ZEBRAFISH
Volume 13, Number 3, 2016

DOI: 10.1089/zeb.2015.1213

Integrated Cytogenetic and Mitochondrial DNA Analyses Indicate That Two Different Phenotypes of *Hypancistrus* (L066 and L333) Belong to the Same Species

Adauto Lima Cardoso,[1] Hagi Lopescu Silva Carvalho,[1] Thayse Cristine Melo Benathar,[1] Suellen Maria Gales Serrão,[2] Cleusa Yoshiko Nagamachi,[1,*] Julio Cesar Pieczarka,[1,*] Leandro Melo de Sousa,[3] Jonathan Stuart Ready,[2] and Renata Coelho Rodrigues Noronha[1]

Abstract

The diversity of *Hypancistrus* species in the Xingu River is remarkable and the variation in color morphs represents a real challenge to taxonomists to delimit species boundaries. One of the most recognizable *Hypancistrus* complexes is the worm-lined species, known in the aquarium trade as King Tiger Plec in English, *Hypancistrus* ''*pão*'' in Portuguese or under the L-numbers L066 and L333 that represent two melanic pigment pattern phenotypes. To assess the identity of these two phenotypes, we described their karyotypes and sequenced part of the mitochondrial cytochrome oxidase I gene (DNA barcode). These fishes have 52 chromosomes (40 meta-submetacentric and 12 subtelo-acrocentric) and a strong heteromorphism in chromosome pair 21 was observed, which does not correlate with the two phenotypes or sex. DNA barcodes separated the samples analyzed from *Hypancistrus zebra* and other publicly available sequences of Loricariidae showing no divergence between the two phenotypes. The data set indicates that worm-lined *Hypancistrus* from the Xingu form a single species with clear chromosomal and melanic pigment pattern polymorphisms.

Introduction

The Xingu River is a tributary of the Amazon River with nearly 467 identified species of fish.[1] This ichthyofauna is threatened by several human actions, which have been increasing in intensity since the 1970s with the most recent major impacts due to the beginning of the construction of the Belo Monte dam.[1,2]

The family Loricariidae represents an important component of the Xingu River ichthyofauna, including many endemic species,[1,2] many of them being exploited in the ornamental trade.[3] Therefore, studies that aim to clarify the patterns and processes that caused and maintain this diversity are fundamental for future conservation plans. In this respect, the knowledge about population or species identity and similarities, especially in relation to karyotypic features, is important because karyotypic differences can act as post-zygotic reproductive barriers.[4]

The genus *Hypancistrus* Isbrücker and Nijssen contains six formally described and several undescribed species.[2,5,6] Five of the six valid species are described from the upper Orinoco River basin in Venezuela and Colombia with some species also occurring in the Negro River,[6,7] while the sixth species *Hypancistrus zebra* is known from the great bend in the Xingu River, near Altamira, Pará State, Brazil (Fig. 1).[8] Two phenotypes of *Hypancistrus* with worm-like linear melanic pigment patterns also occur in the Xingu River, and are named as ''L066'' and ''L333'' in the ornamental trade where distinct phenotypes that are believed to represent undescribed species of Loricariidae are given L-number codes (Fig. 2). To assess the identity and degree of genetic similarity of these two phenotypes we characterized their karyotypes and performed a DNA barcode analysis.

Material and Methods

Specimens of *Hypancistrus* L066 and L333 and *H. zebra* were collected in the Xingu River in conjunction with fishermen. Sample locations are indicated in Figure 1 (only localities in the Xingu River). Sample collection was authorized by the

[1]Laboratório de Citogenética and [2]Laboratório de Lepidopterologia e Ictiologia Integrada, Centro de Estudos Avançados da Biodiversidade, Instituto de Ciências Biológicas, Universidade Federal do Pará, Belém, Pará, Brazil.
[3]Faculdade de Ciências Biológicas, Universidade Federal do Pará, Campus de Altamira, Altamira, Pará, Brazil.
*CNPq Researcher.

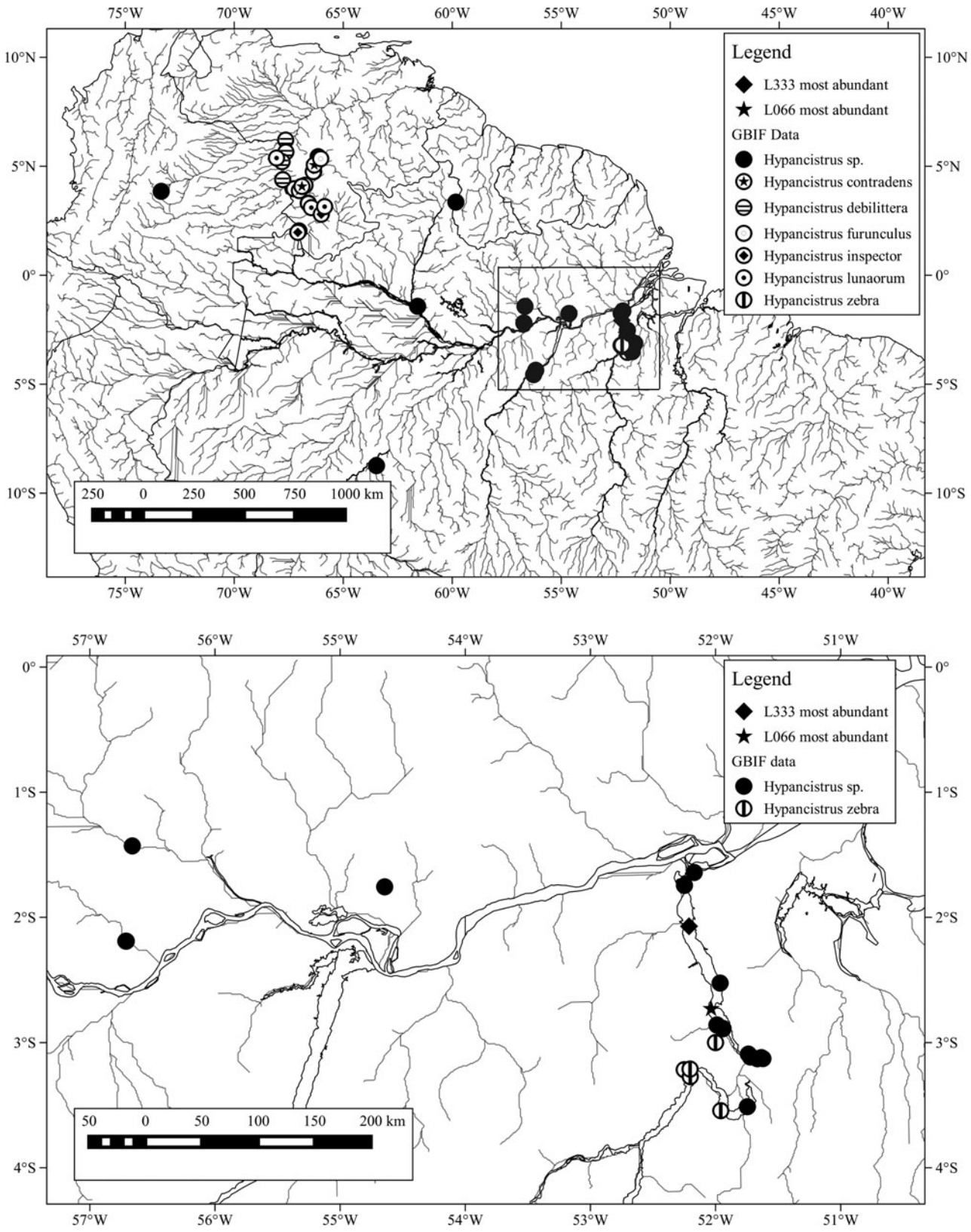

FIG. 1. Localities sampled in this study and known occurrences (GBIF, 2015) of *Hypancistrus* species, with indication of the localities reported to support the main abundance of the phenotypes L066 and L333.

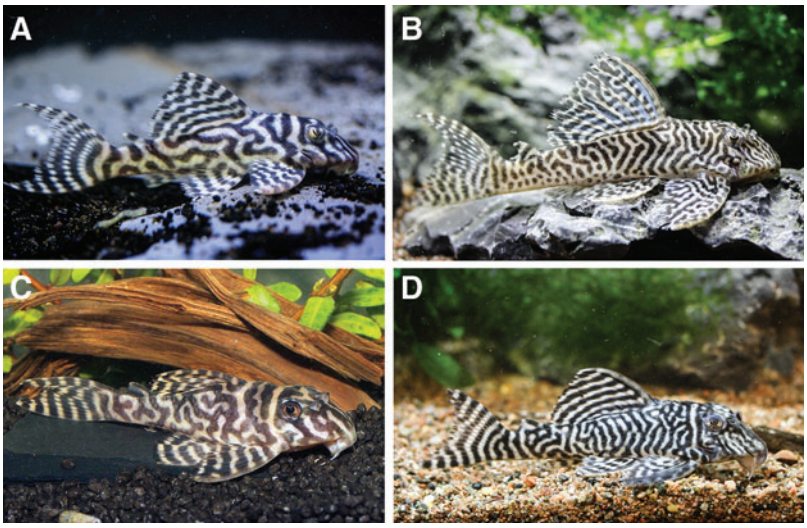

FIG. 2. Phenotypes of worm-lined *Hypancistrus* from the Xingu River: **(A)** juvenile of L066; **(B)** adult of L066; **(C)** juvenile of L333; and **(D)** adult of L333. Photos **(A, B, and D)** by Haakon Haagensen. Photo **(C)** by Daniel Konn-Vetterlein. Color images available online at www.liebertpub.com/zeb

Brazilian licensing institutions: Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) and Secretaria de Estado de Meio Ambiente do Pará (SEMA-PA), permit 020/2005. Animals were anesthetized with eugenol following Brazilian ethical requirements.

Morphological identification was made using the existing literature[6] and observations of samples to assess the potential inclusion of any previously undescribed morphological diagnostic characters, which might correlate with the existing phenotypes. The sex of each sample was identified by examination of the gonads. Voucher specimens are deposited in the UFPA cytogenetic collection, lots UFPACIT2415 to UFPACIT3750.

Chromosomes were obtained from kidney cells[9] and analyzed using conventional staining, C-banding,[10] Nucleolar Organizer Region staining with $AgNO_3$,[11] and fluorescent *in situ* hybridization using probes for *5S* and *18S* ribosomal DNA (rDNA),[12] which were labeled with biotin or digoxigenin by nick translation and detected using avidin-Cy3 or anti-digoxigenin-FITC, respectively.

Sequencing of a part of the mitochondrial cytochrome oxidase I (*coI*) was performed for 10 specimens following the protocols established by the Consortium for the Barcode of Life,[13,14] using DNA extraction methods, primers, amplification conditions, and sequencing techniques previously described.[15]

Sequences were aligned using the Geneious 7.1 (www.geneious.com)[16] and analyzed using MEGA 6.0[17] to provide values of pairwise sequence divergence and produce a K2P neighbor joining tree with support based on 1000 bootstrap replicates. Sequences were submitted to the Barcode of Life Data Systems (BOLD systems) under the Barcoding and Cytogenetics (BCCG) project, subproject Barcoding and Cytogenetics of Siluriformes (BCSIL), Process IDs BCSIL001-15 to BCSIL010-15. Unpublished barcode sequences present in the Barcode of Life (BOL) database are included in this study by using the tree-based identification of samples within BOLD systems.

Results

No morphological differences were found between samples of *Hypancistrus* L066 ($N=20$) and L333 ($N=19$).

All the individuals of *Hypancistrus* L066 ($N=9$) and L333 ($N=7$) karyotyped in this study have a diploid number (2n) of 52 chromosomes and a karyotypic formula (KF) with 20 meta-submetacentric (m-sm) pairs and 6 subtelo-acrocentric (st-a) pairs (Fig. 3A). Morphologically differentiated sex chromosomes were not detected.

Constitutive heterochromatin was detected in centromeric region of all the chromosomes, in large blocks in 2q, 3p, 4q, and 5q and in small bands in distal regions of most chromosomes (Fig. 3C).

Active sites of *18S* rDNA were detected in the distal regions of 2q (Fig. 3D) and additional and inactive sequences were detected in the distal region of the pair 21 (Fig. 3D, green). 5S rDNA genes were located at the interstitial region of 1p and in the distal region of 4q (Fig. 3D, red).

Chromosome pair 21 showed a heteromorphism represented by two types of chromosomes (Figs. 3A, B and 4): the A type is a large chromosome, which presents a heterochromatic band in the distal region of the short arm and inactive sites of *18S* rDNA that are adjacent to a heterochromatic block in the distal region of the long arm; the B type is a small chromosome without the large heterochromatic block and without *18S* rDNA sites. Two genotypes related to this chromosome were found: the homozygous AA ($N=11$) and the heterozygous AB ($N=5$). The homozygous genotype BB was not detected. This heteromorphism does not correlate with sex or phenotype. It may therefore represent either a loss of genetic material or a chromosomal rearrangement.

The *coI* barcode sequences revealed that all the specimens identified as ''L066'' ($N=6$) and ''L333'' ($N=3$) grouped together with a mean of 0.3% K2P divergence within the group, indicating no consistent divergence between the two phenotypes. This data also indicated a clear separation of this single monophyletic group of worm-lined *Hypancistrus* from their congener *H. zebra*, though only by 1.5%–2.2% (mean = 1.7%) K2P divergence (Fig. 5).

Unpublished data in the form of the BOLD sample identification tree (Fig. 6) confirms this very low variability in samples of worm-lined *Hypancistrus*, including a single publicly available record (Fig. 6, sample 63) from Nhamundá. Including the sequence for this sample resulted in an increase

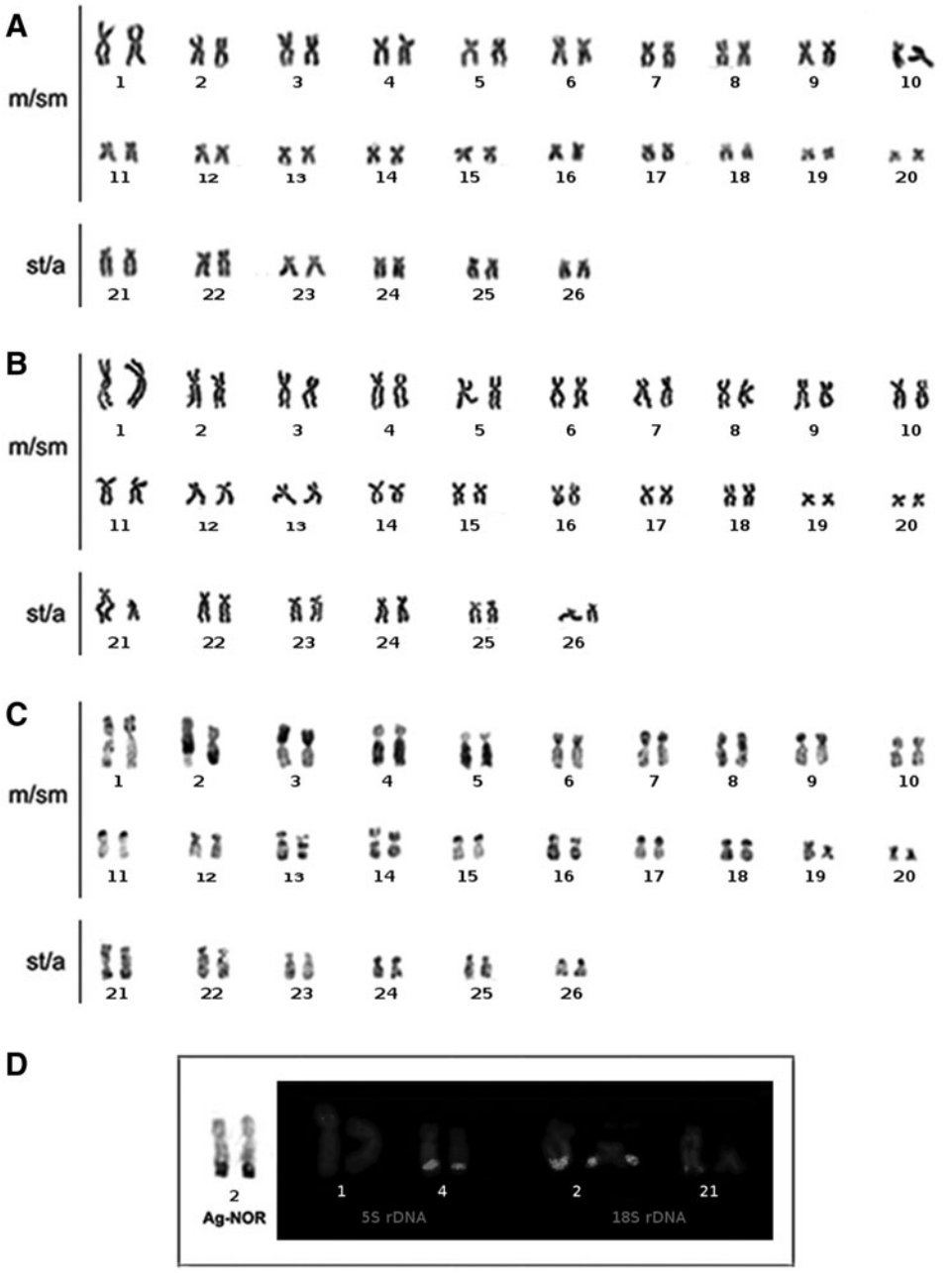

FIG. 3. Karyotypes of *Hypancistrus* sp. L066 and L333: conventional staining in L066 **(A)**; conventional staining in L333 **(B)**; C banding **(C)**; sites of ribosomal genes **(D)**.

of only 0.1% (up to 0.4%) for the mean K2P divergence within the worm-lined group containing L066 and L333. This identification tree also indicates probable phylogenetic organization, with worm-lined *Hypancistrus* most closely related to ''*H. debilittera*'' from Pará state, Brazil, and both of these forming a sister group with just over 1% K2P divergence from a group containing *Hypancistrus* sp. ''L260'' and *Hypancistrus* sp. ''L262'' from the lower Tapajós River (Fig. 6). *H. zebra* and *Hypancistrus furunculus* are then more distantly related with $\sim$1.5% and 2.5% K2P divergence, respectively.

Discussion

The fact that no morphological characters were found to correlate with the distinct melanic phenotypes suggests that either morphology is static (if speciation had occurred), or that the differences indicate a pigmentation polymorphism within the species that may represent phenotypic plasticity with adaptation and/or selection of local populations to their environment, or environmentally induced changes in the development of melanic pigment patterns.

The 2n = 52 of the worm-lined *Hypancistrus* is shared with most species in the tribe Ancistrini. This feature represents a probable synapomorphy of the tribe,[18] with chromosome inversions being the probable driving force of karyotype evolution.[18–20] Despite this, the *Hypancistrus* samples analyzed here have a combination of chromosomes types (40m-sm/12st-a) that diverges from their congeners *H. zebra* (38m-sm/14st-a) and *H.* cf. *debilittera* from the Uatumã River (34m-sm/18st-a).[20]

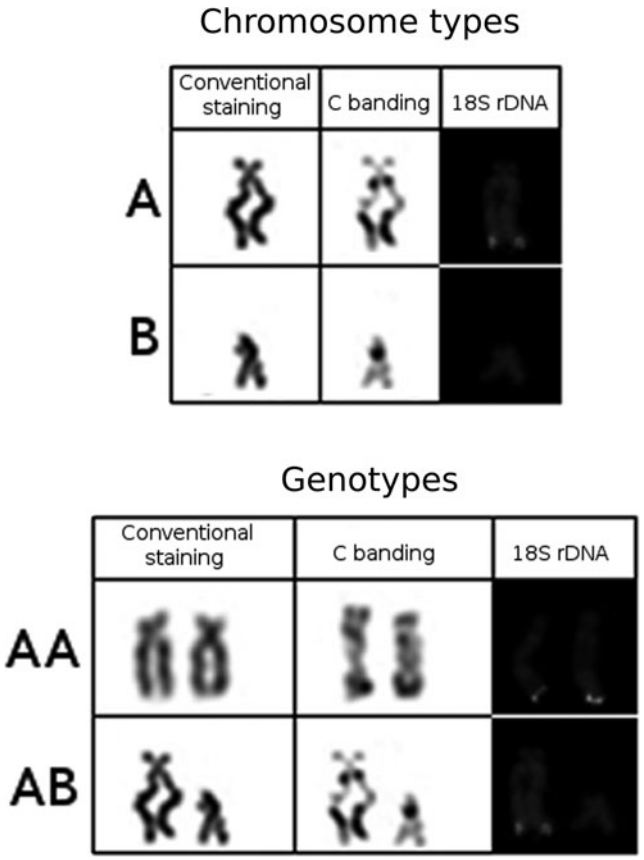

FIG. 4. Chromosome 21 polymorphism of *Hypancistrus* sp. L066 and L333.

The location of active sites of *18S* rDNA (NOR) in the distal region of 2q is similar to others species of *Hypancistrus*.[20] Moreover, an extra and heteromorphic site in 21q was also found. It is similar to the condition denominated ''P3'' in *H.* cf. *debilittera* from the Uatumã River, that is believed to have arisen through a transposition event.[20] This could indicate that these two species are more closely related than either is with *H. zebra*, which does not have this extra site.

The chromosome pair 21 presents a heteromorphism within worm-lined *Hypancistrus* in relation to the occurrence of inactive sites of *18S* rDNA that are adjacent to a heterochromatic block in the distal region of the long arm. This heteromorphism, which is found in both the L066 and L333 phenotypes, is most frequently encountered as the AA genotype. The absence of the genotype BB in the sample may be due to the sample size, which might not be large enough to obtain individuals with the BB genotype.

If this heteromorphism was caused by chromosomal rearrangement, chromosome painting would be needed to confirm this with low sample numbers. This is extremely difficult to perform given the size similarity of the smaller version of chromosome 21 and other chromosomes in these organisms. On the other hand, it is possible that this represents a deleterious condition with the total loss of a large chromosome segment. Although the lost segment is heterochromatic and only presents inactive *18S* rDNA sites, it is likely that such a large amount of genetic material does play some kind of regulatory role essential to development.[21,22]

The location of *5S* rDNA sites is similar to that found in *H. zebra* and *H.* cf. *debilittera* from the Uatumã River.[20] Both species have a small cluster in the interstitial region of chromosome pair 1. However, *H. zebra* and *H.* cf. *debilittera* have two clusters of these genes in pair 9, while worm-lined

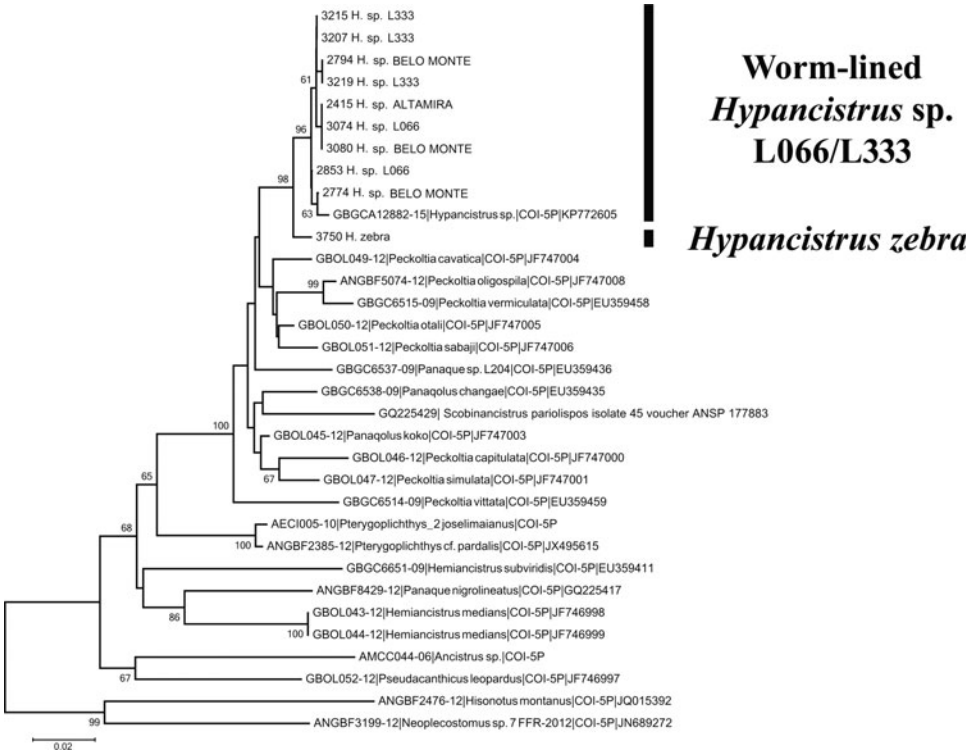

FIG. 5. Kimura 2-parameter neighbor-joining tree for samples in this study along with publicly available *coI* barcode sequences for the family Loricariidae in BOLD. Support values based on 1000 bootstrap pseudoreplicates with only values >50% shown.

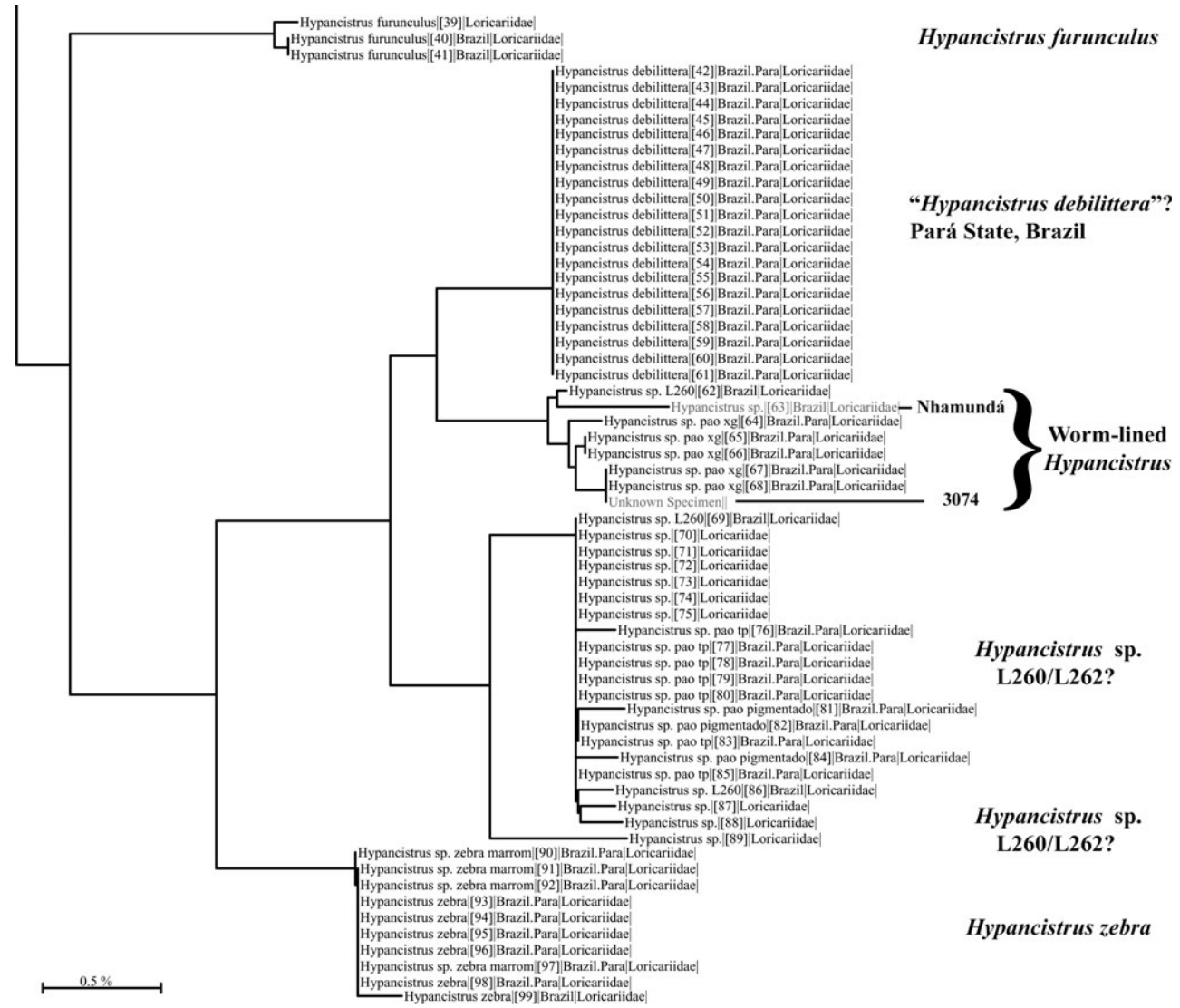

FIG. 6. Cropped species identification tree obtained from submitting sample 3074 (L066 phenotype) to the BOLD tree identification system with annotations to relate ornamental trade names, local common names, and L-number codes to species where possible.

Hypancistrus present only one cluster in pair 4. Intraspecific heteromorphisms related to these sequences were identified only in *H.* cf. *debilittera*.[20]

Barcode data also support the monophyly and single species identity of L066 and L333 samples. The public barcode record from Nhamundá indicates a much wider distribution for this species than the Xingu basin alone with only a slight increase in the within-group divergence values when including this sequence in analyses, compared to the divergence between these samples and the valid species *H. zebra*. The minimum divergence of 1.5% between the L066/L333 samples and *H. zebra* is only slightly lower than the usually cited 2% divergence between species found in other barcode studies on fishes,[23–25] and it suggests that the phylogeographic variation within the more closely related but unpublished BOLD samples (L260, L262, ''*H. debilittera*'') in Figure 6 will require more careful analysis.

The greater similarity of *Hypancistrus* L066/L333 with ''*H. debilittera*'' from Pará state, Brazil, compared to the clade with *Hypancistrus* ''L260'' and *Hypancistrus* ''L262'' indicated in Figure 6 raises doubt on the identity of the identification of the ''*H. debilittera*'' samples in this tree. *H. debilittera* is described only from the upper Orinoco basin in Venezuela.[6] Occurrence in the Rio Negro in Brazil may be possible via the Casiquiare channel, which links the upper reaches or the Orinoco with the Rio Negro. An extended distribution in Pará state is theoretically possible, but may be considered unlikely.

This incongruence will only be resolvable when a review of the samples is made after the data becomes public and after samples from the Orinoco are sequenced. It serves to highlight the possibility of overeager interpretation of unpublished data in BOLD species identification trees. What can be confirmed is that the greater similarity of these samples appears to makes sense in geographic terms. Additionally, misidentification may be based largely on pigmentation patterns that may correlate partially with phylogenetic similarity (all these species present linear patterns with varying intensities and degrees

of reticulation vs. spotted patterns in other described *Hypancistrus* species[6]), or may be linked to ecological constraints related to camouflage[26] or even thermal regulation.[27]

There is no chromosomal or molecular evidence that suggests divergence between the two worm-lined *Hypancistrus* phenotypes analyzed here. The karyotype of these worm-lined *Hypancistrus* from the Xingu River is divergent from other species of *Hypancistrus* at the macrostructural level. Combined with DNA barcode data that show *coI* sequence divergence from *H. zebra* and other species, this strongly supports the single species identity of these fishes. With this result, formal description of new species of *Hypancistrus* from the Xingu River will be facilitated.

Acknowledgments

Financial support for this research was from FAPESPA (Edital Vale–Proc2010/110447) and Banco Nacional de Desenvolvimento Econômico e Social–BNDS (Operação 2.318.697.0001), on a project coordinated by J.C.P.; CNPq (BRBol Project, Edital MCT/CNPq/FNDCT 50/2010) supported J.S.R.; FAPESPA (Pará Foundation for Supporting Science, through the PPP program Edital 011/2009) and NSF (Lower Amazon Aquatic Faunal Survey DEB-1146374) supported J.S.R.; UFPA/FAPESPA (PIBIC undergraduate study grants) supported H.L.S.C. and S.M.G.S; and CNPq (Universal—Proc. 475013/2012-3) supported a project coordinated by C.Y.N. C.Y.N. (308428/20013-7) and J.C.P. (308401/2013-1) are grateful to CNPq for Productivity Grants. The authors thank Projeto Arapaima Ltda (Pará) for help with field sampling, the hobbyist community for the enthusiastic help and support and for providing live pictures and sharing behavioral habits for better understanding of these fishes, Cesar Martins UNESP-Botucatu for primers for cytogenetic probes, and ICMBio (Instituto Chico Mendes de Conservação da Biodiversidade) for the collection permit (ICMBio Registration: 21078-10).

Disclosure Statement

No competing financial interests exist.

References

1. Camargo M, Giarrizzo T, Isaac V. Review of the geographic distribution of fish fauna of the Xingu River basin, Brazil. Ecotropica 2004;10:123–147.
2. Giarrizzo T, Oliveira RRS, Gonçalves AP, Barbosa TAP, Martins AR, Marques DK, *et al.* Length-weight and length-length relationships for 135 species from the Xingu River (Amazon Basin, Brasil). J Appl Ichthyol 2015;31:415–424.
3. Armbruster JW. Phylogenetic relationships of the suckermouth armoured catfishes (Loricariidae) with emphasis on the Hypostominae and the Ancistrinae. Zool J Linn Soc Lond 2004;141:1–80.
4. King M. Species Evolution. Cambridge University Press, Cambridge, United Kingdom, 1993.
5. Isbrücker IJH, Nijssen H. *Hypancistrus zebra*, a new genus and species of uniquely pigmented ancistrine loricariid fish from the Rio Xingu, Brazil (Pisces: Siluriformes: Loricariidae). Ichthyol Explor Freshw 1991;1:345–350.
6. Armbruster JW, Lujan NK, Taphorn DC. Four new *Hypancistrus* (Siluriformes: Loricariidae) from Amazonas, Veneuzela. Copeia 2007;1:62–79.
7. Armbruster JW. *Hypancistrus inspector*: a new species of suckermouth armored catfish (Loricariidae: Ancistrinae). Copeia 2002;1:86–92.
8. GBIF.org (October 5, 2015) GBIF Occurrence Download. Available at http://doi.org/10.15468/dl.nmlxam Accessed October, 2015.
9. Bertollo LAC, Takashi CS, Moreira-Filho O. Cytotaxonomic considerations on *Hoplias lacerdae*. Cytogenet Cell Genet 1978;63:215–220.
10. Sumner AT. A simple technique for demonstrating centromeric heterochromatin. Exp Cell Res 1972;75:304–306.
11. Howell WM, Black DA. Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 1980;36:1014–1015.
12. Martins C, Galetti PM. Chromosome localization of 5S rDNA genes in *Leporinus* (Anostomidae, Characiformes). Chromosome Res 1999;7:363–365.
13. Ivanova NV, de Waard JR, Hajibabaei M, Hebert PDN. Protocols for high volume DNA barcoding. Draft submission to: DNA working group Consortium for the Barcode of Life. 2005. Available atwww.dnabarcoding.ca/ Accessed August, 2014.
14. Ivanova NV, de Waard JR, Hebert PDN. An inexpensive, automation-friendly protocol for recovering high-quality DNA. Mol Ecol Notes 2006;6:998–1002.
15. Cardoso AL, Ready JS, Pieczarka JC, Milhomem SSR, Figueiredo-Ready WMBF, Silva FHR, *et al.* Chromosomal Variability Between Populations of *Electrophorus electricus* Gill, 1864 (Pisces: Gymnotiformes: Gymnotidae). Zebrafish 2015;12:440–447.
16. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, *et al.* Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 2012;28:1647–1649.
17. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol 2013;30:2725–2729.
18. Artoni RF, Bertollo LAC. Trends in the karyotype evolution of Loricariidae fish (Siluriformes). Hereditas 2001;134: 201–210.
19. Cardoso AL, Sales KAH, Nagamachi CY, Pieczarka JC, NoronhaRCR. Comparative cytogenetics of two species of genus *Scobinancistrus* (Siluriformes, Loricariidae, Ancistrini) from the Xingu River, Brazil. Comp Cytogenet 2013;7:43–51.
20. Silva M, Ribeiro ED, Matoso DA, Sousa LM, Hrbek T, Py-Daniel LR, *et al.* Chromosomal polymorphism in two species of *Hypancistrus* (Siluriformes: Loricariidae): an integrative approach for understanding their biodiversity. Genetica 2014;142:127–139.
21. Yamamoto M. Interchromosomal effects of heterochromatin deletions on recombination in *Drosophila melanogaster*. Genetics 1979;93:437–448.
22. Hilliker AJ, Appels R. Pleiotropic effects associated with the deletion of heterochromatin surrounding rDNA on the X chromosome of *Drosophila*. Chromosoma 1982;86: 469–490.
23. Carvalho DC, Oliveira DAA, Pompeu PS, Leal CG, Oliveira C, Hanner R. Deep barcode divergence in Brazilian freshwater fishes: the case of the São Francisco River basin. Mitochondrial DNA 2011;22:80–86.
24. Castro Paz FP, Batista Jda S, Porto JIR. DNA barcodes of Rosy Tetras and allied species (Characiformes: Characidae: Hyphessobrycon) from the Brazilian Amazon basin. PLoS One 2014;9:e98603.

25. Pereira LHG, Pazian MF, Hanner R, Foresti F, Oliveira C. DNA barcoding reveals hidden diversity in the Neotropical freshwater fish *Piabina argentea* (Characiformes: Characidae) from the Upper Paraná Basin of Brazil. Mitochondrial DNA 2011;22 Suppl 1:87–96.
26. Pasteur G. A classification review of mimicry systems. Annu Rev Ecol Syst 1982;13:169–199.
27. Watt WB. Adaptive significance of pigment polymorphisms in *Colias* butterflies I. Variation in melanin pigment in relation to thermoregulation. Evolution 1968;22:437–458.

Address correspondence to:
Renata Coelho Rodrigues Noronha, PhD
Laboratório de Citogenética
Centro de Estudos Avançados da Biodiversidade
Instituto de Ciências Biológicas
Universidade Federal do Pará
Rua Augusto Corrêa, 01
Belém
Pará 66075-110
Brazil

E-mail: renatarcrn@gmail.com