NICOLE ESTEFANIA IBAGON ESCOBAR

DIVERSITY OF *Hoplias malabaricus* (BLOCH, 1794) IN SOUTH AMERICA: AN EVOLUTIONARY AND ECOLOGICAL APPROACH

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ecologia, para obtenção do título de *Doctor Scientiae.*

Orientadora: Karla Suemy Clemente Yotoko

VIÇOSA - MINAS GERAIS
2019

Ficha catalográfica preparada pela Biblioteca Central da Universidade Federal de Viçosa - Câmpus Viçosa

T

Ibagon Escobar, Nicole Estefania, 1987-

I12d
2019

Diversity of *Hoplias malabaricus* (Bloch, 1794) in South America : an evolutionary and ecological approach / Nicole Estefania Ibagon Escobar. – Viçosa, MG, 2019.

95 f. : il. (algumas color.) ; 29 cm.

Texto em inglês.
Orientador: Karla Suemy Clemente Yotoko.
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Erythrinidae. 2. Characiformes. 3. Peixes de água doce. I. Universidade Federal de Viçosa. Departamento de Biologia Geral. Programa de Pós-Graduação em Ecologia. II. Título.

CDD 22 ed. 596

NICOLE ESTEFANIA IBAGON ESCOBAR

DIVERSITY OF *Hoplias malabaricus* (BLOCH, 1794) IN SOUTH AMERICA: AN EVOLUTIONARY AND ECOLOGICAL APPROACH

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Ecologia, para obtenção do título de *Doctor Scientiae.*

APROVADA: 16 de julho de 2019.

Nicole Estefania Ibagon Escobar
Autora

Karla Suemy Clemente Yotoko
Orientadora

a mi mamá

ACKNOWLEDGEMENTS

A lo largo de los ultimos años muchas personas contribuyeron para la reaización de mi doctorado y de este manuscrito. Seria imposible nombrarlos a todos, pero espero que sepan este trabajo también es logro suyo. Quiero agradecer la oportunidad que todas esas personas me dieron al conversar, recibirme en sus casas/colecciones y apoyarme en algunos momentos difíciles no tiene precio.

Agradeço à Universidade Federal de Viçosa, especialmente ao Departamento de Biologia Geral, aos técnicos-administrativos que com sua colaboração permitiram meu trabalho diário.

A pessoa que mais contribuiu para meu proceso de formação doutoral foi a mina orientadora Karla Yotoko, eu lhe agradeço pelos momentos de conversa, e sobre tudo por ser um grande exemplo de mulher em ciencia. Sua entrega pelo Laboratório de Bioinformática e Evolução e as pessoas que nele trabalhamos, rende frutos todo dia.

El Laboratorio de Bioinformatica y Evolución fue mi casa por todos estos años. Distrute especialmente la compañía de Camilo y Marina, con ustedes me siento segura para reir y llorar, espero que sepan que cuentan conmigo siempre.

O laboratorio de Bioinformática e Evolução foi a minha casa durante todos estes anos. Eu curti muito a companhia do Camilo e da Marina, com vocês eu me sinto segura para rir e chorar, espero que saibam que vocês contam comigo sempre.

Vou sentir saudades do nossos cafés Marininha e Naty. Se alguém me perguntar qual é meu lugar feliz sempre vou falar que é como me sinto quanto estou com vocês.

A mí querida amiga Hanna, gracias por transmitirme tranquilidad.

Eu agradeço ao programa de pós-graduação em Ecologia por me receber e pelo suporte econômico e acadêmico. À Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) pela bolsa concedida. Eu agredeço ao

governo brasileiro por me receber e receber outros miles de estrangeiros em processo de formação.

I would like to thank all the curators of the collections that allow me to visit the museums: Carlos Luis DoNascimiento Montoya and Carlos Montaña from the Instituto de Investigación de Recursos Biológicos Alexander von Humboldt, Marcelo Britto and Cristiano Moreira from the Museu Nacional/UFRJ, Saúl Prada Pedreros and Javier Maldonado Ocampo from the Pontificia Universidad Javeriana, Jorge Dergam from the MZUFV, Alesio Datovo and Oswaldo Oyakawa from the MZUSP, Carla Simone Pavanelli from the Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura.

I would like to thank to Elizabeth Boenigk for the language editing of part of this manuscript.

Ao meu companheiro Gonimar, obrigada por me deixar parte da tua familia e me permitir ser livre, eu acho que essa é a verdadeira demonstração de amor.

Finalmente me gustaria agradecerle a mi familia, mi mamá y mi papá, que en la distancia me apoyaron y permitieron seguir mis sueños. Gracias por darme la libertad de volar y por estar siempre presentes.

ABSTRACT

IBAGON, Nicole, D.Sc., Universidade Federal de Viçosa, July, 2019**, Diversity of *Hoplias malabaricus* (Bloch, 1794) in South America: an evolutionary and ecological approach**. Adviser: Karla Yotoko.

The erythrinid *Hoplias malabaricus* (Bloch, 1794) is a freshwater fish species widely distributed from Panama to northern Argentina, occupying most of the cis and trans-Andean hydrographic basins. This species complex includes seven karyomorphs, with 40 or 42 chromosomes, with different chromosome morphology, and sex chromosome systems. *H. malabaricus* also includes several molecular independent lineages. The low vagility of this species facilitates the fixation of chromosomal rearrangements and genetic differentiation in independent molecular lineages. The lack of an extensive taxonomic revision for *H. malabaricus* and the existence of several nominal species considered junior synonyms, make this group one of the most complex taxonomic problems of Neotropical fishes. The three papers that compose this thesis are the result of extensive sampling and three methodological approaches. We used geometric morphometric (1,942 samples), cytogenetics (32 samples), and phylogeography (74 samples) to explore the current diversity and historical biogeography of *H. malabaricus* in both regional and continental scales. Our geometric morphometrics analyses from the Brazilian Continental Margin and South America, comprising 1,942 samples, aimed to explore the head shape of *H. malabaricus*. We tested the effect of allometry, long-term evolutionary isolation, adaptation to different aquatic systems and latitude in the shape of the head of *H. malabaricus*. We found that all the variables have a significant response. However, the effect of allometry is the only one that shows a clear pattern of correlation. Thus, we suggested that environmental adaptation constraints morphometric differentiation between independent molecular clades that maintain distinct morphological shapes, hampering the identification of new species within the *H. malabaricus* complex. We also used samples from Lower Central America and Northern South America to trace the influence of the uplift of the Northern Andes in *H. malabaricus* diversity using cytogenetics, geometric morphometrics, and molecular phylogeny. We found a clear Miocene/Pliocene vicariance between the cis and trans-Andean and a sharp discontinuity along the Magdalena River. Upper/Middle and Lower Magdalena samples exhibit different karyomorphs with a contrasting fluorescence pattern. Lower Magdalena karyomorph is similar to the other cis-Andean populations. The shape of the head of those groups also differs significantly. Finally, the molecular data show two principal clades separating Upper/Middle Magdalena from Lower Magdalena. While Upper/Middle Magdalena groups with other trans-Andean specimens, Lower Magdalena groups with cis-Andean samples. We hypothesized that the emergence of the Eastern Cordillera of the Andes worked as a vicariant event that initially separated the two principal clades, but posterior colonization of the Lower Magdalena generated a discontinuity along the Magdalena River.

RESUMO

IBAGON, Nicole, D.Sc., Universidade Federal de Viçosa, julho de 2019, **Diversidade de *Hoplias malabaricus* (Bloch, 1794) na America do Sul: Uma abordagem evolutiva e ecologica**. Orientadora: Karla Yotoko

O eritrínideo *Hoplias malabaricus* (Bloch, 1794) é um peixe de água doce com ampla distribuição geográfica que vai do Panamá até o norte da Argentina, ocupando a maior parte das bacias hidrográficas *cis* e *trans*-andinas. Este complexo de espécies inclui sete cariomorfos, com 40 ou 42 cromossomos, com diferentes morfologias cromossômicas e sistemas de cromossomos sexuais. *H. malabaricus* também inclui várias linhagens moleculares independentes. A baixa vagilidade dessa espécie facilita a fixação de rearranjos cromossômicos e a diferenciação genética em linhagens moleculares independentes. A falta de uma extensiva revisão taxonômica e a existência várias espécies nominais consideradas sinônimos júnior fazem desse grupo um dos problemas taxonômicos mais complicados dos peixes neotropicais. Os três artigos que compõem a presente tese são resultado de uma extensa amostragem em três abordagens metodológicas. Utilizamos morfometria geométrica (1.942 amostras), citogenética (32 amostras) e filogeografia (74 amostras) para explorar a diversidade atual e a biogeografia histórica de *H. malabaricus* nas escalas regional e continental. Nossas análises de morfometria geométrica da Margem Continental Brasileira (375amostras) e da América do Sul (1.942 amostras) tiveram como objetivo explorar a forma da cabeça de *H. malabaricus*. Nós testamos o efeito da alometria, do isolamento evolutivo a longo prazo, da adaptação a diferentes sistemas aquáticos e da latitude na forma da cabeça de *H. malabaricus*. Os resultados sugerem que todas as variáveis têm uma resposta significativa. No entanto, o efeito da alometria é o único que mostra um padrão claro de correlação. Inferimos então que a adaptação ambiental restringe a diferenciação morfométrica entre clados moleculares independentes, mantendo uma enorme sobreposição de formas, o que dificulta a identificação de novas espécies dentro do complexo *H. malabaricus*. Nós também utilizamos amostras da América Central Inferior e do Norte da América do Sul para traçar a influência da elevação dos Andes na diversidade de *H. malabaricus* usando citogenética, morfometria geométrica e filogenia molecular. Encontramos evidências de vicariância entre as populações do lado cis e trans da Cordilheira dos Andes que datam da transição entre o Mioceno e o Plioceno. Dentro do Rio Magdalena, encontramos também uma clara descontinuidade: enquanto as amostras do Alto e Médio Magdalena exibem um cariomorfo, as do Baixo Madalena exibem outro, similar às encontradas no lado cis-andino. A forma da cabeça desses dois grupos também difere significativamente. Finalmente, os dados moleculares mostraram dois clados principais separando Alto/Médio e Baixo Magdalena: as amostras do Alto/Médio Magdalena agruparam com outras populações trans-andinas enquanto as do Médio Magdalena agruparam com populações cis-andidas. Nossa hipótese diante destes resultados é que o surgimento da Cordilheira Oriental dos Andes funcionou como um evento vicariância que inicialmente separou os dois clados principais, mas posteriormente permitiu uma colonização do Baixo Magdalena e gerou uma descontinuidade ao longo do rio Magdalena.

LIST OF FIGURES

LIST OF TABLES

LIST OF CONTENTS

1. INTRODUCTION

Recent landscape modifications events, such as the emergence of the Andes and the closure of the Isthmus of Panama, shaped the current diversification of the freshwater fishes in the Neotropical region (Bermingham et al., 1998; Sivasundar et al., 2001; Reis et al., 2003). In this region the ichthyofauna is dominated by Characiformes, Siluriformes and Gymnotiformes (Albert et al., 2011).

The Characiformes contains 2000 species that are distributed in the Neotropical and Paleotropical regions (Nelson et al., 2016; Eschmeyer et al., 2019) and divided into 23 families. Erythrinidae and Tarumaniidae are grouped in the superfamily Erythrinoidea (Pinna et al., 2017; Arcila et al., 2018). Lebiasinidae, Ctenoluciidae, and Hepsetus are sister groups of Erythrinoidea (Vari, 1995).

The Erythrinidae contains three extant genera: *Erythrinus* Scopoli 1777, *Hoplerythrinus* Gill 1895 and *Hoplias* Gill 1903, with a total of 16 valid species (Oyakawa, 2003; Oyakawa & Mattox, 2009; Nelson et al., 2016). It also contains the extinct genus *PaleoHoplias*, with a single species † *PaleoHoplias assisbrasiliensis* Bocquentin & Negri 2003 (Gayet et al., 2003). *Erythrinus* and *Hoplerythrinus* include only species of medium size, while *Hoplias* includes species of medium and large sizes (Oyakawa, 2003). There are well defined diagnostic characters between the three genera.

Within the Erythrinidae, the genus *Hoplias* contains species described in a vague and imprecise way, i.e., with weak diagnostic characters (Oyakawa & Mattox, 2009). Research on the taxonomy of the genus started with the identification of species presenting easy-to-recognize diagnostic traits, such as *Hoplias* aimara and *Hoplia lacerdae* (Mattox et al., 2006, 2014, Oyakawa & Mattox, 2009). Currently, three distinct groups: *Hoplias* macrophthalmus group, *Hoplias* lacerdae group, and *Hoplias malabaricus* group, contain twelve species (Fig. 1.1).

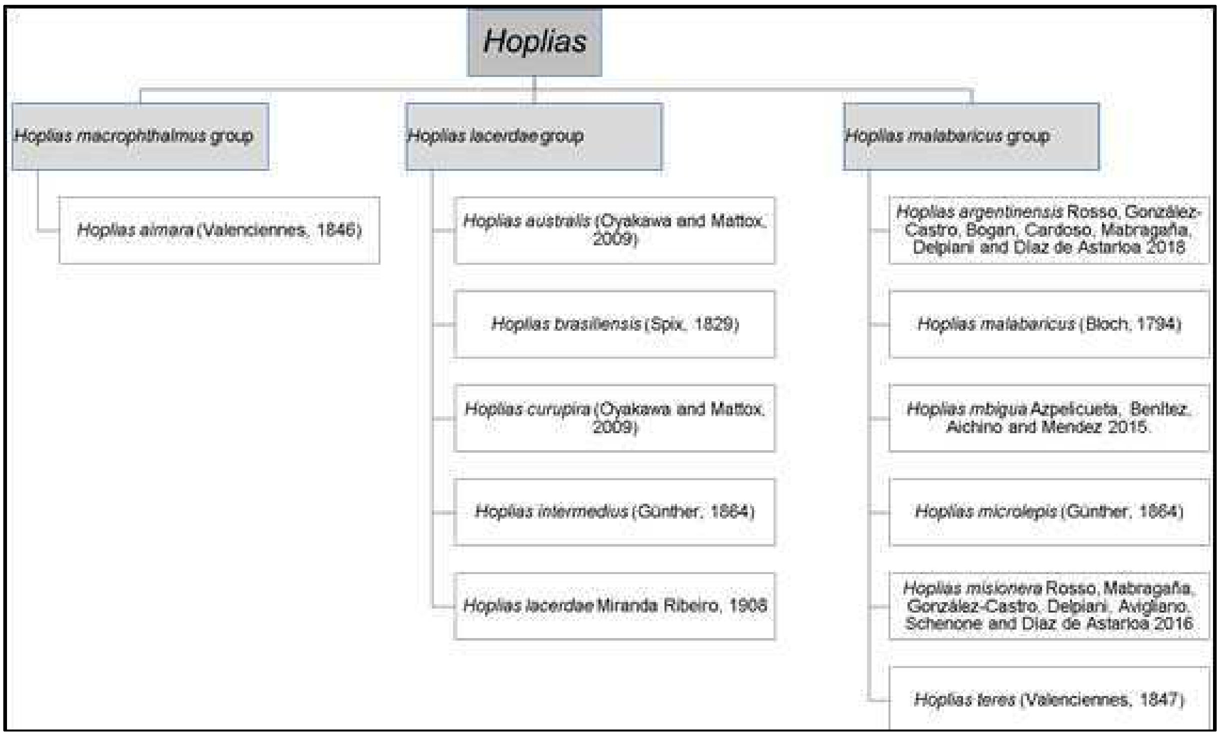


Figure 1.1 Species groups of the genus *Hoplias*

The diagnostic characters of *H. malabaricus* group are having the medial margins of the contralateral teeth converging V shape as well the presence of tooth plates in the fleshy tissue of the dorsal surface of the basihyal and basibranchial or simply, tongue with teeth (Oyakawa & Mattox, 2009). The group has six species described so far: *Hoplias microlepis* (Günther, 1864), *Hoplias teres* (Valenciennes in Cuvier and Valenciennes, 1847), *Hoplias mbigua* (Azpelicueta et al., 2015), *Hoplias misionera* (Rosso et al., 2016), *Hoplias argentinensis* (Rosso et al., 2018), and *Hoplias malabaricus* (Bloch, 1794).

Hoplias malabaricus (Bloch, 1794) is a widely distributed complex of species occurring from Northern Panama to Argentina (Oyakawa, 2003), including river basins from both sides of the Andes (Mattox et al., 2014). It is considered to be one of the most complicated taxon among the Neotropical fishes (Oyakawa, 2003; Oyakawa & Mattox, 2009) as the traditional external morphology traits fail to distinguish between independent lineages (Oyakawa, 2003; Oyakawa & Mattox, 2009). Several nominal species, considered to be junior synonyms (Oyakawa 2003), support the hypothesis that *H. malabaricus* is a species complex (Mattox et al. 2014).

The systematic challenge represented by *H. malabaricus* started with the name of the taxon. It was formerly described as *Esox malabaricus* by Bloch, 1794 because the museum specimens first examined were labeled as being from Malabar (SouthWest Coast of India). Later, circumstantial evidence indicated that the most likely place of origin of the type specimen was Suriname (Bertollo et al., 2000).

Cytogenetic studies performed since 1970 allowed the recognition of seven karyomorphs of *H. malabaricus* in Argentina, Brazil, and Colombia (Bertollo et al., 1979, 2000; Ibagón, 2015; Grassi et al., 2017). The seven karyomorphs differ in chromosome number and the absence or presence of different sex chromosome systems. The low vagility of small isolated populations facilitated the fixation of chromosomal rearrangements (Bertollo et al., 2000; Sember et al., 2018).

Most of the molecular studies of *H. malabaricus* take a phylogeographic approach, suggesting the structuring of *H. malabaricus* occurs in independent evolutionary units between adjacent rivers, lending support to dispersion and vicariousness processes between the hydrogeographic basins. (Dergam et al., 2002; Jacobina et al., 2009, 2011; Santos et al., 2009; Pereira et al., 2012; Marques et al., 2013; Rincon-Sandoval et al., 2019). The use of DNA barcoding allowed for the recognition independent lineages of *H. malabaricus* on a regional (Marques et al., 2013; Rosso et al., 2018) and continental scale (Cardoso et al., 2018; Jacobina et al., 2018).

With a wide distribution, vast karyotypic diversity, several junior synonyms and evidence of deep molecular differentiation, *H. malabaricus* is considered as an excellent model to understand the mechanisms of genomic diversity and evolution of sex chromosomes using traditional and molecular techniques cytogenetic (Cioffi et al., 2012). Many questions about the evolution and distribution of the karyotypes of *H. malabaricus*, remain to be answered. These answers would allow us to better understand the evolutionary patterns in these and other Neotropical fishes.

In the next chapters, you will find various methodological approaches that explore the diversity of *H. malabaricus* on a continental scale in South America, as well as on a regional level for Northern South America and the Brazilian Continental

Margin. The ecological (Type of systems and latitude) and evolutionary (Allometry, independent molecular lineages and major river basins) factors tested in this manuscript provide insights into the evolutionary history and the current diversity of the species complex *H. malabaricus*.

References

Albert J.S., Petry P., & Reis R. (2011) Major Biogeographic and Phylogenetic Patterns. Historical Biogeography of Neotropical Freshwater Fishes (ed. by J.S. Albert and R. Reis), pp. 21–58. University of California Press, Berkeley Los Angeles London.

Arcila D., Petry P., & Ortí G. (2018) Phylogenetic relationships of the family Tarumaniidae (Characiformes) based on nuclear and mitochondrial data. Neotropical Ichthyology, 3, 16–19.

Bermingham E., Martin P., & Moritz C. (1998) Comparative mtDNA phylogeography of Neotropical freshwater fishes: Testing shared history to infer the evolutionary landscape of lower Central America. Molecular Ecology, 7, 499–517.

Bertollo L.A.C., Born G.G., Dergam J.A., Fenocchio A.S., & Moreira-Filho O. (2000). A biodiversity approach in the neotropical Erythrinidae fish, *Hoplias malabaricus*. Karyotypic survey, geographic distribution of cytotypes and cytotaxonomic considerations. Chromosome Research, 8, 603–613.

Cardoso Y.P., Rosso J.J., Mabragana E., Gonzalez-Castro M., Delpiani, M., Avigliano E., Bogan S., Covain R., Schenone N.F., Díaz de Astarloa J.M. (2018) A continental-wide molecular approach unraveling mtDNA diversity and geographic distribution of the Neotropical genus *Hoplias*. PLoS ONE, 13, 1–25.

Cioffi M.D.B., Molina W.F., Artoni R.F., Antonio L., & Bertollo C. (2012) Chromosomes as Tools for Discovering Biodiversity – The Case of

Erythrinidae Fish Family. Recent Trends in Cytogenetic Studies - Methodologies and Applications (ed. by W.F. Molina). IntechOpen, Rijeka.

Dergam J.A., Paiva S.R., Schaeffer C.E., Godinho A.L., & Vieira F. (2002) Phylogeography and RAPD-PCR variation in *Hoplias malabaricus* (Bloch, 1794) (Pisces, Teleostei) in southeastern Brazil. Genetics and Molecular Biology, 25, 379–387.

Gayet M., Jégu M., Bocquentin J., & Negri F.R. (2003). New characoids from the Upper Cretaceous and Paleocene of Bolivia and the Mio-Pliocene of Brazil: phylogenetic position and paleobiogeographic implications. Journal of Vertebrate Paleontology, 23, 28–46.

Grassi D.J., Swarça A.C., Dergam J.A., Pastori M.C., & Fenocchio A.S. (2017) Cytogenetic characterization of *Hoplias malabaricus* (Bloch, 1794) from the Ctalamochita River (Córdoba, Argentina): first evidence for southernmost populations of this species complex and comments on its biogeography. Comparative Cytogenetics, 11, 15–28.

Ibagón N. (2015) Alto nível de variação genética em populações de *Hoplias malabaricus* (Bloch, 1794) (Teleostei, Erythrinidae) da Colômbia. Universidade Federal de Viçosa,

Jacobina U.P., Affonso P.R.A.D.M., Carneiro P.L., & Dergam J.A. (2009) Biogeography and comparative cytogenetics between two populations of *Hoplias malabaricus* (Bloch, 1794) (Ostariophysi: Erythrinidae) from coastal basins in the State of Bahia, Brazil. Neotropical Ichthyology, 7, 617–622.

Jacobina U.P., Lima S.M.Q., Maia D.G., Souza G., Batalha-Filho H., & Torres R.A. (2018) DNA barcode sheds light on systematics and evolution of neotropical freshwater trahiras. Genetica, 146, 505–515.

Jacobina U.P., Paiva E., & Dergam J.A. (2011) Pleistocene karyotypic divergence in *Hoplias malabaricus* (Bloch, 1794) (Teleostei: Erythrinidae) populations in southeastern Brazil. Neotropical Ichthyology, 9, 325–333.

Marques D., Santos F., Silva S., Sampaio I., & Rodrigues L. (2013) Cytogenetic and DNA barcoding reveals high divergence within the trahira, *Hoplias malabaricus* (Characiformes: Erythrinidae) from the lower Amazon River. Neotropical Ichthyology, 11, 459–466.

Mattox G.M.T., Bifi A.G., & Oyakawa O.T. (2014) Taxonomic study of *Hoplias microlepis* (Günther, 1864), a trans-Andean species of trahiras (Ostariophysi: Characiformes: Erythrinidae). Neotropical Ichthyology, 12, 343–352.

Mattox G.M.T., Toledo-Piza M., & Oyakawa O.T. (2006) Taxonomic Study of *Hoplias aimara* (Valenciennes, 1846) and *Hoplias* macrophthalmus (Pellegrin, 1907) (Ostariophysi, Characiformes, Erythrinidae). Copeia, 2006, 516–528.

Nelson J.S., Grande T., & Wilson M. (2016) Fishes of the World. Wiley-Blackwell, New Jersey.

Oyakawa O.T. (2003) Family Erythrinidae (Trahiras). Check List of the Freshwater Fishes of South and Central América (ed. by R. Reis, S. Kullander, and J.C. Ferraris), pp. 238–240. EDIPUCRS, Porto Alegre.

Oyakawa O.T. & Mattox G. (2009) Revision of the Neotropical trahiras of the *Hoplias lacerdae* species-group (Ostariophysi: Characiformes: Erythrinidae) with descriptions of two new species. Neotropical Ichthyology, 7, 117–140.

Pereira T.L., Santos U., Schaefer C.E., Souza G.O., Paiva S.R., Malabarba L.R., Schmidt E.E., & Dergam J.A. (2012) Dispersal and vicariance of *Hoplias malabaricus* (Bloch, 1794) (Teleostei, Erythrinidae) populations of the Brazilian continental margin. Journal of Biogeography, 40, 905–914.

Pinna M.D.E., Zuanon J., Py-daniel L.R., & Petry P. (2017) A new family of neotropical freshwater fishes from deep fossorial Amazonian habitat, with a reappraisal of morphological characiform phylogeny (Teleostei : Ostariophysi). 1–31.

Reis R.E., Kullander S.O., Ferraris C.J., & do Rio Grande do Sul. Museu de Ciências e Tecnologia P.U.C. (2003) Check List of the Freshwater Fishes of South and Central America. EDIPUCRS,

Rincon-Sandoval M., Betancur-R R., & Maldonado-Ocampo J.A. (2019) Comparative Phylogeography of Trans-Andean Freshwater Fishes Based on Genome-Wide Nuclear and Mitochondrial Markers. Molecular Ecology, 0–2.

Rosso J.J., González-Castro M., Bogan S., Cardoso Y.P., Mebragaña E., Delpiani M.S., & Díaz de Astarloa J.M. (2018) Integrative taxonomy reveals a new species of the *Hoplias malabaricus* species complex (Teleostei: Erythrinidae). Ichthyological Exploration of Freshwaters, IEF-1076, 1–18.

Santos U., Völcker C., Belei F., Cioffi M.B., & Bertollo L.A.C. (2009) Molecular and karyotypic phylogeography in the Neotropical *Hoplias malabaricus* (Erythrinidae) fish in eastern Brazil. Journal of Fish Biology, 75, 2326–2343.

Sember A., Bertollo L.A.C., Ráb P., Yano C.F., Hatanaka T., de Oliveira E.A., & Cioffi M. de B. (2018) Sex chromosome evolution and genomic divergence in the fish *Hoplias malabaricus* (Characiformes, Erythrinidae). Frontiers in Genetics, 9, 1–12.

Sivasundar A., Bermingham E., & Ortí G. (2001) Population structure and biogeography of migratory freshwater shes (*Prochilodus*: Characiformes) in major South American rivers. Molecular Ecology, 10, 407–417.

Vari R.P. (1995). The Neotropical Fish Family Ctenoluciidae (Teleostei: Ostariophysi: Characiformes): Supra and Intrafamilial Phylogenetic Relationships, with a Revisionary Study.

2. ALLOMETRY, LONG-TERM EVOLUTIONARY ISOLATION, AND ADAPTATION TO DIFFERENT ENVIRONMENTS AFFECTING SHAPE DIVERSITY IN THE TRAHIRA *Hoplias malabaricus* (TELEOSTEI: ERYTHRINIDAE)

Manuscript in review in Journal of Fish biology

N. Ibagon[1,2], B. Shalaguti[1], K. Yotoko[1]

1. Laboratorio de Bioinformatica e Evolução. Departamento de Biologia Geral, Av. PH Rolfs s/n, Universidade Federal de Viçosa, Viçosa, Minas Gerais CEP 36570-900, Brazil.
2. Programa de Pós-Graduação em Ecologia, Universidade Federal de Viçosa, Av. P.H. Rolfs, s/n, Campus Universitário, 36570-900 Viçosa, MG, Brazil

Correspondence:
Karla Yotoko
Laboratório de Bioinformática e Evolução. Departamento de Biologia Geral, Av. PH Rolfs s/n, Universidade Federal de Viçosa, Viçosa, Minas Gerais CEP 36570-900, Brazil.
Telephone: 55 31 3899 3363
karla@ufv.br

Abstract

Shape diversity of the trahira *Hoplias malabaricus* heads from Brazilian Continental Margin comprises at least three components: i. diversity induced by an allometric process: the shape changes over the growth of individuals in a predictable manner; ii. shape differentiation induced by long-term evolutionary isolation: we found a small but significant difference between the three clades based on a previous molecular study; iii. adaptation to different aquatic systems generated by divergent selection or phenotypic plasticity: we found significant differences between samples of lentic and lotic systems. Based on our results, we suggest that such environmental adaptation restricts morphometric differentiation between populations or species in well-defined molecular clades that maintain distinct morphological shapes, and thus, hampers the identification of new species within the *H. malabaricus* complex.

Keywords: Brazilian Continental Margin; Geometric morphometrics; Freshwater; Species Complex.

Significance statement

Trahira is a Neotropical fish species complex comprising several karyomorphs and independent molecular lineages. Using geometric morphometrics of the heads of specimens from the Brazilian Continental Margin, we observed morphological diversity related to static allometry, differentiation induced by long-term isolation and differentiation between aquatic systems. We suggest that adaptation to different environments restricts morphometric differentiation between well-defined molecular clades, which maintains distinct morphological shapes in different clades, hampering the identification of new species of trahiras.

Introduction

Hoplias malabaricus (Bloch, 1794), also referred to as trahira, is a widespread fish species that inhabits lentic and lotic ecosystems (Petry et al., 2003) ranging from northern Panama to Argentina (Oyakawa, 2003). The species karyotype diversity comprises at least seven karyomorphs (A–G) with 40 or 42 chromosomes (Bertollo et al., 2000). The karyomorphs differ regarding chromosome morphology and sex chromosome systems (Bertollo et al., 2000; Santos et al., 2009; Cioffi & Bertollo, 2010) and have been reported to occasionally hybridize (Utsunomia et al., 2014). Karyotype differentiation in this species leads several researchers to consider *H. malabaricus* as a species complex. Indeed, recent taxonomic reviews proposed three new species within this complex that inhabit the La Plata River basin: *Hoplias mbigua* (Azpelicueta et al., 2015), *Hoplias misionera* (Rosso et al., 2016), and *Hoplias argentinensis* (Rosso et al., 2018).

Studies based on molecular data also suggested the structuring of *H. malabaricus* in independent evolutionary units (Santos et al., 2009; Pereira et al., 2012; Marques et al., 2013; Cardoso et al., 2018; Jacobina et al., 2018). In a study on samples from several neotropical basins, four independent molecular lineages were described; each lineage contains several karyomorphs while the same karyomorph occurs in different clades, suggesting that the karyotypes are homoplastic characters (Jacobina et al., 2018).

Geometric morphometrics (GM) is another potential method to examine biological variation, and this approach has been used previously to investigate morphological diversity of other fish species complexes (Klingenberg et al., 2003; Maderbacher et al., 2008; Santos & Araújo, 2014; Francesco et al., 2016). GM uses landmark coordinates to capture shape variables that can be used to study biological and evolutionary processes such as long-term evolutionary diversification mediated by genetic drift or natural selection (Zastavniouk et al., 2017), allometry (Castro et al., 2018), or adaptation to local environmental factors (Foster et al., 2015).

In a study of trahira from the Brazilian Continental Margin (Figure 2.1), Pereira *et al.* (2012) delimited three molecular lineages and found substantial genetic divergence between the Northeastern ($2n$ = 40) and other genetic groups (Eastern, 2n=42; and Southeastern, $2n$ = 42), and interpreted this divergence as evidence for the vicariant effect of the Abrolhos Formation. They also found evidence for divergence between the Eastern and Southeastern groups and suggested that the Cabo Frio Magmatic Lineament may have separated these groups (Figure 1). Based on these assumptions, we examined shape diversity in *H. malabaricus* taking the suggested clades into account and investigated potential consequences of local adaptation to lentic and lotic aquatic systems. Furthermore, we measured the effects of allometry that was observed in this species by Castro *et al*. (2018).

Materials and methods

In this study, we photographed 375 samples from the Brazilian Continental Margin from three Brazilian ichthyological collections: Museu de Zoologia da USP (MZUSP), Museu de Zoologia João Moojen - UFV (MZUFV), and Museu Nacional do Rio de Janeiro - UFRJ (MNRJ) (Appendix S1).

We examined shape variation in *H. malabaricus* using the cephalic region, which is less likely to deform than the rest of the body during formaldehyde fixation. We used samples with a standard length of more than 15 cm and eliminated all distorted samples from the analyses.

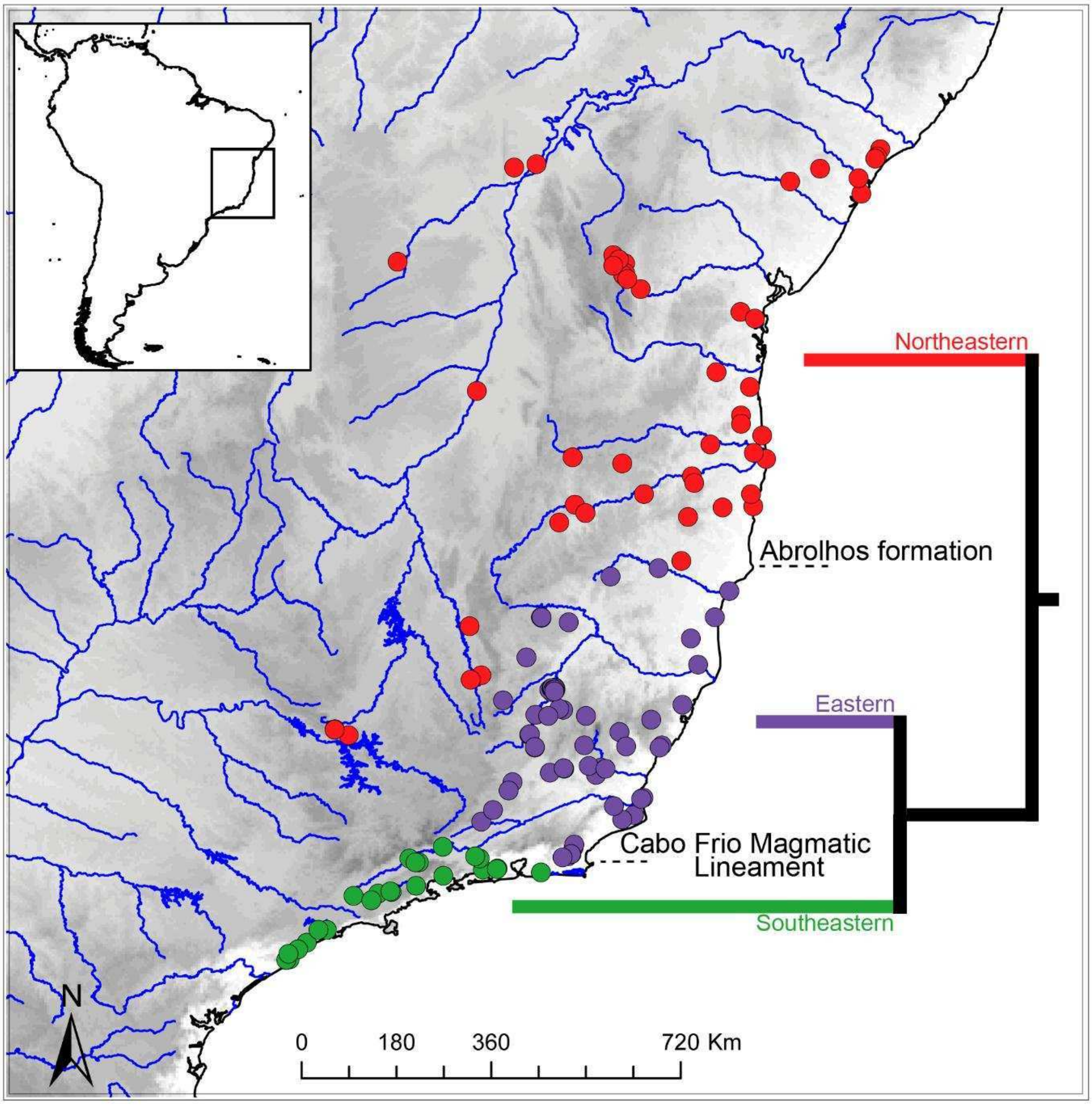


Figure 2.1. Map of the Brazilian Continental Margin with *Hoplias malabaricus* sampling locations. Colored dots indicate individuals (red: Northeastern; purple: Eastern; green: Southeastern regions), based on the phylogenetic tree by Pereira et al. (2012) shown on the right.

The left lateral side of the head of each specimen was photographed using a Coolpix P530 camera (Nikon, Japan) at different focal distances and using a range scale for later size corrections. We did not differentiate samples by sex due to the lack of secondary sexual characteristics (Hauser & Benedito, 2012). The images were processed as tps files using tpsUtil 1.74 software (Rohlf, 2015). We compiled the coordinate data using tpsDIG 2.29 software (Rohlf, 2015) and selected 13 homologous landmarks (Figure 2.2), classified as type I (n = 8), II (n = 4), and III (n = 1), according to Bookstein (1991). The following characteristics were assessed: snout position (landmarks 1 and 2), eye shape (landmarks 3-6; determined using a plus sign aligned with the nostril), opercle formation (landmarks 7-12), and the intersection between the dentary and the maxilla (landmark 13). Data analyses were performed using MorphoJ 1.06 software (Klingenberg, 2011).

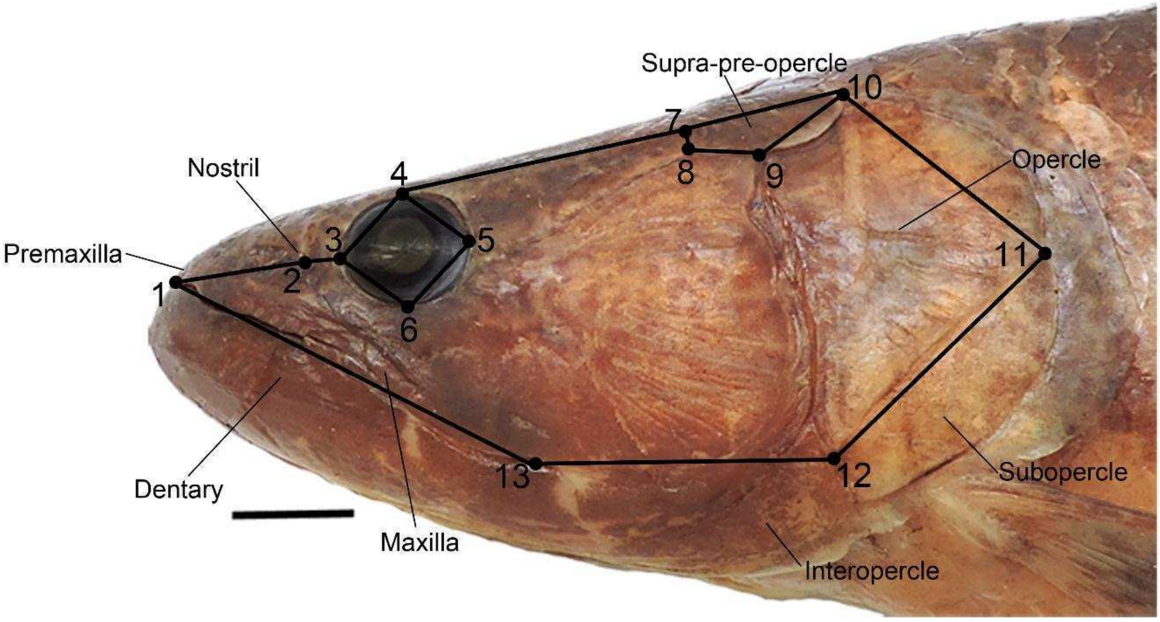


Figure 2.2. Position of the landmarks on the lateral view of the head of *Hoplias malabaricus*. Description of the landmarks (roman numerals represent landmark types according to Bookstein, 1991): 1. most anterior point of the premaxilla (iii); 2. second nostril (i); 3. most anterior point of the orbital margin (ii); 4. most dorsal point of the orbital margin (ii); 5. most posterior point of the orbital margin (ii); 6. most ventral point of the orbital margin (ii); 7. intersection between the infraorbital 4, supra-pre-opercle, and pterotic (i); 8. intersection between the infraorbital 4, 5 and the supra-pre-opercle (i); 9. most posterior point between the supra-pre-opercle and the infra-orbital 5 (i); 10. most posterior intersection between the supra-pre-opercle and the opercle (i); 11. most posterior point of the opercle (i); 12. ventral intersection between the intra-opercle and the subopercle (i); 13. most posterior intersection between the dentary and the maxilla (i).

To evaluate shape diversity, we used a Procrustes superimposition to eliminate variation in size, translation, and rotation. After that, we tested for static allometry (the relationship between shape and size in mature individuals) effects using the multivariate regression of the shape (regression score) as a dependent variable, and the log-transformed centroid size as an independent variable in a test of 10,000 permutations. A significant correlation can be interpreted as a signal of allometry, and its effect can be extracted to compare shapes for other purposes. The residuals of this regression contain the non-allometric component of shape variation (Sidlauskas et al., 2011).

Shape variation was analyzed using a principal component analysis (PCA) on the covariance matrix of the residuals of the regression; the specimens were labeled according to either the three clades proposed by Pereira *et al.* (2012) (79 samples from the Northeastern, 264 from the Eastern, and 32 from the Southeastern regions of the Brazilian Continental Margin), or to the two putative environmental systems in three categories: lentic (143 samples), lotic (85 samples), or unknown (147 samples). The type of the putative aquatic system was inferred from descriptions of the sampling location, which were associated with the catalog number of each ichthyological collection. As lotic systems we considered locations described by the terms ‘stream’ (*riacho*), ‘creek’ (*córrego*), or ‘river under a bridge’ (*rio sob ponte*), and lentic systems by the terms ‘lagoon’ (*lagoa*) and ‘dam’ (*represa*; Appendix S1). Locations with descriptions referring to a large basin or with insufficient information were categorized as ‘unknown’.

We evaluated whether there are significant shape differences between the clades (*sensu* Pereira et al. 2012) or types of system (lentic/lotic) based on the non-allometric component of shape variation (residuals of allometry). Therefore, we made one linear model for each hypothesis (clades and systems) and performed an Analysis of Variance (ANOVA) of each linear model with the permutation procedure and the Ordinary Least Squares as an estimation method using the “procD.lm” function of the Geomorph package (Adams et al., 2013). We also calculated the pairwise differences between groups (for each hypothesis) using the “pairwise” function of the package RRPP (Collyer & Adams, 2018). Both tests (ANOVA and pairwise differences) were performed using the R software (R Core Team, 2018), with 95% of confidence.

Results

The regression between shape (Procrustes coordinates) and size (the log centroid size) indicated significant static allometry ($P < 0.001$), i.e., the variables are correlated in a linear pattern, and shape changes predictably during the growth of an individual. Allometry explains 23.23% of shape variation. The results suggest that smaller heads are dorso-ventrally flattened, whereas larger heads are dorso-ventrally expanded. Moreover, eyes are proportionally larger in specimens with smaller heads than in those with larger heads (Figure 2.3).

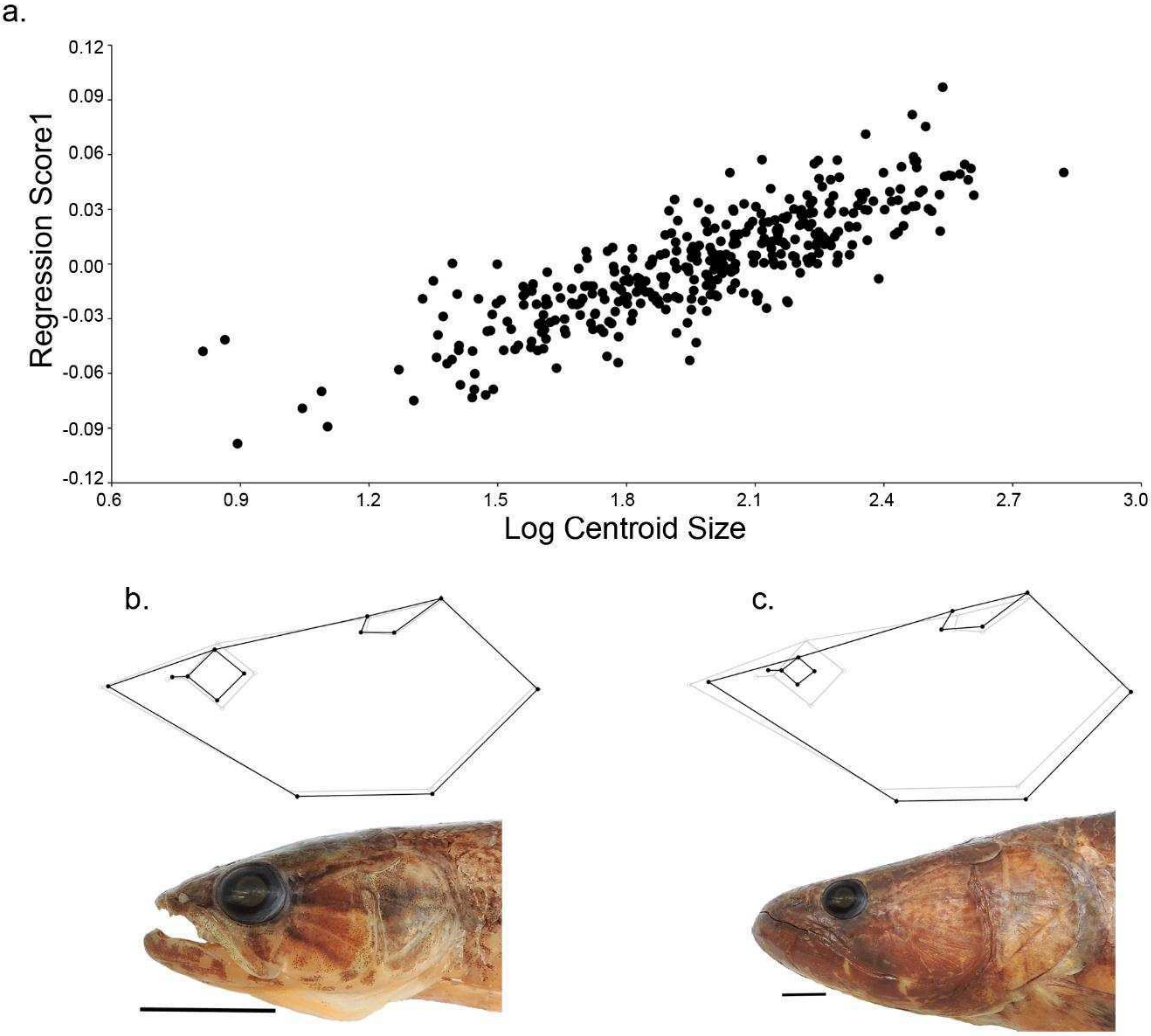


Figure 2.3. (a) Scatterplot of shape (regression score 1) and log-transformed centroid size of the lateral view of the head of *Hoplias malabaricus*. Wire frames show most negative (b) and positive (c) (-3.0 and +3.0) values (black) compared with the average shape (grey). Picture of AN0001MZUFV3625 (b) and AN0001MZUSP58742 (c) (Appendix S1). Scale bars = 1.0 cm.

The PCA, based on the residuals of the previous regression, produced 22 shape components. The first three of these components explain 53.28% of the overall shape variation (Figure 2.4), revealing subtle differentiation between the three clades proposed by Pereira et al. (2012) (Figure 2.4a), and between the two types of systems (Figure 2.4b). In both cases, the variation within each group is higher than between groups.

The results of the multivariate ANOVA show significant differences between clades [$F_{(2,272)}$=3.97, Rsq=0.02, Effect size (Z)=4.04, p=0.001]. Furthermore, pairwise comparisons show significant differences between all the clades with bigger effect size between Northeastern and Eastern groups (Table 2.1). The Average shapes of the three groups differ in the position of the opercle and the snout: the Southeastern clade exhibits a hide opercle region and a snout pointing to the ventral side, while Northeastern and Eastern clades differ in the eye position and position of the opercle (Figure 2.4c).

Table 2.1. Pairwise distances between means of the clades of *Hoplias malabaricus* in the Brazilian Continental Margin, according to Pereira et al. (2012): Northeastern, Eastern, and Southeastern. Z: effect size in the upper diagonal. P values in lower diagonal

	Northeastern	Eastern	Southeastern
Northeastern		5.869	2.713
Eastern	0.001		2.358
Southeastern	0.017	0.025	

Regarding the different types of system, the results of the multivariate ANOVA also show significant differentiation between specimens from lotic and lentic systems [F(1,226)=7.90, Rsq=0.03, Effect size (Z)=4.45, p=0.001], and the pairwise comparisons [Z(lentic – lotic)= 7.41, P=0.001]. The Average shapes of the two groups differ in their snout position and l length: compared to specimens from lotic systems, those from lentic systems on average have shorter snouts. (Figure 2.4d).

Discussion

We aimed to explore the shape diversity of a regional sample of *H. malabaricus* to infer the effect of allometry, long-term evolutionary diversification, and type of aquatic system (lentic or lotic) on such diversity. We found that static allometry explains 23.23% of shape variation of the cephalic region of *H. malabaricus*. Most of such variation concentrates on the head depth and the opening of the orbit, which was also reported by Castro et al. (2018) in 18 species of five orders of bony fish. Indeed, a functional restriction for eye growth in adult animals is expected because “the rods and cones do not vary with the size of the animal” (Thompson, 1942).

Apart from the effect of allometry on shape diversity, we found significant differences between the three clades suggested by Pereira *et al.* (2012). Such differences between clades support the expectation of higher divergence between the Northeastern ($2n$ = 40 chromosomes) and the other samples ($2n$ = 42 chromosomes), than between Eastern and Southeastern samples. Pereira et al. (2012) showed that the Abrolhos Formation and the Cabo Frio Magmatic Lineament were effective barriers for gene flow among *H. malabaricus* populations, which resulted in clear differentiation observed in a nuclear marker (RAG2), which requires a long evolutionary time period or a profound effect of genetic drift in small isolated populations to produce significant differences (Brown et al., 1979). Such long periods of isolation were enough to produce minor shape differences among the clades. However, variation within each clade is considerably higher than between clades, and we observed a substantial overlap of shapes (Figure 2.4), suggesting that evolutionary or ecologic factors act to maintain the shape variation within each clade.

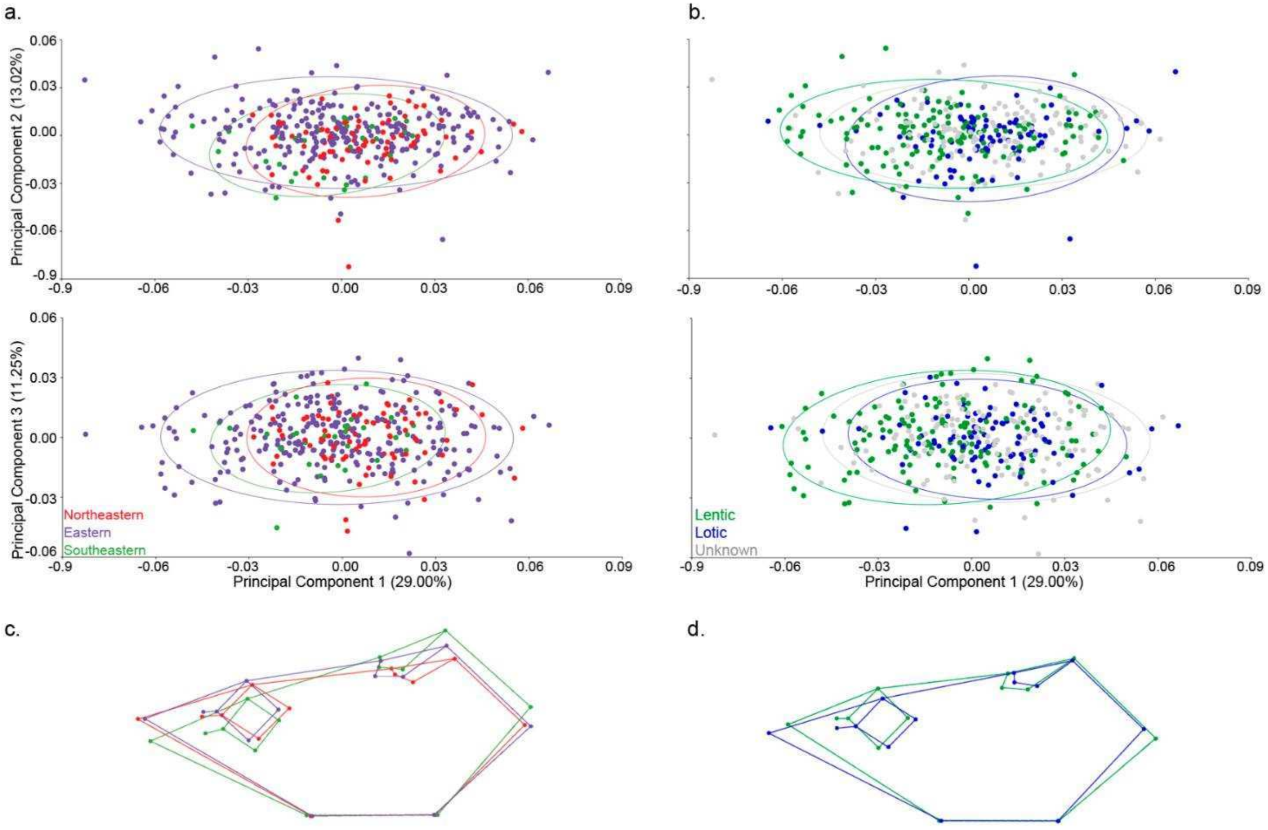


Figure 2.4. Scatterplots of the first three components of the principal component analysis based on geometric morphometrics of the lateral view of *Hoplias malabaricus* heads from the Brazilian Continental Margin. (a) Colored by clades according to Pereira et al. (2012): Northeastern, Eastern, and Southeastern. (b) Colored by type of system: lentic, lotic, or unknown. Ellipses represent 90% confidence levels of each group. (c) Average shapes by clades according to Pereira et al. (2012), or (d) type of system, scale factor 5.0.

Therefore, we tested whether head shape diversity in *H. malabaricus* responds to different types of systems and found significant differences between samples from lentic and lotic systems, which is in line with the results of the GM studies of Haas *et al.* 2015), Berbel-Filho *et al.* (2015), and Santos & Araújo (2014). Our results show that specimens associated with lotic environments exhibit a more hydrodynamic, torpedo-shaped head, which may increase locomotory steadiness (Figure 2.4) (Langerhans & Reznick, 2010). Such differences result from genetic divergence, phenotypic plasticity, or both (Langerhans, 2008), and laboratory experiments should be performed to distinguish between these alternatives.

We concluded that the adaptation (via divergent selection or phenotypic plasticity) (Grenier et al., 2016) to different types of systems restricts the morphometric differentiation between populations or species in these well-defined molecular clades, therefore maintaining their morphology, and hampered the identification of new species in the *H. malabaricus* complex. Other factors that could not be addressed in this study may also contribute to shape diversity, such as potential differences between sexes, other environmental traits, and trophic variables (Lostrom et al., 2015; Bohórquez-Herrera et al., 2017).

Acknowledgements

The authors thank M. Britto and C. Moreira from the Museu Nacional/UFRJ, J. Dergam from the MZUFV, A. Datovo and O. Oyakawa from the MZUSP. The authors thank Editage for English language editing. This study was financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) (Finance Code 001) through the doctoral fellowship of Nicole Ibagón and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG).

Contributions

NI and KY designed the study. NI and BS collected the data and performed the analyses. All authors wrote, reviewed, edited and approved the manuscript.

References

Adams D.C., Otárola-Castillo E., Adriaens D., & Otárola-Castillo E. (2013) Geomorph: An r package for the collection and analysis of geometric morphometric shape data. Methods in Ecology and Evolution, 4, 393–399.

Azpelicueta M. de las M., Benítez M., Aichino D., & Mendez D. (2015) A new species of the genus *Hoplias* (Characiformes, Erythrinidae), a tararira from the lower Paraná River , in Misiones , Argentina. Acta Zoologica Lilloana, 59, 71–82.

Berbel-Filho W.M., Martinez P.A., Ramos T.P.A., Torres R.A., Lima S.M.Q.Q., Pablo W.M.B., Telton A.M., Torres R.A., & Lima S.M.Q.Q. (2015) Inter- and intra-basin phenotypic variation in two riverine cichlids from northeastern Brazil: potential eco-evolutionary damages of Sao Francisco interbasin water transfer. Hydrobiologia, 766, 43–56.

Bertollo L.A.C., Born G.G., Dergam J.A., Fenocchio A.S., & Moreira-Filho O. (2000) A biodiversity approach in the neotropical Erythrinidae fish, *Hoplias malabaricus*. Karyotypic survey, geographic distribution of cytotypes and cytotaxonomic considerations. Chromosome Research, 8, 603–613.

Bohórquez-Herrera J., Aurioles-Gamboa D., Hernández-Camacho C., & Adams D.C. (2017) Variability in the Skull Morphology of Adult Male California Sea Lions and Galapagos Sea Lions. Tropical Pinnipideds: Bio-Ecology Threats and Conservation (ed. by J.J. Alava), pp. 22–49. CRC Press, Boca Raton.

Bookstein F.L. (1991) Morphometric Tools for Landmark Data. Geometry and Biology. Cambridge University Press, Cambridge.

Brown W., George M., & Wilson A. (1979) Rapid evolution of animal mitochondrial DNA. Proceedings of the National Academy of Sciences, 76, 1967–1971.

Cardoso Y.P., Rosso J.J., Mabragana E., Gonzalez-Castro M., Delpiani M., Avigliano E., Bogan S., Covain R., Schenone N.F., Díaz de Astarloa J.M., (2018) A continental-wide molecular approach unraveling mtDNA diversity and geographic distribution of the Neotropical genus *Hoplias*. PLoS ONE, 13, 1–25.

Castro K.M.D.S.A., Santos M.P.D., Brito M.F.G., Bidau C.J., & Martinez P.A. (2018) Ontogenetic allometry conservatism across five teleost orders. Journal of Fish Biology, 93, 745–749.

Cioffi M.B. & Bertollo L.A.C. (2010) Initial steps in XY chromosome differentiation in *Hoplias malabaricus* and the origin of an X(1)X(2)Y sex chromosome system in this fish group. Heredity, 105, 554–561.

Collyer M.L. & Adams D.C. (2018) RRPP : An r package for fitting linear models to high-dimensional data using residual randomization. Methods in Ecology and Evolution, 9, 1772–1779.

Foster K., Bower L., & Piller K. (2015) Getting in shape: Habitat-based morphological divergence for two sympatric fishes. Biological Journal of the Linnean Society, 114, 152–162.

Francesco L., Kirschbaum F., Ernst A., Feulner P.G., Mamonekenee V., Paul C., & Tiedemann R. (2016) Species delimitation and phylogenetic relationships in a genus of African weakly-electric fishes (Osteoglossiformes, Mormyridae, *Campylomormyrus*). Molecular Phylogenetics and Evolution, 101, 8–18.

Grenier S., Barre P., & Litrico I. (2016) Phenotypic Plasticity and Selection : Nonexclusive Mechanisms of Adaptation. Scientifica, 2016, 9.

Haas T.C., Heins D.C., & Blum M.J. (2015) Predictors of body shape among populations of a stream fish (*Cyprinella venusta,* Cypriniformes: Cyprinidae). Biological Journal of the Linnean Society, 115, 842–858.

Hauser M. & Benedito E. (2012) Species of the *Hoplias* aff *malabaricus* complex (Characiformes: Erythrinidae): An investigation of coexistence in a Neotropical floodplain. Zoologia, 29, 59–69.

Jacobina U.P., Lima S.M.Q., Maia D.G., Souza G., Batalha-Filho H., & Torres R.A. (2018) DNA barcode sheds light on systematics and evolution of neotropical freshwater trahiras. Genetica, 146, 505–515.

Klingenberg C.P. (2011) MorphoJ: an integrated software package for geometric morphometrics. Molecular Ecology Resources, 11, 353–357.

Klingenberg C.P., Barluenga M., & Meyer A. (2003) Body shape variation in cichlid fishes of the *Amphilophus citrinellus* species complex. Biological Journal of the Linnean Society, 80, 397–408.

Langerhans R. & Reznick D. (2010) Ecology and Evolution of Swimming Performance in Fishes. Fish Locomotion: An Eco-ethological Perspective (ed.

by O. Domenici and B. Kapoor), pp. 200–248. Enfield, Science Publishers, New Hampshire.

Langerhans R.B. (2008) Predictability of phenotypic differentiation across flow regimes in fishes. Integrative and Comparative Biology, 48, 750–768.

Lostrom S., Evans J.P., Grierson P.F., Collin S.P., Davies P.M., & Kelley J.L. (2015) Linking stream ecology with morphological variability in a native freshwater fish from semi-arid Australia. Ecology and Evolution, 5, 3272–3287.

Maderbacher M., Bauer C., Herler J., Postl L., Makasa L., & Sturmbauer C. (2008) Assessment of traditional versus geometric morphometrics for discriminating populations of the *Tropheus moorii* species complex (Teleostei: Cichlidae), a Lake Tanganyika model for allopatric speciation. Journal of Zoological Systematics and Evolutionary Research, 46, 153–161.

Marques D., Santos F., Silva S., Sampaio I., & Rodrigues L. (2013) Cytogenetic and DNA barcoding reveals high divergence within the trahira, *Hoplias malabaricus* (Characiformes: Erythrinidae) from the lower Amazon River. Neotropical Ichthyology, 11, 459–466.

Oyakawa O.T. (2003) Family Erythrinidae (Trahiras). Check List of the Freshwater Fishes of South and Central América (ed. by R. Reis, S. Kullander, and J.C. Ferraris), pp. 238–240. EDIPUCRS, Porto Alegre.

Pereira T.L., Santos U., Schaefer C.E., Souza G.O., Paiva S.R., Malabarba L.R., Schmidt E.E., & Dergam J.A. (2012) Dispersal and vicariance of *Hoplias malabaricus* (Bloch, 1794) (Teleostei, Erythrinidae) populations of the Brazilian continental margin. Journal of Biogeography, 40, 905–914.

Petry A.C., Agostinho A.A., & Gomes L.C. (2003) Spatial variation of the fish assemblage structure from the upper Rio Paraná floodplain, Brazil , in a dry year. Acta Limnol. Bras, 15, 1–13.

R Core Team (2018) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. .

Rohlf F.J. (2015) The tps series of software. Hystrix, 26, 1–4.

Rosso J.J., González-Castro M., Bogan S., Cardoso Y.P., Mebragaña E., Delpiani M.S., & Díaz de Astarloa J.M. (2018) Integrative taxonomy reveals a new species of the *Hoplias malabaricus* species complex (Teleostei: Erythrinidae). Ichthyological Exploration of Freshwaters, IEF-1076, 1–18.

Rosso J.J., Mabragaña E., González-Castro M., Delpiani M.S., Avigliano E., Schenone N., & Díaz de Astarloa J.M. (2016) A new species of the *Hoplias malabaricus* species complex (Characiformes: Erythrinidae) from the La Plata River basin. Cybium, 40, 199–208.

Santos A.B.I. & Araújo F.G. (2014) Evidence of morphological differences between *Astyanax bimaculatus* (Actinopterygii: Characidae) from reaches above and below dams on a tropical river. Environmental Biology of Fishes, 98, 183–191.

Santos U., Völcker C., Belei F., Cioffi M.B., & Bertollo L.A.C. (2009) Molecular and karyotypic phylogeography in the Neotropical *Hoplias malabaricus* (Erythrinidae) fish in eastern Brazil. Journal of Fish Biology, 75, 2326–2343.

Sidlauskas B.L., Mol J.A.N.H., & Vari R.P. (2011) Dealing with allometry in linear and geometric morphometrics : a taxonomic case study in the *Leporinus* cylindriformis group (Characiformes : Anostomidae) with description of a new species from Suriname. Zoological Journal of the Linnean Society, 162, 103–130.

Thompson D.W. (1942) On growth and form. Cambridge University Press, Cambridge.

Utsunomia R., Pansonato Alves J.C., Paiva L.R.S., Costa Silva G.J., Oliveira C., Bertollo L.A.C., & Foresti F. (2014b) Genetic differentiation among distinct karyomorphs of the wolf fish *Hoplias malabaricus* species complex (Characiformes, Erythrinidae) and report of unusual hybridization with natural triploidy. Journal of Fish Biology, 85, 1682–1692.

Zastavniouk C., Weir L.K., & Fraser D.J. (2017) The evolutionary consequences of habitat fragmentation: Body morphology and coloration differentiation among brook trout populations of varying size. Ecology and Evolution, 7, 6850–6862.

3. THE SHAPE VARIATION IN *Hoplias malabaricus*: AN EFFECT OF MULTIPLE ECOGEOGRAPHIC CAUSES

Manuscript in preparation, to be submitted to Hydrobiologia

Nicole Ibagon, Karla Yotoko

Departamento de Biologia Geral, Universidade Federal de Viçosa, Viçosa, Minas Gerais Brasil

Abstract

Hoplias malabaricus is a complex of cryptic species widely distributed, with several karyomorphs and independent molecular lineages. On the one hand, it confirms the group status of complex of species and on the other hand hampers the proper differentiation between species. In a previous study, using a regional sampling of trahiras evaluated with geometric morphometrics tools to try to shade light in this systematic problem. That study indicated that the shape diversity of this taxon is more complicated than predicted. *H. malabaricus* shape variation is affected by multiple factors, such as size, independent evolutionary lineages, and the type of ecosystem (lentic of lotic environments). Except for the size, the other variables explain the variation in a weak but significant way. Therefore, we suggested that other environmental factor should also affect the shape of trahiras. In this work, we expanded the sample to include specimens of the seven major river basins of South America, applied geometric morphometrics, confirmed the differences of shapes due to the type of ecosystems, and detected shape differences related to the isolation provided by the river basin of origin of the specimens. We also measured the effect of latitude (that we treated as a proxy of temperature) in the shape diversity of *H. malabaricus*. Again, all the factors show weakly but significant effects, suggesting then that the shape variation in this species complex results from multiple causes and contributing then to the lack of morphological differences between species, which consequently prevents taxonomic identification of species.

Key words: Characiformes, Erythrinidae, freshwater, landmarks, lentic, lotic

Introduction

The Neotropical Region, a portion extending from Mexico to southern South America, is one of the most diverse and complex areas of the planet (Antonelli & Sanmartín, 2011; Brown et al., 2014). Several historical and ecological theories were proposed to explain why tropical regions, especially the Neotropics, are more diverse than other areas (Brown et al., 2014). One of such theories explicitly suggests that higher temperatures accelerate the evolutionary rates, leading to the current diversity (Brown et al., 2014; Orton et al., 2019). Some authors indicated that the latitude affects the shape of animals (Viguier, 2002; Frost et al., 2003; Monteiro et al., 2003).

Additionally, freshwater fish diversity is higher in the Neotropical Region (Albert & Reis, 2011). The Characiforms fishes present an extreme body shape diversification in the Neotropics when compared to the Old World (Burns & Sidlauskas, 2018). The trahira *Hoplias malabaricus* (Bloch, 1794), a Neotropical characiform, inhabiting 46 of the 69 ecoregions of Central and South America (Abell et al., 2008), from Panama to Argentina (Oyakawa, 2003). Its karyotypic and molecular diversity is so high that several authors consider that *H. malabaricus* as species complex (Bertollo et al., 2000; Cardoso et al., 2018; Jacobina et al., 2018). Recently, various taxonomic studies delimited three new species of the complex at the La Plata basin (Azpelicueta et al., 2015; Rosso et al., 2016, 2018). Such extensive distribution associated with the extreme diversity and the low vagility of *H. malabaricus* (Bialetzki et al., 2002) may facilitate the population isolation and make this species complex an excellent model to study fish diversity in the Neotropical Region (Jacobina et al., 2009; Sember et al., 2018).

In previous work, Ibagon et al. (2019), explored the shape diversity of *H. malabaricus* in a regional approach using geometric morphometrics (GM) tools on specimens sampled from the Brazilian Continental Margin. They found that the shape diversity is related to static allometry (the shape changes with the size of mature individuals), long-term evolutionary isolation (testing whether the three independent molecular clades suggested by Pereira et al., 2012) show significant shape differentiation), and adaptation to different types of ecosystems (samples collected in lentic and lotic environments present significant shape differentiation).

In the present study, we expanded the sampling to include specimens of the seven major river basins of South America (Fig.3.1). We tested whether the isolation of samples in different hydrographic basins contributed to the shape variation of *H. malabaricus*. We also tested if the effect of the type of ecosystem (lotic or lentic) is significant in such an expanded sample. Based on the hypothesis that other environmental factors could contributed to the shape variation of the complex (proposed by Ibagon et al. 2019), we tested the effect of latitude on the shape diversity of *H. malabaricus*, adding an additional piece to understand shape variation puzzle of this complex of species

Materials and methods

We examined a total of 1,942 specimens of *H. malabaricus* deposited at seven ichthyological collections: Instituto de Investigación de Recursos Biológicos Alexander von Humboldt (IAvH-P – 251 samples), Instituto de Ciencias Naturales (ICN-MHN-Ic – 92 samples), Museu Nacional Universidade Federal do Rio de Janeiro (MNRJ- 112 samples), Museo Javeriano de Historia Natural (MPUJ- 35 samples), Museu de Zoologia João Moojen Universidade Federal de Viçosa (MZUFV – 378 samples), Museo de Zoología de la Universidad de São Paulo (MZUSP – 571 samples), Núcleo de Pesquisas em Limnologia Ictiologia e Aquicultura (NUPELIA- 503 samples).

Following the same sample process methodology described in Ibagon et al. (2019), we photographed the cephalic region of trahira samples larger than 15 cm and eliminated all distorted samples from the analyses. The heads were laterally photographed, and the same 13 landmarks from Ibagon et al (2019) were marked. We transformed the landmarks removing position, scale, and orientation by using the Procrustes fit. Finally, we performed a multivariate regression of the Procrustes coordinates and the logarithm of the centroid size with 10,000 permutations to discount the static allometry of the shapes, using the residuals of the regression for further analyses (Sidlauskas et al., 2011).

We calculated a principal component analysis (PCA) on the covariance matrix of the residuals of the regression between shape and size (static allometry). The specimens were labeled according to their sampling locations, such as according to major River Basins (Amazon: 555 specimens, Guianas: 19, La Plata: 605, Northeastern Atlantic: 75, Orinoco: 178, Southeastern Atlantic: 404 or Trans-Andean: 106); or according to type of system (lentic: 573, lotic: 402 or unknown 967). The type of the putative aquatic system (lentic or lotic) was inferred following the same criteria of Ibagon et al. (2019). Data analyses were performed using MorphoJ 1.06 software (Klingenberg, 2011).

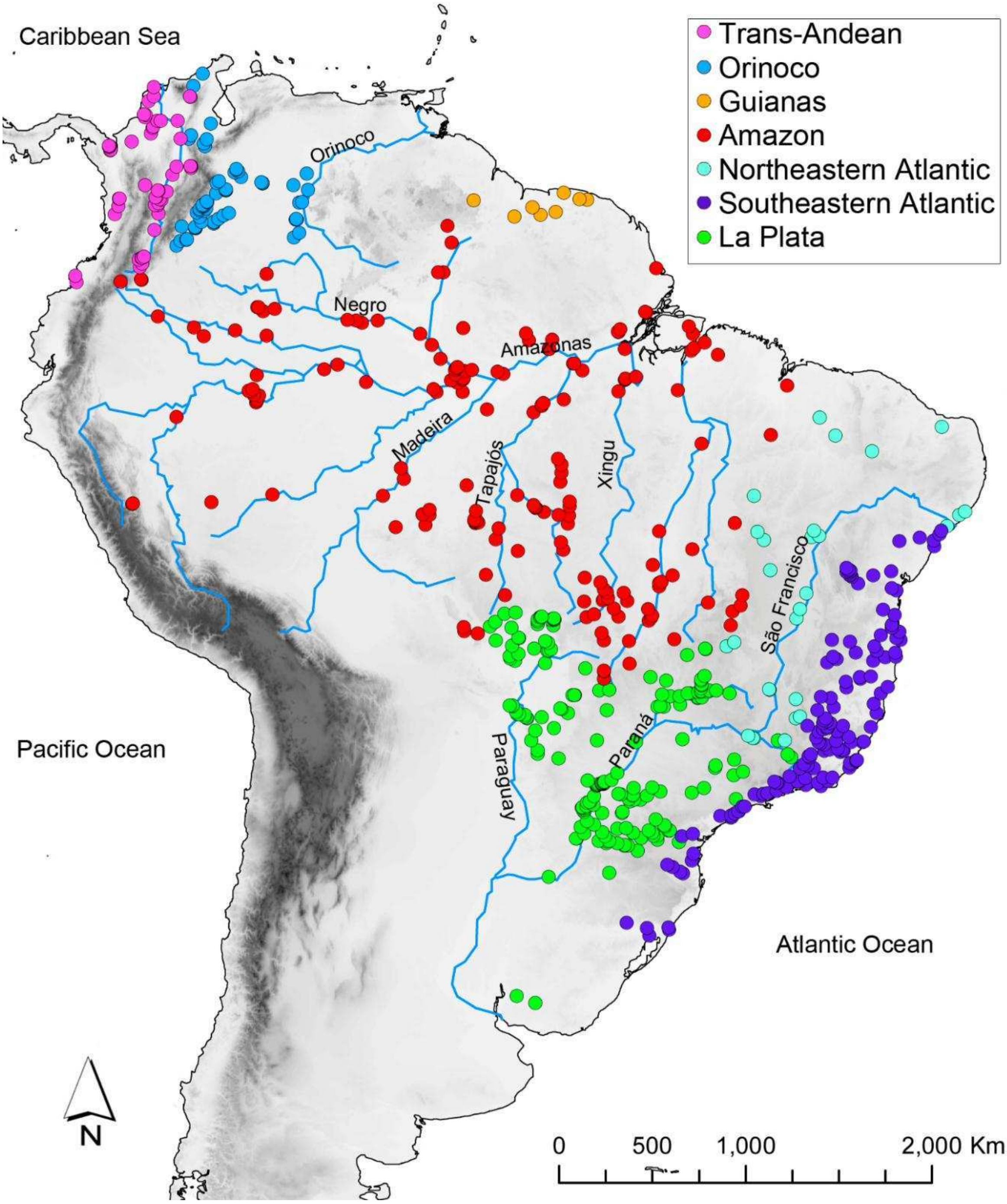


Figure 3.1 Sample distribution of *Hoplias malabaricus* in South America. Colors represents major river basins

To test the differences between groups (major river basins or type of system), we did a linear model using the “procD.lm” function of the Geomorph package (Adams et al., 2013). We completed 1,000 permutations, allowing us to perform an Analysis of Variance (ANOVA). We also did pairwise comparisons of the groups using the “pairwise” function of the RRPP package (Collyer & Adams, 2018). Both tests were completed using the R software, while using a 95% confidence interval and the R software (R Core Team, 2018).

Samples included in this study are distributed geographically from 33° latitude South in Uruguay to 11° latitude North in Colombia. To test the effect of latitude on the trahira heads shapes, we performed a multivariate regression of the residuals from the allometry test with the latitude of each sampling size. We then used the 10,000 permutations to calculate how much of the shape variation could be explained by the latitude.

The effect of external climate pressures is much stronger in size than in organisms shape (Cardini et al., 2007). Bergmann's rule applied to endotherm vertebrates describes a pattern where organism body size increase in colder temperatures (Bergmann, 1847). We were unable to test the effect of size in this paper due to our collecting procedures. The differences in collection techniques from each museum could have influenced the size in the specimens, thus we were not able to evaluate for Bergmann's rule.

Results

Multivariate regression between size (Log centroid size) and shape (Regression score was comparable with Ibagon et al. (2019) result. We found the correlation to be significant ($P<0.001$) and that allometry accounts for 25.7% of shape variation (Fig. 3.2).

The main shape variation of the lateral view of the head among all specimens was explored in the morphospace defined by Principal Component (PC) 1 and PC2, which accounted for 44.01% of the total shape variance (Fig. 3.3).

When labeled according to major river basins, the PCA shows considerable overlap between the samples (Fig. 3.3a). However, the ANOVA [$F(6,1941)=11.072$, $Rsq=0.332$, Effect size $(Z)=10.803$, $p=0.001$] and pairwise distances show significant differences between all groups tested. We found a higher effect size between the

Amazon and La Plata and lower effect between La Plata and Northeastern Atlantic groups (Table 3.1). Major shape changes in the first two PC (Principal Components) concentrates on the head depth, snout position (dorsal, ventral, shortened or elongated) and opercular region expansion (Fig. 3.3c).

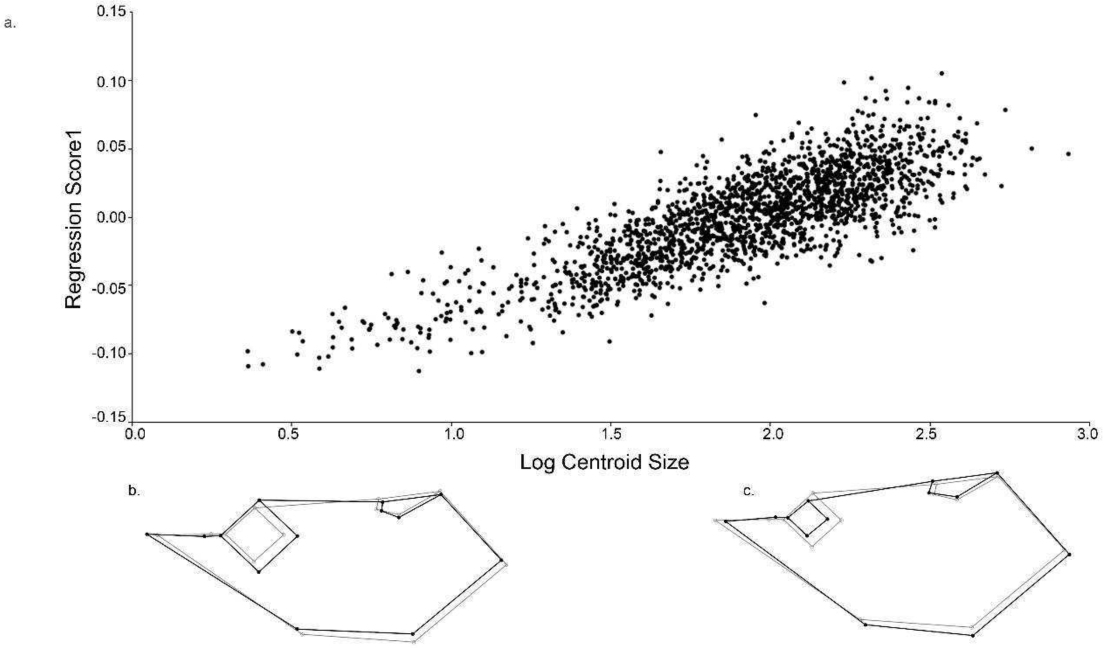


Figure 3.2 (a) Scatterplot of shape (regression score 1) and log-transformed centroid size of the lateral view of the head of *Hoplias malabaricus*. Wire frames show most negative (b) and positive (c) (-1.0 and +1.0) values (black) compared with the average shape (grey).

Table 3.1 Pairwise differences between means of *Hoplias malabaricus* from the major river basins. Z: effect size in the upper diagonal. P values in lower diagonal.

	1	2	2	4	5	6	7
1.Amazon	X	4.733	14.601	4.498	9.924	12.863	11.164
2. Guianas	0.001	X	4.001	5.112	3.772	5.042	6.494
3. La Plata	0.001	0.004	X	3.768	5.417	12.104	8.684
4. Notheastern	0.001	0.001	0.001	X	4.004	4.418	6.017
5. Orinoco	0.001	0.005	0.001	0.003	X	6.527	7.079
6.Southeastern	0.001	0.001	0.001	0.001	0.001	X	9.766
7.Trans-Andean	0.001	0.001	0.001	0.001	0.001	0.001	X

When labeled according to the type of ecosystem, the PCA shows a subtle and significant differentiation between lentic and lotic environments (Fig. 3.3b). This is supported by the results of the ANOVA [F(1,973)=10.764, Rsq=0.011, Effect size (Z)=5.020, p=0.001] and pairwise distances [Z(lentic – lotic)= 9.70, P=0.001]. Shapes differed mainly regarding snout position and length, showing that specimens from lentic systems on average had shorter snouts when compared to specimens from lotic systems (Fig 3.3d)

The multivariate regression between the shape (Regression Score) and latitude revealed that the distance from the Equator accounts for 5.6% of the shape variation and is significant (P-value:<0.0001) (Fig. 3.4).

Discussion

Different factors affect the shape of organisms. GM is the statistical tool to study the covariance between shape changes and their casual factors (Bookstein, 1991; Zelditch et al., 2004). GM had been used to explore cryptic species diversity (Klingenberg et al., 2003; Maderbacher et al., 2008; Santos & Araújo, 2014; Lamanna et al., 2016) and clinal variation (Cardini et al., 2007; Morales et al., 2018). In this study, we explored the *H. malabaricus* shape diversity, trying to understand the patterns of shape variation of freshwater fishes in the Neotropical region.

According to Ibagon et al. (2019), static allometry accounts for 23.3% of the head shape variation of *H. malabaricus* from the Brazilian Continental Margin. By adding a vast number of samples that account for almost the entire distribution of *H. malabaricus*, this study found that allometry accounts for 25,7% of their head shape variation. We found that the smaller animals present wider eyes than larger ones due to functional restrictions of the internal structures of the eye (Thompson, 1942), a pattern that is observed in other Characifomes (Castro et al., 2018).

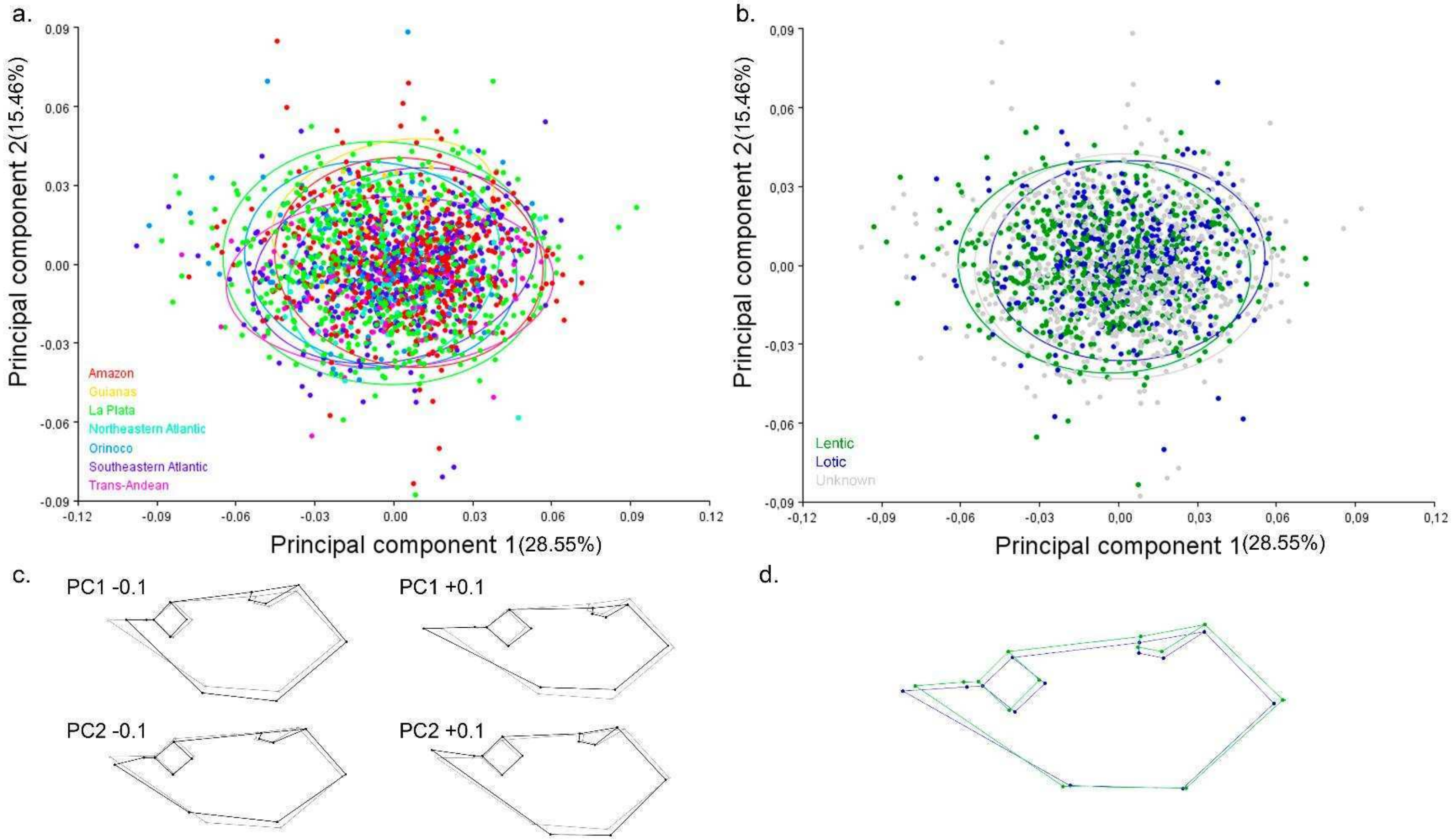


Figure 3.3 Morphospace of the lateral view of the head of *Hoplias malabaricus* from South America. Scatterplot of the first two components of the principal component analysis. (a) Labeled according to major river basins, (b) labeled by type of system and, (c) Principal Component (PC) shape changes, scale factor 0.1, (d) average shape of the residuals for type of system Lentic (green) or Lotic (blue), scale factor 3.

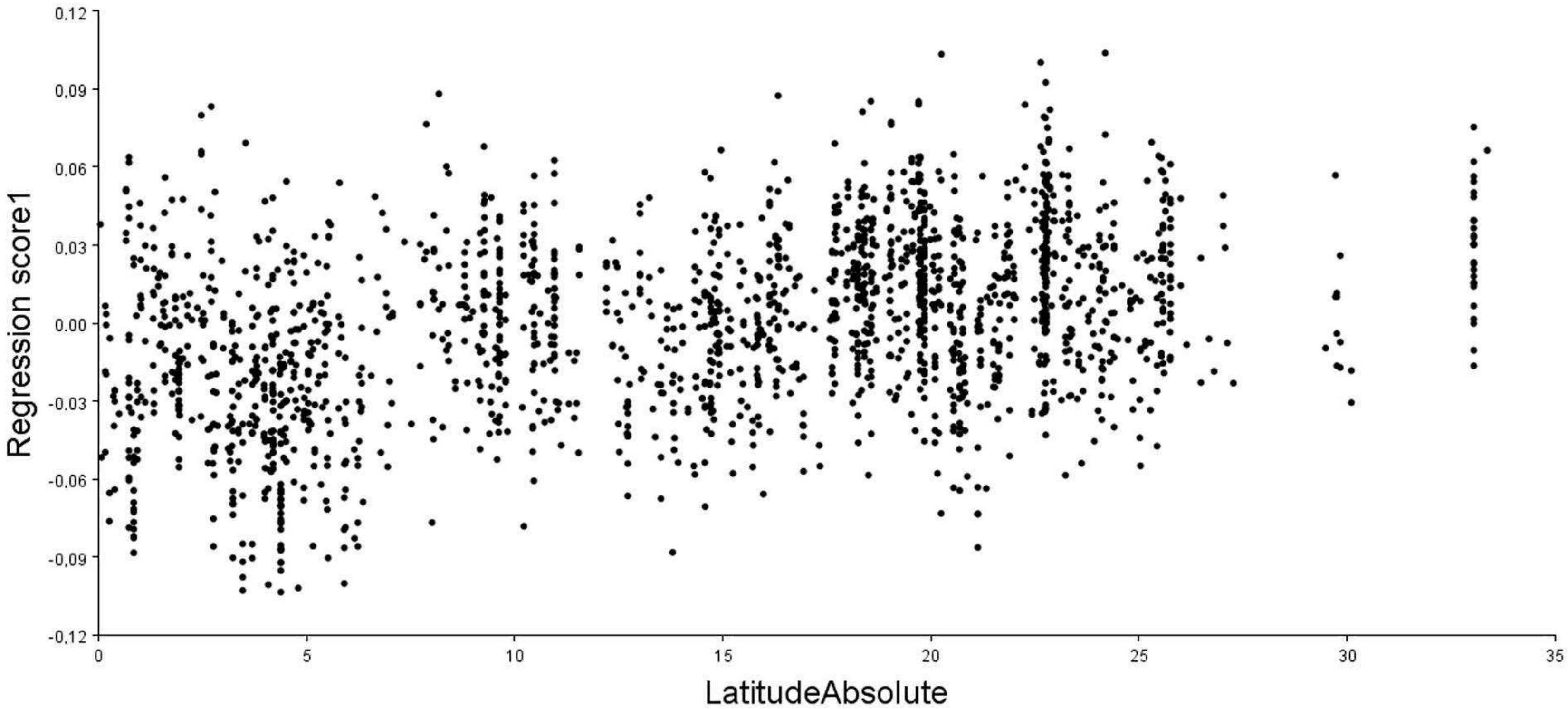


Figure 3.4 (a) Scatterplot of shape (regression score 1) of the lateral view of the head of *Hoplias malabaricus* and absolute value of latitude of the sample local.

The current configuration of the major river basins of South America results from the geomorphological evolution of the area since the drifting apart of Africa and South America, including the rise of the Andes during the last 20 Ma (Schaefer et al., 2011). The uplift of this mountain chain produced several modifications at the river courses over time, including headwater captures that allowed gene flow between populations inhabiting adjacent rivers and basins (Albert & Carvalho, 2011; Lima & Ribeiro, 2011).

Our results indicate a significant yet weak differentiation and a substantial overlap between the head shapes from the major river basins, a finding which can be affected by headwater capture events. Phylogeographic (Santos et al., 2009; Pereira et al., 2012) and phylogenetic (Cardoso et al., 2018; Jacobina et al., 2018) studies also found clues of the headwater capture events in South America, as they show that different hydrographic basins share the same evolutionary independent lineages.

The PCA labeled with the seven major river basins was very similar to the PCA labeled with the three phylogenetic clades found by Pereira et al. (2012) in a previous study (Ibagon et al., 2019). In both results, the variation within each group is much larger than between groups. We argue then that the long-term evolutionary isolation was not sufficient to produce a substantial change in the head shape, suggesting then that environmental factors have played essential roles in the maintenance of the shape diversity in the whole distribution of *H. malabaricus*.

A previous study found that the head shape differs according to the type of ecosystem (lentic or lotic) of their respective sample points (Ibagon et al. 2019), which is in line with other GM results (Santos & Araújo, 2014; Berbel-Filho et al., 2015; Haas et al., 2015). We interpreted this result as a consequence of phenotypic plasticity or divergence selection, as proposed by Langerhans (2008). The present work confirms that the effect of type of ecosystem is a consistent pattern o throughout the distribution of *H. malabaricus.* Future studies, including lab experiments, should distinguish if this pattern results from phenotypic plasticity, divergence selection, or a combination of both.

The present study sampling effort allowed us to test whether the latitude or the distance to the Equator affects the head shape of *H. malabaricus*. Our results indicate that the correlation was weakly significant, as each latitude corresponds to several different shapes. It is well-known that the latitude affects the shape of animals as we see in various organismal groups (Viguier, 2002; Frost et al., 2003; Monteiro et al., 2003), and our results confirm this. Latitude and temperature are correlated variables once high latitude places show lower annual temperature means than low latitude places. Habitat temperature has also ben shown to influence fishes body shape, as samples of the same species present deeper bodies in warmer waters and more elongated bodies in colder waters (Loy et al., 1996; Marcil et al., 2006). Our hypothesis considered latitude as a temperature proxy, but future studies should also take into account that rivers flow from high altitude towards low altitude places, as the water temperature results from a combination of latitude and elevation. Hence, it is possible that the several different shapes associated with each latitude are related to several altitude values within each river basin, blurring the results.

Our results show that the shape variation in *H. malabaricus* has multiple causes, including but not limited to: size, long-term isolation (Ibagon et al., 2019), river basin of origin, type of ecosystem and distance to the Equator. Since all of which produce significant yet effects (except size), we conclude that several other factors should contribute to the shape variation of this species complex. It is also possible that our findings result from the fact that *H. malabaricus* includes more than one species. Most of the species are likely widely distributed and maintain the same general shape, making it difficult to find the leading causes of shape variation.

Acknowledgements

The authors thank Carlos Luis DoNascimiento Montoya and Carlos Montaña from the Instituto de Investigación de Recursos Biológicos Alexander von Humboldt, Marcelo Britto and Cristiano Moreira from the Museu Nacional/UFRJ, Saúl Prada Pedreros and Javier Maldonado Ocampo from the Pontificia Universidad Javeriana, Jorge Dergam from the MZUFV, Alesio Datovo and Oswaldo Oyakawa from the MZUSP, Carla Simone Pavanelli from the Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura. This study was financed by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) (Finance Code 001) through the doctoral fellowship of Nicole Ibagón and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG).

References

Abell R., Thieme M.L., Revenga C., Bryer M., Kottelat M., Bogutskaya N., Coad B., Mandrak N., Balderas S.C., Bussing W., Stiassny M.L.J., Skelton P., Allen G.R., Unmack P.J., Naseka A., Ng R., Sindorf N., Robertson J., Armijo E., Higgins J. V., Heibel T.J., Wikramanayake E., Olson D., López H.L., Reis R.E., Lundberg J.G., Sabaj Pérez M.H., & P. Petry, 2008. Freshwater Ecoregions of the World: A New Map of Biogeographic Units for Freshwater Biodiversity Conservation. BioScience, 58, 403–414.

Adams D.C., Otárola-Castillo E., Adriaens D., & E. Otárola-Castillo, 2013. Geomorph: An r package for the collection and analysis of geometric morphometric shape data. Methods in Ecology and Evolution, 4, 393–399.

Albert J.S. & T. Carvalho, 2011. Neogene assembly of modern faunas. Historical Biogeography of Neotropical Freshwater Fishes (ed. by J.S. Albert and R.E. Reis), pp. 119–136. University of California Press, Berkeley Los Angeles London.

Albert J.S. & R.E. Reis, 2011. Historical Biogeography of Neotropical Freshwather Fishes. University of California Press, Berkeley Los Angeles London.

Antonelli A. & I. Sanmartín, 2011.Why are there so many plant species in the Neotropics? Taxon, 60, 403–414.

Azpelicueta M. de las M., Benítez M., Aichino D., & D. Mendez, 2015. A new species of the genus *Hoplias* (Characiformes, Erythrinidae), a tararira from the lower Paraná River , in Misiones , Argentina. Acta Zoologica Lilloana, 59, 71–82.

Berbel-Filho W.M., Martinez P.A., Ramos T.P.A., Torres R.A., Lima S.M.Q.Q., Pablo W.M.B., Telton A.M., Torres R.A., & S.M.Q.Q Lima, 2015. Inter- and intra-basin phenotypic variation in two riverine cichlids from northeastern Brazil: potential eco-evolutionary damages of Sao Francisco interbasin water transfer. Hydrobiologia, 766, 43–56.

Bergmann C, 1847. Über die Verhältnisse der Wärmeökonomie der Thiere zu ihrer Grösse. Gottinger Studien, 3, 595–708.

Bertollo L.A.C., Born G.G., Dergam J.A., Fenocchio A.S., & O Moreira-Filho., 2000. A biodiversity approach in the neotropical Erythrinidae fish, *Hoplias malabaricus*. Karyotypic survey, geographic distribution of cytotypes and cytotaxonomic considerations. Chromosome Research, 8, 603–613.

Bialetzki A., Nakatani K., Sanches P. V, & G, Baumgartner 2002. Spatial and temporal distribution of larvae and juveniles of *Hoplias* aff. *malabaricus* (Characiformes, Erythrinidae) in the Upper Paraná River floodplain, Brazil. Brazilian Journal of Biology, 62, 211–222.

Bookstein F.L. , 1991. Morphometric Tools for Landmark Data. Geometry and Biology. Cambridge University Press, Cambridge.

Brown J.H., Banks J., Belt T., Von A., Hooker J.D., & C. Darwin, 2014. Why are there so many species in the tropics? Journal of Biogeography, 1905, 8–22.

Burns M.D. & B.L Sidlauskas, 2018. Ancient and contingent body shape diversification in a hyperdiverse continental fish radiation. Evolution, 73, 569–587.

Cardini A., Jansson A., & S. Elton, 2007. A geometric morphometric approach to the study of ecogeographical and clinal variation in vervet monkeys. Journal of Biogeography, 34, 1663–1678.

Cardoso Y.P., Rosso J.J., Mabragana E., Gonzalez-Castro M., Delpiani M.S., Avigliano E., Bogan S., Covain R., Schenone N.F., & J.M. Díaz de Astarloa, 2018. A continental-wide molecular approach unraveling mtDNA diversity and geographic distribution of the Neotropical genus *Hoplias*. PLoS ONE, 13, 1–25.

Castro K.M.D.S.A., Santos M.P.D., Brito M.F.G., Bidau C.J., & P.A. Martinez, 2018. Ontogenetic allometry conservatism across five teleost orders. Journal of Fish Biology, 93, 745–749.

Collyer M.L. & D.C. Adams, 2018. RRPP : An r package for fitting linear models to high- - dimensional data using residual randomization. Methods in Ecology and Evolution, 9, 1772–1779.

Frost S.R., Marcus L.F., Bookstein F.L., Reddy D.P., & E. Delson, 2003. Cranial Allometry, Phylogeography, and Systematics of Large-Bodied Papionins (Primates: Cercopithecinae) Inferred From Geometric Morphometric Analysis of

Landmark Data. The Anatomical Record, 1072, 1048–1072.

Haas T.C., Heins D.C., & M.J. Blum, 2015. Predictors of body shape among populations of a stream fish (*Cyprinella venusta*, Cypriniformes: Cyprinidae). Biological Journal of the Linnean Society, 115, 842–858.

Ibagon N., Shalaguti B., & K. Yotoko, 2019. Allometry, long-term evolutionary isolation, and adaptation to different environments affecting shape diversity in the trahira *Hoplias malabaricus* (Teleostei: Erythrinidae). Journal of Fish Biology, in review.

Jacobina U.P., Affonso P.R.A.D.M., Carneiro P.L., & J.A. Dergam, 2009. Biogeography and comparative cytogenetics between two populations of *Hoplias malabaricus* (Bloch, 1794) (Ostariophysi: Erythrinidae) from coastal basins in the State of Bahia, Brazil. Neotropical Ichthyology, 7, 617–622.

Jacobina U.P., Lima S.M.Q., Maia D.G., Souza G., Batalha-Filho H., & R.A. Torres, 2018. DNA barcode sheds light on systematics and evolution of Neotropical freshwater trahiras. Genetica, 146, 505–515.

Klingenberg C.P., 2011. MorphoJ: an integrated software package for geometric morphometrics. Molecular Ecology Resources, 11, 353–357.

Klingenberg C.P., Barluenga M., & A. Meyer, 2003. Body shape variation in cichlid fishes of the *Amphilophus citrinellus* species complex. Biological Journal of the Linnean Society, 80, 397–408.

Lamanna F., Kirschbaum F., Ernst A.R.R., Feulner P.G.D., Mamonekene V., Paul C., & R. Tiedemann, 2016. Species delimitation and phylogenetic relationships in a genus of African weakly-electric fishes (Osteoglossiformes, Mormyridae, *Campylomormyrus*). Molecular Phylogenetics and Evolution, 101, 8–18.

Langerhans R.B., 2008. Predictability of phenotypic differentiation across flow regimes in fishes. Integrative and Comparative Biology, 48, 750–768.

Lima F.C.T.F. & A.C.A.A.C Ribeiro, 2011. Continental-scale tectonic controls of biogeography and Ecology. Historical Biogeography of Neotropical Freshwater Fishes (ed. by J.S. Albert and R.E. Reis), pp. 145–164. University of California Press, Berkeley Los Angeles London.

Loy A., Ciccotti E., Ferrucci L., & S. Cataudella, 1996. An Application of Automated Feature Extraction and Geometric Morphometrics : Temperature-related Changes in Body Form of Cyprinus curpio Juveniles. Aquacultural Engineering, 15, 301–311.

Maderbacher M., Bauer C., Herler J., Postl L., Makasa L., & C. Sturmbauer, 2008. Assessment of traditional versus geometric morphometrics for discriminating populations of the *Tropheus moorii* species complex (Teleostei: Cichlidae), a Lake Tanganyika model for allopatric speciation. Journal of Zoological Systematics and Evolutionary Research, 46, 153–161.

Marcil J., Swain D.P., & J.A. Hutchings, 2006. Genetic and environmental components of phenotypic variation in body shape among populations of Atlantic cod (*Gadus morhua*). Journal of the Linnean Society, 88, 351–365.

Monteiro L.R., Duarte L.C., & S. dos Reis, 2003. Environmental correlates of geographical variation in skull and mandible shape of the punar ´ e rat *Thrichomys apereoides* (Rodentia : Echimyidae). J. Zool. Lond, 261, 47–57.

Morales A.E., de la Mora M., & D. Piñeiro, 2018). Spatial and environmental factors predict skull variation and genetic structure in the cosmopolitan bat *Tadarida brasiliensis*. Journal of Biogeography, 1–12.

Orton M.G., May J.A., Lee D.J., & S.J. Adamowicz, 2019. Is molecular evolution faster in the tropics? Here, 122, 513–524.

Oyakawa O.T., 2003. Family Erythrinidae (Trahiras). Check List of the Freshwater Fishes of South and Central América (ed. by R. Reis, S. Kullander, and J.C. Ferraris), pp. 238–240. EDIPUCRS, Porto Alegre.

Pereira T.L., Santos U., Schaefer C.E., Souza G.O., Paiva S.R., Malabarba L.R., Schmidt E.E., & J.A .Dergam,2012. Dispersal and vicariance of *Hoplias malabaricus* (Bloch, 1794) (Teleostei, Erythrinidae) populations of the Brazilian continental margin. Journal of Biogeography, 40, 905–914.

R Core Team, 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. .

Rosso J.J., González-Castro M., Bogan S., Cardoso Y.P., Mebragaña E., Delpiani

M.S., & J.M. Díaz de Astarloa, 2018. Integrative taxonomy reveals a new species of the *Hoplias malabaricus* species complex (Teleostei: Erythrinidae). Ichthyological Exploration of Freshwaters, IEF-1076, 1–18.

Rosso J.J., Mabragaña E., González-Castro M., Delpiani M.S., Avigliano E., Schenone N., & J.M. Díaz de Astarloa, 2016. A new species of the *Hoplias malabaricus* species complex (Characiformes: Erythrinidae) from the La Plata River basin. Cybium, 40, 199–208.

Santos A.B.I. & F.G. Araújo, 2014. Evidence of morphological differences between *Astyanax bimaculatus* (Actinopterygii: Characidae) from reaches above and below dams on a tropical river. Environmental Biology of Fishes, 98, 183–191.

Santos U., Völcker C., Belei F., Cioffi M.B., & L.A.C. Bertollo, 2009. Molecular and karyotypic phylogeography in the Neotropical *Hoplias malabaricus* (Erythrinidae) fish in eastern Brazil. Journal of Fish Biology, 75, 2326–2343.

Schaefer S., Albert J.S., & R. Reis, 2011. The Andes: riding the tectonic uplift. Historical Biogeography of Neotropical Freshwater Fishes (ed. by J.S. Albert and R.E. Reis), pp. 259–279. Berkeley Los Angeles London.

Sember A., Bertollo L.A.C., Ráb P., Yano C.F., Hatanaka T., de Oliveira E.A., & M. de B. Cioffi,2018. Sex chromosome evolution and genomic divergence in the fish *Hoplias malabaricus* (Characiformes, Erythrinidae). Frontiers in Genetics, 9, 1–12.

Sidlauskas B.L., Mol J.A.N.H., & R.P Vari, 2011. Dealing with allometry in linear and geometric morphometrics : a taxonomic case study in the *Leporinus cylindriformis* group (Characiformes : Anostomidae) with description of a new species from Suriname. Zoological Journal of the Linnean Society, 162, 103–130.

Viguier B., 2002. Is the morphological disparity of lemur skulls (Primates) controlled by phylogeny and / or environmental constraints ? Biological Journal of the Linnean Society, 76, 577–590.

Zelditch M.L., Swiderski D.L., Sheets H.D.H.D., & W.L. Fink, 2004. Geometric Morphometrics for Biologist.

4. TRACING THE INFLUENCE OF THE NORTHERN ANDES UPLIFT IN *Hoplias malabaricus* (BLOCH, 1794) (TELEOSTEI: ERYTHRINIDAE) DIVERSIFICATION

Manuscript in preparation, to be submitted to Journal of Biogeography

Nicole Ibagón[1,2], Javier A. Maldonado-Ocampo[3], Gustavo A. Ballen[4], Marcelo de Bello Cioffi[5], Karla Yotoko[1], Jorge A. Dergam[2]

[1]. Departamento de Biologia Geral, Universidade Federal de Viçosa, Viçosa, Minas Gerais Brasil. [2]. Departamento de Biologia Animal, Universidade Federal de Viçosa, Viçosa, Minas Gerais Brasil. [3]. Laboratorio de Ictiología. Unidad de Ecología y Sistemática (UNESIS), Departamento de Biología, Facultad de Ciencias. Pontificia Universidad Javeriana. Bogotá, D.C., Colombia. [4]. Museu de Zoologia da Universidade de São Paulo, São Paulo,SP, Brazil. [5] .Departamento de Genética e Evolução, Universidade Federal de São Carlos, São Carlos, Brazil.

Correspondence: Nicole Ibagón, Departamento de Biologia Geral. Universidade Federal de Viçosa Av. P.H. Rolfs, s/n. 36570-000, Viçosa Minas Gerais, Brazil.

Email: nicoleibagon@gmail.com

Abstract

Aim: We explored the karyotype, morphometrics and molecular diversity of *Hoplias malabaricus* in Northern South America and evaluated the influence of the uplift of the Northern Andes in this diversity.

Location: Northern South America and Lower Central America, with emphasis on the Magdalena River.

Methods: We applied traditional and molecular cytogenetics techniques for samples collected along the Magdalena basin. We also used 2D geometric morphometrics tools to explore the shape diversity of the head of museum samples of *H. malabaricus* collected along the Magdalena basin, and proposed a dated phylogeny based on three gene partitions (two mitochondrial and one nuclear) of samples from Northern South America and Lower Central America .

Results: The Upper and Middle Magdalena populations have 2*n*=42 with a unique in situ fluorescence pattern, whereas the Lower Magdalena population has 2*n*=40, with a widespread fluorescent pattern. The head shapes of *H. malabaricus* from Upper/Middle significantly differ from those head shapes from Lower Magdalena. Molecular data exhibit two allopatric groups, diverged 5.46 mya. The first group contains Upper and Middle Magdalena with the Colombian Pacific slope and Lower Central America sequences. The second group includes Lower Magdalena and other Caribbean basins, Trinidad-Tobago, Guianas and the Orinoco and Amazon sequences.

Main conclusions: The Eastern Cordillera, in the Northern Andes, played a vicariance barrier role that split the *cis* and *trans*-Andean populations of *Hoplias malabaricus*. Later, during the last glaciations, populations from the cis-Andean side colonized the Caribbean region (including the Lower Magdalena) through connections between the rivers due to the low level of the sea.

Keywords: Cytogenetics, geometric morphometrics, freshwater fishes, trahira, species complex.

Introduction

The Neotropical region has the highest richness of freshwater fish in the world, with most of its diversity concentrated in few orders, and most taxa endemic to few basins (Albert et al., 2011). The Andes orogeny that started on the late Cretaceous and continued along the Cenozoic shaped the evolution and diversification of Neotropical fishes and determined such an endemic pattern (Lundberg et al., 1998; Schaefer et al., 2011). The Andes uprising caused drastic ecological transformations in the local fish fauna, determining the extinction of the former fishes of the basins (Lundberg et al., 1998). In each basin, the faunal composition is a result of extinction, speciation, vicariance, and dispersal events (Albert et al., 2011; Dagosta & Pinna, 2017).

In Northern South America (NSA), the Magdalena River Basin is the largest *trans*-Andean basin with 1,612 km. Its main river flows between the Central and Eastern Cordillera. The formation of the Magdalena Riber Basin coincides with the final uplift of the Eastern Cordillera, when this drainage separated from Orinoco-Amazon system (Lundberg et al., 1998; Albert et al., 2006; Wesselingh & Hoorn, 2011). The maximum elevation of this basin is 3,685 m above the sea level (Restrepo & Syvitski, 2006). This system harbors 222 fish species, or 15% of the Colombian ichthyofauna (DoNascimiento et al., 2017), from which 50% are endemic of the system. Magdalena River has a low richness of species when compared with its adjacent drainages, Orinoco and Amazon, harboring 1,000 and 2,000 fish species, respectively (Albert et al., 2011). Paleontological data suggest the extinction of at least four genera typical of the Amazon-Orinoco region: *Lepidosiren, Colossoma, Brachyplatystoma,* and *Arapaima* (Lundberg et al., 1986; Lundberg & Chernoff, 1992). Therefore, the Magdalena River is an intriguing scenario for studying the effects of the Andean orogeny and its consequences for speciation, extinction, and immigration of Neotropical freshwater fishes.

The predatory characiform *Hoplias malabaricus* (Bloch, 1794) is one of the few widespread species of the continent. It occurs in 41 out of the 52 ecoregions in South America and five out of 17 in Central America (Abell et al., 2008), from northern Argentina to Panama, on both sides of the Andes (Oyakawa, 2003). *H. malabaricus* is a species complex (Bertollo et al., 2000) containing at least seven karyomorphs (A-G) with 40 or 42 chromosomes, that differ in chromosome morphology, sex chromosomes systems (Bertollo et al., 2000; Santos et al., 2009; Cioffi & Bertollo, 2010), and occasionally hybridize (Utsunomia et al., 2014). Molecular studies pointed out that *H. malabaricus* contains several evolutionary units yet undescribed (Pereira et al., 2012; Marques et al., 2013).

Until now, studies involving phylogeography (Santos et al., 2009; Jacobina et al., 2011; Pereira et al., 2012; Marques et al., 2013) and cytogenetics (Bertollo et al., 1979, 2000; Jacobina et al., 2009; Blanco et al., 2010b) of *H. malabaricus* concentrated on populations of the oriental (*cis*) side of the Andes.

In this study, we used cytogenetics, geometric morphometrics (GM), and molecular biology to access the diversity of *H. malabaricus* from the Magdalena River and to evaluate the influence of the uplift of the Eastern Cordillera of Northern Andes in this species diversification.

Materials and methods

Specimens and Taxon Sampling

We sampled 213 individuals of *H. malabaricus* from NSA and Lower Central America (LCA) (Fig. 4.1, Appendix S1), from which 199 are from the Magdalena River. Upper Magdalena (UM) and Middle Magdalena (MM) sample points share the same physiographic characteristics: small, fast-flowing and clear water creeks (Galvis & Iván Mojica, 2007). Lower Magdalena (LM) sample points consist of lowland flooding plains, with marshes and lagoons (Galvis & Iván Mojica, 2007). Voucher specimens of cytogenetic were deposited in MPUJ (Museo de la Pontificia Univesidad Javeriana – Bogota, Colombia). The specimens were identified following Oyakawa (2003) and Oyakawa & Mattox (2009). The Ethical Committee on Animal Use guidelines (CEUA 29/2013) of Federal University of Viçosa approved the Protocols of this study.

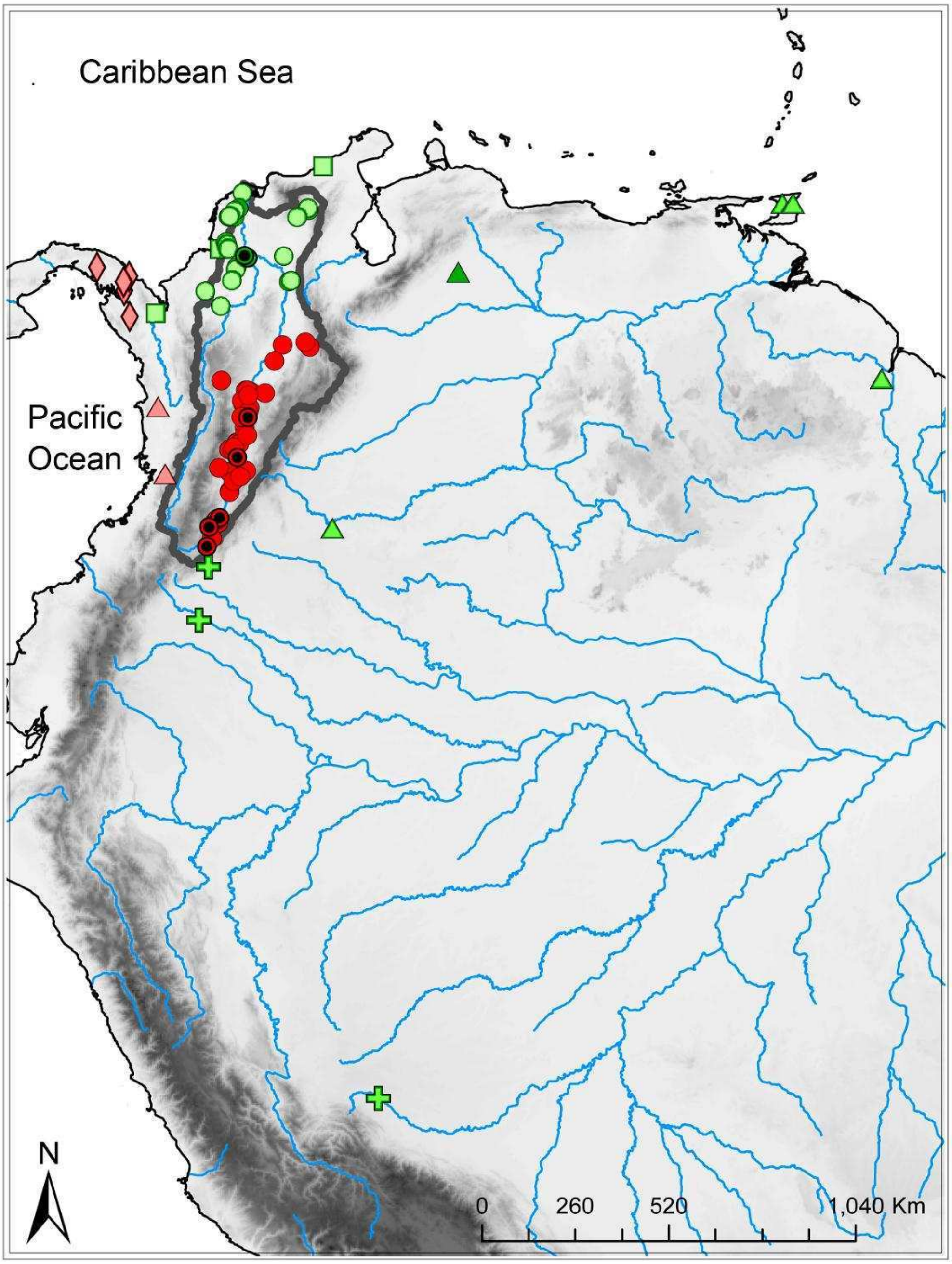


Figure 4.1 Collection sites of *Hoplias malabaricus* for molecular phylogeny, cytogenetics (black dot points) and Geometric morphometrics; Lower Central America (red diamonds), Pacific Slope of Colombia (red triangles), Upper Magdalena (UM)/Middle Magdalena (MM) (red circles), Lower Magdalena (LM) (green circles), other Caribbean basins (green squares), Orinoco (green triangles), the Magdalena river basin is circled in gray.

Cytogenetic analyses

We carried out cytogenetics with 32 specimens (10 from UM, nine from MM and 13 from LM) collected alive. We stimulated cell division with yeast 24 hours before processing (Molina, 2001). Fishes were euthanized with clove oil (Griffiths, 2000). Mitotic chromosomes were obtained from anterior kidney cell suspension using the conventional air-drying method (Bertollo et al., 2015). Chromosomes were stained with Giemsa 0.5% and images were obtained with an Olympus BX53 microscope using cellSens Dimensions software (Olympus). We analyzed at least 30 metaphase spreads per individual to confirm diploid number, karyotypic structure and FISH patterns. Chromosomes were classified as metacentric (m), submetacentric (sm) or acrocentric (a), based in arm ratios (Levan et al., 1964).

Fluorescence in situ hybridization (FISH) probes included three multigene families isolated from the *H. malabaricus* genome, and some microsatellite motif repeat probes. The first probe is a 5S rDNA copy containing 120 base pairs (bp) of the 5S rRNA encoding gene and 200 bp of the non-transcribed spacer (NTS) (Martins et al., 2006). The second probe contain a copy of the repetitive satellite 5S*HindIII*-DNA sequence with 360 bp composed by 95 bp segment similar to the 5S rRNA gene of the first probe and a 265 bp segment similar to the NTS of the first probe (Martins et al., 2006). The third probe correspond to a 1,400 bp segment of the 18S rRNA gene obtained via PCR from nuclear DNA (Cioffi et al., 2009a). The 5S*Hind*III-DNA and 5S rDNA were labelled with Biotin-14-dUTP by nick translation (Roche, Basel, Switzerland), while 18S rDNA was labelled with digoxinenin-11-dUTP, also by nick translation (Roche, Basel, Switzerland). Microsatellite motifs with sequences $(CA)_{15}$ and $(GA)_{15}$ were synthesized according to Kubat et al. (2008) and commercially labelled with Cy3 at the 5’ ends during synthesis (Sigma, St. Louis, MO, USA). All FISH experiments were carried out according to Yano et al., (2017). The 5S*Hind*III-DNA and 5S rDNA probes were detected with avidin-FITC (Sigma), while 18S DNA probe was detected with anti-digoxigenin-rhodamine (Roche).

Geometric Morphometrics

We studied the shape variation of the head of *H. malabaricus*, using 34 specimens from the UM, 36 from the MM, and 43 from the LM, deposited in four Colombian museums: CZUT-IC, IavH-P, ICNMHN and MPUJ (Fig. 4.1. Appendix S1). We choose the cephalic region because the formalin fixation cannot affect it. We selected specimens larger than 15 cm of standard length. We photographed the left side of the heads using a Nikon Coolpix P530 digital camera at different focal length, including a scale for size inference. All images were edited to overlap a plus-sign on the eye-aligned nostril, to define the location of the landmarks located around the eye. We built a tps file from images using TPS utility program Version 1.76 (Rohlf, 2015). We collected 13 landmarks according to Ibagon et al. (2019) using the tpsDIG2 2.31 (Rohlf, 2015). We aligned the landmarks coordinates using procrustes superimposition to eliminate variation in translation, and rotation. The coordinates were size-corrected by conducting a pooled within-group regression of the body shape data on log10 centroid size. Shape variation was explored with a PCA based on the covariance matrix of the residuals of the regression. Discriminant function analyses were performed using cross-validation between UM/MM and LM. All statistical analysis was performed in MorphoJ software 1.6 version (Klingenberg, 2011).

Phylogenetic analysis and Divergence Time Estimation

We extracted the total DNA of 74 samples of *H. malabaricus* (Appendix S1), using ethanol-preserved tissues (liver, heart or gill filaments) following Boyce et al. (1989). The PCR reaction was used to amplify and sequence two mitochondrial, ATP synthetase 6 (*ATPase 6*), Cytochrome oxidase subunit I gene (*COI*); and a nuclear gene: Recombination activating gene 2 (*RAG2*). The PCR products were purified using PEG8000 (20% polyethylene glycol, 2.5M NaCl) and sequenced on the platform of Macrogen (Macrogen, Seoul, South Korea). Sequences were deposited in GenBank (Appendix S1).

We also included 24 sequences available in GenBank of sister taxa (*Hoplias lacerdae, Hoplias intermedius, Hoplerythrinus unitaeniatus, Erythrinus erythrinus*, Lebiasinidae, Hepsetidae, Ctenolucidae) to calibrate and date the molecular hypothesis (Appendix S1). Sequences were aligned separately using Clustal W algorithm (Thompson et al., 1994) as implemented in MEGA 7.0 software (Kumar et al., 2016). We chose the best model of nucleotide evolution for each gene with MrModeltest 2.4 (Nylander, 2004) using the Akaike information criterion.

The phylogeny and divergence time estimation were carried out in Beast v2.4.6 (Bouckaert et al., 2014) considering the three genes as three partitions. We based the molecular clock on three calibration points, the first two based on fossil records: *Hoplias* sp. (Lundberg, 1997) cf. *Hoplias* sp. (Roberts, 1975) and the third based on the the date of the separation between South America and Trinidad island. Prior parameter specification and test for the isochron hypothesis were carried out with the Bayestools package under R v3.4.4 (R Core Team, 2018) using a relaxed lognormal clock model, and a birth-death tree prior. The Markov Chain Monte Carlo was set to run for 40.000.000 generations sampling each 5.000 and discarding the initial 25% as burn-in. We ran four independent analyses that attained convergence as assessed through ESS values (always above 200 for all parameters). We combined the post-burn-in samples afterwards, obtaining ESS values mostly above 1000, and always above 600. An additional round of analyses was carried out without data in order to sample from the prior and compare with the posterior distributions. Parameter-log and sampled-tree files were combined with logcombiner v2.4.6 (Bouckaert et al., 2014), and a summary tree prepared with treeannotator v2.4.6 (Bouckaert et al., 2014), the latter using the maximum clade-credibility tree and node heights corresponding to the median height.

We estimated the number of genetically diverged groups using a Bayesian analysis of population genetic structure BAPS (Corander et al., 2013) for the mitochondrial dataset, testing from 1 to 20 clusters with two replicates.

Results

Cytogenetics

We found two different karyotypes in the Magdalena River (Fig. 4.2). UM and MM samples present 2*n* = 42, FN = 84, and a karyotype composed of 22m +20sm, while

LM samples present 2*n* = 40, FN = 80, and a karyotype composed of 22m +18sm. Both karyomorphs present no morphologically differentiated sex chromosomes. UM and MM show 5S*Hind*III-DNA satellite probes restricted to chromosome pairs number 5 (in subtelomeric position) and 6 (in pericentromeric position). In sharp contrast, LM samples present eleven chromosome pairs hybridized with 5S*Hind*III-DNA probe in their pericentromeric regions (Fig. 4.2).

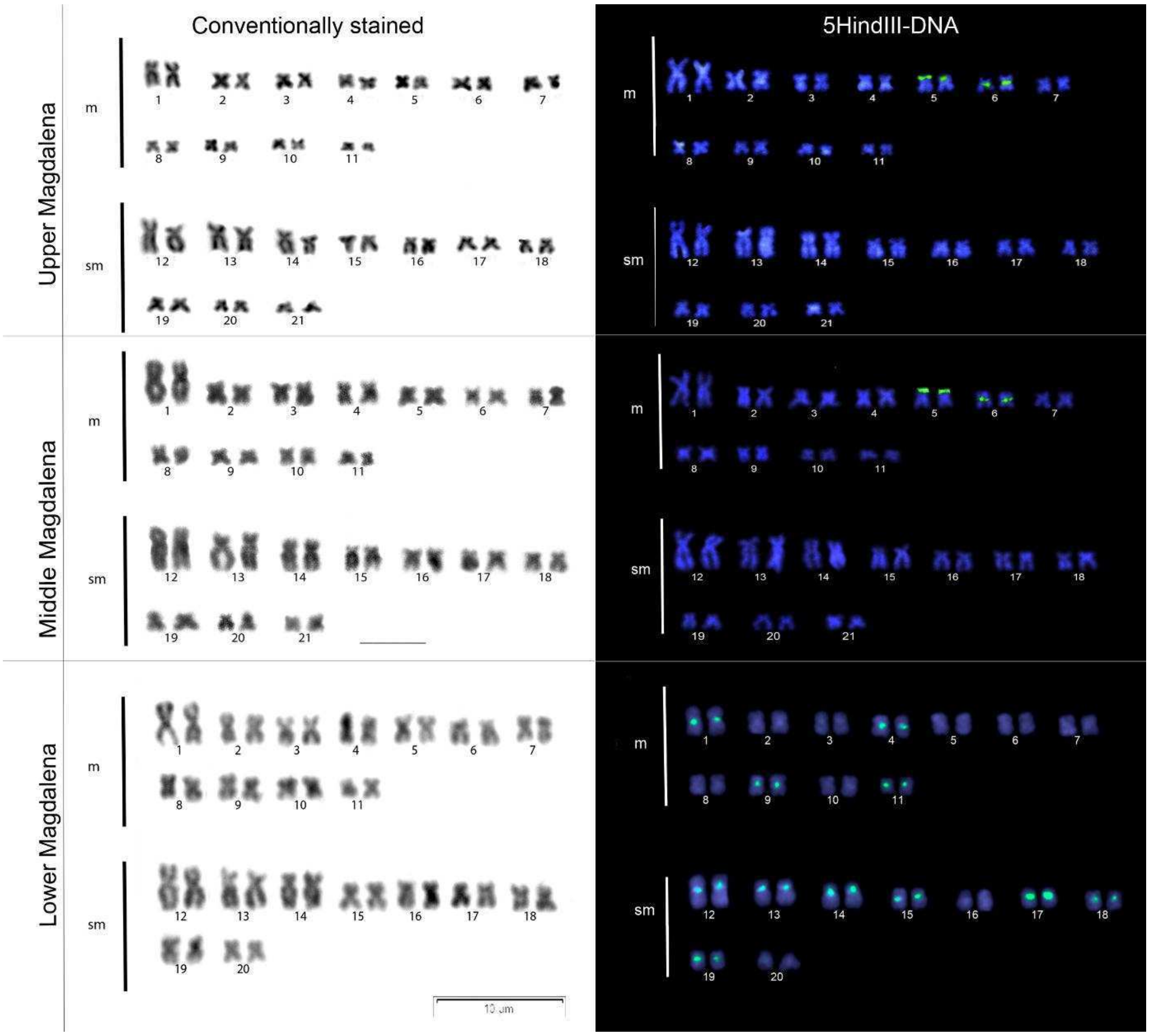


Figure 4.2 Conventionally stained karyomorphs and 5SHindIII-DNA probe of *Hoplias malabaricus* from the Upper (2n=42A), Middle (2*n*=42A) and Lower Magdalena Basin (2*n* =40C) in Colombia

Probes 5SrRNA and 18SrDNA shows differences between UM, MM and LM samples (Figure 4.3), as well as the repetitive $(CA)_{15}$ and $(GA)_{15}$ probes (Figure 4.4). The UM samples present 5SrRNA marks on the subtelomeric region of the chromosome pair number 4 and the pericentromeric region of chromosome pair number 8. The MM samples present marks on the subpericentromeric pair number 4 and two conspicuous subtelomeric and pericentromeric marks on chromosome pair number 17. The LM samples present pericentromeric 5S rDNA sites on the chromosome pair number 6 and 10. For the 18S rDNA probe, the UM and LM samples have subtelocentric marks, in different chromosomes. In UM, the 18S rDNA probe marks both homologs of chromosome pairs 12 and 16, and one chromosome of the pair 21. In MM, this probe marks both homologs of chromosome pairs 15, 19, and 21. In LM, the 18S rDNA probe hybridized on the pericentromeric and telomeric region of chromosome pair number 4. The chromosome pair number 6 has a syntenic mark of 5S rDNA and 18S rDNA probes (Figure 4.3).

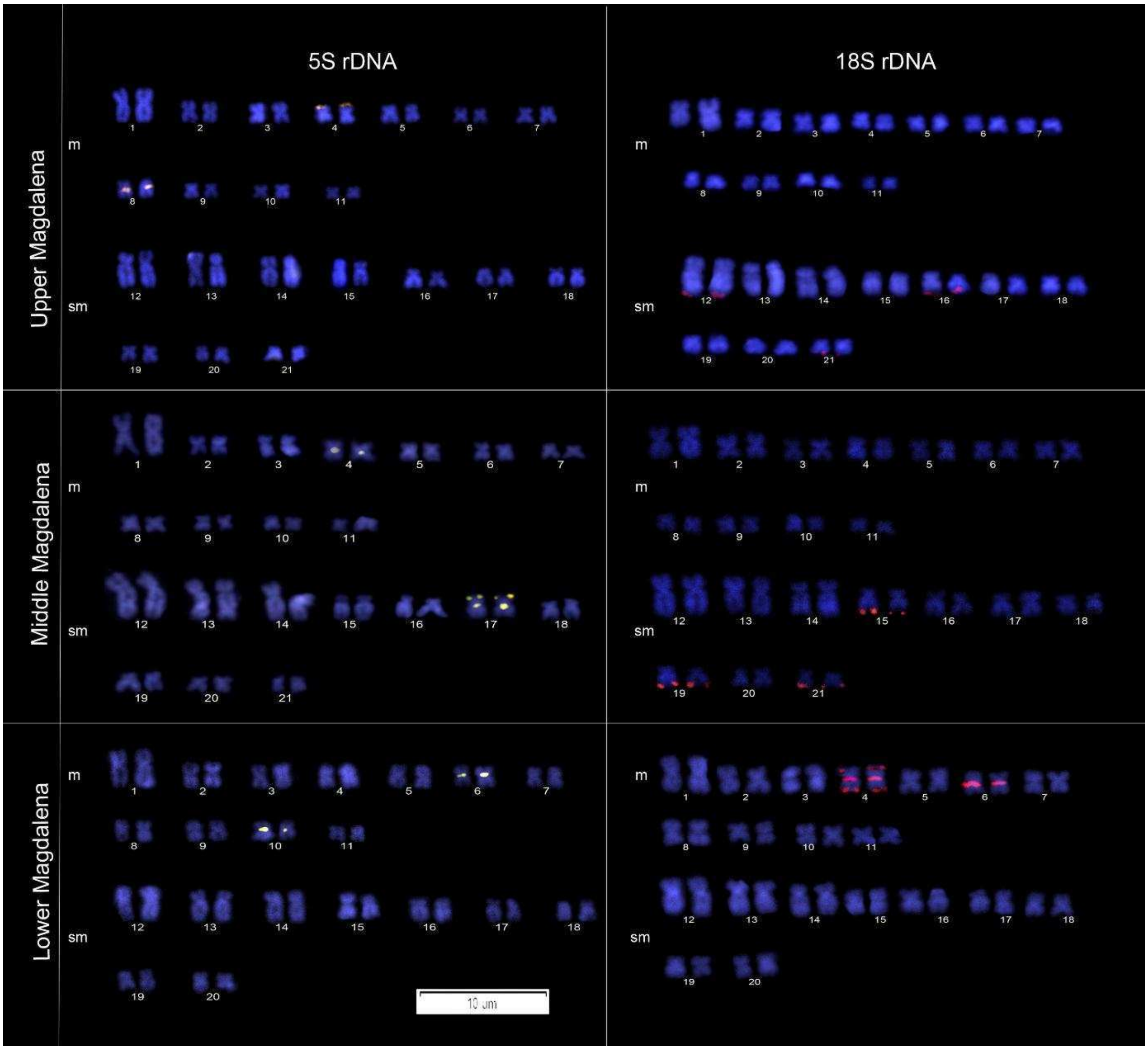


Figure 4 3. Karyomorphs with fluorescent in situ hybridization (FISH) using 5S rDNA and 18S rDNA probe of *Hoplias malabaricus* from a. the Upper (2n=42A), Middle (2n=42A) and Lower Magdalena River (2n =40C) in Colombia.

$(CA)_{15}$ probes were stronger on larger chromosomes, whereas $(GA)_{15}$ was more homogeneous among chromosome pairs. Additionally, conspicuous interstitial sites were observed in the largest two submetacentric pairs using both probes (**Figure 4.4**).

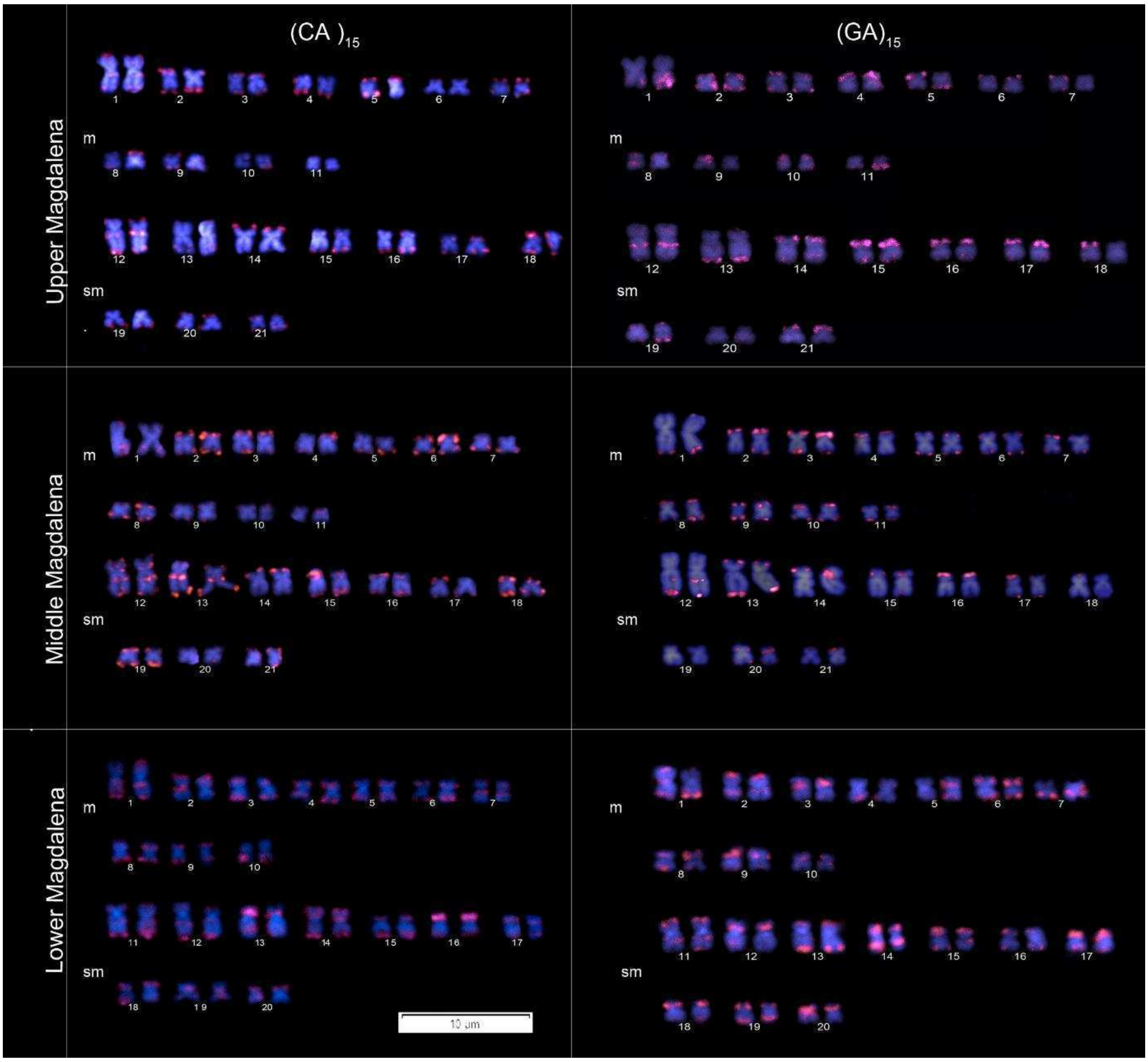


Figure 4.4. Karyomorphs with fluorescent in situ hybridization (FISH) using repetitive DNA probes $(CA)_{15}$ and $(GA)_{15}$ of *Hoplias malabaricus* from a. the Upper (2n=42A), Middle (2n=42A) and Lower Magdalena River (2n =40C) in Colombia.

Geometric morphometrics

Discriminant Function Analysis with the two sections from the Magdalena River, given as a prior group (UM/MM and LM) outcomes a graphic exhibiting the shape differences between them (Mahalanobis distance: 2.7, P=<.0001) (Fig 4.3.). Head deformation represented as wireframes indicates differences between UM/MM vs. LM. UM/MM samples exhibit a more fusiform shape, with an anterior-pointing rostrum. On the other hand, LM specimens displayed a deeper head and more dorsally pointing rostrum. Both populations also differed in the relative position of the posterior part of the head (opercular region), which was also deeper in the LM specimens. (Fig 4.3.).

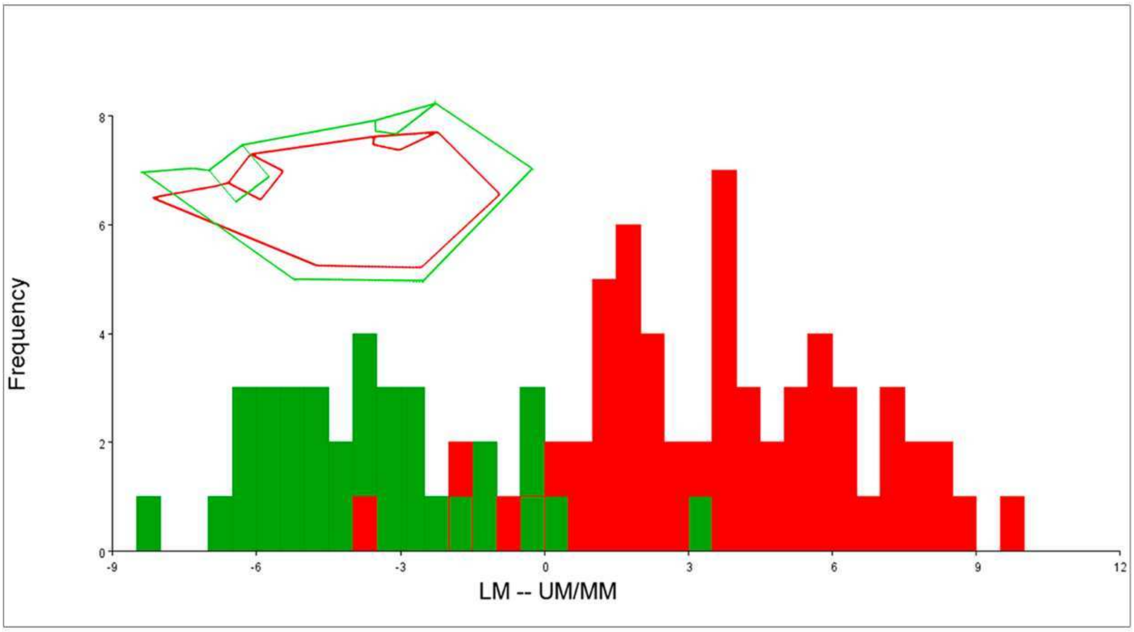


Figure 4.5 Scatter plot of discriminant scores between the two a priori groups UM/MM (red) and LM (green) of *Hoplias malabaricus*, wireframe graph for shape difference scale factor -3 and 3.

Phylogenetic analysis and Divergence Time Estimation

The phylogenetic hypothesis based on the concatenated mitochondrial and nuclear markers (*ATPase*6 = 1-587; *COI* = 588-1183; *RAG*2 = 1184-1203) recovered the Erytrinidae as a monophyletic group containing a clade including all the represented species of the genus *Hoplias* (Fig. 4.6). Inside *Hoplias*, we found a monophyletic group including all *H. malabaricus* sequences. The sequences of *H. malabaricus* from the Magdalena River (UM, MM, and LM - see methods) grouped into two different monophyletic clades, including other basins samples and diverged from 3.3 to 8.2 mya. The first clade (the green groups in Fig. 4.6) diverged from 1.85

to 4.9 mya and contains the *cis*-Andean basins: Amazon, Caribbean, Guyana, Orinoco, Trinidad samples, and the LM population. LM and the Caribbean basins samples form a monophyletic clade of 0.78 – 2.13 my. The second clade (the red groups of Fig. 4.6) has two monophyletic groups: the first containing the other *trans*-Andean samples: the Pacific slope of Colombia and LCA; and the second containing the samples from UM and MM. These groups diverged from 1.2 to 4.2 mya.

Discussion

We found congruence among cytogenetic, geometric morphometrics, and molecular data. The overall pattern shows an unexpectedly sharp discontinuity between UM/MM and LM samples along the Magdalena River.

Cytogenetics

Hoplias malabaricus is the Neotropical species with more cytogenetic studies, all of them concerning the *cis*-Andean populations. Such studies detected seven karyomorphs (A-G) with 40 or 42 chromosomes (Bertollo et al. 2000). The present study is the first one conducted on *trans*-Andean populations. The UM/MM karyomorph was identified as 2*n*=42A, whereas the LM karyomorph was identified as a 2*n*=40C (*sensu* Bertollo et al., 2000)) (Fig. 4.2). Da Rosa et al. (2014) showed that these karyomorphs are the most widespread in the Brazilian rivers: they listed all the locations where *H. malabaricus* was cytogenetically studied and found that 2*n*=42A and 2*n*=40C occur in 37 and 21 out of 79 localities, respectively. Indeed, the 2*n*=42A occurs in Argentina, at the southernmost distribution range of *H. malabaricus* (Grassi et al., 2017).

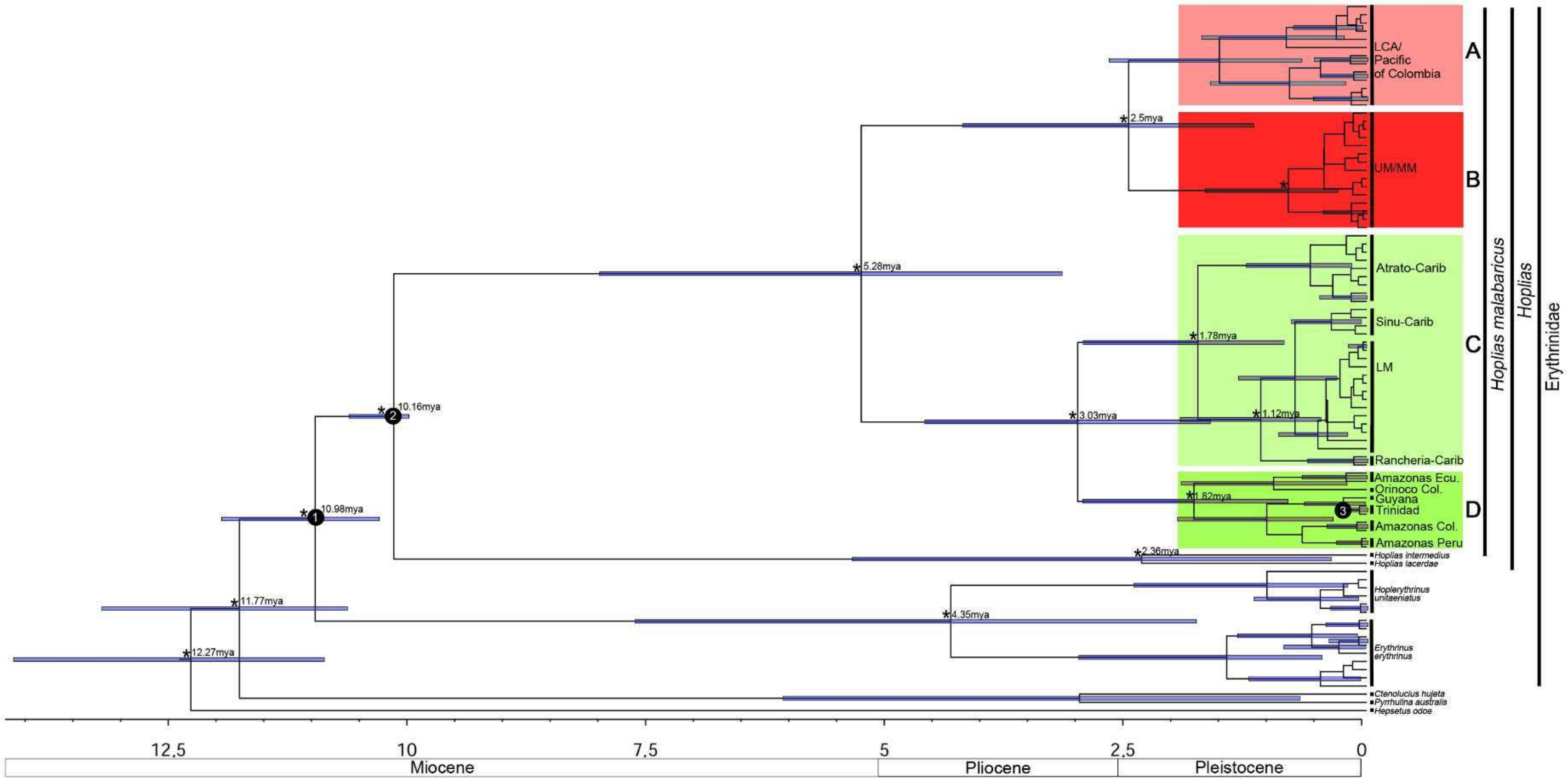


Figure 4.6 Time-Calibrated phylogeny of *Hoplias malabaricus* from Northern South America and Lower Central America, inferred by Bayesian analyses based in three gene partitions (Atpase6, COI, and RAG2). The four colours indicate BAPS clusters. Calibration points: 1) cf. *Hoplias* sp. (Roberts, 1975) calibrating the node for the Erythrinidae, 2) *Hoplias* sp. (Lundberg, 1997) calibrating the node for the genus *Hoplias* and 3. Date of the Separation between South America and Trinidad island and the continent. Values next to nodes indicate ages. Horizontal bars indicate 95% HPD. * indicates nodes with posterior probability of 1. A, B, C and D are genetically diverged groups according with BAPS.

FISH probes show sharp distinction between UM/MM and LM populations. The 5S*Hind*III-DNA probe is a satellite DNA family exclusive of *H. malabaricus* that, in *cis*-Andean karyomorphs, occurs in a large number of centromeric sites, ranging from 16 to 24 chromosomes in each metaphase, and always involves the largest three submetacentric pairs (Martins et al., 2006; Ferreira et al., 2007; Santos et al., 2009; Blanco et al., 2010b; Cioffi & Bertollo, 2010; Cioffi et al., 2011). The LM population also shows this general pattern. However, the UM and MM population exhibits telomeric and centromeric 5S*Hind*III-DNA marks on only two chromosome pairs (Fig. 4.2 and 4.7).

Typically, 5S rDNA sites are located in only one or two chromosomal pairs, showing high levels of variation within and among karyomorphs from different basins (Born & Bertollo, 2000; Ferreira et al., 2007; Santos et al., 2009; Blanco et al., 2010b, 2010a). The 18S rDNA probe marks from two to six chromosome pairs in the *cis*-Andean populations (Cioffi et al., 2009b; Blanco et al., 2010b). According, to our results, both markers allowed to differentiate samples within the UM/MM population and distinguish this population from the LM. Notably, the LM (2*n* = 40C) population presents a syntenic condition for both markers. Cioffi et al., 2009b observed this uncommon trait for the first time in a 2*n*=42A population from the Cuiabá River in Central Brazil. These syntenic conditions may represent rare and independent events in *H. malabaricus*.

Geometric Morphometrics

The body shape is a crucial aspect of the locomotor ability in fishes (Langerhans & Reznick, 2010). The two *H. malabaricus* populations of the Magdalena River (UM/MM and LM) show different head shapes. The UM/MM population exhibits a flattened and streamlined shape facilitating the swim in the fast-flowing and clear water creeks of these regions of the Magdalena River. In contrast, LM population shows a deeper head shape which does not make much difference for swimming in a lentic system as the environments of the LM. LM shows the mouth in a dorsal position, similar to the shape found in other species living in lentic habitats (Foster et al., 2015). Other studies, using *H. malabaricus* already indicated differences in between samples collected in lentic or lotic ecosystems, for regional or continental scales, the shape changes found by those authors also showed the same pattern (Ibagon et al. 2019a,b). We do not know whether shape differentiation along the Magdalena River is a product of phenotypic plasticity or natural selection acting on the divergence of these groups. Considering the differences in the physical structure along the Magdalena River, those modifications could represent an advantage when reproducing or feeding in the place where they live (Langerhans & Reznick, 2010).

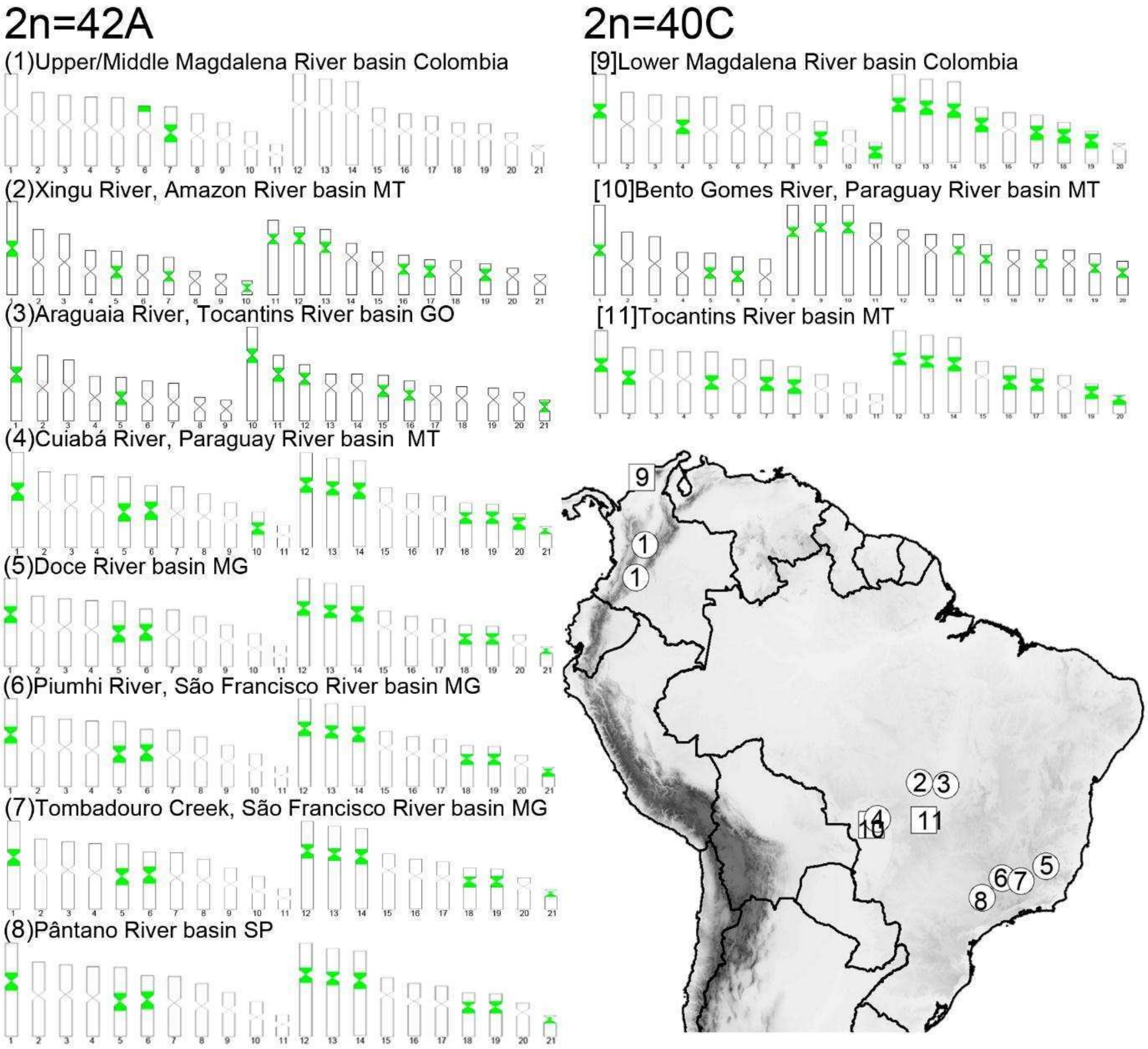


Figure 4 7. Geographical variation of 5SHindIII-DNA probe patterns in *Hoplias malabaricus*. Ideograms 1 and 9 correspond to the present study*, UM/MM samples exhibits the karyomorph 2n=42A, and two chromosomes stain, Lower Magdalena samples have 2n=40C karyomorph and several chromosomes stained. 2, 3 and 6 from Blanco et al 2010; 4, 5 and 8 from Cioffi et al 2009; 7 in Santos et al 2009; 10

from Cioffi et al 2009; 11 in Vitorino et al 2011. Circles in the map represents populations with karyomorph 2n=42A, squares show karyomorph 2n=40C from Bertollo et al. 2000.

Phylogenetic Analysis

Our phylogenetic hypothesis indicates that UM/MM and LM populations have independent evolutionary histories, revealing an unexpected sharp discontinuity along the Magdalena River despite the current absence of physical barriers along the river. Other sedentary aquatic organisms from the Magdalena River as the turtle *Podocnemis lewyana* also exhibit genetic differentiation between Upper and Lower Magdalena (Vargas-Ramírez et al., 2012). However, migrant fishes such as *Prochilodus magdalenae* and *Pseudoplatystoma fasciatum* exhibit a single large panmictic population along the river (Gallo & Díaz-Sarmiento, 2003; Aguirre-Pabón et al., 2013).

Hoplias malabaricus is a predator species with ambush strategy, highly adapted to long periods of starvation, which allow the surviving in small habitats for several generations. In general, these fishes occupy structured environments with an abundance of small fishes looking for refuge (Loureiro-crippa & Hahn, 1996; Pompeu & Godinho, 2001; Luz-Agostinho et al., 2009; Montenegro et al., 2013). Besides, *H. malabaricus* exhibits low vagility and lentic water preferences (Luz-Agostinho et al., 2006). Therefore, most populations remain isolated, and the main channel of the Magdalena River could work as an ecological barrier for *H. malabaricus*, inducing genetic discontinuity along the Magdalena River.

The divergence between the two principal clades (3.3 - 8.2 mya) *of H. malabaricus* (Fig. 4.6) was probably the consequence of the discrete uplift of the Eastern Cordillera (See Appendix S2), which timing is still controversial. Some authors suggest that the Eastern Cordillera in Colombia and the Mérida Andes were positive by middle to late Miocene (16.0 – 5.0 mya) (Gamero, 1996; Lundberg et al., 1998). In contrast, other authors suggest a complicated tectonic history for the Andes of NSA, arguing that the Eastern Cordillera in Colombia uplifted in pulses, with some areas being positive during the late Paleocene (58.0-55.0 mya) (e.g., Santander Massif, Bayona et al., 2013) and other regions uplifting from middle Eocene to middle

Miocene (47.0 - 5.0 mya) (e.g., Perijá Ranges and central Eastern Cordillera; (Bayona et al., 2010, 2013; Caballero & Parra, 2010; Ayala et al., 2012; Ochoa et al., 2012) or even during the Pliocene (5.0 – 2.0 mya, Gregory-wodzicki, 2000). Given such differences, it is not recommended to calibrate the phylogeny using one or other set of ages associated with the uplift of these mountain chains. Still, the divergence time between clades I and II agrees with the most recent periods of both time estimations. Studies involving terrestrial vertebrates and invertebrates also dated the divergence caused by the uplift of the NSA Andes since the late Miocene until the Pleistocene (Weir & Price, 2011; Ferreira et al., 2017; Bartoleti et al., 2018; Salgado-Roa et al., 2018).

In the Lower Magdalena, Miocene-Pleistocene arid conditions during the glacial periods caused profound changes in the neotropical rivers (Wesselingh & Hoorn, 2011), causing the extinction of freshwater biota (Galvis, 1997), including *H. malabaricus*. At the same time, marine regressions could cause freshwater faunal exchange between NSA basins by coastal stream capture along the coastline or across the Perijá Range (Albert et al., 2006). Our data suggest a dispersal process from some *cis*-Andean Basins to Caribbean basins (Atrato, Sinu, Rancheria Rivers, LM, among others). The shallow and quiet waters of the Amazonas and Orinoco basins resemble those of the Caribbean basins (Lundberg et al., 2010), allowing the surviving of *H. malabaricus* in the new environment. The limit of such dispersion seems to be the Atrato River, which presents a high degree of fauna turnover with LCA, probably because of the Baudó Range, which is the southernmost portion of the Cordillera of Lower Meso America (See Appendix S2) (Smith & Bermingham, 2005).

The first clade (red groups in Fig. 4.6) joins all the *trans*-Andean samples: LCA-Pacific, and UM/MM, which are currently separated by the Western and Central Cordillera (see Appendix S2). Maldonado-Ocampo (unpublished results) found a genetic similarity between Pacific and UM populations of some groups of fishes (*Bryconamericus, Creagrutus, Pesudopimelodus,* and *Pimelodella*), suggesting a fluvial connection between the upper portion of the Cauca and the Magdalena rivers (paleo Cauca-Magadalena) (Camilo Montes, personal communication). According to our divergence time estimation, such fluvial connection closed 4.16 to 1.19 mya. Regarding the LCA-Pacific cluster, our phylogenetic hypothesis contains two clades. The first includes only LCA samples, while the second one comprises LCA and Pacific samples. Further studies would be necessary to address the relationship of *H. malabaricus* in those geographic regions.

Conclusions

Our results show a clear Miocene/Pliocene vicariance between the *cis* and *trans*-Andean *H. malabaricus* groups, caused by the uplift of the Eastern Cordillera. Within the Magdalena Basin, we found both groups, an evidence of the dispersion from the *cis*-Andean populations to the Caribbean basins (including the Lower Magdalena region).

Acknowledgements

This research was supported by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). We are grateful to the Smithsonian Tropical Research Institute (STRI) in Panamá for sharing tissues, Pedro Caraballo (Universidad de Sucre - Colombia) and Cristhian Conde (Universidade Federal de Viçosa - Brazil) for logistic support in the field. Gustavo A. Ballen is supported by FAPESP (processes 2014/11558-5 and 2016/02253-1) Marcelo de Bello Cioffi is supported by FAPESP (2016/22196-2) and CNPQ (304992/2015-1). Divergence time estimation was performed on the *Zungaro* server at LBP-UNESP funded by FAPESP (process 2014/26508-3).

Author contributions

J.D. and N.I. conceived and designed the study, G.A.B., J. D., K.Y., M.C., N.I. and W. A. analysed the data, J.D., G.A.B., K.Y. and N.I.wrote the paper. All authors reviewed, edited and approved the final version of the manuscript.

Supporting information

Supplementary information accompanies this paper:

Competing financial interests

The authors declare no competing financial interests.

References

Abell R., Thieme M.L., Revenga C., Bryer M., Kottelat M., Bogutskaya N., Coad B., Mandrak N., Balderas S.C., Bussing W., Stiassny M.L.J., Skelton P., Allen G.R., Unmack P.J., Naseka A., Ng R., Sindorf N., Robertson J., Armijo E., Higgins J. V., Heibel T.J., Wikramanayake E., Olson D., López H.L., Reis R.E., Lundberg J.G., Sabaj Pérez M.H., & Petry P. (2008) Freshwater Ecoregions of the World: A New Map of Biogeographic Units for Freshwater Biodiversity Conservation. BioScience, 58, 403–414.

Aguirre-Pabón J., Narváez Barandica J., & Castro García L. (2013) Mitochondrial DNA variation of the bocachico *Prochilodus magdalenae* (Characiformes, Prochilodontidae) in the Magdalena River Basin, Colombia. Aquatic Conservation: Marine and Freshwater Ecosystems, 23, 594–605.

Albert J.S., Lovejoy N.R., & Crampton W.G.R.R. (2006) Miocene tectonism and the separation of cis- and trans-Andean river basins: Evidence from Neotropical fishes. Journal of South American Earth Sciences, 21, 14–27.

Albert J.S., Petry P., & Reis R. (2011) Major Biogeographic and Phylogenetic Patterns. Historical Biogeography of Neotropical Freshwater Fishes (ed. by J.S. Albert and R. Reis), pp. 21–58. University of California Press, Berkeley Los Angeles London.

Ayala R.C., Bayona G., Cardona A., Ojeda C., Montenegro O.C., Montes C., Valencia V., & Jaramillo C. (2012) The paleogene synorogenic succession in the northwestern Maracaibo block: Tracking intraplate uplifts and changes in sediment delivery systems. Journal of South American Earth Sciences, 39, 93–111.

Bartoleti L.F. de M., Peres E.A., Fontes F. von H.M., da Silva M.J., & Solferini V.N. (2018) Phylogeography of the widespread spider *Nephila clavipes* (Araneae: Araneidae) in South America indicates geologically and climatically driven lineage diversification. Journal of Biogeography, .

Bayona G., Cardona A., Jaramillo C., Mora A., Montes C., Caballero V., Mahecha H., Lamus F., Montenegro O., Jimenez G., Mesa A., & Valencia V. (2013) Onset of fault reactivation in the Eastern Cordillera of Colombia and proximal Llanos Basin; response to Caribbean–South American convergence in early Palaeogene time. Geological Society, London, Special Publications, 377, 285–314.

Bayona G., Montenegro O., Cardona A., Jaramillo C., Lamus F., Morón S., Quiroz L., Ruiz M., Valencia V., Parra M., & Stockli D. (2010) Estratigrafía , procedencia , subsidencia y exhumación de las unidades Paleógenas en el Sinclinal de Usme , sur de la zona axial de la Cordillera Oriental. Geología Colombiana, 35, 5–35.

Bertollo L.A.C., Born G.G., Dergam J.A., Fenocchio A.S., & Moreira-Filho O. (2000) A biodiversity approach in the neotropical Erythrinidae fish, *Hoplias malabaricus*. Karyotypic survey, geographic distribution of cytotypes and cytotaxonomic considerations. Chromosome Research, 8, 603–613.

Bertollo L.A.C., Cioffi M.D.B., & Moreira-Filho O. (2015) Direct chromosome preparations from Freshwater teleost fishes. Fish techniques, Ray-Fin Fishes and Chondrichthyans (ed. by C. Ozouf-Costaz, E. Pisano, F. Foresti, and L.F. de Almeida-Toledo), pp. 21–26. CRC Press, Boca Raton.

Bertollo L.A.C., Takahashi C.S., & Moreira-Filho O. (1979) Karyotypic studies of two allopatric populations of the genus *Hoplias*. Revista Brasileira de Genética, II, 17–37.

Blanco D.R., Lui R.L., Bertollo L.A.C., Diniz D., & Moreira-Filho O. (2010a) Characterization of invasive fish species in a river transposition region: Evolutionary chromosome studies in the genus *Hoplias* (Characiformes, Erythrinidae). Reviews in Fish Biology and Fisheries, 20, 1–8.

Blanco D.R., Lui R.L., Bertollo L.A.C., Margarido V.P., & Moreira-Filho O. (2010b) Karyotypic diversity between allopatric populations of the group *Hoplias malabaricus* (Characiformes: Erythrinidae): Evolutionary and biogeographic considerations. Neotropical Ichthyology, 8, 361–368.

Born G.G. & Bertollo L.A.C. (2000) An XX/XY sex chromosome system in a fish species, *Hoplias malabaricus*, with a polymorphic NOR-bearing X chromosome. Chromosome Research, 8, 111–118.

Bouckaert R., Heled J., Kühnert D., Vaughan T., Wu C.-H., Xie D., Suchard M. a., Rambaut A., & Drummond A.J. (2014) BEAST 2: A Software Platform for Bayesian Evolutionary Analysis. PLoS Computational Biology, 10, 1–6.

Boyce T., Zwick M., & Aquadro C. (1989) Mitochondrial DNA in the bark weevils: size, structure and heteroplasmy. Genetics, 123, 825–836.

Caballero V. & Parra M. (2010) Levantamiento De La Cordillera Oriental De Colombia Durante El Eoceno Tardío – Oligoceno Temprano : Proveniencia Sedimentaria En El Sinclinal De Nuevo Mundo. Boletín de Geología, 32, 45–77.

Cioffi M.B. & Bertollo L.A.C. (2010) Initial steps in XY chromosome differentiation in *Hoplias malabaricus* and the origin of an X(1)X(2)Y sex chromosome system in this fish group. Heredity, 105, 554–561.

Cioffi M.B., Martins C., & Bertollo L.A.C. (2009a) Comparative chromosome mapping of repetitive sequences. Implications for genomic evolution in the fish, *Hoplias malabaricus*. BMC genetics, 10, 11.

Cioffi M.B., Molina W.F., Moreira-Filho O., & Bertollo L.A.C. (2011) Chromosomal distribution of repetitive DNA sequences highlights the independent differentiation of multiple sex chromosomes in two closely related fish species. Cytogenetic and Genome Research, 134, 295–302.

Corander J., Cheng L., Marttinen P., & Tang J. (2013) BAPS : Bayesian Analysis of Population Structure. Manual v 6.0. Bioinformatics, 1–28.

Dagosta F.C.P. & Pinna M. de (2017) Biogeography of Amazonian fishes: deconstructing river basins as biogeographic units. Neotropical Ichthyology, 15, 1–24.

DoNascimiento C., Herrera-collazos E.E., Herrera-r G.A., Ortega-lara A., Villa-navarro F.A., Saulo J., Oviedo U., Maldonado-Ocampo J.A., Herrera E.E., Herrera G., Ortega-lara A., Villa-navarro F.A., Usma J.S., & Maldonado-Ocampo J.A. (2017) Checklist of the freshwater fishes of Colombia: a Darwin Core alternative to the updating problem. ZooKeys, 708, 25–138.

Ferreira I.A., Bertollo L.A.C., & Martins C. (2007) Comparative chromosome mapping of 5S rDNA and 5SHindIII repetitive sequences in Erythrinidae fishes (Characiformes) with emphasis on the *Hoplias malabaricus* “species complex.” Cytogenetic and Genome Research, 118, 78–83.

Ferreira M., Aleixo A., Ribas C.C., & Santos M.P.D. (2017) Biogeography of the Neotropical genus *Malacoptila* (Aves: Bucconidae): the influence of the Andean orogeny, Amazonian drainage evolution and palaeoclimate. Journal of Biogeography, 44, 748–759.

Foster K., Bower L., & Piller K. (2015) Getting in shape: Habitat-based morphological divergence for two sympatric fishes. Biological Journal of the Linnean Society, 114, 152–162.

Gallo H. & Díaz-Sarmiento J. (2003) Variabilidad genética del bagre rayado *Pseudoplatystoma fasciatum* (Pisces: Pimelodidae) en el río Magdalena (Colombia). Revista de la Academia Colombiana de las Ciencias, 27(105), 559–606.

Galvis G. (1997) Peces del Catatumbo. Asociación Cravo Norte, Santa Fe de Bogotá.

Galvis G. & Iván Mojica J. (2007) The Magdalena River fresh water fishes and fisheries. Aquatic Ecosystem Health & Management, 10, 127–139.

Gamero M.L.D. De (1996) The changing course of the Orinoco River during the Neogene: a review. Palaeogeography, Palaeoclimatology, Palaeoecology, 123, 385–402.

Grassi D.J., Swarça A.C., Dergam J.A., Pastori M.C., & Fenocchio A.S. (2017) Cytogenetic characterization of *Hoplias malabaricus* (Bloch, 1794) from the Ctalamochita River (Córdoba, Argentina): first evidence for southernmost populations of this species complex and comments on its biogeography. Comparative Cytogenetics, 11, 15–28.

Gregory-Wodzicki K. (2000) Uplift history of the Central and Northern Andes : A review. GSA Bulletin2, 112, 1091–1105.

Griffiths S. (2000) The use of clove oil as an anaesthetic and method for sampling intertidal rockpool fishes. Journal of Fish Biology, 57, 1453–1464.

Ibagon N., Shalaguti B., & Yotoko K. (2019a) Allometry, long-term evolutionary isolation, and adaptation to different environments affecting shape diversity in the trahira *Hoplias malabaricus* (Teleostei: Erythrinidae). Journal of Fish Biology, *in review*.

Ibagon, N. Yotoko, K. (2019b). The shape variation in *Hoplias malabaricus*: an effect of multiple ecogeographic causes.

Jacobina U.P., Affonso P.R.A.D.M., Carneiro P.L., & Dergam J.A. (2009) Biogeography and comparative cytogenetics between two populations of *Hoplias malabaricus* (Bloch, 1794) (Ostariophysi: Erythrinidae) from coastal basins in the State of Bahia, Brazil. Neotropical Ichthyology, 7, 617–622.

Jacobina U.P., Paiva E., & Dergam J.A. (2011) Pleistocene karyotypic divergence in *Hoplias malabaricus* (Bloch, 1794) (Teleostei: Erythrinidae) populations in southeastern Brazil. Neotropical Ichthyology, 9, 325–333.

Klingenberg C.P. (2011) MorphoJ: an integrated software package for geometric morphometrics. Molecular Ecology Resources, 11, 353–357.

Kumar S., Stecher G., & Tamura K. (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Molecular biology and evolution, 33, 1870–1874.

Langerhans R. & Reznick D. (2010) Ecology and Evolution of Swimming Performance in Fishes. Fish Locomotion: An Eco-ethological Perspective (ed. by O. Domenici and B. Kapoor), pp. 200–248. Enfield, Science Publishers, New Hampshire.

Levan A., Fredga K., & Sandberg A. (1964) Nomenclature for Centromeric Position on Chromosomes. Hereditas, 52, 201–220.

Loureiro-crippa V.E. & Hahn N.S. (1996) Dieta e Atividade Alimentar da Traira *Hoplias malabaricus* (Bloch, 1794) (Osteichthyes, Erythrinidae) nos primeiros anos de formação do reservatório de segredo - Pr. Acta Liminologica Brasiliensia, 8, 195–205.

Lundberg J.G. (1997) Freshwater fishes and their paleobiotic implications. Vertebrate Paleonotology in the Neotropics: The Miocene Fauna of La Venta (ed. by M. Kay, R.H. Hadden, R.L. Cifelli, and J.J. Flynn), pp. 67–92. Smithsonian Press, Washington D.C.

Lundberg J.G. & Chernoff B. (1992) A Miocene Fossil of the Amazonian Fish *Arapaima* (Teleostei, Arapaimidae) from the Magdalena River Region of Colombia - Biogeographic and Evolutionary Implications. Biotropica, 24, 2–14.

Lundberg J.G., Machado-Allison A., & Kay R.F. (1986) Miocene characid fishes from Colombia: evolutionary stasis and extirpation. Science, 234, 208–209.

Lundberg J.G., Marshall L.G., Guerrero J., Horton B.K., Malabarba M.C.S.L., & Wesselingh F. (1998) The Stage for Neotropical Fish Diversification: A History of Tropical South American Rivers. Phylogeny and Classification of Neotropical

Fishes (ed. by R. Reis, R.P. Vari, Z.M. Lucena, and C.A.S. Lucena), pp. 14–48. Porto Alegre.

Lundberg J.G., Pérez M.H.S., Dahdul W.M., & Aguilera O.A. (2010) The Amazonian Neogene Fish Fauna. Amazonia: Landscape and Species Evolution: A look into the past, 1 (ed. by C. Hoorn and F.P. Wesselingh), pp. 281–301. Wiley-Blackwell Publishing Ltd, Oxford.

Luz-Agostinho K., Agostinho A., Gomes L., Júlio-Jr. H., & Fugi R. (2009) Effects of flooding regime on the feeding activity and body condition of piscivorous fish in the Upper Paraná River floodplain. Brazilian Journal of Biology, 69, 481–490.

Luz-Agostinho K.D.G., Bini L.M., Fugi R., Agostinho A.A., & Julio J.H.F. (2006) Food spectrum and trophic structure of the ichthyofauna of Corumba reservoir, Parana river Basin, Brazil. Neotropical Ichthyology, 4, 61–68.

Marques D., Santos F., Silva S., Sampaio I., & Rodrigues L. (2013) Cytogenetic and DNA barcoding reveals high divergence within the trahira, *Hoplias malabaricus* (Characiformes: Erythrinidae) from the lower Amazon River. Neotropical Ichthyology, 11, 459–466.

Martins C., Ferreira I.A., Oliveira C., Foresti F., & Galetti P.M. (2006) A tandemly repetitive centromeric DNA sequence of the fish *Hoplias malabaricus* (Characiformes: Erythrinidae) is derived from 5S rDNA. Genetica, 127, 133–141.

Molina W.F. (2001) An alternative method for mitotic stimulation in fish cytogenetics. Chromosome Science, 5, 149–152.

Montenegro A.K.A., Vieira A.C.B., Cardoso M.M.L., Souza J. enisa R.T. de, & Crispim M.C. (2013) Piscivory by *Hoplias* aff. *malabaricus* (Bloch, 1974): a question of prey availability? Acta Limnologica Brasiliensia, 25, 68–78.

Nylander J.A.A. (2004) MrModeltest. Program distributed by the author. Evolutionary Biology Centre, Uppsala University.

Ochoa D., Hoorn C., Jaramillo C., Bayona G., Parra M., & De la Parra F. (2012) The final phase of tropical lowland conditions in the axial zone of the Eastern Cordillera of Colombia: Evidence from three palynological records. Journal of South American Earth Sciences, 39, 157–169.

Oyakawa O.T. (2003) Family Erythrinidae (Trahiras). Check List of the Freshwater Fishes of South and Central América (ed. by R. Reis, S. Kullander, and J.C. Ferraris), pp. 238–240. EDIPUCRS, Porto Alegre.

Oyakawa O.T. & Mattox G. (2009) Revision of the Neotropical trahiras of the *Hoplias lacerdae* species-group (Ostariophysi: Characiformes: Erythrinidae) with descriptions of two new species. Neotropical Ichthyology, 7, 117–140.

Pereira T.L., Santos U., Schaefer C.E., Souza G.O., Paiva S.R., Malabarba L.R., Schmidt E.E., & Dergam J.A. (2012) Dispersal and vicariance of *Hoplias malabaricus* (Bloch, 1794) (Teleostei, Erythrinidae) populations of the Brazilian continental margin. Journal of Biogeography, 40, 905–914.

Pompeu P.D.S. & Godinho A.L. (2001) Mudança na dieta da traíra *Hoplias malabaricus* (Bloch) (Erythrinidae, Characiformes) em lagoas da bacia do rio Doce devido à introdução de peixes piscívoros. Revista Brasileira de Zoologia, 18, 1219–1225.

R Core Team (2018) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. .

Roberts T.R. (1975) Characoid fish teeth from Miocene deposits in the Cuenca Basin, Ecuador. Journal of Zoology, 175, 259–271.

Rohlf F.J. (2015) The tps series of software. Hystrix, 26, 1–4.

da Rosa R., Vicari M.R., Dias A.L., & Giuliano-Caetano L. (2014) New insights into the biogeographic and karyotypic evolution of *Hoplias malabaricus*. Zebrafish, 11, 198–206.

Salgado-Roa F., Pardo-Diaz C., Lasso E., Arias C., Solferini V.N., & Salazar C. (2018) Gene flow and Andean uplift shape the diversification of *Gasteracantha cancriformis* (Araneae: Araneidae) in Northern South America. bioRxiv, 250977,

Santos U., Völcker C., Belei F., Cioffi M.B., & Bertollo L.A.C. (2009) Molecular and karyotypic phylogeography in the Neotropical *Hoplias malabaricus* (Erythrinidae) fish in eastern Brazil. Journal of Fish Biology, 75, 2326–2343.

Schaefer S., Albert J.S., & Reis R. (2011) The Andes: riding the tectonic uplift. Historical Biogeography of Neotropical Freshwater Fishes (ed. by J.S. Albert and R.E. Reis), pp. 259–279. Berkeley Los Angeles London.

Smith S. a. & Bermingham E. (2005) The biogeography of lower Mesoamerican freshwater fishes. Journal of Biogeography, 32, 1835–1854.

Thompson J.D., Higgins D.G., & Gibson T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic acids research, 22, 4673–4680.

Utsunomia R., Pansonato Alves J.C., Paiva L.R.S., Costa Silva G.J., Oliveira C., Bertollo L. a. C., & Foresti F. (2014a) Genetic differentiation among distinct karyomorphs of the wolf fish *Hoplias malabaricus* species complex (Characiformes, Erythrinidae) and report of unusual hybridization with natural

Vargas-Ramírez M., Stuckas H., Castaño-Mora O.V., & Fritz U. (2012) Extremely low genetic diversity and weak population differentiation in the endangered Colombian river turtle *Podocnemis lewyana* (Testudines: Podocnemididae). Conservation Genetics, 13, 65–77.

Weir J.T. & Price M. (2011) Andean uplift promotes lowland speciation through vicariance and dispersal in Dendrocincla woodcreepers. Molecular Ecology, 20, 4550–4563.

Wesselingh F. & Hoorn C. (2011) Geological development of Amazon and Orinoco basins. Historical Biogeography of Neotropical Freshwater Fishes pp. 59–68.

Yano C.F., Bertollo L.A.C., & Cioffi M.D.B. (2017) Fish-FISH: Molecular Cytogenetics in Fish Species. Fluorescence In Situ Hybridization (FISH) Application Guide pp. 429–443. Springer Berlin Heidelberg,

Appendix

Appendix S1. Specimens used with collection code, species, region.CT: Cytogenetics. GM: Geometric morphometrics

CODE	SPECIES	REGION/BASIN	CT	Molecular Biology (Access Number)			GM
				ATP6	COI	*RAG2*	
AM-139	*Hoplias malabaricus*	Amazon		OK	OK	OK	
AM-181	*Hoplias malabaricus*	Amazon		OK	OK	OK	
STRI-366	*Hoplias malabaricus*	Amazon		OK	OK	OK	
STRI-474	*Hoplias malabaricus*	Amazon		OK	OK	OK	
STRI-9449	*Hoplias malabaricus*	Amazon		OK	OK	OK	
STRI-9450	*Hoplias malabaricus*	Amazon		OK	OK	OK	
CZUTIC011771_1D4	*Hoplias malabaricus*	Caribbean					OK
CZUTIC011771_2D4	*Hoplias malabaricus*	Caribbean					OK
CZUTIC011771_3D4	*Hoplias malabaricus*	Caribbean					OK
CZUTIC011771_4D4	*Hoplias malabaricus*	Caribbean					OK
CZUTIC011859_1D1	*Hoplias malabaricus*	Caribbean					OK
CZUTIC014881_1D1	*Hoplias malabaricus*	Caribbean					OK
IAvHP3387_NN0001	*Hoplias malabaricus*	Caribbean					OK
IAvHP3387_NN0002	*Hoplias malabaricus*	Caribbean					OK
IAvHP3387_NN0003	*Hoplias malabaricus*	Caribbean					OK
IAvHP6374_NN0001	*Hoplias malabaricus*	Caribbean					OK
IAvHP6375_NN0001	*Hoplias malabaricus*	Caribbean					OK
IAvHP6375_NN0002	*Hoplias malabaricus*	Caribbean					OK
IAvHP6376_NN0001	*Hoplias malabaricus*	Caribbean					OK
IAvHP6378_NN0001	*Hoplias malabaricus*	Caribbean					OK
IAvHP6379_N0001I	*Hoplias malabaricus*	Caribbean					OK
IAvHP6380_NN0001	*Hoplias malabaricus*	Caribbean					OK
IAvHP7260_NN0001	*Hoplias malabaricus*	Caribbean					OK
IAvHP7289_NN0001	*Hoplias malabaricus*	Caribbean					OK

IAvHP7290_NN0001	*Hoplias malabaricus*	Caribbean	OK
IAvHP7450_NN0001	*Hoplias malabaricus*	Caribbean	OK
IAvHP7450_NN0002	*Hoplias malabaricus*	Caribbean	OK
IAvHP7451_NN0001	*Hoplias malabaricus*	Caribbean	OK
IAvHP7451_NN0002	*Hoplias malabaricus*	Caribbean	OK
IAvHP7452_NN0001	*Hoplias malabaricus*	Caribbean	OK
IAvHP7453_NN0001	*Hoplias malabaricus*	Caribbean	OK
IAvHP7454_NN0001	*Hoplias malabaricus*	Caribbean	OK
ICNMHN000022_1D1	*Hoplias malabaricus*	Caribbean	OK
ICNMHN000384_1D1	*Hoplias malabaricus*	Caribbean	OK
ICNMHN000391_1D1	*Hoplias malabaricus*	Caribbean	OK
ICNMHN000829_1D1	*Hoplias malabaricus*	Caribbean	OK
ICNMHN002224_1D1	*Hoplias malabaricus*	Caribbean	OK
ICNMHN006815_1D2	*Hoplias malabaricus*	Caribbean	OK
ICNMHN006815_2D2	*Hoplias malabaricus*	Caribbean	OK
ICNMHN008892_1D1	*Hoplias malabaricus*	Caribbean	OK
ICNMHN008904_1D1	*Hoplias malabaricus*	Caribbean	OK
ICNMHN009615_1D1	*Hoplias malabaricus*	Caribbean	OK
ICNMHN009659_1D1	*Hoplias malabaricus*	Caribbean	OK
ICNMHN009681_1D1	*Hoplias malabaricus*	Caribbean	OK
ICNMHN009717_1D2	*Hoplias malabaricus*	Caribbean	OK
ICNMHN009717_2D2	*Hoplias malabaricus*	Caribbean	OK
ICNMHN009737_1D2	*Hoplias malabaricus*	Caribbean	OK
ICNMHN009737_2D2	*Hoplias malabaricus*	Caribbean	OK
ICNMHN009745_1D1	*Hoplias malabaricus*	Caribbean	OK
ICNMHN009774_1D1	*Hoplias malabaricus*	Caribbean	OK
ICNMHN009783_1D1	*Hoplias malabaricus*	Caribbean	OK
ICNMHN009786_1D1	*Hoplias malabaricus*	Caribbean	OK
ICNMHN009801_1D1	*Hoplias malabaricus*	Caribbean	OK
ICNMHN009822_1D1	*Hoplias malabaricus*	Caribbean	OK
ICNMHN009828_1D3	*Hoplias malabaricus*	Caribbean	OK

ICNMHN009828_2D3	*Hoplias malabaricus*	Caribbean				OK
ICNMHN009828_3D3	*Hoplias malabaricus*	Caribbean				OK
ICNMHN010843_1D1	*Hoplias malabaricus*	Caribbean				OK
ICNMHN017262_1D1	*Hoplias malabaricus*	Caribbean				OK
ICNMHN019446_1D2	*Hoplias malabaricus*	Caribbean				OK
ICNMHN019446_2D2	*Hoplias malabaricus*	Caribbean				OK
JAM1383	*Hoplias malabaricus*	Caribbean	OK	OK	OK	
JAM1384	*Hoplias malabaricus*	Caribbean	OK	OK	OK	
JAM1385	*Hoplias malabaricus*	Caribbean	OK	OK	OK	
NI052	*Hoplias malabaricus*	Caribbean	OK		OK	
NI053	*Hoplias malabaricus*	Caribbean	OK		OK	
NI054	*Hoplias malabaricus*	Caribbean	OK	OK	OK	
NI055	*Hoplias malabaricus*	Caribbean	OK	OK	OK	
NI056	*Hoplias malabaricus*	Caribbean	OK	OK	OK	
NI057	*Hoplias malabaricus*	Caribbean	OK	OK	OK	
STRI-11560	*Hoplias malabaricus*	Central America	OK		OK	
STRI-1762	*Hoplias malabaricus*	Central America	OK	OK	OK	
STRI-26846	*Hoplias malabaricus*	Central America	OK	OK	OK	
STRI-26847	*Hoplias malabaricus*	Central America	OK		OK	
STRI-3644	*Hoplias malabaricus*	Central America	OK	OK	OK	
STRI-4174	*Hoplias malabaricus*	Central America	OK	OK	OK	
STRI-7682	*Hoplias malabaricus*	Central America	OK	OK	OK	
STRI-7688	*Hoplias malabaricus*	Central America	OK	OK	OK	
ROM-4410	*Hoplias malabaricus*	Guyana	OK	OK		
IAvHP11321_NN0001	*Hoplias malabaricus*	Magdalena - LM				OK
IAvHP11322_NN0001	*Hoplias malabaricus*	Magdalena - LM				OK
IAvHP11323_NN0001	*Hoplias malabaricus*	Magdalena - LM				OK
IAvHP12742_NN0001	*Hoplias malabaricus*	Magdalena - LM				OK
IAvHP13494_NN0001	*Hoplias malabaricus*	Magdalena - LM				OK
IAvHP7838_NN0001	*Hoplias malabaricus*	Magdalena - LM				OK
IAvHP7838_NN0002	*Hoplias malabaricus*	Magdalena - LM				OK

IAvHP7850_NN0001	*Hoplias malabaricus*	Magdalena - LM					OK
IAvHP7850_NN0002	*Hoplias malabaricus*	Magdalena - LM					OK
IAvHP7850_NN0003	*Hoplias malabaricus*	Magdalena - LM					OK
IAvHP7877_NN0001	*Hoplias malabaricus*	Magdalena - LM					OK
ICNMHN0000608_1D1	*Hoplias malabaricus*	Magdalena - LM					OK
ICNMHN000275_1D1	*Hoplias malabaricus*	Magdalena - LM					OK
ICNMHN003670_1D1	*Hoplias malabaricus*	Magdalena - LM					OK
ICNMHN010956_1D1	*Hoplias malabaricus*	Magdalena - LM					OK
ICNMHN017983_1D1	*Hoplias malabaricus*	Magdalena - LM					OK
ICNMHN018396_1D2	*Hoplias malabaricus*	Magdalena - LM					OK
ICNMHN018396_2D2	*Hoplias malabaricus*	Magdalena - LM					OK
MPUJ013214_NI047	*Hoplias malabaricus*	Magdalena - LM	OK	OK	OK	OK	OK
MPUJ013215_NI040	*Hoplias malabaricus*	Magdalena - LM	OK				OK
MPUJ013215_NI041	*Hoplias malabaricus*	Magdalena - LM	OK				OK
MPUJ013215_NI042	*Hoplias malabaricus*	Magdalena - LM	OK	OK	OK	OK	OK
MPUJ013215_NI043	*Hoplias malabaricus*	Magdalena - LM	OK		OK	OK	OK
MPUJ013215_NI044	*Hoplias malabaricus*	Magdalena - LM	OK				OK
MPUJ013215_NI045	*Hoplias malabaricus*	Magdalena - LM	OK	OK	OK	OK	OK
MPUJ013215_NI046	*Hoplias malabaricus*	Magdalena - LM	OK				OK
MPUJ013216_NI048	*Hoplias malabaricus*	Magdalena - LM	OK				OK
MPUJ013216_NI049	*Hoplias malabaricus*	Magdalena - LM	OK				OK
MPUJ013216_NI050	*Hoplias malabaricus*	Magdalena - LM	OK				OK
MPUJ013216_NI051	*Hoplias malabaricus*	Magdalena - LM	OK				OK
MPUJ013217_NI036	*Hoplias malabaricus*	Magdalena - LM					OK
MPUJ013217_NI037	*Hoplias malabaricus*	Magdalena - LM					OK
MPUJ013217_NI038	*Hoplias malabaricus*	Magdalena - LM					OK
MPUJ013217_NI039	*Hoplias malabaricus*	Magdalena - LM	OK				OK
MPUJ013222_NI018	*Hoplias malabaricus*	Magdalena - LM					OK
MPUJ013222_NI019	*Hoplias malabaricus*	Magdalena - LM					OK
MPUJ013223_NI017	*Hoplias malabaricus*	Magdalena - LM		OK		OK	OK
MPUJ013224_NI011	*Hoplias malabaricus*	Magdalena - LM		OK		OK	OK

MPUJ013225_NI015	*Hoplias malabaricus*	Magdalena - LM	OK	OK	OK	OK
MPUJ013225_NI016	*Hoplias malabaricus*	Magdalena - LM	OK		OK	OK
MPUJ013227_NI012	*Hoplias malabaricus*	Magdalena - LM	OK		OK	OK
MPUJ013227_NI013	*Hoplias malabaricus*	Magdalena - LM	OK		OK	OK
MPUJ013227_NI014	*Hoplias malabaricus*	Magdalena - LM	OK		OK	OK
CZUT-IC 15626	*Hoplias malabaricus*	Magdalena - LM	OK	OK	OK	
JAM1373	*Hoplias malabaricus*	Magdalena - LM		OK	OK	
JAM1374	*Hoplias malabaricus*	Magdalena - LM	OK	OK	OK	
JAM580	*Hoplias malabaricus*	Magdalena - LM	OK	OK	OK	
JAM581	*Hoplias malabaricus*	Magdalena - LM	OK	OK	OK	
JAM582	*Hoplias malabaricus*	Magdalena - LM	OK	OK	OK	
JAM607	*Hoplias malabaricus*	Magdalena - LM	OK	OK	OK	
JAM608	*Hoplias malabaricus*	Magdalena - LM	OK	OK	OK	
JAM609	*Hoplias malabaricus*	Magdalena - LM	OK	OK	OK	
CZUTIC001266_1D1	*Hoplias malabaricus*	Magdalena - MM				OK
IAvHP10388_NN0001	*Hoplias malabaricus*	Magdalena - MM				OK
IAvHP10391_NN0001	*Hoplias malabaricus*	Magdalena - MM				OK
IAvHP11513_NN0001	*Hoplias malabaricus*	Magdalena - MM				OK
IAvHP11513_NN0002	*Hoplias malabaricus*	Magdalena - MM				OK
IAvHP12337_NN0001	*Hoplias malabaricus*	Magdalena - MM				OK
IAvHP13676_NN0001	*Hoplias malabaricus*	Magdalena - MM				OK
IAvHP13676_NN0002	*Hoplias malabaricus*	Magdalena - MM				OK
IAvHP4305_NN0001	*Hoplias malabaricus*	Magdalena - MM				OK
IAvHP4306_NN0001	*Hoplias malabaricus*	Magdalena - MM				OK
IAvHP8188_NN0001	*Hoplias malabaricus*	Magdalena - MM				OK
IAvHP8450_NN0001	*Hoplias malabaricus*	Magdalena - MM				OK
IAvHP8451_NN0001	*Hoplias malabaricus*	Magdalena - MM				OK
ICNMHN006490_1D2	*Hoplias malabaricus*	Magdalena - MM				OK
ICNMHN006490_2D2	*Hoplias malabaricus*	Magdalena - MM				OK
ICNMHN010406_1D1	*Hoplias malabaricus*	Magdalena - MM				OK

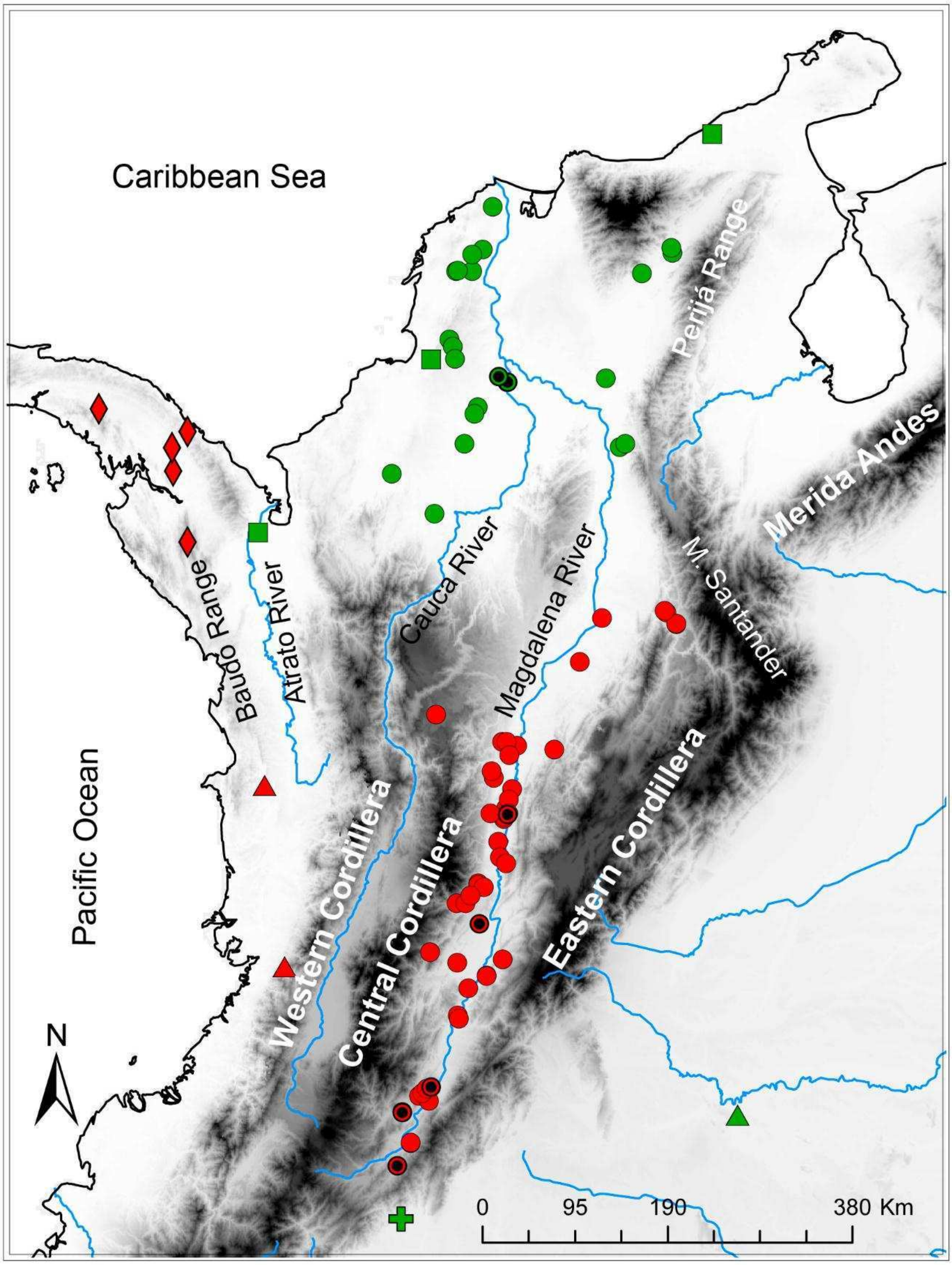
ICNMHN011074_1D1	*Hoplias malabaricus*	Magdalena - MM				OK
ICNMHN011091_1D1	*Hoplias malabaricus*	Magdalena - MM				OK
ICNMHN016339_1D1	*Hoplias malabaricus*	Magdalena - MM				OK
MPUJ013218_NI034	*Hoplias malabaricus*	Magdalena - MM	OK	OK	OK	OK
MPUJ013220_NI027	*Hoplias malabaricus*	Magdalena - MM	OK	OK	OK	OK
MPUJ013220_NI028	*Hoplias malabaricus*	Magdalena - MM	OK	OK	OK	OK
MPUJ013220_NI030	*Hoplias malabaricus*	Magdalena - MM	OK	OK	OK	OK
MPUJ013218_NI033	*Hoplias malabaricus*	Magdalena - MM	OK			
MPUJ013218_NI035	*Hoplias malabaricus*	Magdalena - MM	OK			
MPUJ013220_NI029	*Hoplias malabaricus*	Magdalena - MM	OK	OK	OK	
MPUJ013220_NI031	*Hoplias malabaricus*	Magdalena - MM	OK	OK	OK	
MPUJ013220_NI032	*Hoplias malabaricus*	Magdalena - MM	OK			
CZUTIC000042_2D2	*Hoplias malabaricus*	Magdalena - UM				OK
CZUTIC000101_1D1	*Hoplias malabaricus*	Magdalena - UM				OK
CZUTIC000106_1D1	*Hoplias malabaricus*	Magdalena - UM				OK
CZUTIC000191_1D1	*Hoplias malabaricus*	Magdalena - UM				OK
CZUTIC000256_1D1	*Hoplias malabaricus*	Magdalena - UM				OK
CZUTIC000528_1D1	*Hoplias malabaricus*	Magdalena - UM				OK
CZUTIC000531_1D1	*Hoplias malabaricus*	Magdalena - UM				OK
CZUTIC000837_1D1	*Hoplias malabaricus*	Magdalena - UM				OK
CZUTIC000868_1D1	*Hoplias malabaricus*	Magdalena - UM				OK
CZUTIC001071_1D3	*Hoplias malabaricus*	Magdalena - UM				OK
CZUTIC001071_2D3	*Hoplias malabaricus*	Magdalena - UM				OK
CZUTIC001071_3D3	*Hoplias malabaricus*	Magdalena - UM				OK
CZUTIC001424_1D1	*Hoplias malabaricus*	Magdalena - UM				OK
CZUTIC002321_1D3	*Hoplias malabaricus*	Magdalena - UM				OK
CZUTIC002321_2D3	*Hoplias malabaricus*	Magdalena - UM				OK
CZUTIC002321_3D3	*Hoplias malabaricus*	Magdalena - UM				OK
CZUTIC002417_1D2	*Hoplias malabaricus*	Magdalena - UM				OK

CZUTIC002670_1D1	*Hoplias malabaricus*	Magdalena - UM					OK
CZUTIC016073_1D1	*Hoplias malabaricus*	Magdalena - UM					OK
ICNMHN001758_2D3	*Hoplias malabaricus*	Magdalena - UM					OK
ICNMHN001758_3D3	*Hoplias malabaricus*	Magdalena - UM					OK
ICNMHN003784_1D1	*Hoplias malabaricus*	Magdalena - UM					OK
ICNMHN020101_1D1	*Hoplias malabaricus*	Magdalena - UM					OK
ICNMHN020147_1D1	*Hoplias malabaricus*	Magdalena - UM					OK
ICNMHN020266_1D1	*Hoplias malabaricus*	Magdalena - UM					OK
ICNMHN020877_1D1	*Hoplias malabaricus*	Magdalena - UM					OK
MPUJ013219_NI023	*Hoplias malabaricus*	Magdalena - UM				OK	OK
MPUJ013219_NI025	*Hoplias malabaricus*	Magdalena - UM	OK				OK
MPUJ013221_NI020	*Hoplias malabaricus*	Magdalena - UM	OK		OK	OK	OK
MPUJ013226_NI01	*Hoplias malabaricus*	Magdalena - UM	OK	OK	OK	OK	OK
MPUJ013228_NI05	*Hoplias malabaricus*	Magdalena - UM	OK	OK		OK	OK
MPUJ013229_NI02	*Hoplias malabaricus*	Magdalena - UM	OK	OK	OK	OK	OK
MPUJ013229_NI03	*Hoplias malabaricus*	Magdalena - UM	OK	OK	OK	OK	OK
MPUJ013229_NI04	*Hoplias malabaricus*	Magdalena - UM	OK	OK	OK	OK	OK
MPUJ013219_NI024	*Hoplias malabaricus*	Magdalena - UM	OK				
MPUJ013221_NI021	*Hoplias malabaricus*	Magdalena - UM	OK				
MPUJ013221_NI022	*Hoplias malabaricus*	Magdalena - UM	OK	OK			
STRI-9519	*Hoplias malabaricus*	Magdalena - UM		OK	OK	OK	
STRI-9520	*Hoplias malabaricus*	Magdalena - UM		OK	OK	OK	
STRI-9545	*Hoplias malabaricus*	Orinoco		OK	OK	OK	
W1	*Hoplias malabaricus*	Orinoco		OK		OK	
W2	*Hoplias malabaricus*	Orinoco		OK		OK	
W4	*Hoplias malabaricus*	Orinoco		OK		OK	
CZUTIC001142_1D1	*Hoplias malabaricus*	Pacific slope of Colombia					OK
ICNMHN000174_1D1	*Hoplias malabaricus*	Pacific slope of Colombia					OK
ICNMHN019286_1D1	*Hoplias malabaricus*	Pacific slope of Colombia					OK
ICNMHN019684_1D1	*Hoplias malabaricus*	Pacific slope of Colombia					OK

JAM647	*Hoplias malabaricus*	Pacific slope of Colombia			OK
JAM648	*Hoplias malabaricus*	Pacific slope of Colombia			OK
JAM649	*Hoplias malabaricus*	Pacific slope of Colombia	OK		OK
STRI-1406	*Hoplias malabaricus*	Pacific slope of Colombia	OK	OK	OK
STRI-1407	*Hoplias malabaricus*	Pacific slope of Colombia	OK	OK	OK
STRI-4258	*Hoplias malabaricus*	Trinidad	OK	OK	OK
STRI-4285	*Hoplias malabaricus*	Trinidad	OK	OK	OK
JD2265	*Hoplias lacerdae*		OK	OK	
US103	*Hoplias intermedius*		OK	OK	
	Ctenolucius hujeta		AP011987.1	AP011987.1	AY804059.1
	Hepsetus odoe		AP011991.1	KX911961.1	AY804086.1
	Pyrrhulina australis			JN989187.1	HQ289390.1
	Erythrinus erythrinus			KY348936.1	
	Erythrinus erythrinus			KY348937.1	
	Erythrinus erythrinus			KY348940.1	
	Erythrinus erythrinus			KY348941.1	
	Erythrinus erythrinus			JN988855.1	
	Erythrinus erythrinus			JN988856.1	
	Erythrinus erythrinus			JN988858.1	
	Erythrinus erythrinus			JN988857.1	

	Erythrinus erythrinus	KR491518.1	
	Hoplerythrinus unitaeniatus	JN988900.1	HQ289501.1
	Hoplerythrinus unitaeniatus	JN988901.1	
	Hoplerythrinus unitaeniatus	JN988902.1	
	Hoplerythrinus unitaeniatus	JN988903.1	
	Hoplerythrinus unitaeniatus	JN988904.1	
	Hoplerythrinus unitaeniatus	HM064996.1	

Appendix S2. Collection sites of *Hoplias malabaricus* for molecular phylogeny, cytogenetics (circle dot in middle) and Geometric morphometrics; Panama (red diamonds), Pacific Slope of Colombia (red triangles), UM/MM (red circles), LM (green circles), other Caribbean basins (green squares), Orinoco (green triangles), the Magdalena river basin is highlighted in gray. This map is a zoom of the NSA populations, exhibing the Cordilleras and principal rivers.



5. GENERAL CONCLUSIONS

- In this work, we aimed to distinguish the causes hampering the identification of the component species of the complex. Also, we explored the role of the Andean uplift in this complex diversification.
- In our first two manuscripts, we found that the shape variation is mainly related to allometry - different sizes of adults of trahira present significantly and predictably different shapes. Considering only the residuals of this correlation, we found that the ecogeographic and evolutionary causes explored here have weak but significant effects on the shape variation of *H. malabaricus*.
- We evaluated the effect of historical causes by comparing *H. malabaricus* shape variation between independent molecular lineages and specimens sampled from different hydrographic basins. In both approaches, the groups show considerable shape overlap, leading us to speculate that different cryptic species within the *H. malabaricus* complex could also contain the same shape variation, impeding the diagnosis of well-defined new species using morphological characteristics.
- We also demonstrated the effect of the type of ecosystem (lentic or lotic) in the shape of the trahiras and concluded that these organisms adapt to the type of ecosystem in which they inhabit, either by phenotypic plasticity or by divergence selection.
- Our results show a significant correlation between latitude and *H. malabaricus* head shape. We suggest further studies exploring the effect of the elevation on the shape, which could combine the effect of temperature and type of environment, as elevation determines the position of these sedentary fishes in the river.
- In our third manuscript, we found a clear Miocene/Pliocene vicariance between the cis and trans-Andean *H. malabaricus* groups, caused by the uplift of the Eastern Cordillera. Notably, we found both groups within the Magdalena Basin: the trans-Andean group at the upper and middle part of the river, and the cis-Andean group at the lower part of the river. We interpreted it as evidence of the dispersion from the cis-Andean populations to the Caribbean basins (including

the Lower Magdalena region) because of the sea-level lowering during the last glaciations that allowed the connection of river mouths.

- Within the Magdalena River, we found two cytogenetic fluorescence patterns: the first related to individuals from upper and middle river samples and the second from individuals from lower river samples. Reinforcing our findings, we also found a shape and molecular differentiation between the individuals of these two stocks. We need mention that the samples from the upper and medium part of the river were sampled exclusively in lotic systems, while the samples of the lower part of the river were sampled exclusively in lentic systems, suggesting that we need to explore the impact of elevation in the shape of *H. malabaricus* in the Magdalena Basin.