Molecular Analysis and Chromosome Mapping of Repetitive DNAs in the Green Terror *Andinoacara rivulatus* (Cichlidae: Cichlasomatini)

Mauro Nirchio,^{1,2} Fabilene Gomes Paim,³ Ricardo Britzke,^{2,3} Anna Rita Rossi,⁴ Valentina Milana,⁴ and Claudio Oliveira³

Abstract

Neotropical cichlids include hundreds of species whose taxonomy has benefited of molecular phylogeny and whose karyotype evolution has been related to the amount and distribution of different classes of repetitive sequences. This study provides the first integrative molecular (cytochrome c oxidase subunit 1 and 16S sequences) and cytogenetic analyses of wild samples of the green terror *Andinoacara rivulatus*, a cichlid naturally distributed in Ecuador and spread throughout the world as an aquarium pet. Molecular data revealed that sequences of green terror constitute a single monophyletic clade within the genus and allowed species attribution of uncertain samples previously cytogenetically analyzed. Chromosome number ($2n=48$) conforms to the general trend observed within neotropical cichlids. However, mapping of different classes of repeated sequences (18S rDNA, 5S rDNA, U1 snDNA and telomeric) revealed the presence of features uncommon among representatives of these fishes, like multiple major rDNA sites, and suggested a recent occurrence of rearrangements (fusion/inversion) in two chromosome pairs.

Keywords: Cichliformes, Ecuador, heterochromatin, *in situ* hybridization, ITS, NORs

Introduction

THE FAMILY CICHLIDAE, which presently includes 1723 valid species,¹ is the most species-rich nonostariophysan freshwater fish family and one of the major vertebrate group.² Fishes of this family are widespread distributed in Africa, Latin America, and Madagascar, and are also present (with a low number of species) in South India and the Middle East.³ Neotropical cichlids belong to Cichlinae, one of the four cichlids subfamilies, whose taxonomy has long and repeatedly been revised.⁴ The 571 species of this subfamily¹ are organized into 7 tribes.⁵ The tribe Cichlasomatini has >70 species and 11 genera, one of which, *Andinoacara*⁶ is widely distributed throughout NW South America and includes 8 valid species.⁷ Among these, *Andinoacara rivulatus*⁸ is one of the 38 native cichlid species present in Ecuador.⁹ It is commonly known as green terror, but this name is not univocal as it was originally associated with *Aequidens rivulatus*, now split into two species, *An. rivulatus* (distributed in Ecuador and northwestern Peru) and *A. stalsbergi*¹⁰ (present in trans-

Andean rivers and lakes at the Peruvian Pacific coast).^{11,12} Molecular phylogeny showed that these two sister taxa diverged ~2.9 million years ago.⁷ The green terror is commercialized throughout the world as an aquarium pet, sometimes crossed with *Andinoacara* cf. *pulcher* to obtain hybrids with the size and color of *A. pulcher*, and the orange seams of the *An. rivulatus*, both on the dorsal and caudal fin. In addition, *An. rivulatus* represents an important source of food and employment for populations of the northwestern Peru (Piura province) on which many fishermen and fish traders depend,¹³ and in Ecuador, it is one of the native wild species most consumed by the population in all the western provinces (Guayas, Manabí, Los Ríos, El Oro, Santo Domingo, and Esmeraldas).¹⁴ Although this species has not yet been assessed in the IUCN Red List, currently its capture without any control has generated problems of over exploitation with the consequent risk of affecting natural populations.¹⁵

Fish cytogenetics is a very active research area that provides information for taxonomy and for the understanding of the phylogenetic relationships among taxa.^{16–23} The most

¹Departamento de Acuicultura, Escuela de Ciencias Aplicadas del Mar, Núcleo de Nueva Esparta, Universidad de Oriente, Porlamar, Venezuela.

²Departamento de Acuicultura, Facultad de Ciencias Agropecuarias, Universidad Técnica de Machala, Machala, Ecuador.

³Departamento de Morfologia, Instituto de Biociências Universidade Estadual Paulista, UNESP, Botucatu, Brazil.

⁴Dipartimento di Biologia e Biotecnologie “C. Darwin,” Sapienza—Università di Roma, Rome, Italy.

recent checklist of cytogenetically analyzed fish species reveals that only ~8% of cichlid fishes are included.²⁴ The diploid number in the group ranges from $2n=32$ to $2n=60$, but in neotropical cichlids, the modal diploid number is $2n=48$.²⁵ Other information on the karyotype of these species includes mapping of 45S or 5S rDNA or description of heterochromatin patterns.^{24–26} Indeed, karyotypic evolution of neotropical cichlids has been related to the amount and distribution of these classes of repetitive sequences that define different genomic organization.^{25,26}

Data on *Andinoacara* spp. are scanty and mostly from aquarium source. Marescalchi²⁷ published a report on the diploid number and the karyotype formula of aquarium trade samples of *A. pulcher*²⁸ ($2n=48$; 4m-sm + 44st-a) and of green terror ($2n=48$; 8m-sm + 40st-a). At that time, the scientific name of green terror was still *Ae. rivulatus*, so it is not clear whether the karyotype formula reported actually refers to *An. rivulatus* or *A. stalsbergi*, a doubt that needs to be unraveled. Thus, the aim of this article is to perform a molecular analysis on the green terror *An. rivulatus* (Cichlidae: Cichlasomatini) from its native area (Ecuador) using mitochondrial sequences of two genes (16S and cytochrome c oxidase subunit 1 [COI]) for the molecular identification of samples and for the comparison with *Ae. rivulatus* previously analyzed by Marescalchi.²⁷ In addition, classical staining (Giemsa, silver nitrate, C-banding) and fluorescence *in situ* hybridization (FISH) mapping of different classes of repetitive sequences (18S rDNA, 5S rDNA, U1 snDNA, and telomeric repeats) were applied to deeply define the karyotype of the species.

Materials and Methods

Specimens collection and identification

Fifteen specimens of *An. rivulatus* (three males, six females, and six unsexed) were collected with a seine net in several locations of El Oro and Guayas Provinces Ecuador (Fig. 1) (Supplementary Table S1). Identification of samples was performed following morphological criteria.²⁹ Fish were transported to the laboratory in sealed plastic bags (32 inches) containing 2 gallons of water and replacing the air with pure oxygen, and finally kept in an aquarium until their utilization for cytogenetic preparations (see the Cytogenetic analysis section of Materials and Methods). The experiments followed ethical conducts, in accordance with Institutional Authorization Research Project 396/2016, Universidad Técnica de Machala (UTMACH), and approved by the Bioscience Institute/Universidade Estadual Paulista (UNESP) Ethics Committee on use of animals (Protocol No. 1058/2017) through the cooperation agreement signed on March 18, 2015. A piece of muscle was collected from each specimen and preserved in 95% ethanol for molecular analysis. Voucher specimens fixed in 10% formalin are deposited in the Ichthyology Collection of the Laboratório de Biologia e Genética de Peixes of UNESP and in the UTMACH (Supplementary Table S1).

Molecular analysis

Total DNA was extracted from ethanol-preserved muscle samples using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Partial sequences of mitochondrial genes COI and 16S rRNA were amplified using polymerase chain reaction (PCR) with the following primers: Fish F1 and

Fish R1 for the COI gene,³⁰ and 16Sar and 16Sbr for the 16S rRNA gene.³¹ PCRs were carried out in an Applied Biosystems thermocycler (Applied Biosystems, Foster City, CA) following protocols reported by Milana *et al.*³² The BigDye sequencing kit cycle terminators (Applied Biosystems) were used as reagent component in all sequencing reactions, according to the manufacturer's instructions, and sequences were analyzed using ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

Sequences were aligned using the software Clustal X.³³ The basic local alignment search tool (BLAST, <https://blast.ncbi.nlm.nih.gov/Blast>) was used to search on GenBank database for similar sequences to confirm species assignment. Sequence of *Andinoacara* spp. available in GenBank database was retrieved and included in analysis (Supplementary Table S1); *Acaronia nassa*³⁴ was used as out-group.

Phylogenetic reconstruction was obtained from the data set with neighbor joining (NJ), maximum likelihood (ML) and Bayesian inference (BI) analyses. NJ and ML analyses (1000 bootstrap replicates) were performed using MEGA5³⁵ and PhyML 3.0,³⁶ respectively. Bayesian analyses were carried out as implemented in MrBayes 3.1.2.³⁷ Two independent runs of four Markov chains, each for 1,000,000 generations, were performed. ModelTest 3.7³⁸ and MrModelTest 2.3³⁹ were used to select the evolutionary models that best fit the data set for the ML and the BI analyses, respectively. The Akaike information criterion was used.

Cytogenetic analysis

Fish were injected intramuscularly with a yeast–glucose solution for mitosis stimulation⁴⁰; after 24 h, each fish received an intra-abdominal injection of 0.0125% colchicine (1.0 mL/100 g of body weight) 50 min before being sacrificed by a numbing overdose of benzocaine (250 mg/L) as recommended by the American Veterinary Medical Association.⁴¹ Mitotic chromosomes were obtained following the conventional air-drying method from kidney cell suspension.⁴²

For the conventional karyotype, slides were stained for 20 min with 10% Giemsa in phosphate buffer at pH 6.8. Active nucleolus organizer regions (NORs) were revealed by silver (Ag) staining.⁴³ For C-banding, metaphase plates were exposed to depurination of chromatin with HCl, denaturation of the DNA with BaOH, and removal of the chains of denatured DNA in hot saline sodium citrate 2X concentrate.⁴⁴

The 5S rDNA and 18S rDNA (ribosomal genes), U1 snRNA gene clusters (U1 snRNA is a noncoding RNA that forms part of the spliceosome),⁴⁵ and telomeric repeats were mapped onto chromosomes by FISH.⁴⁶ Probes were obtained and labeled by PCR from the genome of *Hypsolebias flagellatus*⁴⁷ using the primers 5SA (5'-TCA ACC AAC CAA AAA GAC ATT GGC AC-3') and 5SB (5'-TAG ACT TCT GGG TGG CCA AAG GAA TCA-3') for 5S rDNA,⁴⁸ 18S6F (5'-CTC TTT CGA GGC CCT GTA AT-3') and 18S6R (5'-CAG CTT TGC AAC CAT ACT CC-3') for 18S rDNA,⁴⁹ U1F (5'-GCA GTC GAG ATT CCC ACA TT-3') and U1R (5'-CTT ACC TGG CAG GGG AGA TA-3') for U1 snDNA,⁵⁰ and (TTAGGG)₅ and (CCCTAA)₅ for telomeric repeats.⁵¹ The 5S rDNA and telomeric probes were labeled with biotin-16-dUTP (2'-deoxyuridine 5'-triphosphate), and the 18S rDNA and U1 snDNA probes were labeled by including digoxigenin-11-dUTP in the PCR. Hybridization was detected with fluorescein-

FIG. 1. Sampling area of *Andinoacara rivulatus*: stars indicate the different sampling sites.

conjugated avidin (Sigma-Aldrich, www.sigma-aldrich.com) and antidioxigenin–rhodamine conjugate (Roche Diagnostics, www.roche.com), respectively. Chromosomes were counterstained with 4,6-diamidino-2-phenylindole included in the Vectashield mounting medium (Vector Laboratories, Ltd., Peterborough, UK).

The chromosome spreads were analyzed under an Olympus BX61 epifluorescence microscope (Olympus, www.olympus.com) and images were captured with an Olympus DP70 digital camera using Image-Pro plus 6.0 software (Media Cybernetics). Images were merged and edited for optimization of brightness and contrast using Photoshop CS5 program (Adobe Systems, www.adobe.com). Chromosomes were arranged in decreasing order and classified as metacentric (m), submetacentric (sm), and subtelocentric/acrocentric (st/a). For calculating the number of chromosome arms (fundamental number, FN), the st/a chromosomes were considered as unarmed and m-sm elements as biarmed.

Results

Molecular analysis

Sequences obtained in this study for COI (651 base pairs) and 16S rRNA (542 base pairs) were deposited in GenBank under accession numbers provided in Supplementary Table S1. When 16S rDNA sequences obtained in this study were

BLASTed in GenBank, they showed 99.8% similarity with those provided by Marescalchi.²⁷ In addition, the phylogenetic reconstructions based on 16S (Supplementary Fig. S1) and COI (Fig. 2) data, which include sequences of other certainly identified *Andinoacara* spp. retrieved from the databases, show that *An. rivulatus* constitutes a single monophyletic clade. The genetic COI K2P distance between *An. rivulatus* and the other species of the genus ranges from 8.8% to 13% with *Andinoacara biseriatus*⁵² and *Andinoacara coeruleopunctatus*,⁵³ respectively.

Cytogenetic analysis

The analysis of 345 well-spread metaphases (20–25 per individual) of *An. rivulatus* showed a modal (98%) diploid number of $2n=48$ and a karyotype composed by 2 small m, 10 sm, and 36 st/a chromosomes (Fig. 3a) with FN of 60. Chromosomes are very similar in shape and gradually decrease in size. This makes it difficult to identify the homologues with certainty, with the exception of the unique m chromosome pair, identified as number 1, and of chromosome pair number 2, which is the largest in the sm series. No morphologically differentiated chromosomes between males and females were observed. The analysis of the C-banding pattern (10 metaphases per individual) revealed constitutive heterochromatin restricted to the centromeric regions of all chromosomes (Fig. 3b).

FIG. 2. NJ tree based on mitochondrial sequences of cytochrome c oxidase subunit 1 of *Andinoacara*. At each node, bootstrap values >70% (NJ and maximum likelihood) and posterior probabilities >0.9 (Bayesian inference) are shown. Specimens sequenced in this study and cytogenetically examined are indicated by stars. NJ, neighbor joining.

Twenty silver nitrate-stained metaphases were photographed from each individual, and two to three actively transcribing NORs, localized on the short arms of an sm chromosome pair and in pericentromeric position of an st/a chromosome pair, were identified (Fig. 4a–c). Most of the metaphases show only two chromosomes positively silver stained, either in homozygous or heterozygous combination, although some metaphases showing three or four Ag–NOR-bearing chromosomes were observed (Fig. 4d, e). A maximum of four silver-stained nucleoli were observed also in interphasic cells (Fig. 4f).

Double FISH experiments using rDNA probes (Fig. 5) confirmed the presence of two pairs of 18S rDNA positive sites and a single pair of 5S rDNA sites. The analysis of ~15 metaphases per individual showed the major ribosomal genes localized consistently on the short arms of a medium sized sm chromosome pair (pair 5) and on the pericentromeric position of an st/a chromosome pair (pair 19) (Fig. 5a); in a single individual (collected at Arenillas), two additional and small 18S rDNA positive sites were observed on a pair of st/a chromosomes (Fig. 5b). The minor ribosomal genes are localized terminally on the tip of the unique m chromosome pair (pair 1) (Fig. 5a). U1 snDNA is localized on the tip of the

short arms of a medium-sized sm pair, likely chromosome pair 3 (Fig. 5c).

In situ hybridization with the telomeric probe (five well-defined metaphases per individual) provided positive signals at the ends of all chromosomes and interstitial positive sites (ITSs) on two chromosome pairs: in the pericentromeric region on the unique m chromosome pair (pair 1) and on an sm chromosome pair (pair 2) (Fig. 6).

Discussion

Molecular data demonstrated that specimens examined in this survey belong to the same species examined by Marescalchi²⁷ and actually correspond to *An. rivulatus*. This species is unambiguously defined within the genus as a monophyletic group, according to the previous phylogenetic data by Musilová *et al.*⁷ where it represented one of the four *Andinoacara* taxa belonging to the southern clade.

Cytogenetic data provided here show that *An. rivulatus* has a chromosomal complement of $2n=48$ (12m-sm +36st-a, FN=60), which differs in the karyotype formula reported by Marescalchi²⁷ ($2n=48$; 8m-sm +40st-a). However, these differences could be due to a different degree of condensation

FIG. 3. Conventional Giemsa-stained (a) and C-banded (b) karyotype of *Andinoacara rivulatus*. m, metacentric; sm, submetacentric; st/a, subtelocentric/acrocentric.

of chromosomes that make their classification and comparison difficult. A diploid number $2n=48$ is the most common in fishes,^{51,54} and it is also the one typically observed in neotropical cichlids.^{55–58} However, many species of this group, despite sharing this chromosome number, differ in the karyotype formula, an evidence that could be explained by intrachromosomal rearrangements like centromeric shifts. As a result of these processes, karyotypic diversity in neotropical cichlids has a higher rate of evolution than that observed in cichlids from Old World.²⁵ Among the 25 species of the tribe

Cichlasomatini (Supplementary Table S2), the same chromosome number is associated with many different number of chromosome arms and it is not possible to identify a general trend; however, some of the genera (*Laetacara*,⁵⁹ *Nannacara*⁶⁰) seem to be more prone to chromosome number reduction than others. Within the genus *Andinoacara*, the only other species cytogenetically examined to date, *A. pulcher*, share the same chromosome number of *An. rivulatus*. Although *A. pulcher* is characterized by the presence of a large m chromosome pair, it is much larger than the others of the

FIG. 4. Metaphases of *Andinoacara rivulatus* stained with AgNO_3 showing (a–c) two (d) three, and (e) four positive sites (arrows). Enlargements and schematic representation of the silver-stained NOR-bearing chromosomes are shown. (f) Interphasic nuclei showing one to four silver-stained nucleoli.

FIG. 5. FISH/DAPI. (a) Karyotypes of *Andinoacara rivulatus* under double FISH with 5S rDNA (positive signals on pair 1) and 18S rDNA (positive signals on pairs 5 and 19) probes. (b) metaphase plates showing four (left) and six (right) 18S rDNA sites (arrows). (c) Karyotypes of *An. rivulatus* under single FISH with U1 snDNA probe. FISH, fluorescence *in situ* hybridization; DAPI, 4,6-diamidino-2-phenylindole.

complement, and it is not comparable with any chromosome of *An. rivulatus*. The analysis of other species of the genus will be necessary to clarify whether and how much this large m chromosome pair is spread among species of *Andinoacara* and whether there is any relationship between large and small m chromosomes.

The presence of ITS in two chromosome pairs of *An. rivulatus*, reported also in representatives of different orders of fishes⁶¹ including those of the subfamily Cichlinae within Cichliformes,⁶² suggests a recent occurrence of chromosome rearrangement (fusions and inversions) during the karyotype diversification in cichlids. Indeed, telomeric repeats when present in sites other than those at the ends of the chromosomes, tend to shorten, to be lost over time, or to become inactive,^{61,63,64} and the detection of residual sequences could be difficult⁶⁵; as a consequence, ITS could be considered not only a marker of chromosome rearrangements but also of their time of occurrence.

The heterochromatin distribution pattern in the centromeric or pericentromeric chromosome regions is common to

FIG. 6. Metaphase of *Andinoacara rivulatus* assayed by FISH using telomeric probes and counterstained by DAPI. Interstitial positive sites on chromosome pair 1 (m) and pair 2 (sm) are indicated by arrows and arrowheads, respectively.

the other neotropical cichlids^{25,62} and thus it seems to be not informative. This pattern suggests the absence of sex chromosomes, as in fish these chromosomes tend to accumulate repeated sequences partly or completely heterochromatic,^{66–68} which could be detected as heterochromatic blocks.⁶⁹

Location of ribosomal genes has been investigated in thousands of species, making these chromosomal regions a preferred marker in comparative cytogenetics and systematic studies.⁷⁰ *An. rivulatus* FISH assays with 18S rDNA probe demonstrated that the polymorphism observed after silver staining was due to a different activity of NORs that was already reported in many animals⁶⁸ including fishes⁷² and Cichlidae.⁷³ Usually the mechanisms of inactivation of NORs include elimination of rDNA, DNA methylation, and gene silencing due to position effects induced by heterochromatin (C-bands) and/or telomeres and transposable elements.^{71,74,75} It has also been shown that different rDNA units could be present in the genome and they are not randomly expressed.⁷⁶ The presence of two additional sites in one individual requires a more detailed analysis, on a wider number of individuals, to assess whether an interpopulation variation in constitutive ribosomal genes exists or, on the contrary, it is an isolated case and/or it might be linked to the difficulty in detecting inactive major rDNA sites with a low copy number of genes.⁷⁷ Indeed, the copy number of genes at these sites can change easily⁷⁸ as an adaptive response to genome stress.⁷⁹

As far as the presence of multiple rDNA sites is concerned, this condition is not very commonly detected in fishes. Indeed, in this group of vertebrates, the presence of a single NOR-bearing chromosome pair is considered as the plesiomorphic condition.⁸⁰ It characterizes ancient actinopterygian fishes,⁸¹ most primitive actinopterygians not originated by polyploidization, and is the most common conditions in teleost species, being shared by 72% of the taxa.⁸² Cichlids generally conform to this picture: the animal rDNA database (www.animalrdnadatabase.com) reports 39 records for 45S rDNA in this family and a single site of these rDNA clusters is the general trend, according to the

observation of a single pair of Ag–NORs.^{24,25} This was described as the probable symplesiomorphic condition in the group.⁵⁶ Few exceptions have been reported in *Cichlasoma sanctifranciscense*,^{83,84} *Geophagus brasiliensis*,^{82,85} *Uaru amphiacanthoides*,³⁴ and *Pterophyllum leopoldi*,^{62,86} and *An. rivulatus* (this study): they would represent a derived character in neotropical cichlids.

An. rivulatus minor ribosomal genes are localized on a single chromosome pair, with no synteny with major ribosomal genes. This pattern corresponds to what is generally observed in fishes,^{87,88} including Cichlidae,^{84,89} being shared by 26 out of the 38 analyzed species of the family (www.animalrdbnadb.com), with few exceptions.⁶² Indeed, these genes usually show high variability at molecular level, as they are associated with other genes, microsatellites or transposable elements, and low variability at chromosomal level.⁸⁸

Regarding U1 snRNA, chromosomal localization of coding genes has been investigated so far only in a few teleost fishes of the family Hepsetidae,¹⁶ Mugilidae,²⁰ Merlucciidae,⁹⁰ and Characidae,⁵⁰ and in the latter they are often associated with 5S ribosomal genes. In Cichlidae,⁹¹ mapping of the U1 snRNA in 19 species belonging to different subfamilies had shown positive signals located on a single chromosome pair, mainly (14 out of 19 species) terminally on the long arms. This is also the case of the unique species belonging to the Cichlasomatini tribe *Aequidens tetramerus*.³⁴ Thus the terminal location on short chromosomal arms detected in *An. rivulatus* could be a derived condition, from chromosome rearrangements.

We provide here new data on the cytogenetic profile of *An. rivulatus*, the first on wild samples and thus the reference for the species, with mapping of different gene families, and the molecular identification of specimens. The molecular phylogeny provides clear relationships within *Andinoacara*; further in-depth cytogenetic studies on congeneric species are required to better understand the karyotype evolutionary history of this group within neotropical cichlids.

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Supplementary Material

Supplementary Figure S1
Supplementary Table S1
Supplementary Table S2

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Address correspondence to:

Anna Rita Rossi, PhD

Dipartimento di Biologia e Biotechnologie “C. Darwin”

Sapienza—Università di Roma

Via Alfonso Borelli 50

Rome 00161

Italy

E-mail: annarita.rossi@uniroma1.it