation, reproductive isolation or migration mechanisms (Larmuseau et al., 2010; Lemaire et al., 2005):

- Direct natural selection acting on mtDNA or, indirect selection against epistatic relationships between mitochondrial genome and nuclear genes were invoked by Rand (2001). However, this hypothesis is highly speculative as we have no indication whatsoever that such complex disruptive selection might be at work within the Yata-Iténez system.

- Mitochondrial markers are more efficient for detecting differentiation among populations with few migrant exchanges because of a lower effective population size (more sensitive to genetic drift), which is one-quarter that of nDNA (Shaw et al., 2004). Moreover, the faster genetic drift is combined with a faster mutation rate, which is about five to ten orders of magnitude that of nDNA (Brown et al., 1979). These characteristics, however, cannot explain

### Table 3

Results of AMOVA (nuclear DNA) analysis according to ecological and geographical groups a priori formed for six geographic samples of Tucunaré from the Bolivian Amazon following Renno et al. (2006).

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Percentage of variation</th>
<th>Fixation index</th>
<th>Significances tests</th>
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</thead>
<tbody>
<tr>
<td>Ecological criteria, three water origin groups a priori formed: (Manuripi-Yata) from lowland waters, (Sécure-Ichilo) from Andean waters and (Middle-Upper Iténez) from the Brazilian Shield</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Among groups</td>
<td>36.96</td>
<td>ΦCT = 0.27</td>
<td>P = 0.11, d.f.=2</td>
</tr>
<tr>
<td>Among populations within groups</td>
<td>31.41</td>
<td>ΦST = 0.43</td>
<td>P = 0.00, d.f.=3</td>
</tr>
<tr>
<td>Within populations</td>
<td>41.64</td>
<td>ΦRT = 0.58</td>
<td>P = 0.00, d.f.=128</td>
</tr>
<tr>
<td>Geographical criteria, three distant groups a priori formed: (Manuripi) Madre de Dios watershed, (Sécure-Ichilo) Mamoré watershed, and (Yata-Middle and Upper Iténez) Yata-Iténez watershed</td>
<td></td>
<td></td>
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<tr>
<td>Among groups</td>
<td>39.79</td>
<td>ΦCT = 0.40</td>
<td>P = 0.01, d.f.=2</td>
</tr>
<tr>
<td>Among populations within groups</td>
<td>20.94</td>
<td>ΦST = 0.35</td>
<td>P = 0.00, d.f.=3</td>
</tr>
<tr>
<td>Within populations</td>
<td>39.27</td>
<td>ΦRT = 0.61</td>
<td>P = 0.00, d.f.=128</td>
</tr>
</tbody>
</table>
alone the observed discrepancy between markers as the panmictic Yata-Iténez system indicates that important random gene flow, hence migrant exchanges, exist among the sampled localities. As mtDNA is matrilineally inherited, a sex-biased ratio with predominance of males, could increase the differences of the effective population size between mitochondrial and nuclear DNA, leading to a much larger estimate of 0 at the cytoplasmic loci than at the nuclear loci as a result of a stronger drift of mtDNA. The hypothesis of male-biased sex-ratio, however, is unlikely as out of the 2300 specimens collected (mainly by hook and line) for life history studies in the Yata-Iténez system, the sex ratio was not significantly skewed (0.48 F / 0.52 M) despite the fact that males are more prone to capture due to their higher aggressiveness (Duponchelle et al. unpublished data).

- Length homoplasy could explain an underestimation of genetic differentiation between populations in the case of hyper variable nuclear markers like microsatellites (Estoup et al., 2002). Higher mutation rates and larger effective population sizes are likely to produce more homoplasy in hypervariable markers (Balloux et al., 2000). Here, intronic length polymorphism concerned only a few alleles (between 2 and 3) and the variation in genetic differentiation among populations was independent of their level of polymorphisms (see: Manuripi / Ichilo with low polymorphism, but also Sécure / Yata-Iténez with high polymorphism). Hence, it is unlikely that homoplasy could have acted to reduce the genetic differences between populations.

- A sex-biased reproductive isolation mechanism involving a counter selection of the allochthonous female could explain the limited dispersion of mtDNA from a population to another. Lemaire et al. (2005) proposed this mechanism to explain the mode of hybridization observed between Atlantic and Mediterranean populations of sea bass. This hypothesis might apply to our case, but is highly speculative hereby as there is no indication that such counter selection might occur.

- Another hypothesis could lie in sex-biased dispersal rates (Cano et al., 2008; Fitzsimmons et al., 1997). This bias is known to occur in fish species showing an intense male-male competition for female access (Consuegra and García de Leániz, 2007). In C. temensis a mark-recapture study has shown that the larger males were able to migrate tens of kilometers, whereas females and small male's movements were usually restricted to within one kilometer of their original marking site (Hoeinghaus et al., 2003). There is no direct information about differential dispersal behavior between sexes in C. pleiozona. However, as for C. temensis, males reach significantly larger sizes than females (Muñoz et al., 2006) and are likely more capable to migrate. Then, a likely explanation for the nuclear and mitochondrial discrepancies observed for C. pleiozona in the Bolivian Amazon is a variable sex-biased migration rate, with sedentary female and a dispersion capacity of some large males depending on geographical distance and ecological parameters such as water quality. Indeed, in muddy water systems, the Tucunaré are seldom found in the main channels but live in habitats with better transparency, such as adjacent lakes. In these systems, the main river channels seem to act as a barrier limiting the Tucunaré's movements. On the contrary, in clear-waters, such as in the Yata-Iténez system, Tucunaré are abundant in lakes, but also in the main river channels (Muñoz et al., 2006). Water transparency and quality might explain why the discrepancy between nDNA and mtDNA results apparently only appears in the Yata-Iténez systems. On the one hand, the higher number of migrants observed (highest Nm, with both nDNA and mtDNA markers) between the geographically distant, but genetically non-differentiated Yata, Middle and Upper Iténez samples (following nDNA information) can be explained by the water quality, which favors fish movements and hence gene flows. On the other hand, the white-muddy waters in the other river systems sampled likely restrict the fish movements and inhibit the superior migratory behavior of Tucunaré males.

The review of the possible explanations for the markers discrepancy observation suggested that no unique cause is involved. The most probable explanation should be a sex-biased dispersal augmented by effective population sizes differences between nuclear and mitochondrial markers.

However, the geographical structure of C. pleiozona clearly showed a watershed heterogeneity. The more likely explanation for the observed patterns of differentiation among the geographical populations would be that the small populations (Manuripi, Sécure, Ichilo), restricted to adjacent habitats, because of unfavorable water quality conditions in the main river channels, are isolated and hence more subjected to genetic drift. Contrastingly, within the Yata-Iténez system, the fish movements are facilitated by the more favorable clear-waters, which allow the expression of probable sex-biased dispersal abilities, resulting in the observed discrepancy between nuclear and cytoplasmic markers in this system.

Acknowledgments

This research was supported by IRD-UR 175, France, and partially by the consortium BirdLife International-WCS-BP-CI Fauna & Flora International (Conservation Leadership Programme) during the sampling period in the Upper Iténez. It was carried out within the framework of the network RIIA (Red de Investigación de la Ictiofauna Amazónica: http://www.riiaamazonia.org/). We would like to thank Carvajal-Vallejos P.K. for suggestions to improve the manuscript and Zeballoes Fernández A.J. for the maps and geographic distances.

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