Aquatic Conservation-marine And Freshwater Ecosystems
August 2020, Volume 30 Issue 8 Pages 1521-1539
https://doi.org/10.1002/aqc.3373
https://archimer.ifremer.fr/doc/00695/80725/

The Amazonian dwarf cichlid *Apistogramma agassizii* (Steindachner, 1875) is a geographic mosaic of potentially tens of species: Conservation implications

Estivals Guillain [1, 2, 3, 4, *] , Duponchelle Fabrice [2, 3, 4, 5], Romer Uwe [2, 3, 4, 6], Garcia-Davila Carmen [2, 3, 4, 7], Airola Etienne [2, 3, 4], Deleglise Margot [2, 3, 4], Renno Jean-François [2, 3, 4, 8, *]

[1] Sorbonne Univ, UPMC, Ecole Doctorale Sci Nat & Homme ED 227, Paris, France.
[2] Inst Invest Amazonia Peruana IIAP Peru, Lab Mixte Int Evolut & Domesticat Ichtyofaune Ama, Iquitos, Peru.
[3] Univ Autonoma Gabriel Rene Moreno UAGRM Bolivia, Santa Cruz, Bolivia.
[4] IRD Inst Rech Dev, Marseille, France.
[5] Univ Montpellier, CNRS, MARBEC, IRD, Montpellier, France.
[6] Univ Trier, Inst Biogeog UTIB, Anim Res Grp, Trier, Germany.
[7] Inst Invest Amazonia Peruana IIAP, AQUAREC, Lab Biol & Genet Mol, Iquitos, Peru.
[8] IRD, UMR Diversite Adaptat & Dev Plantes DIADE, Montpellier, France.

* Corresponding authors : Guillain Estivals, email address : ichtyos3134@hotmail.fr ; Jean-François Renno, email address : jean-francois.renno@ird.fr

Abstract :

1. Assessing biodiversity and understanding how it works is a prerequisite for species conservation. The Amazon basin is one of the main biodiversity hotspots where fish are heavily exploited for ornamental purposes.

2. The ornamental trade heavily exploits the genusApistogramma, which is one of the most species-rich among Neotropical cichlids with 94 formally described species. This number is certainly underestimated owing to the limitations of conventional taxonomy, which is still too often based solely on morphological criteria and sometimes on few individuals.

3. Most species of this genus have a high degree of endemism and are highly prized on the ornamental market, which could put them at risk. A few species are supposed to have extensive distributions, and in particularApistogramma agassizii, present from the Amazon estuary up to the Ucayali and Maranon rivers in Peru.

4. This study assessed the taxonomic status of 1,151 specimens ofA. agassiziicollected from 35 sites around Iquitos in the Peruvian Amazon. On the basis of molecular analyses (nuclear and mitochondrial DNA) and mate choice experiments, at least three biological species within the nominalA. agassiziiwere evidenced in the sampling area, which is extremely small compared with the known distribution of the species as initially described.

5. According to the molecular calibrations, these three species would have diverged during the Plio-Pleistocene. Two of them seem to be endemic from small sub-basins, one from the Nanay River and the other from the Apayacu/Ampiyacu systems. A possible scenario that may explain the evolutionary history of these species is proposed.

6. The conservation implications of these results on the estimation of the diversity ofA. agassizii, ofApistogrammaspecies in general, and of other Amazonian cichlids are discussed.

Keywords : biodiversity, biogeography, fish, fishing, floodplain, genetics, river

1 INTRODUCTION

Anthropogenic pressures on biodiversity, and aquatic biodiversity in particular, are increasing alarmingly in the Amazon basin (Castello et al., 2013; Castello & Macedo, 2016). The main threats are deforestation (Houghton et al., 2000; Morton et al., 2006), the construction of infrastructures such as hydroelectric dams (Anderson et al., 2018; Finer & Jenkins, 2012; Forsberg et al., 2017) and overexploitation (Castello et al., 2013), although pollution (Finer, Jenkins, Pimm, Keane & Ross, 2008; Kingston, 2002; Swenson, Carter, Domec & Delgado, 2011) and species introductions (Latini & Petrere, 2004; Vitule, Freire & Simberloff, 2009; Vitule et al., 2017) are also involved. Many of these threats result in habitat degradation or loss directly affecting aquatic communities including fish. As a traditional major source of protein and income in the Amazon basin, fish additionally have to cope with threats associated with fishing exploitation (Barthem & Goulding, 2007; Castello, Arantes, McGrath, Stewart & Sousa, 2015; Castello et al., 2013), including for ornamental purposes (Bayley & Petrere, 1989).

Of the more than 2700 fish species formally described for the Amazon basin, (Dagosta & Pinna, 2019), 697 are exported from Brazil for ornamental purposes (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis, 2017) and more than 300 from Peru (Gerstner, Ortega, Sanchez & Graham, 2006). These estimates are certainly below reality due to problems of taxonomic identification by exporters. Indeed, many fish are exported under generic terms that sometimes include dozens of species (Moreau & Coomes, 2007). In addition, there are species that are not described and others that require taxonomic revisions. Indeed, the vast majority of current descriptions have been made only on morpho-meristic characteristics. At the time of molecular biology, these descriptions show their limits, particularly in the case of complexes of morphologically very close, and thus by phenotype, confusable species. Many fish are therefore exploited without knowing their taxonomic status and vulnerability. Many ornamental fishes have restricted distributions, or compared to species used for human consumption, live in areas with high rates of endemism (Moreau & Coomes, 2007; Olivier, 2001). They are therefore particularly vulnerable to the threats already mentioned and to ornamental fishing, the impact of which has received little attention (Alho, Reis & Aquino, 2015; Crampton, 1999; Gerstner, Ortega, Sanchez & Graham, 2006; Moreau & Coomes, 2007; Römer, 1995).

The genus *Apistogramma* is the most diversified of the Neotropical cichlids: it is composed of 94 species that are considered valid and is subject to regular new descriptions (Britzke, Oliveira & Kullander, 2014; Römer, 2006; Römer & Hahn, 2008, 2013; Römer et al.,

2011, 2012, 2015, 2017). All of its species are valued as ornamental fish species. They generally have small distribution areas. The most extreme case is *Apistogramma cinilabra* (Römer et al., 2011; formerly known as *A.* sp. 'Roterpunkt' in the ornamental fish trade), which exclusively lives in a small isolated lake near the city of Nauta (Peru). In 2014, it was added to the IUCN Red List and assessed as vulnerable. Today, it is probably in critical danger due to deforestation and chemical fishing in its habitat. According to the comment of an ornamental fisherman, it has become so scarce that it is no longer interesting to fish for it.

The genus *Apistogramma* does not escape the known problems of taxonomic identification in other ornamental species (Britzke, 2015; Römer, 2006; Tougard et al., 2017), especially since some descriptions have been made on a limited number of individuals (f. e. Mesa & Lasso, 2011), not allowing to account for their distribution and intraspecific variability (Schindler & Staeck, 2013). The number of species belonging to this genus is probably significantly underestimated (Römer, 2006), as evidenced by the many new descriptions in recent years. Within this genus, only three nominal species, *A. agassizii*, *A. cacatuoides* and *A. bitaeniata*, are reported to have large distribution ranges. Fishes placed into the species *A. agassizii* are found from the Marañon and Ucayali rivers of Peru, along the main Amazon River, in some of its tributaries, and downstream to its estuary at the Atlantic Ocean. They can be found throughout the floodplain of the Amazon basin and live in all types of water, whether white, clear or black (Kullander, 1986; Römer, 2006). The species prefers areas with little current where plant debris accumulates, particularly dead leaves (Römer, 2000). Different geographical populations, based on morphological characteristics, have been identified by Römer (2000, 2006) and Koslowski (2002). A recent molecular study has shown that the taxon *A. agassizii* is not monophyletic at the Amazon basin scale: the species *A. gephyra* is located within the taxon, which is divided into four clades, including one from the Peruvian Amazon (Britzke, 2015). It is therefore likely that within this taxon, described on morpho-meristic characteristics and colour patterns, there are several distinct biological or phylogenetic species as suggested by Britzke (2015), Römer (2000, 2006) and Tougard et al. (2017). It is of paramount importance to precisely define the species boundaries in the genus *Apistogramma* and, in particular, within what is presently considered *A. agassizii* in order to assess the true species diversity of the genus *Apistogramma* as a tool to identify the number of individuals extracted per species for the ornamental fish trade. That will facilitate regulation of their harvests and help to ensure their conservation.

The objectives of this study are to evaluate genetic variation and taxonomic status of *A. agassizii* in a portion of the Loreto region (Peru). The variation of the allelic frequencies of

microsatellite markers and the sequence polymorphism of a fragment of the Cytochrome c oxidase subunit I (COI) gene were analysed, and mate choice experiments to assess pre-zygotic reproductive isolation were performed. The genetic and biological boundaries of *A. agassizii* species from the study area are delimited on the basis of three species concepts: genetic entities defined by clusters (e. g. Mallet 1995), monophylogenetic clades (e. g. Cracraft, 1989; Hennig, 1966; Mayden & Wood, 1995), and reproductive isolation (e. g. Mayr, 1942). The evolutionary processes that might have caused the observed genetic variation are discussed. Finally, the implications of the existence of a biodiversity that is probably largely underestimated, are discussed for the conservation: a) of the *A. agassizii* "complex", b) of the genus *Apistogramma*, and c) of Amazonian cichlids in general. Overall, the monotypy hypothesis for Peruvian *Apistogramma* presently considered to belong to the nominal taxon *A. agassizii* is tested.

2 MATERIALS AND METHODS

2.1 SAMPLING

First study areas have been defined from three regions where *A. agassizii* was known to be abundant based on information by ornamental fishermen (Figure 1a). The first study area corresponds to the surroundings of Jenaro Herrera (Loreto, Peru). This region is drained by a complex network of forest streams, which flow into various parts of the Ucayali River. The streams surrounding Jenaro Herrera (at the right bank of the Ucayali) are located on "tierra firme", i.e. they are not submerged by the floods of the Ucayali (Figure 1b). A second study area corresponds to the surroundings of Orán (Figure 1c). The Orán region, hillier than Jenaro Herrera, is also drained by a complex network of forest streams where *A. agassizii* is found exclusively in lowland areas near the Amazon floodplain. The third study area is the Nanay River, representing the largest black water river in the Peruvian Amazon. *A. agassizii* is mainly distributed in its floodplain. All of the three regions have been sampled consecutively in order to have a global vision to evaluate the genetic structure of the species at different geographical scales.

Between 2015 and 2018, 1151 samples were collected from 35 sites near the rivers Ucayali, Marañón, Amazon and Nanay (Figure 1) using a beach seine or landing nets. The sample (all the fish) collected from a single site will thereafter be named "popsite" (the contraction of the words population and site). The fish were anaesthetized with eugenol using the protocol defined by Chanseau, Bosc, Galiay & Oules (2002) until respiratory arrest, then

were stored in coded tubes one by one and preserved with 96% ethanol. Most fully mature males were photographed after sedation before being preserved in ethanol. The alcohol was renewed after 24 hours. The tubes with voucher specimens are kept in the collection of the "Laboratorio de Biología y Genética Molecular del Instituto de Investigaciones de la Amazonía Peruana" (LBGM-IIAP, Iquitos, Peru).

To evaluate the evolutionary history of *A. agassizii* in the study area, the mitochondrial marker Cytochrome c oxidase subunit I (COI) and 10 nuclear markers (microsatellite loci) were used. The COI allows to identify clade differentiation events and microsatellite loci reveal genetic entities structured in clusters, and hence, that do not share geneflow.

2.2 DNA EXTRACTION, AMPLIFICATION AND GENOTYPING OF MICROSATELLITE LOCI

DNA extraction was performed from ~ 10 mg of caudal fin using a modified CTAB extraction protocol (Doyle & Doyle 1987). The amplification of the 10 microsatellite loci was performed with primers developed by Quérouil, Vela Diaz, García-Dávila, Römer & Renno (2015) using the Qiagen Multiplex PCR kit. For each multiplex, a fluorescent primer was marked with one of the three dyes 6-FAM, HEX or NED (Applied Biosystems). The amplification program used was the one recommended by the kit manufacturer (hybridisation temperature 57 °C and 28 cycles). The analysis of the fragment's length was performed on an ABI 3130 XL sequencer with 16 capillaries (Applied Biosystems). For each capillary, 1 µL of the diluted PCR product, added to 8.85 µl of formamide and 0.15 µl of ROX 500™ denatured for 10 min at 70 °C were used. The size of each allele was obtained using the Geneious 9.1.8 program (Kearse et al., 2012).

2.3 AMPLIFICATION AND SEQUENCING OF THE CYTOCHROME C OXIDASE SUBUNIT I (COI) GENE

The individuals sequenced were chosen after microsatellite genotyping. A total of 32 individuals from 14 popsites were selected summarizing both the information provided by the nuclear data and the sampling area. The COI was amplified using the L5698-Asn (F) 5' -AGG CCT CGA CGA TCC TAC AAA GKT TTA GTT AAC-3' (Miya & Nishida, 2000) and H7271 (R) 5' -GTG GTG GGC TCA TAC AAT AAA-3' (Ramos, 2007). For both fragments, amplification was performed in a 10 µl volume containing: 1 µl buffer 10 x for Kod Hot Start DNA Polymerase (Novagen®), 1.7 µl dNTP (25 mM), 0.8 µl MgSO4 (25 mM), 0.7 µl Kod Hot

Strat DNA Polymerase (1.0 U / µl), 3.8 µl H_2O, 0.5 µl for each primer, and 1 µl ADN (100 ng / µl). The amplification program used included a polymerase activation phase of 2 min at 94 °C and then 35 cycles: 30 s at 94 °C, 40 s at 54 °C and 1 min at 72 °C, to complete a final extension of 10 min at 72 °C. Sequence reactions were performed in both directions using the same primers as for amplification. The sequence analysis was performed on an ABI 3130XL sequencer with 16 capillaries (Applied Biosystems).

2.4 MATE CHOICE (REPRODUCTIVE ISOLATION)

The objectives of these experiments were to test for reproductive isolation (pre-zygotic barrier) through mate choice pairing between the genetic entities previously determined by the molecular analyses. The popsites selected for mate choice experiments were those where the larger number of fishes with similar sizes (and colour) could be sampled. To determine whether females preferentially reproduce with males of their own genetic entity, mate choice experiments were performed in eighteen 100x30x30 cm aquariums (Figure 2). Females from different genetic entities were presented two males (one of their own genetic entity and one of a different genetic entity) of the same colour (according to our visual criteria) to choose as potential mate. In each combined set, the males were similar in length to avoid size assortative mating by females (Beeching & Hopp 1999). Females were placed in the central compartment (50 x 30 x 30 cm) and, males in lateral compartments (25 x 30 x 30 cm). The glass dividers separating the compartments of the female and the two males were drilled (26 holes of 8 mm diameter) in their lower part to allow the passage of possible olfactory and / or acoustic signals. Spawning sites consisted of PVC tubes of 10 cm length and 4.8 cm diameter cut lengthwise and positioned to each side of the dividers (Figure 2). To exclude habituation, learning, or general preference by females for a particular end of a tank during the experiments, males from the two tested genetic entities were placed alternately on the right or the left side of the tank in the 18 experimental tanks. Females of each genetic entity were offered the same number of male combinations (their genetic entity either on the right or on the left side of the aquarium). When a female had spawned in one of the PVC spawning sites, it was considered it had sexually selected the male adjacent to that site (Couldridge & Alexander, 2001, 2002; Römer & Beisenherz, 2005). The female and eggs were then removed; the female was placed in a recuperation tank and replaced by another female of the same genetic entity. Each fish had a specific code to prevent putting a female twice in contact with the same male to exclude any learning effects on the test results, as indicated by Römer, Engelking & Beisenherz (2014) for

another species of the genus. Aquariums were separated by black plastic film to avoid inter-tank interaction.

Each aquarium was filtered using internal sponge filters, maintained at a temperature of ~25 °C using air-conditioner and illuminated by LED strip light on a 12 L: 12 D cycle. Fish behavior and potential spawning events were observed twice a day (morning and afternoon). Fish were fed commercial pellets (Aquatech® Trucha Pre-inicio 45) following these observation periods.

2.5 DATA ANALYSIS

2.5.1 NUCLEAR DATA

2.5.1.1 ALLELIC AND GENETIC DIVERSITY

Initially, the analyses of microsatellite data were carried out using popsites as taxonomic units. Each popsite was characterized by: (1) allele diversity, average number of alleles per locus, allele richness and number of private alleles (TNA, MNA, AR and NPA, respectively) using Fstat software version 1.2 (Goudet, 1995) and GENETIX 4.05 (Belkhir, Borsa, Chikhi, Raufaste & Bonhomme, 2004), (2) genetic diversity such as expected heterozygosity, expected unbiased heterozygosity and observed heterozygosity (H exp., H n.b. and H obs., respectively) with the software GENETIX 4.05 (Belkhir et al., 2004), and (3) the panmixia deviations, evaluated according to the values of the F_{IS} estimator (Weir & Cockerham, 1984), using the program GENETIX 4.05 (Belkhir et al., 2004).

2.5.1.2 DISTANCES AND GENETIC GROUPS

The distance matrix from Nei (1972) was used to create an unrooted Neighbor Joining dendrogram to establish genetic distance relationships among “popsites”. The robustness of the nodes was tested by “bootstrap” from 1000 draws. The analyses were performed using R’s “adegenet” library (Jombart, 2008). From this dendrogram, we identified the clusters and then alleles with a frequency higher than 0.50 present in at least one of the clusters using GENETIX 4.05 (Belkhir et al., 2004).

A discriminant analysis of principal components (DAPC) was performed on the individuals in order to identify clusters using R’s “adegenet” library (Jombart, 2008; Jombart, Devillard & Balloux, 2010). Popsite information was taken into account to identify clusters

within each popsite. This method uses K-means and model selection to infer genetic clusters and combines both, the advantages of discriminant analysis (DA, which attempts to synthesize genetic differentiation between clusters), and of principal component analysis (PCA, which aims to synthesize overall variability among individuals, as well as variation occurring within clusters) (Jombart et al., 2010). In our case, this method has proven to be more efficient compared to the Bayesian method used by the "STRUCTURE" software (Pritchard, Stephens & Donnelly, 2000). Indeed, we considered that the best method was the one that was able to find the smallest and totally disjointed clusters (i.e. that cluster all the individuals of a popsite to a single branch). It has been shown by the authors of the DAPC method that this analytic strategy is more effective than using STRUCTURE in unravelling complex population structures (Jombart et al., 2010). Then the degrees of genetic differentiation were calculated in each cluster between pairs of popsites from the values of the F_{ST} θ estimator (Weir & Cockerham, 1984) using GENETIX 4.05 (Belkhir et al., 2004).

2.5.2 MITOCHONDRIAL DATA

2.5.2.1 ANALYSIS OF POLYMORPHISM

The complete sequence fragments were obtained by aligning the forward and reverse fragments using the MEGA-X v.10.0.5 program (Kumar, Stecher, Li, Knyaz & Tamura et al., 2018). No gaps or missing data occurred. The numbers of haplotypes, haplotypic diversity, variance of haplotypic diversity and nucleotide diversity (Nhap, Hd and π, respectively) were calculated using the DnaSP v.6 program (Rozas et al., 2017).

2.5.2.2 PHYLOGENETIC RELATIONSHIPS AND EVOLUTIONARY HISTORY

Two maximum likelihood (ML) trees were produced. The first (haplotype tree) was constructed from 32 sequences of *A. agassizii* rooted with two outgroups, *A. bitaeniata* and *A. eremnopyge*, which are the most closely related species to *A. agassizii* (Tougard et al., 2017). The results were included in a part of the phylogeny of Tougard et al. (2017) (species tree), which corresponds to the species of the A2, A3 and A4 clades as well as 6 haplotypes of *A. agassizii* sampled in Peru. The approach was first to determine if *A. agassizii* is monophyletic in the Peruvian Amazon and second to evaluate if the Peruvian haplotypes of *A. agassizii* would correspond to the clades we highlighted. The ML trees were produced using the program MEGA-X v.10.0.5 (Kumar et al., 2018), taking into account the best evolutionary models

estimated with the corrected AIC of Burnham & Anderson (2004). The strengths of the tree nodes were estimated by percentage of bootstraps after 1000 replications.

In order to reconstruct the evolutionary history between clades, the molecular clock was assumed to be constant for the comparison between species. Then the differences between clades have been calculated from the number of nucleotide substitutions using the same model used for the construction of the tree (MEGA-X v.10.0.5; Kumar et al., 2018). The substitution rate and the divergence times of the marker between clades were then estimated using two types of calibrations from the publication of Genner et al. (2007). The first calibration considers that the origin of cichlids predates the fragmentation of Gondwana, the second calibration takes into account the fossil dating of cichlids, which implies a transatlantic dispersion. The divergence dates considered were those established by the authors between the genus *Apistogramma* and *Crenicichla*; in the first case the divergence is estimated at 50.2 Ma and in the second 17.3 Ma ago. In order to have a better estimate of the average substitution rate between *Apistogramma* and *Crenicichla* all our *A. agassizii* sequences have been used as well as *Apistogramma* and *Crenicichla* sequences from GeneBank (Table S1).

2.5.3 MATE CHOICE EXPERIMENTS

One-tailed binomial statistical tests were performed to test for deviation from random mating between genetic entities.

3 RESULTS

3.1 NUCLEAR DNA

3.1.1 GENETIC VARIABILITY

A sample of 1151 fish was analysed distributed among 35 popsites (Figure 1). Genetic diversity between popsites is very heterogeneous (Table 1). For example, the two popsites "Or7" and "Or8", which have the same number of individuals, have a total number of alleles of 24 and 101, respectively. The allelic richness for all popsites is between 2.40 and 9.82 for an average value of 5.86 ± 1.90 (SD). The "An" popsite has the highest allelic richness, followed by the "JH4", "JH3" and "JH5" popsites with values of 9.54, 9.51 and 9.35, respectively. The "Or7" popsite has the lowest allelic richness. The average number of private alleles per popsite is 2 ± 3.3 (SD), although the "An" popsite has 19. The values of the F_{IS} estimator show a

panmixia deviation for seven popsites ("JH3", "Or8", "JH13", "JH2", "JH11", "JH16" and "JH14") over a total of 35 popsites.

3.1.2 STRUCTURING AND GENETIC DISTANCES

The dendrogram of genetic distance shows the presence of three clusters (Figure 3). Clusters 2 versus 1 and 3 are supported by a bootstrap value of 94 and cluster 3 versus 1 and 2 by a value of 98. Cluster 1 is highly structured. Some popsites are very differentiated, such as popsites Na1, Na2 and Na3, which form a group supported by a bootstrap value of 97. Another group is formed by the popsites JH15, JH19 and JH16 supported by a bootstrap value of 72 and the popsites JH12, JH13, JH20, JH18 and JH8 form another group supported by a bootstrap value of 73. This structuring is closely linked to the geographic distribution of popsites. Indeed, popsites sampled on the same stream network are genetically closer to each other than to popsites from neighbouring streams (Figure 1b).

For each of the 10 loci, at least one allele has a high frequency in only one of the 3 clusters (freq. >0.50, Table 2), with the exception of alleles 100 and 142 in locus 01 and 16 whose frequencies are greater than 0.5 for clusters 2 and 3. The allele 123 (locus 11) is diagnostic of cluster 1. Alleles 194 (Locus 28) and 161 (locus 09) are diagnostic of clusters 1 and 3 with frequencies of 0.45 and 0.41, respectively.

The eigenvalues of the discriminant analysis of principal components show that the two main components represent nearly 100% of the genetic structure (Figure 4a). The results show three distinct clusters that are well differentiated and genetically isolated: the distribution of the three clusters is disjoint, with no admixture between them (Figure 4b). The first main component differentiates on the negative side the individuals of cluster 1 (blue) and on the positive side the individuals of cluster 2 (green). The second main component differentiates individuals of cluster 3 (red). All the individuals in a popsite belong exclusively to one of the three clusters (Figure 4b). The structuring into 3 clusters gives the smallest and most differentiated clusters (Figure 4b, c and d). It is worth noting that these three clusters are the same as those identified by the dendrogram of genetic distance.

3.1.3 GENETIC DIFFERENTIATION IN CLUSTERS

The F_{ST} values per pair of popsites within cluster 1 are between 0.00 and 0.36, the popsites Na1, Na2 and Na3 are the most differentiated (Table 3). For cluster 2, the F_{ST} values in

pairs of comparisons are between 0.00 and 0.33, the Or7 popsite is the most differentiated (Table 4). For cluster 3, the value of the F_{ST} between the two popsites is 0.10.

3.2 MITOCHONDRIAL DNA

3.2.1 POLYMORPHISM OF COI SEQUENCES

The Cytochrome c oxidase subunit I gene shows a strong polymorphism in *A. agassizii* (Table 5): the 32 sequences analysed are split into 12 haplotypes (Hd = 0.9173 ± 0.023). These are clustered into three clades, five in clade 1 (Hd = 0.780 ± 0.085, π = 0.00615), four in clade 2 (Hd = 0.733 ± 0.12, π = 0.00137), and three in clade 3 (Hd = 0.679 ± 0.122, π = 0.00089). Of the total of 942 nucleotide sites studied, 37 are polymorphic (π = 0.015).

3.2.2 PHYLOGENETIC RELATIONSHIPS

The maximum likelihood trees were constructed taking into account the best evolutionary model according to AICc, TN93 + G (Log likelihood = -1541.89, gamma parameter = 0.0500 (5 categories + G), transition/transversion ratio = 6.0666) for the haplotype tree (Figure 5a) and TN93 + G (Log likelihood = -2821.79, gamma parameter = 0.3722 (5 categories + G), transition/transversion ratio = 4.4691) for the species tree (Figure 5b). The haplotype tree, rooted with two species close to *A. agassizii* (*A. eremnopyge* and *A. bitaeniata*) confirms the presence of three clades (three clusters with nDNA), each of which is monophyletic (Figure 5a). The three clades are supported by high bootstrap values, 100, 99 and 98, respectively. It is worth noting that haplotype 8 of clade 1 is well differentiated from other haplotypes and has a significant number of own mutations (anagenesis).

Considering that *Apistogramma* and *Crenicichla* would have diverged between 50.2 Ma (fragmentation of Gondwana) and 17.3 Ma (fossil dating of cichlids), the substitution rate obtained was 0.3 % per Ma or 0.9% per Ma for the partial COI marker. Then, *A. agassizii* and *A. bitaeniata*/*A. eremnopyge* would have diverged between 23.2 Ma and 8.01 Ma ago. The most recent common ancestor (MRCA) to the three clades would have a maximum of 3.80 Ma and a minimum of 1.31Ma. Under the same assumptions, clades 2 and 3 would have diverged between 2.27 Ma and 0.78 Ma ago. In cluster 1, haplotype 8 would have diverged between 1.71 Ma and 0.59 Ma ago.

The three entities Sp1, Sp2 and Sp3 (which represent clades 1, 2 and 3) form a monophyletic clade supported by a bootstrap value of 100 when placed in the partial

Apistogramma phylogeny of Tougard et al. (2017; Figure 5b). Of the six haplotypes representing the overall polymorphism observed in the study of Tougard et al. (2017) in *A. agassizii*, five are positioned with Sp1 (including one with haplotype 8) and one with Sp2.

3.3 MATE CHOICE

Females from genetic entities 1 (JH4) and 2 (Or4) were tested for potential mating preferences for males of their own genetic entity. This mating preference would indicate their ability to recognize and intentionally mate with presumptive conspecific. Fourteen out of 15 females tested from entity 1 laid eggs on the side with the male belonging to their own genetic entity; only one laid it on the side of a male from genetic entity 2 ($p < 0.001$). Out of the 11 females tested from genetic entity 2, 10 laid their eggs on the side with males of their own entity and only one on the side of a male of genetic entity 1 ($p = 0.005$). Our results thus indicate highly significant pre-zygotic reproductive isolation between these two genetic entities. Unfortunately, we were not able to test all possible combinations of genetic entities, as test specimens of genetic entity 3 were still too small during the mating experiments.

4 DISCUSSION

4.1 APISTOGRAMMA AGASSIZII A MOSAIC OF SPECIES

As in most animal and plants species, taxonomic descriptions in *Apistogramma* have been made based on morpho-meristic characteristics. In some cases, these descriptions show limitations, particularly in the case of cryptic species complexes where morphological differentiation is not *a priori* sufficient (Römer et al., 2017). Therefore, the risk is evident, that some species described only on morphological criteria are in fact groups or clusters of species. That would, underestimate biodiversity (Briscoe & Tait, 1995; Rocha-Olivares, Fleeger & Foltz, 2001; Frankham, Ballou & Briscoe, 2002) and potentially bias the assessment of species protection status (Bowen & Avise, 1996; Frankham et al., 2002). Indeed, a taxon with poorly defined taxonomic contours could be wrongly considered representing an abundant species having a large distribution. Such a species would not require protection status, whereas it could in reality represent a group of distinct species with small distribution areas, some of which might be threatened (Römer & Hahn, 2013; Römer et al., 2015).

Although many Amazonian lowlands small-sized fish species of relatively sedentary habits (including cichlids) present very wide distributions, which is possibly resulting (at least

388 in part) from passive drifting in floating meadows (Schiesari et al., 2003), the presumptive wide
389 distribution of *A. agassizii* throughout the Amazon is rather atypical for species of the genus.
390 Several authors have suggested that such a large range clearly could encompass more than one
391 distinct species (Britzke, 2015; Koslowski, 2002; Römer, 2000, 2006; Römer et al., 2017;
392 Tougard et al., 2017). Our study in the small portion of Peruvian Amazon investigated, clearly
393 shows that there are at least three entities that are represented as separate "genotypic clusters",
394 or biological and phylogenetic species (Mayr, 1942; Hennig, 1966; Cracraft, 1989; Mayden &
395 Wood, 1995; Mallet 1995), presently confounded under the name of *A. agassizii*. Using the
396 dating of Genner et al. (2007), these three presumptive species would have diverged between
397 3.80 (fragmentation of Gondwana) and 1.31 (fossil dating) Ma ago. If another totally
398 independent calibration from mainly perciform reef fish based on the closure of the Panama
399 Isthmus (DiBattista et al., 2013, Figure 5a) is considered, then the three species would have
400 diverged between 1.17 Ma (mutation rate for COI of 1% per Ma) and 0.59 Ma ago (mutation
401 rate for COI of 2% per Ma). The mutation rate of 1% per Ma found by DiBattista et al. (2013)
402 is consistent with that estimated by Genner et al. (2007) with the fossil calibration.
403 The DAPC shows three totally disjointed clusters and indicates that there have not been
404 any recent nuclear introgression or any migrant between the three genetic entities. The COI
405 phylogeny also does not show any mitochondrial introgression between the three entities and
406 mate choice experiments indicate pre-zygotic isolation between at least two of them. According
407 to the genetic concepts of reproductive isolation and monophyly, these three genetic entities
408 will therefore be considered as different species and referred to as Sp1, Sp2 and Sp3 in the
409 following. These results support the hypothesis of a mosaic distribution of biological species
410 having reached reciprocal monophyly, rather than a complex of species with recent genetic
411 introgressions or current hybridisations.
412
413 4.2 GEOGRAPHICAL DISTRIBUTION OF THE THREE SPECIES

414 Species Sp2 and Sp3 have significantly reduced distribution areas compared to species
415 Sp1. Based on available samples, they are found exclusively on the left bank of the Amazon
416 (going downstream) and are separated from each other by the Napo River (Figure 1). Species
417 Sp1 appears to have a much larger distribution area than the other two; it occurs on the left
418 bank of the Marañón River and the right banks of the Ucayali and Amazonas rivers.
419 The high F_{ST} values found between geographically close popsites (especially in Sp1)
420 belonging to the same stream system (Figure 1b) indicate a low dispersal capacity of *A.* cf.

agassizii (Frankham et al., 2002; Waples, 1987; Ward, Woodwark & Skibinski, 1994). For example, Jenaro Herrera's popsites JH13 and JH20, which are less than 1 km apart from each other (following the stream), have an F_{ST} value of 0.13. Another example, still in the same stream network, the JH20 popsite is less than 2 km from the JH12 popsite, yet the F_{ST} value is 0.21. The intraspecific F_{ST} values observed in species Sp1 are of the same magnitude or higher than populations of *Apistogramma gephyra* (F_{ST} value of 0.128) and *A. pertensis* (F_{ST} value of 0.228) belonging to two catchments of the Negro River, separated since the middle or late Pleistocene and with almost no gene flow (Leitão et al., 2017). They also are similar to those of populations of *Cichla temensis* from the Negro and Orinocco basins (range 0.2-0.5, Willis et al., 2015). Within a same time frame, they are also similar to the values observed between species belonging to different genera in some Haplochromine cichlids: 0.14 between *Astatotilapia nubila* and *Lipochromis maxillaris* for example (Odhiambo, Mautner, Bock & Sturmbauer, 2012). The popsites of Jenaro Herrera (Sp1) city are found exclusively in small stream systems that tend to reduce or even dry out on some sections during the dry season; this phenomenon is amplified by the intense deforestation for livestock and food crops around the city. For instance, popsite JH12 (Figure 1b) was sampled in November 2016, but the following year at the same time (October), the previously sampled section had completely dried out and the sampling of this popsite (n = 16) could not be completed. Between these two years a parcel of forest of several hectares was deforested around this popsite. It is likely that popsites near the city of Jenaro Herrera, which have the lowest allelic richness compared to other popsites of species Sp1, regularly experience population bottlenecks due to seasonal drying out of the habitat and deforestation. Such bottlenecks would directly affect allelic frequencies within the popsites, which could partly explain the high F_{ST} values observed in the same stream networks near the city of Jenaro Herrera.

Looking exclusively at popsites of species Sp1 from the four largest streams (An; JH3+JH4+JH5; Ya2+Ya3; Or8+Or9) with little or no deforestation, allelic richness tends to decrease from upstream (Ucayali, popsite An, AR = 9.818) to downstream (Amazon, posites Or8+Or9, AR = 7.821). Moreover, the Mantel test between the geographical distance and the genetic distance of Rousset (1997) between these four streams indicated a significant isolation by distance (*p-value* = 0.042; Figure 6b). *Apistogramma* Sp1, in the study area, therefore most likely dispersed from upstream (Ucayali) to downstream (Amazon), a result corresponding to proposals on dispersal for another species of the genus by Römer (1992, 2000).

According to molecular dating, the three species started diverging from the MRCA during the Plio-Pleistocene, a period known for its glacial and interglacial cycles. These glacial

events have affected river dynamics, particularly erosion/sedimentation phases (Baker & Fritz, 2015) and species distributions (Dumont, Lamotte & Kahn, 1990; Hubert & Renno 2006). During the Quaternary period, terraces appeared along the Amazon and some of its tributaries like the Nanay River (Dumont & García 1989; Dumont, Lamotte & Fournier, 1988; Dumont, Deza & García, 1991; Dumont, 1992; Gonçlaves, Soares, Tatumi, Yee & Mittani, 2016; Wesselingh & Salo 2006). The popsites where the three species of *A.* cf. *agassizii* were found seem to be restricted to these Quaternary terraces and floodplains. The dispersion of the most recent common ancestor (MRCA) and the formation of the three species may have been favoured by these geologic and climatic events. As an effect of it, the MRCA population may at least temporarily have been split up in different refuges (Ab´Sáber cited in Sick, 1993; Grabert, 1991; Haffer, 1969; Römer, 2006) and undergone further development (and genetic drift) under more or less strict geographic isolation.

Although the role of large rivers as a barrier to species distributions (Wallace, 1854) was recently challenged for many animal groups in the Amazon basin (Santorelli, Magnusson & Deus, 2018), the distribution of the three species is consistent with the barrier effect of large rivers, as previously shown in other Amazonian fish groups (Hubert & Renno 2006). Depending on taxon vagility, large rivers can prevent gene flow between populations of different regions favoring allopatric speciation. Species Sp2 and Sp3, which are phylogenetically closest, are found on the left side of the Amazon River and apparently are separated from each other by the Rio Napo, whereas species Sp1 is observed on the right side of the Amazon River only. Despite several samplings in the main rivers between their respective distribution areas (Figure S1), these three species have never been, so far, encountered in strict sympatry, i.e. in the same watercourse. Given that popsites Na1, Na2 and Na3 with species Sp1 are located on the left bank of the Marañon River could *a priori* seem inconsistent with the barrier effect of large rivers. Concomitant with glacial events, however, the reactivation of tectonics, particularly in the Ucamara depression (delimited by the Marañón and Ucayali rivers), has favoured rapid changes in the course of the Ucayali and Marañon rivers (i.e. avulsion; Sébrier, Mercier, Mégard, Laubacher & Carey-Gailhardis, 1985; RADAMBRASIL, 1977; Räsänen, Salo & Kalliola, 1987). Furthermore, according to Dumont (1991), the Ucayali was once connected to the Marañón River above the city of Nauta (above the popsites Na1, 2 and 3, Figure 1), which could explain the presence of species Sp1 on the left side of the Marañon.

4.3 VARIATION OF COLOUR PATTERNS

The three species are distinguished at genetic (nDNA and mtDNA), behavioral (mate choice), and geographic levels (i.e., allopatric distributions). Once the three species were clearly identified on genetic criteria, phenotypic variation could also be observed *a posteriori* among the genetically identified species, particularly on color patterns of the caudal fin (Figure 7). Further studies will be needed to investigate whether distinct colour and morpho-meristic patterns are diagnosable, and possibly, working as diagnostic phenotypic characters, may be used to segregate these new species.

4.4 BIODIVERSITY OF THE GENUS APISTOGRAMMA IN THE AMAZON REGION AND CONSERVATION IMPLICATIONS

In eastern Amazonia, Ready et al. (2006) demonstrated the existence of three species in what was previously identified as *A. caetei* from unconnected small river basins. Here, a similar situation is described in *A. agassizii*, but in a much smaller area of interconnected streams and river network. At least three species, and potentially four (popsites Na1, Na2 and Na3) were observed in a small area of the Peruvian Amazon. This clearly demonstrates that *A. agassizii* cannot be considered a widespread species any longer; this nominal taxon appears to represent a mosaic of many species whose precise geographical distribution remain undefined. Considering the highly dynamic geo-hydrological changes occurring in its extensive distribution area, it is most likely that the nominal *A. agassizii* actually holds tens of species. As the type locality for *A. agassizii* (Steindachner, 1875) is in Manacapuru area near the city of Manaus in central Amazonia, all three species identified in the Peruvian Amazon could actually be new species. The present results also suggest that two out of the three newly identified species could be endemic to very localized regions: the Apayacu and Ampiyacu systems for species Sp2 and the Nanay basin for species Sp3. Despite several samplings in rivers and streams around the Nanay (Figure S1), *A.* Sp3 was never observed and seems to be endemic to the Nanay, which is a small basin but the largest black water river in the Peruvian Amazon (Ortega & Castro, 1998; Sioli, 1984). The Nanay is also the most heavily exploited river for the ornamental fish trade owing to its close proximity to the city of Iquitos, where most Peruvian fish exporters are operating (Gerstner, Ortega, Sanchez & Graham, 2006; Tello & Cánepa, 1991). This situation puts further pressure on *A.* Sp3, which currently supports most of the local ornamental fishing for *A. agassizii*. Although the aquarium trade has the advantage of attracting the general public's awareness on these species that often inhabit environments that are almost invisible to the public attention when compared to large rivers (Frederico, Zuanon & De Marco

Júnior, 2018; Olden, Hogan & Zanden, 2007), it may also imperil their survival by over-harvesting when their distribution or population sizes are limited (Raghavan, Ali, Philip & Dahanukar, 2018). The present results should foster adaptive management actions to take this situation into account and avoid local extirpations and loss of genetic variability, which is one of conservation's ultimate goals. Potential conservation actions could be the implementation of quotas or specified licenses for extraction/exportation. For such controls to be easier to implement, this study needs to be extended and the new species described to provide export inspectors with clear diagnostic characters, although this study shows that simple barcoding could already be used. Officially describing the new species identified and advertising their presence to aquarium exporters and fishermen in Iquitos could also help diverting part of the pressure exerted on Sp3 in the Nanay, by offering new species for export. An additional measure could be incentives to culture the species and export certified, sustainably bred F1 specimens.

The ornamental fish trade probably has fewer effects on *A.* Sp1 and Sp2. Both species, however, and especially species Sp1, live in forest streams that are highly sensitive to primary effects of deforestation, as previously emphasized for popsite JH12 in Jenaro Herrera. This further emphasizes the interdependence of freshwater and terrestrial habitat for conservation (Frederico, Zuanon & De Marco Júnior, 2018). Increasing water temperatures resulting from deforestation can also affect *Apistogramma* species (Beninde, Römer, Vela Diaz & Duponchelle, in press). Deforestation will likely further impact the ability of *Apistogramma* species and other stream fishes to support expected climate-induced changes in water quality and quantity (Frederico, Olden & Zuanon, 2016; Freitas, Siqueira-Souza, Humston & Hurd, 2013). Additionally, *Apistogramma* species are facing other threats: during our field trips, recipients/canisters used by fishermen to fish with barbasco, an ichthyotoxic substance naturally found in plants of the genera *Lonchocarpus*, *Paraderris*, and *Tephrosia* (Andel, 2000) were often observed. Rural communities in Amazonia traditionally use barbasco in small to medium-sized streams to catch food fishes quickly and indiscriminately. The problem is that it kills almost all fishes in the influence area, although its active ingredient seems to degrade quickly. Its use appears widespread in the Peruvian Amazon, and its consequences on aquatic communities have not been assessed.

The small geographic area sampled during the present study appears to be a hotspot of biodiversity and endemism for *Apistogramma* species, given the existence of at least 3 new species within the nominal *A. agassizii* and the presence of a complex of closely related species in the Pebas area (Fig. 1), most of them not described, albeit known of the aquarium trade

(Tougard et al., 2017). Centres of vertebrate endemism are excellent surrogates for identifying conservation priorities in the Amazon (Loyola, Kubota & Lewinsohn, 2007). This highlights the critically urgent need for further research in a biodiversity and endemism hotspot located to the immediate vicinity of one the most rapidly expending cities of the Amazon basin, Iquitos. Large cities in the Amazon basin can cast defaunation shadows over hundreds of km (Tregidgo, Barlow, Pompeu, de Almeida Rocha & Parry, 2017). The rapid expansion of deforestation, pollution, habitat degradation, overexploitation, invasive species (such as tilapia or gourami, which could compete with *Apistogramma* in small streams) around Iquitos could lead to undetected and irreversible biodiversity losses, particularly for restricted range species such as *Apistogramma* and other stream-dwelling species, even before they have the chance to be discovered, let alone described. As restricted range species also are key indicators of biogeographic patterns and underlying evolutionary mechanisms (Nogueira et al., 2010) they should *de facto* warrant research and conservation prioritization (Rosenfield, 2002). Further studies emphasizing the extent of biodiversity and endemism in the potential hotspot around Iquitos might foster the interest of conservation NGOs and public awareness, and ultimately trigger actions from the regional government to limit the already mentioned threats exerting on the small streams around Iquitos, and particularly local-scale deforestation.

The present study focused on the nominal *A. agassizii*, but it is also very likely that the two other nominal *Apistogramma* species known to have very wide distributions in the Amazon basin, *A. bitaeniata* and *A. cacatuoides*, also encompass many species, as suggested by Römer (2000, 2006), Römer et al. (2015, 2017), and Britzke (2015). The genus *Apistogramma* could then be made up of hundreds of species if we add the numerous species whose taxonomical status has not been molecularly assessed (e.g. *A. caetei*, Ready et al., 2006). This situation might also apply to the other highly diversified cichlid taxa such as *Crenicichla* (Burress et al., 2018; Piálek, Říčan, Casciotta, Almirón & Zrzavý, 2012; Říčan, Piálek, Dragová & Novák, 2016). Such estimates have no reference value, but may illustrate the limitations of our knowledge regarding the biodiversity of Amazonian stream fishes (Frederico, Zuanon & De Marco Júnior, 2018; Nogueira et al., 2010). Knowledge of aquatic biodiversity patterns is an essential prerequisite for the conservation of freshwater ecosystems, which are the most threatened in the world (Abell, Thieme & Revenga, 2008). This also holds true for the Amazon basin (Nogueira et al., 2010; Castello et al., 2013; Castello & Macedo 2016) and even more so for streams (Frederico, Zuanon & De Marco Júnior, 2018), where most species-rich taxa, such as *Apistogramma* live (Römer, 2000, 2006). Freshwater fish are among the most threatened vertebrates in the world (Pimm et al., 2014), which also is likely the case in the Amazon basin

owing to habitat degradation or loss and the still limited knowledge about fish ecology and systematics (Nogueira et al., 2010; Castello et al., 2013; Castello & Macedo 2016). Small streams are among the most vulnerable ecosystems in the Amazon basin and the lack of information on stream fishes biodiversity hampers conservation efforts (Frederico, Zuanon & De Marco Júnior, 2018). The vulnerability of stream fishes is further aggravated by the inherent connectedness structure of river networks that can propagate anthropogenic disturbances over long distances (Dudgeon et al., 2006). Further in depth studies are urgently needed to understand the extent of biodiversity in the many small-bodied, species-rich, yet understudied Amazonian taxa (Barletta et al., 2010), such as *Apistogramma* or other highly speciose cichlid groups (e.g. *Crenicichla*, Piálek et al., 2012; Říčan, Piálek, Dragová & Novák, 2016; Burress et al., 2018). By providing detailed information about the distribution of three new taxa in a localized region of the Peruvian Amazon, this study contributes to improving the biodiversity knowledge in the Loreto region and fuels baselines for conservation actions.

ACKNOWLEDGEMENTS

Guillain Estivals was supported by a scholarship of the Franco-Peruvian School of Life Sciences. Research was funded by and carried out within the framework of the LMI-EDIA (International Mixt Laboratory - Evolution and Domestication of Amazonian Ichthyofauna), IIAP-IRD-UAGRM. The authors declare to have no conflict of interest regarding the publication of this article.

REFERENCES

Abell, R., Thieme, M. L., Revenga, C., Bryer, M., Kottelat, M., Bogutskaya, N., . . . Petry, P. (2008). Freshwater Ecoregions of the World: A New Map of Biogeographic Units for Freshwater Biodiversity Conservation. *BioScience*, 58, 403-414. doi:10.1641/b580507

Alho, C. J., Reis, R. E., & Aquino, P. P. (2015). Amazonian freshwater habitats experiencing environmental and socioeconomic threats affecting subsistence fisheries. *Ambio*, 44, 412-425. doi:10.1007/s13280-014-0610-z

Andel, T. (2000). The diverse uses of fish-poison plants in Northwest Guyana. *Economic Botany*, 54. doi:10.1007/BF02866548

624 Anderson, E. P., Jenkins, C. N., Heilpern, S., Maldonado-Ocampo, J. A., Carvajal-Vallejos, F.
625 M., Encalada, A. C., . . . Tedesco, P. A. (2018). Fragmentation of Andes-to-Amazon
626 connectivity by hydropower dams. *Science Advances*, 4, eaao1642. doi:10.1126/sciadv.aao1642
627
628 Baker, P. A., & Fritz, S. C. (2015). Nature and causes of Quaternary climate variation of
629 tropical South America. *Quaternary Science Reviews*, 124, 31-47.
630 doi:10.1016/j.quascirev.2015.06.011
631
632 Barletta, M., Jaureguizar, A. J., Baigun, C., Fontoura, N. F., Agostinho, A. A., Almeida-Val, V.
633 M. F., . . . Corrêa, M. F. M. (2010). Fish and aquatic habitat conservation in South America: a
634 continental overview with emphasis on neotropical systems. *Journal of Fish Biology,* 76, 2118-
635 2176. doi:10.1111/j.1095-8649.2010.02684.x
636
637 Barthem, R. & Goulding, M. (2007) *Un ecosistema inesperado: la Amazonía revelada por la*
638 *pesca*. Perú : Asociación para la Conservación de la Cuenca Amazónica (ACCA): Amazon
639 Conservation Association (ACA).
640
641 Bayley, P., & Petrere, M. (1989). Amazon fisheries: Assessment methods, current status and
642 management options. *Proceedings of the International Large River Symposium*, 106, 385-398.
643
644 Beeching, S., & Hopp, A. B. (1999). Male mate preference and size-assortative pairing in the
645 convict cichlid. *Journal of Fish Biology*, 55, 1001-1008. doi:10.1111/j.1095-
646 8649.1999.tb00735.x
647
648 Belkhir K., Borsa P., Chikhi L., Raufaste N., Bonhomme F. (2004). Genetix, logiciel sous
649 Windows TM pour la génétique des populations. In Laboratoire Génome, Populations,
650 Interactions CNRS UMR 5000, Université de MontpellierII, Montpellier (France).
651
652 Beninde, J., Römer, U., Vela Díaz, A. & Duponchelle, F. (in press). Changes of water
653 temperature caused by deforestation are of major concern for the future of many species of
654 *Apistogramma* (Regan, 1913) (Teleostei; Perciformes; Cichlidae; Geophaginae). *Bulletin of*
655 *Fish Biology,* in press.
656

Bowen, B. W., & J. C. Avise. (1996). Conservation genetics of marine turtles. J. C. Avise, & J. L. Hamrick (Eds.), *Conservation genetics: Case histories from nature* (pp. 190-237), New York: Chapman and Hall.

Briscoe, D. A., & Tait, N. N. (1995). Allozyme evidence for extensive and ancient radiations in Australian Onychophora. *Zoological Journal of the Linnean Society*, 114, 91-102. doi:10.1111/j.1096-3642.1995.tb00114.x

Britzke, R. (2015) Relações filogenéticas do gênero *Apistogramma* (Teleostei, Cichlidae) e filogeografia da espécie *Apistogramma agassizii*. 187 f. (PhD thesis). Universidade Estadual Paulista Júlio de Mesquita Filho, Instituto de Biociências de Botucatu, Botucatu, Brazil.

Britzke, R., Oliveira, C., & Kullander, S. (2014). *Apistogramma ortegai* (Teleostei: Cichlidae), a new species of cichlid fish from the Ampyiacu River in the Peruvian Amazon basin. *Zootaxa*, 3869, 409-419. doi:10.11646/zootaxa.3869.4.5

Burnham, K. P., & Anderson, D. R. (2004). Multimodel Inference. *Sociological Methods & Research*, 33, 261-304. doi:10.1177/0049124104268644

Burress, E. D., Pialek, L., Casciotta, J. R., Almiron, A., Tan, M., Armbruster, J. W., & Říčan, O. (2018). Island- and lake-like parallel adaptive radiations replicated in rivers. *Proceedings of the Royal Society B: Biological Sciences*, 285: 20171762. doi:10.1098/rspb.2017.1762

Castello, L., Arantes, C. C., Mcgrath, D. G., Stewart, D. J., & Sousa, F. S. D. (2015). Understanding fishing-induced extinctions in the Amazon. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 25, 587-598. doi:10.1002/aqc.2491

Castello, L., & Macedo, M. N. (2016). Large-scale degradation of Amazonian freshwater ecosystems. *Global Change Biology*, 22, 990-1007. doi:10.1111/gcb.13173

Castello, L., McGrath, D. G., Hess, L. L., Coe, M. T., Lefebvre, P. A., Petry, P., . . . Arantes, C. C. (2013). The vulnerability of Amazon freshwater ecosystems. *Conservation Letters*, 6, 217-229. doi:10.1111/conl.12008

Chanseau, M., Bosc, S., Galiay, E., & Oules, G. (2002). L'utilisation de l'huile de clou de girofle comme anesthésique pour les smolts de saumon atlantique (*Salmo salar* L.) et comparaison de ses effets avec ceux du 2-phenoxyethanol. *Bulletin Français de la Pêche et de la Pisciculture*, 365-366, 579-589. doi:10.1051/kmae:2002054

Couldridge, V. (2001). Does the time spent near a male predict female mate choice in a Malawian cichlid? *Journal of Fish Biology,* 59, 667-672. doi:10.1111/j.1095-8649.2001.tb02370.x

Couldridge, V. C. K., & Alexander, G. J. (2002). Color patterns and species recognition in four closely related species of Lake Malawi cichlid. *Behavioral Ecology*, 13, 59-64. doi:10.1093/beheco/13.1.59

Cracraft, J. (1989) Speciation and its ontology: The empirical consequences of alternative species concepts for understanding patterns and processes of differentiation. In: Otte, D. & Endler, J.A. (Eds.). *Speciation and Its Consequences* (pp. 25-59). Sinauer, Sunderland.

Crampton, W. G. R. (1999). Plano de manejo para o uso sustentável de peixes ornamentais na Reserva de Desenvolvimento Sustentável Mamirauá. In: Queiroz, H. L. & W. G. R. Crampton (Eds.). *Estratégias para Manejo de Recursos Pesqueiros em Mamirauá* (pp. 159-176). Brasília. Sociedade Civil Mamirauá/MCT, CNPq.

Dagosta, F., & de pinna, M. (2019). The Fishes of the Amazon: Distribution and Biogeographical Patterns, with a Comprehensive List of Species. *Bulletin of the American Museum of Natural History*, 431, 1. doi:10.1206/0003-0090.431.1.1

DiBattista, J. D., Berumen, M. L., Gaither, M. R., Rocha, L. A., Eble, J. A., Choat, J. H., . . . McClain, C. (2013). After continents divide: comparative phylogeography of reef fishes from the Red Sea and Indian Ocean. *Journal of Biogeography*, 40, 1170-1181. doi:10.1111/jbi.12068

Doyle, J. J., & Doyle, J. L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemistry Bulletin*, 19, 11-15.

724 Dudgeon, D., Arthington, A. H., Gessner, M. O., Kawabata, Z.-I., Knowler, D. J., Lévêque, C.,
725 . . . Sullivan, C. A. (2006). Freshwater biodiversity: importance, threats, status and conservation
726 challenges. *Biological Reviews*, 81, 163-182. doi:10.1017/s1464793105006950
727
728 Dumont, J.-F. (1992). Rasgos morfoestructurales de la llanura amazónica del Perú: efecto de la
729 neotectónica sobre los cambios fluviales y la delimitación de las provincias morfológicas.
730 *Bulletin de l'Institut français d'études andines*, 21, 801-833.
731
732 Dumont, J. F. (1991). Cambio del curso de los rios Marañon et Ucayali en la depresión
733 Ucamara durante el cuaternario reciente. *VII Congreso Peruano de Geología*, Lima, Peru.
734
735 Dumont, J. F., Deza, E., & García, F. (1991). Morphostructural provinces and neotectonics in
736 the Amazonian lowlands of Peru. *Journal of South American Earth Sciences*, 4, 373-381.
737
738 Dumont, J.-F. & García, F. (1989). Pleistocene deposits in Amazonian Peru: are lithological
739 characteristics related to glacial interstages? First meeting in La Paz, Bolivia, *IGCP project*
740 *281*, 1-4.
741
742 Dumont, J. F., Lamotte, S., & Fournier, M. (1988). Neotectonica dela arco de Iquitos (Jenaro
743 Herrera, Peru). *Boletin de la Sociedad Geológica del Perú*, 77, 7-17.
744
745 Dumont, J. F., Lamotte, S., & Khan, F. (1990). Wetland and upland forest ecosystems in
746 Peruvian Amazonia: plant species diversity in the light of some geological and botanical
747 evidence. *Forest Ecology and Management*, 33-34, 125-139.
748
749 Finer, M., & Jenkins, C. N. (2012). Proliferation of Hydroelectric Dams in the Andean Amazon
750 and Implications for Andes-Amazon Connectivity. *PLoS One*, 7, e35126.
751 doi:10.1371/journal.pone.0035126
752
753 Finer, M., Jenkins, C. N., Pimm, S. L., Keane, B., & Ross, C. (2008). Oil and gas projects in
754 the Western Amazon: threats to wilderness, biodiversity, and indigenous peoples. *PLoS One*, 3,
755 e2932. doi:10.1371/journal.pone.0002932
756

Forsberg, B. R., Melack, J. M., Dunne, T., Barthem, R. B., Goulding, M., Paiva, R. C. D., . . . Weisser, S. (2017). The potential impact of new Andean dams on Amazon fluvial ecosystems. *PLoS One*, 12, e0182254-e0182254. doi:10.1371/journal.pone.0182254

Frankham, R., Ballou, J. D. & Briscoe, D.A. (2002). *Introduction to Conservation Genetics*. Cambridge University Press.

Frederico, R. G., Olden, J. D., & Zuanon, J. (2016). Climate change sensitivity of threatened, and largely unprotected, Amazonian fishes. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 26, 91-102. doi:10.1002/aqc.2658

Frederico, R. G., Zuanon, J., & De Marco, P. (2018). Amazon protected areas and its ability to protect stream-dwelling fish fauna. *Biological Conservation*, 219, 12-19. doi:https://doi.org/10.1016/j.biocon.2017.12.032

Freitas, C. E. C., Siqueira-Souza, F. K., Humston, R., & Hurd, L. E. (2013). An initial assessment of drought sensitivity in Amazonian fish communities. *Hydrobiologia*, 705, 159-171. doi:10.1007/s10750-012-1394-4

Genner, M. J., Seehausen, O., Lunt, D. H., Joyce, D. A., Shaw, P. W., Carvalho, G. R., & Turner, G. F. (2007). Age of cichlids: new dates for ancient lake fish radiations. *Molecular Biology and Evolution*, 24, 1269-1282. doi:10.1093/molbev/msm050

Gerstner, C. L., Ortega, H., Sanchez, H., & Graham, D. L. (2006). Effects of the freshwater aquarium trade on wild fish populations in differentially-fished areas of the Peruvian Amazon. *Journal of Fish Biology*, 68, 862–875. doi:10.1111/j.1095-8649.2006.00978.x

Gonçalves Júnior, E. S., Soares, E. A. A., Tatumi, S. H., Yee, M., & Mittani, J. C. R. (2016). Pleistocene-Holocene sedimentation of Solimões-Amazon fluvial system between the tributaries Negro and Madeira, Central Amazon. *Brazilian Journal of Geology*, 46, 167-180.

Goudet, J. (1995). FSTAT (Version 1.2): A Computer Program to Calculate F-Statistics. *Journal of Heredity*, 86, 485-486. doi:10.1093/oxfordjournals.jhered.a111627

Grabert, H. (1991). *Der Amazonas: Geschichte und Probleme eines Stromgebietes zwischen Pazifik und Atlantik*: Springer-Verlag.

Haffer, J. (1969). Speciation in Amazonian Forest Birds. *Science*, 165, 131-137. doi:10.1126/science.165.3889.131

Hennig, W. (1966). *Phylogenetic Systematics*. University of Illinois Press, Urbana.

Houghton, R. A., Skole, D. L., Nobre, C. A., Hackler, J. L., Lawrence, K. T., & Chomentowski, W. H. (2000). Annual fluxes of carbon from deforestation and regrowth in the Brazilian Amazon. *Nature*, 403, 301-304.

Hubert, N., & Renno, J.-F. (2006). Historical biogeography of South American freshwater fishes. *Journal of Biogeography*, 33, 1414-1436. doi:10.1111/j.1365-2699.2006.01518.x

Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (2017) normativa interministerial n°01/2012, https://www.ibama.gov.br/component/phocadownload/file/2331-instrucao-normativa-interministerial-n-01-2012.

Jombart, T. (2008). adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics*, 24, 1403-1405. doi:10.1093/bioinformatics/btn129

Jombart, T., Devillard, S., & Balloux, F. (2010). Discriminant analysis of principal components a new method for the analysis of genetically structured populations. *BMC Genetics*, 11, 94.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., . . . Drummond, A. (2012). Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, 28, 1647-1649. doi:10.1093/bioinformatics/bts199

Kingston, P. F. (2002). Long-term Environmental Impact of oil spills. *Spill Science & Technology Bulletin*, 7, 53-61.

824 Koslowski, I. (2002). *Die Buntbarsche Amerikas Band 2 Apistogramma & Co*. Ulmer,
825 Stuttgart.
826
827 Kullander, S.O. (1986). *Cichlid fishes of the Amazon River drainage of Peru*. Swedish Museum
828 of Natural History, Stockholm.
829
830 Kumar, S., Stecher, G., Li, M., Knyaz, C., & Tamura, K. (2018). MEGA X: Molecular
831 Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and*
832 *Evolution*, 35, 1547-1549. doi:10.1093/molbev/msy096
833
834 Latini, A. O., & Petrere Jr., M. (2004). Reduction of a native fish fauna by alien species: an
835 example from Brazilian freshwater tropical lakes. *Fisheries Management and Ecology*, 11, 71-
836 79. doi:10.1046/j.1365-2400.2003.00372.x
837
838 Leitão, C. S. S., Santos, C. H. A., Souza, É. M. S., Vilarinho, G. C. C., Paula-Silva, M. N., Val,
839 P., . . . de Almeida-Val, V. M. F. (2017). Development and characterization of microsatellite
840 loci in Amazonian dwarf cichlids *Apistogramma* spp. (Perciformes: Cichlidae): Uncovering
841 geological influence on Amazonian fish population. *Journal of Applied Ichthyology*, 33, 1196-
842 1199. doi:10.1111/jai.13490
843
844 Loyola, R. D., Kubota, U., & Lewinsohn, T. M. (2007). Endemic vertebrates are the most
845 effective surrogates for identifying conservation priorities among Brazilian ecoregions.
846 *Diversity and Distributions*, 13, 389-396. doi:10.1111/j.1472-4642.2007.00345.x
847
848 Mallet, J. (1995). A species definition for the modern synthesis. *Trends in Ecology &*
849 *Evolution*, 10, 294-299. doi:https://doi.org/10.1016/0169-5347(95)90031-4
850
851 Mayden, R.L. & Wood, R.M. (1995) Systematics, species concepts and the evolutionarily
852 significant unit in biodiversity and conservation biology. *American Fisheries Society*
853 *Symposium*. 17, 58-113.
854
855 Mayr, E. (1942) *Systematics and the Origin of Species*. Harvard University Press, Cambridge,
856 MA.
857

Mesa, S. L. M. & Lasso, C. A. (2011). Revisión del género *Apistogramma* Regan, 1913 (Perciformes, Cichlidae) en la cuenca del río Orinoco (Eds.). *Recursos Hidrobiológicos y Pesqueros Continentales de Colombia III* (pp. 1-192). Instituto Humboldt. Bogotá, Colombia.

Miya, M., & Nishida, M. (2000). Use of mitogenomic information in teleostean molecular phylogenetics: a tree-based exploration under the maximum-parsimony optimality criterion. *Molecular Phylogenetics and Evolution*, 17, 437-455. doi:10.1006/mpev.2000.0839

Moreau, M.-A., & Coomes, O. T. (2007). Aquarium fish exploitation in western Amazonia: conservation issues in Peru. *Environmental Conservation*, 34, 12-22. doi:10.1017/s0376892907003566

Morton, D. C., DeFries, R. S., Shimabukuro, Y. E., Anderson, L. O., Arai, E., del Bon Espirito-Santo, F., . . . Morisette, J. (2006). Cropland expansion changes deforestation dynamics in the southern Brazilian Amazon. *Proceedings of the National Academy of Sciences U S A*, 103, 14637-14641. doi:10.1073/pnas.0606377103

Nei, M. (1972). Genetic Distance between Populations. *The American Naturalist*, 106, 283-292.

Nogueira, C., Buckup, P. A., Menezes, N. A., Oyakawa, O. T., Kasecker, T. P., Ramos Neto, M. B., & da Silva, J. M. C. (2010). Restricted-Range Fishes and the Conservation of Brazilian Freshwaters. *PLoS One*, 5, e11390. doi:10.1371/journal.pone.0011390

Odhiambo, E. A., Mautner, S. I., Bock, O., & Sturmbauer, C. (2012). Genetic distinction of four haplochromine cichlid fish species in a satellite lake of Lake Victoria, East Africa. *Journal of Zoological Systematics and Evolutionary Research*, 50, 51-58. doi:10.1111/j.1439-0469.2011.00641.x

Olden, J. D., Hogan, Z. S., & Zanden, M. J. V. (2007). Small fish, big fish, red fish, blue fish: size-biased extinction risk of the world's freshwater and marine fishes. *Global Ecology and Biogeography*, 16, 694-701. doi:10.1111/j.1466-8238.2007.00337.x

Olivier, K. (2001). The Ornamental Fish Market. FAO/Globefish, *United Nations Food and Agriculture Organization*, Research Programme 67.

Ortega H. & Castro, E., (1998). Hidrobiología de la Cuenca del Río Nanay. *Evaluación de la Ictiofauna, Diagnostico de la Pesquería y Propuesta de Manejo de los Recursos Hidrobiológicos* (pp. 60). CEDIA. Lima, Perú.

Piálek, L., Říčan, O., Casciotta, J., Almirón, A., & Zrzavy, J. (2012). Multilocus phylogeny of *Crenicichla* (Teleostei: Cichlidae), with biogeography of the *C. lacustris* group: species flocks as a model for sympatric speciation in rivers. *Molecular Phylogenetics and Evolution*, 62, 46-61. doi:10.1016/j.ympev.2011.09.006

Pimm, S. L., Jenkins, C. N., Abell, R., Brooks, T. M., Gittleman, J. L., Joppa, L. N., . . . Sexton, J. O. (2014). The biodiversity of species and their rates of extinction, distribution, and protection. *Science*, 344, 1246752. doi:10.1126/science.1246752

Pritchard, J. K., Stephens, M., & Donnelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics*, 155, 945-959.

Quérouil, S., Vela Diaz, A., García-Dávila, C., Römer, U., & Renno, J. F. (2015). Development and characterization of polymorphic microsatellite markers in neotropical fish of the genus *Apistogramma* (Perciformes: Labroidei: Cichlidae). *Journal of Applied Ichthyology*, 31, 52-56. doi:10.1111/jai.12975

RADAMBRESIL (1977)- Levantamento de recursos naturais. Folha SB.I9 JURUA,. Ministerio das Minas e Energia, Projeto Radambresil.

Raghavan, R., Ali, A., Philip, S., & Dahanukar, N. (2018). Effect of unmanaged harvests for the aquarium trade on the population status and dynamics of redline torpedo barb: A threatened aquatic flagship. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 28, 567-574. doi:10.1002/aqc.2895

923 Ramos, L. R. (2007). Estudo das relações filogenéticas de Trichomycteridae (Teleostei,
924 Siluriformes) com base em evidências cromossômicas e moleculares (PhD thesis).
925 Universidade Estadual Paulista, Instituto de Biociências de Botucatu, Brazil.
926
927 Räsänen, M. E., Linna, A., Irion, G., Rebata Hernani, L., Vargas Huaman, R., & Wesselingh, F.
928 (1998) Geología y geoformas de la zona de Iquitos. En: Kalliola, R. & Flores, S. (eds.),
929 *Geoecología y desarrollo amazónico: estudio integrado en la zona de Iquitos* (pp. 59-137),
930 Perú. Turun, Turku, Annales Universitatis Turkuensis.
931
932 Räsänen, M. E., Salo, J. S., & Kalliola, R. J. (1987). Fluvial perturbance in the Western
933 Amazon basin: regulation by long-term sub-andean tectonics. *Science*, 238, 1398-1401.
934 doi:10.1126/science.238.4832.1398
935
936 Ready, J. S., Sampaio, I., Schneider, H., Vinson, C., Dos Santos, T., & Turner, G. F. (2006).
937 Colour forms of Amazonian cichlid fish represent reproductively isolated species. *Journal of*
938 *Evolutionary Biology*, 19, 1139-1148. doi:10.1111/j.1420-9101.2006.01088.x
939
940 Říčan, O., Piálek, L., & Dragová, K. (2016). Diversity and evolution of the Middle American
941 cichlid fishes (Teleostei: Cichlidae) with revised classification. *Vertebrate Zoology*, 66, 1-102.
942
943 Rocha-Olivares, A., Fleeger, J. W., & Foltz, D. W. (2001). Decoupling of molecular and
944 morphological evolution in deep lineages of a meiobenthic harpacticoid copepod. *Molecular*
945 *Biology and Evolution*, 18, 1088-1102. doi:10.1093/oxfordjournals.molbev.a003880
946
947 Römer, U. (1992). Weitere Beobachtungen zur Verbreitung, Ökologie und Aquarienbiologie
948 von *Laetacara* spec. "Orangenflossen". *DCG-Informationen*, 23, 74-83.
949
950 Römer, U. (1993). Freilandbeobachtungen an *Apistogramma diplotaenia* Kullander, 1987.
951 *Buntbarschjahrbuch* (pp. 58-71), bede, Kollnburg.
952
953 Römer U. (1995). Kritische Bemerkungen über Angaben zu Beständen und
954 Bestandsentwicklungen von neotropischen Kleinfischen am Beispiel von *Apistogramma*
955 *mendezi* und *Paracheirodon*. In H. Sioli (Eds.). Reports of the Workshop and Exhibition.

Gefährdete Süßwasserfische tropischer Ökosysteme (pp. 1-55), Zoologisches Forschungsinstitut und Museum Alexander Koenig, Bonn, Germany.

Römer, U. (2000). *Cichlid Atlas - Volume 1 - Natural History of South American Dwarf Cichlids*. Melle: Mergus Verlag.

Römer U., (2006) *Cichlid Atlas. Volume 2. Natural History of South American Dwarf Cichlids* Melle: Mergus Verlag.

Römer, U., & Beisenherz, W. (2005). Intra- and interspecific mate choice of female *Apistogramma cacatuoides* (Teleostei: Cichlidae). *Ichthyological Exploration of Freshwaters*, 16, 339-345.

Römer, U., Duponchelle, F., Vela Diaz, A., Davila, C., S, S., & Renno, J.-F. (2011). *Apistogramma cinilabra* sp. n.: Description of a potentially endangered endemic cichlid species (Teleostei: Perciformes: Cichlidae) from the Departamento Loreto, Peru. *Vertebrate Zoology*, 61, 3-23.

Römer, U., Engelking, B., & Beisenherz, W. (2014). Genetically determined mate choice can be influenced by learning in *Apistogramma cacatuoides* HOEDEMAN, 1951 (Teleostei, Cichlidae). *Vertebrate Zoology*, 64, 199-206.

Römer, U., & Hahn, I. (2008). *Apistogramma barlowi* sp. N. Description of a new facultative mouth-breeding cichlid species (Teleostei Perciformes Geophaginae) from Northern Peru. *Vertebrate Zoology*, 58, 49-66.

Römer, U., & Hahn, I. (2013). *Apistogramma aguarico* sp. n. A new species of geophagine cichlid fish (Teleostei Perciformes) from the Ecuadorian and Peruvian río Napo system. *Vertebrate Zoology*, 63, 171-181.

Römer, U., Joscha, B., Duponchelle, F., Vela Diaz, A., Davila, C., Sirvas-Cornejo, S., . . . Renno, J.-F. (2012). Description of *Apistogramma allpahuayo* sp. n., a new dwarf cichlid species (Teleostei: Perciformes: Geophaginae) from in and around the Reserva Nacional Allpahuayo Mishana, Loreto, Peru. *Vertebrate Zoology*, 62, 189-212.

Römer, U., Römer, C., Estivals, G., Vela Diaz, A., Duponchelle, F., Davila, C., . . . Renno, J.-F. (2017). Description of a new maternal larvophilic mouth-brooding cichlid species, *Apistogramma megastoma* sp. n. (Teleostei: Perciformes: Geophaginae), from Loreto, Peru *. *Vertebrate Zoology*, 67.

Römer, U., Soares, D., Davila, C., Duponchelle, F., Renno, J.-F., & Hahn, I. (2015). Re-description of *Apistogramma payaminonis* KULLANDER, 1986, with descriptions of two new cichlid species of the genus *Apistogramma* (Teleostei, Perciformes, Geophaginae) from northern Peru. *Vertebrate Zoology*, 65, 287-314.

Römer U. & Wise, M. (2006). Nominal list of known species and local morphs of the genus *Apistogramma* (Regan) imported untill August 2005. in Römer, (Eds.). *Cichlid Atlas. Volume 2* (pp. 378-390). Mergus publishers, Melle.

Rosenfield, J. A. (2002). Pattern and process in the geographical ranges of freshwater fishes. *Global Ecology and Biogeography*, 11, 323-332. doi:10.1046/j.1466-822X.2002.00287.x

Rousset, F. (1997). Genetic differentiation and estimation of gene flow from F-statistics under isolation by distance. *Genetics*, 145, 1219-1228.

Rozas, J., Ferrer-Mata, A., Sanchez-DelBarrio, J. C., Guirao-Rico, S., Librado, P., Ramos-Onsins, S. E., & Sanchez-Gracia, A. (2017). DnaSP 6: DNA Sequence Polymorphism Analysis of Large Data Sets. *Molecular Biology and Evolution*, 34, 3299-3302. doi:10.1093/molbev/msx248

Santorelli, S., Jr., Magnusson, W. E., & Deus, C. P. (2018). Most species are not limited by an Amazonian river postulated to be a border between endemism areas. *Scientific Reports*, 8, 2294. doi:10.1038/s41598-018-20596-7

Schiesari, L., Zuanon, J., Azevedo-Ramos, C., Garcia, M., Gordo, M., Messias, M., & Monteiro Vieira, E. (2003). Macrophyte rafts as dispersal vectors for fishes and amphibians in the Lower Solimões River, Central Amazon. *Journal of Tropical Ecology*, 19, 333-336. doi:10.1017/s0266467403003365

Schindler, I., & Staeck, W. (2013). Description of *Apistogramma helkeri* sp. n., a new geophagine dwarf cichlid (Teleostei Cichlidae) from the lower río Cuao (Orinoco drainage) in Venezuela. *Vertebrate Zoology*, 63, 301-306.

Sick, H. (1993). *Birds in Brazil: a natural history*. Princeton University Press, Princeton & Chichester.

Sioli H. (1984). *The Amazon, Limnology and landscape ecology of a mighty river and its basin*. Dr. W. Junk Publishers. Dordrecht Netherlands and Boston and Hingham, MA, USA.

Steindachner, F. (1875). Beiträge der Kenntniss der Chromiden des Amazonenstromes. Sitzungsberichte der kaiserlichen Akademie der Wissenschaften. *Mathematischnaturwissenschafliche Classe*, 71, 61-137

Swenson, J. J., Carter, C. E., Domec, J. C., & Delgado, C. I. (2011). Gold mining in the Peruvian Amazon: global prices, deforestation, and mercury imports. *PLoS One*, 6, e18875. doi:10.1371/journal.pone.0018875

Tello, J., & Serna, J. (1991). Estado actual de la explotación de los principales peces ornamentales de la Amazonía Peruana. *Folia Amazónica*, 3, 107-128. doi:10.24841/fa.v3i1-2.205

Tougard, C., Garcia Davila, C. R., Romer, U., Duponchelle, F., Cerqueira, F., Paradis, E., . . . Renno, J. F. (2017). Tempo and rates of diversification in the South American cichlid genus *Apistogramma* (Teleostei: Perciformes: Cichlidae). *PLoS One*, 12, e0182618. doi:10.1371/journal.pone.0182618

Tregidgo, D. J., Barlow, J., Pompeu, P. S., de Almeida Rocha, M., & Parry, L. (2017). Rainforest metropolis casts 1,000-km defaunation shadow. *Proceedings of the National Academy of Sciences*, 114, 8655-8659. doi:10.1073/pnas.1614499114

Vitule, J. R. S., Freire, C., & Simberloff, D. (2009). Introduction of non-native freshwater fish can certainly be bad. *Fish and Fisheries*, 10, 98-108. doi:10.1111/j.1467-2979.2008.00312.x

Vitule, J. R. S., da Costa, A. P. L., Frehse, F. A., Bezerra, L. A. V., Occhi, T. V. T., Daga, V. S., & Padial, A. A. (2017). Comment on 'Fish biodiversity and conservation in South America by Reis et al. (2016)'. *Journal of Fish Biology*, 90, 1182-1190. doi:10.1111/jfb.13239

Wallace, A. R. (1854). On the Monkeys of the Amazon. *Annals and Magazine of Natural History*, 14, 451-454. doi:10.1080/037454809494374

Waples, R. S. (1987). A Multispecies Approach to the Analysis of Gene Flow in Marine Shore Fishes. *Evolution*, 41, 385-400. doi:10.2307/2409146

Ward, R. D., Woodwark, M., & Skibinski, D. O. F. (1994). A comparison of genetic diversity levels in marine, freshwater, and anadromous fishes. *Journal of Fish Biology*, 44, 213-232. doi:10.1111/j.1095-8649.1994.tb01200.x

Weir, B. S., & Cockerham, C. C. (1984). Estimating F-Statistics for the Analysis of Population Structure. *Evolution*, 38, 1358-1370. doi:10.2307/2408641

Wesselingh, F., & Salo, J. A. (2006). A Miocene perspective on the evolution of the Amazonian biota. *Scripta Geologica*, 133, 439-458.

Willis, S. C., Winemiller, K. O., Montaña, C. G., Macrander, J., Reiss, P., Farias, I. P., & Ortí, G. (2015). Population genetics of the speckled peacock bass (*Cichla temensis*), South America's most important inland sport fishery. *Conservation Genetics*, 16, 1345-1357. doi:10.1007/s10592-015-0744-y

TABLES

Table 1: Genetic diversity indices in *Apistogramma agassizii* popsite based on 10 microsatellite loci. N, samples size; TNA, total number of alleles; MNA, mean number of alleles; AR, allelic richness; NPA, number of private alleles; Hobs., observed heterozygosity; Hexp., expected heterozygosity, Hn.b., expected heterozygosity without bias; F_{IS}, inbreeding coefficient of cluster; * = *p-value*<0.050.

		Allelic Diversity				Genetic diversity			
Popsite	***N***	**TNA**	**MNA**	**AR**	**NPA**	***H exp.***	***H n.b.***	***H obs.***	***Fis***
JH3	48	140	14.0	9.512	6	0.754	0.762	0.708	0.072*
An	35	138	13.8	9.818	19	0.754	0.765	0.762	0.003
JH4	39	132	13.2	9.537	1	0.749	0.758	0.761	-0.003
JH5	36	130	13.0	9.349	2	0.739	0.749	0.737	0.017
Or8	30	101	10.1	7.904	4	0.710	0.722	0.654	0.096*
Or9	30	98	9.8	7.738	2	0.707	0.719	0.747	-0.040
Ya3	24	92	9.2	8.147	2	0.715	0.730	0.729	0.001
JH7	29	88	8.8	7.146	4	0.644	0.655	0.682	-0.042
JH17	39	88	8.8	6.803	3	0.686	0.695	0.690	0.007
JH10	30	83	8.3	7.259	1	0.706	0.718	0.703	0.021
JH13	52	79	7.9	6.015	2	0.681	0.688	0.650	0.055*
Or4	30	78	7.8	6.337	3	0.588	0.598	0.600	-0.004
Ya2	23	77	7.7	6.991	1	0.694	0.709	0.735	-0.037
Or5	30	75	7.5	6.115	1	0.537	0.547	0.514	0.061
JH2	48	74	7.4	5.738	2	0.636	0.643	0.581	0.096*
JH9	77	74	7.4	6.091	1	0.658	0.662	0.657	0.007
Or3	30	73	7.3	5.910	2	0.555	0.564	0.560	0.007
Or2	30	71	7.1	5.559	0	0.496	0.504	0.493	0.022
Or1	30	66	6.6	5.482	4	0.531	0.540	0.557	-0.032
JH11	30	63	6.3	5.524	0	0.655	0.667	0.621	0.069*
Pe4	27	60	6.0	5.198	5	0.488	0.497	0.500	-0.006
JH16	32	58	5.8	5.166	0	0.632	0.642	0.578	0.101*
JH19	38	56	5.6	4.908	0	0.618	0.627	0.657	-0.050
JH15	26	55	5.5	5.168	0	0.650	0.663	0.700	-0.058
Na2	24	55	5.5	4.675	0	0.391	0.399	0.425	-0.067
JH20	39	53	5.3	4.567	1	0.549	0.556	0.536	0.036
JH14	28	50	5.0	4.647	0	0.613	0.624	0.539	0.138*
PU16	30	48	4.8	4.144	3	0.410	0.417	0.430	-0.032
Na1	24	47	4.7	4.164	0	0.391	0.399	0.408	-0.023
JH18	40	45	4.5	4.184	0	0.611	0.618	0.605	0.022
JH8	30	42	4.2	3.963	0	0.564	0.574	0.563	0.019
JH12	16	37	3.7	3.700	0	0.544	0.562	0.613	-0.093
Na3	22	34	3.4	3.253	0	0.425	0.435	0.436	-0.004
PU5A	25	32	3.2	3.028	1	0.358	0.365	0.364	0.003
Or7	30	24	2.4	2.317	1	0.350	0.356	0.387	-0.089

Table 2: Alleles with frequencies > 0.5 (in bold) for at least one of the three clusters.

		Frequencies ≥ 0.5		
Locus	Allele	Cluster 1	Cluster 2	Cluster 3
Loc01	100	-----	**0.5049**	**0.9182**
Loc06	138	0.0628	**0.6014**	-----
Loc06	144	0.0006	0.0048	**0.5648**
Loc08	138	0.1195	0.0558	**0.7909**
Loc09	129	**0.5209**	0.1293	-----
Loc09	131	0.2863	**0.6024**	-----
Loc11	123	**0.7099**	-----	-----
Loc11	127	0.0655	**0.5145**	-----
Loc11	137	0.0040	0.0942	**0.5833**
Loc14	116	0.0074	**0.7937**	-----
Loc16	142	0.2649	**0.9082**	**0.9364**
Loc16	144	**0.5676**	0.0024	0.0455
Loc28	196	0.1020	-----	**0.9727**
Loc28	200	0.0353	**0.5631**	-----
Loc30	187	0.0045	0.0048	**0.8364**
Loc32	201	0.2035	-----	**0.6636**
Loc32	203	**0.5609**	-----	0.3091
Loc32	209	0.0023	**0.6401**	0.0273

Cluster 1 is composed of popsites JH2, JH3, JH4, JH5, JH7, JH8, JH9, JH10, JH11, JH12, JH13, JH14, JH15, JH16, JH17, JH18, JH19, JH20, An, Ya2, Ya3, Na1, Na2, Na3, Or8, Or9; Cluster 2 of popsites Pe4, Or1, Or2, Or3, Or4, Or5, Or7, and Cluster 3 of popsites PU5A, PU1.

Table 3. Pairwise value of F_{ST} estimator of Weir & Cockerham (1984) between 26 popsites from cluster 1.

	JH3	JH4	JH5	JH7	JH8	JH9	JH10	JH11	JH12	JH13	JH14	JH15	JH16	JH17	JH18	JH19	JH20	An	Ya2	Ya3	Na1	Na2	Na3	Or8	Or9
JH2	0.08	0.07	0.08	0.07	0.16	0.14	0.08	0.12	0.22	0.12	0.12	0.15	0.14	0.14	0.14	0.18	0.16	0.13	0.16	0.11	0.24	0.24	0.21	0.17	0.18
JH3	-----	0.00	0.00	0.05	0.11	0.08	0.03	0.06	0.13	0.07	0.11	0.08	0.08	0.06	0.09	0.10	0.12	0.06	0.07	0.05	0.19	0.18	0.16	0.08	0.09
JH4	0.00	-----	0.00	0.05	0.12	0.09	0.03	0.06	0.15	0.08	0.11	0.08	0.09	0.07	0.09	0.11	0.11	0.07	0.08	0.05	0.19	0.19	0.17	0.10	0.11
JH5	0.00	0.00	-----	0.05	0.12	0.10	0.04	0.08	0.14	0.07	0.11	0.08	0.09	0.07	0.10	0.11	0.12	0.07	0.08	0.05	0.20	0.20	0.18	0.09	0.10
JH7	0.05	0.05	0.05	-----	0.18	0.08	0.06	0.09	0.20	0.11	0.15	0.10	0.12	0.10	0.15	0.16	0.17	0.09	0.12	0.08	0.18	0.17	0.15	0.11	0.13
JH8	0.11	0.12	0.12	0.18	-----	0.20	0.14	0.19	0.17	0.13	0.21	0.18	0.18	0.18	0.05	0.19	0.10	0.20	0.20	0.18	0.32	0.32	0.32	0.21	0.21
JH9	0.08	0.09	0.10	0.08	0.20	-----	0.05	0.07	0.22	0.14	0.20	0.13	0.15	0.13	0.17	0.18	0.21	0.13	0.14	0.13	0.18	0.18	0.18	0.12	0.15
JH10	0.03	0.03	0.04	0.06	0.14	0.05	-----	0.05	0.16	0.09	0.13	0.10	0.09	0.09	0.12	0.12	0.14	0.10	0.12	0.09	0.20	0.20	0.19	0.12	0.13
JH11	0.06	0.06	0.08	0.09	0.19	0.07	0.05	-----	0.20	0.12	0.19	0.13	0.12	0.13	0.16	0.15	0.21	0.11	0.13	0.11	0.21	0.21	0.20	0.15	0.16
JH12	0.13	0.15	0.14	0.20	0.17	0.22	0.16	0.20	-----	0.09	0.24	0.18	0.18	0.22	0.16	0.18	0.21	0.20	0.24	0.21	0.35	0.34	0.36	0.21	0.22
JH13	0.07	0.08	0.07	0.11	0.13	0.14	0.09	0.12	0.09	-----	0.16	0.12	0.14	0.13	0.10	0.17	0.13	0.12	0.15	0.11	0.25	0.25	0.22	0.15	0.15
JH14	0.11	0.11	0.11	0.15	0.21	0.20	0.13	0.19	0.24	0.16	-----	0.17	0.13	0.15	0.17	0.16	0.19	0.14	0.16	0.13	0.30	0.29	0.26	0.18	0.22
JH15	0.08	0.08	0.08	0.10	0.18	0.13	0.10	0.13	0.18	0.12	0.17	-----	0.09	0.12	0.13	0.11	0.19	0.13	0.14	0.11	0.25	0.24	0.23	0.15	0.16
JH16	0.08	0.09	0.09	0.12	0.18	0.15	0.09	0.12	0.18	0.14	0.13	0.09	-----	0.15	0.16	0.05	0.19	0.14	0.16	0.14	0.27	0.26	0.27	0.18	0.19
JH17	0.06	0.07	0.07	0.10	0.18	0.13	0.09	0.13	0.22	0.13	0.15	0.12	0.15	-----	0.14	0.18	0.15	0.10	0.07	0.08	0.24	0.24	0.22	0.12	0.14
JH18	0.09	0.09	0.10	0.15	0.05	0.17	0.12	0.16	0.16	0.10	0.17	0.13	0.16	0.14	-----	0.17	0.08	0.16	0.14	0.13	0.28	0.27	0.27	0.18	0.20
JH19	0.10	0.11	0.11	0.16	0.19	0.18	0.12	0.15	0.18	0.17	0.16	0.11	0.05	0.18	0.17	-----	0.22	0.17	0.18	0.16	0.30	0.29	0.30	0.19	0.20
JH20	0.12	0.11	0.12	0.17	0.10	0.21	0.14	0.21	0.21	0.13	0.19	0.19	0.19	0.15	0.08	0.22	-----	0.18	0.20	0.18	0.35	0.34	0.33	0.23	0.24
An	0.06	0.07	0.07	0.09	0.20	0.13	0.10	0.11	0.20	0.12	0.14	0.13	0.14	0.10	0.16	0.17	0.18	-----	0.11	0.08	0.24	0.23	0.20	0.10	0.12
Ya2	0.07	0.08	0.08	0.12	0.20	0.14	0.12	0.13	0.24	0.15	0.16	0.14	0.16	0.07	0.14	0.18	0.20	0.11	-----	0.04	0.28	0.27	0.25	0.10	0.13
Ya3	0.05	0.05	0.05	0.08	0.18	0.13	0.09	0.11	0.21	0.11	0.13	0.11	0.14	0.08	0.13	0.16	0.18	0.08	0.04	-----	0.26	0.25	0.21	0.08	0.10
Na1	0.19	0.19	0.20	0.18	0.32	0.18	0.20	0.21	0.35	0.25	0.30	0.25	0.27	0.24	0.28	0.30	0.35	0.24	0.28	0.26	-----	0.00	0.16	0.24	0.26
Na2	0.18	0.19	0.20	0.17	0.32	0.18	0.20	0.21	0.34	0.25	0.29	0.24	0.26	0.24	0.27	0.29	0.34	0.23	0.27	0.25	0.00	-----	0.15	0.23	0.26
Na3	0.16	0.17	0.18	0.15	0.32	0.18	0.19	0.20	0.36	0.22	0.26	0.23	0.27	0.22	0.27	0.30	0.33	0.20	0.25	0.21	0.16	0.15	-----	0.22	0.25
Or8	0.08	0.10	0.09	0.11	0.21	0.12	0.12	0.15	0.21	0.15	0.18	0.15	0.18	0.12	0.18	0.19	0.23	0.10	0.10	0.08	0.24	0.23	0.22	-----	0.02

FST values < 0.05 (highlighted in green), 0.05 ≤ yellow < 0.15, 0.15 ≤ Orange < 0.25 and ≥ 0.25 (blue).

Table 4. Pairwise value of F_{ST} estimator of Weir and Cockerham (1984) between 7 popsites from cluster 2.

	Pe4	Or1	Or2	Or3	Or4	Or5	Or7
Pe4	-----	0.13	0.08	0.12	0.08	0.07	0.33
Or1	0.13	-----	0.07	0.11	0.06	0.07	0.23
Or2	0.08	0.07	-----	0.05	0.01	0.00	0.27
Or3	0.12	0.11	0.05	-----	0.03	0.04	0.28
Or4	0.08	0.06	0.01	0.03	-----	0.02	0.21
Or5	0.07	0.07	0.00	0.04	0.02	-----	0.26

FST values < 0.05 (highlighted in green), 0.05 ≤ yellow < 0.15, 0.15 ≤ Orange < 0.25 and ≥ 0.25 (blue).

Table 5. Genetic parameters for COI sequences in *Apistogramma agassizii*, clade 1, clade 2 and clade 3.

	Apistogramma. agassizii	Clade 1	Clade 2	Clade 3
Number of sequencies (N)	32	14	10	8
Number of haplotypes (H)	12	5	4	3
Haplotypic diversity (Hd) ± SD(Hd)	0.917 ± 0.023	0.780 ± 0.085	0.733 ± 0.12	0.679 ± 0.122
Nucleotic diversity (π) ± SD(π)	0.015 ± 0.00059	0.00615 ± 0.00055	0.00137 ± 0.000354	0.00089 ± 0.00022
Total number of sites	924	924	924	924
Total number of polymophic sites	37	13	4	2
Mean nucleotidic differenciation between pairwise sequences (K)	13.857	5.681	1.267	0.821

FIGURE LEGEND

Figure 1: Elevation map of: a) the complete study area, Loreto (Peru) with the 35 population collecting sites (popsites), **b)** the sampling area in Jenaro Herrera region, **c)** the sampling area in Orán region. Popsites with different colours: blue (JH2, JH3, JH4, JH5, JH7, JH8, JH9, JH10, JH11, JH12, JH13, JH14, JH15, JH16, JH17, JH18, JH19, JH20, Pe9, An, Ya2, Ya3, Na1, Na2, Na3, Or8 and Or9), green (Pe4, Or1, Or2, Or3, Or4, Or5 and Or7) and red (PU5A and PU16) correspond to distinct genetic units. Main river channels are highlighted in brown.

Figure 2: Illustration of the aquarium setting for mate choice experiments. Each aquarium (100x30x30cm) is divided in three compartments: central one for females (50x30x30cm) and each extremity (25x30x30cm) for one male of each reproductive unit. Separations between the female and the male's compartments are perforated with 8mm holes to allow for all three types of communication: sound, chemical and visual. Filtering is performed with an internal sponge-filter and lightning with LED tubes on a 12 L : 12 D cycle. Spawning sites are 10cm long and 4.8 cm diameter PVC tubes cut lengthwise and fixed to each side of the glass separations.

Figure 3. Unrooted neighbor-joining dendrogram of pairwise genetic distances of Nei (1972). Codes on branches are the popsites and numbers on nodes are the bootstrap value ≥ 60. Each popsite is identified by a colour code corresponding to a distinct genetic group, as in Figure 1: blue for cluster 1, green for cluster 2 and red for cluster 3. The map is the same as in the figure 1 a).

Figure 4. a) Scatterplot of the DAPC of 1151 individual's genotypes (based on 10 microsatellite loci) collected from 35 popsites of *Apistogramma* cf. *agassizii*. It shows the first two principal components of the DAPC for k=3. Each cluster has a colour code (blue, green and red for clusters 1, 2 and 3, respectively) and an inertia ellipse (dots represent individual genotypes). **b)**, **c)** and **d)** Contingency tables between popsites and clusters for k=3, k=2 and k=4, respectively. Square size reflects the number of individuals.

Figure 5. Maximum likelihood trees of COI gene. **a)** Tree of haplotypes, including 32 *Apistogramma* specimens of *agassizii* phenotype from the Loreto region (Peruvian Amazon) and rooted with two species as outgroups (*A. eremnopyge* and *A. bitaeniata*) using the TN93+G model (LogL= -1541.89). Each haplotype is identified with a colour corresponding to a clade:

Hap. 1 to 4, clade 2 (green); Hap. 5 to 7, clade 3 (red) and Hap. 8 to 12, clade 1 (blue). Below the nodes, years before present (Ma) are indicated: dates in bold estimated from Genner et al. (2007) taking in to account the Gondwana fragmentation (left) and fossil data (right); in italic bold datation estimated from DiBattista et al. (2013) taking into account a mutation rate of 1% (left) and 2% (right). **b)** Tree of species. The three entities (species Sp1-clade 1 in blue, Sp2-clade 2 in red, Sp3-clade 3 in green) and the Haplotype 8 from species Sp1 (clade 1) are placed in the genus *Apistogramma* partial phylogeny of Tougard et al. (2017), using the TN93+G model (LogL= -2821.79). Both trees are drawn to scale, with branch lengths measured in number of substitution per site. Bootstrap values established after 1000 replicates are indicated with black circle when value is >90. The real and bootstrap consensus trees have the same topology.

Figure 6. a) Data distribution simulated from Mantel correlation under the null hypothesis of no isolation by distance. The dot represents the observed correlation. **b)** Relationship between the geographic distance (following the river channels) and the genetic distance of Rousset (1997) between popsites from specie Sp1: *p-value* = 0.042 based on 1000000 permutations.

Figure 7. Colour morph “Blue” and “Yellow” of *Apistogramma* cf. *agassizii* from different localities. Colour code used in boxes are the same as those used in all the other figures: blue for species Sp1, green for species Sp2 and red for species Sp3. The last picture in the “blue” colour morph of species Sp2 is a pattern from the Ampiyacu River identified by Koslowski (2002). Specific phenotypes identified *a posteriori* for each of the three species, on colour pattern of the caudal fin are: All males of Sp1 have a blue (white in picture) "V"-shaped band between two relatively wide dark bands (distal and proximal) at the submarginal part of the caudal. By contrast, the blue band is particularly visible. The dark proximal band is not always clearly defined, so it partially mixes with blue (blue colour moph) or yellow (yellow colour moph) patterns distributed down to the proximal part of the caudal fin. In species Sp2, the thin blue "V"-shaped band is very irregular in blue individuals while it is non-existent or sparse in yellow individuals. Individuals of this specie exhibit only one dark band on the distal part of the caudal fin. All blue males have very well defined blue patterns on the central and proximal part of the caudal fin. These patterns tend to merge with the blue stripe giving a "lace" pattern. These blue patterns are generally absent or spotted in yellow individuals. This colour pattern has been identified by Koslowski (2002). In species Sp3, for the blue colour moph, the blue "V"-shaped band always seems to be present; it is thin, straight and always seems to be between two dark

bands (distal and proximal). While in the yellow colour moph, the blue band may be absent and the proximal dark band is absent. In blue males, the blue patterns of the central and proximal part of the caudal are diffuse and tend to follow the rays of the fin. In yellow males, the colour is solid.