Tracking the Evolutionary Trends Among Small-Size Fishes of the Genus Pyrrhulina (Characiforme, Lebiasinidae): New Insights From a Molecular Cytogenetic Perspective

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Miniature fishes have always been a challenge for cytogenetic studies due to the difficulty in obtaining chromosomal preparations, making them virtually unexplored. An example of this scenario relies on members of the family Lebiasinidae which include miniature to medium-sized, poorly known species, until very recently. The present study is part of undergoing major cytogenetic advances seeking to elucidate the evolutionary history of lebiasinids. Aiming to examine the karyotype diversification more deeply in Pyrrhulina, here we combined classical and molecular cytogenetic analyses, including Giemsa staining, C-banding, repetitive DNA mapping, comparative genomic hybridization (CGH), and whole chromosome painting (WCP) to perform the first analyses in five Pyrrhulina species (Pyrrhulina aff. marilynae, Pyrrhulina sp., P. obermulleri, P. marilynae and Pyrrhulina cf. laeta). The diploid number (2n) ranged from 40 to 42 chromosomes among all analyzed species, but P. marilynae is strikingly differentiated by having 2n = 32 chromosomes and a karyotype composed of large meta/submetacentric chromosomes, whose plesiomorphic status is discussed. The distribution of microsatellites does not markedly differ among species, but the number and position of the rDNA sites underwent significant changes among them. Interspecific comparative genome hybridization (CGH) found a moderate divergence in the repetitive DNA content among the species’ genomes. Noteworthy, the WCP reinforced our previous hypothesis on the origin of the X₁X₂Y multiple sex chromosome system in P. semifasciata. In summary, our data suggest that the karyotype differentiation in Pyrrhulina has been driven by major structural rearrangements, accompanied by high dynamics of repetitive DNAs.

Keywords: fishes, repetitive DNAs, karyotype evolution, sex chromosomes, evolution
INTRODUCTION

Characiformes comprise a very diverse and abundant freshwater order (Nelson et al., 2016), in which the family Lebiasinidae is represented by 75 valid species (Fricke et al., 2021) widely distributed across South and Central America (Weitzman and Weitzman, 2003). The phylogenetic relationships of the Lebiasinidae remained in doubt for a long time, but more recent phylogenetic analysis indicate their proximity to the Ctenoluciidae (Calcagnotto et al., 2005; Oliveira et al., 2011), which was also reinforced by the different studies (Arcila et al., 2017; Betancur-R et al., 2019; Melo et al., 2021). Most Lebiasinidae species reach about 60 mm of Standard Length (SL), but miniature species, not surpassing a maximum of 26 mm SL, is found within the Pyrrhulinae, whereas medium-sized species up to 150 mm SL can be found within Lebiasininae (Weitzman and Weitzman, 2003).

Because of their small sizes and difficulties in obtaining good chromosomal preparations, species of Lebiasinidae were, for a long time, little analyzed in terms of cytogenetics, with scarce references mainly on the chromosomal number of few species (Scheel, 1973; Oliveira et al., 1991; Arai, 2011). However, this scenario has recently undergone significant changes with the methodological advance of cytogenetics and its applicability among small to miniature fishes of Pyrrhulina, Lebiasina, Copina, and Nannostomus genus (de Moraes et al., 2017, de Moraes et al., 2019; Sassi et al., 2019; Toma et al., 2019; Sassi et al., 2020; Sember et al., 2020).

*Pyrrhulina* is one of the most speciose genera of the subfamily Pyrrhulinae, with 19 valid small species (Fricke et al., 2021), ranging from 30.4 to 85 mm SL (Weitzman and Weitzman, 2003; Netto-Ferreira and Marinho, 2013). The genus is among the most problematic, with many poorly known species, species complexes, and old taxonomic problems (Netto-Ferreira and Marinho, 2013). The first *Pyrrhulina* species to have some chromosomal data evidenced was *Pyrrhulina cf. australis*, with 2n = 40 chromosomes, mainly acrocentric ones (Oliveira et al., 1991). Taxonomic boundaries of *P. australis* are still poorly defined, demonstrated in subsequent studies (de Moraes et al., 2017, de Moraes et al., 2019) of two morphotypes. Both *P. australis* and *Pyrrhulina aff. australis* showed similar data 2n = 40 (4st + 36a), distinct from *P. brevis*, 2n = 42 (2sm + 4st + 36a), with no evidence of heteromorphic sex chromosomes in the three species (de Moraes et al., 2017, de Moraes et al., 2019). Another species, *P. semifasciata*, was analyzed, presenting 2n = 42 (4st + 38a) in females, and 2n = 41 (1m + 4st + 36a) in males, the latter with three unpaired chromosomes because of a multiple X1X1X2Y/X1X1Y sex chromosome system (de Moraes et al., 2019). This occurrence was also confirmed by comparative genomic hybridizations (CGH) and whole-chromosome painting (WCP), with some indications that the Y chromosome originated by centric fusions of non-homologous acrocentric chromosomes (de Moraes et al., 2019).

To improve the knowledge of the evolutionary processes within the genus *Pyrrhulina*, we combined classical and molecular cytogenetic analyses, including Giemsa staining, C-banding, repetitive DNA mapping, comparative genomic hybridization (CGH), and whole chromosome painting (WCP) to perform the first analyses in five *Pyrrhulina* species (*Pyrrhulina aff. marilynae*, *Pyrrhulina sp.*, *P. obermulleri*, *P. marilynae* and *Pyrrhulina cf. laeta*). The results highlighted relationships and particular evolutionary paths at the chromosomal and genomic levels among the species. In addition, the hypothesis on the origin of the multiple sex chromosome system in *P. semifasciata* is validated.

MATERIALS AND METHODS

Animals

The collection sites, number, and sex of the specimens investigated are presented in Figure 1, Table 1. Part of the sampling (Figure 1, white circles) resembles the one previously analyzed by de Moraes et al. (2017), de Moraes et al. (2019) with different cytogenetic and molecular methods. Animals were collected with the authorization of the Brazilian environmental agency ICMBIO/SISBIO (license no. 48628-14) and SISGEN (A96FF09). All species were properly identified by morphological criteria, and the specimens were deposited in the fish collection of the Museu de Zoologia da Universidade de São Paulo (MZUSP) under the voucher numbers (119077, 119079, 123073, 123080) and the Universidade Federal da Paraíba (UFPB) museum under the voucher number (12079, 12080, 12082 and 12083). Experiments followed ethical and anesthesia conducts and were approved by the Ethics Committee on Animal Experimentation of the Universidade Federal de São Carlos (process number CEUA 1853260315).

Chromosomal Preparations and Analysis of the Constitutive Heterochromatin

Mitotic chromosomes were obtained from kidney cells by the protocol described in Bertollo et al. (2015). The distribution of constitutive heterochromatin was observed by the C-banding method according to (Sumner, 1972).

Repetitive DNA Mapping with Fluorescence in situ Hybridization (FISH)

The 5S rDNA probe included 120 base pairs (bp) of the 5S rDNA gene coding region and 200 bp of non-transcribed spacer (NTS) (Pendas et al., 1994). The 18S rDNA probe was composed of a 1,400-bp-long segment of the 18S rDNA coding region (Cioffi et al., 2009). Both probes were directly labeled with the Nick-Translation Mix Kit (Jena Bioscience, Jena, Germany)—18S rDNA with ATTO488-dUTP and 5S rDNA with ATTO550-dUTP, according to the manufacturer’s instructions. The (CA)15, (GA)15, (CGG)15 microsatellite probes were directly labeled with Cy3 during the synthesis, according to Kubat et al. (2008). In addition, since it contains the lowest 2n, telomeric (TTAGGG)n sequence was also used as probe in *P. marilynae*. This probe was generated by PCR in the absence of a template according to Ijdo et al. (1991) and later labeled with ATTO550-dUTP with the Nick-Translation Mix Kit (Jena Bioscience, Jena, Germany).
Bioscience, Jena, Germany). FISH experiments followed the methodology described in Yano et al. (2017). Metaphase chromosomes were treated with RNase A (40 μg/ml) for 1.5 h at 37°C and the DNA denatured in 70% formamide/2× SSC at 72°C for 3.15 min. A hybridization mixture (2.5 ng/μL probes, 50% deionized formamide, 10% dextran sulfate) was then dropped on the slides, and the hybridization process was performed overnight at 37°C in a moist chamber. The first post-hybridization wash was performed with 1× SSC for 5 min at 65°C, followed by the second one performed with 4× SSC/Tween for 5 min, at room temperature. Chromosomes were then counterstained with DAPI, and the slides were mounted with an antifade solution (Vectashield from Vector Laboratories, Burlingame, CA).

**FISH for Whole Chromosome Painting**

As *P. semifasciata* represents the only *Pyrrhulina* species that harbors an X<sub>1</sub>X<sub>2</sub>Y multiple sex system, a Y-chromosome probe, named PSEMI-Y, was previously prepared by microdissection, as described in (de Moraes et al., 2019) Male and female metaphases of *P. marilynae*, *Pyrrhulina aff. marilynae*, *Pyrrhulina* sp., *P. obermulleri*/*Pyrrhulina* cf. *laeta* were used for Zoo-FISH experiments with the PSEMI-Y probe, according to procedures described in Yano et al. (2017). The hybridization was performed for 72 h at 37°C in a moist chamber, with post-hybridization washes with 1× SSC for 5 min at 65°C, and in 4× SSC/Tween (RT). 10 μg of male-derived C<sub>0</sub>-1 DNA from *P. semifasciata* was used as suppressor in each experiment. Chromosomes were stained with DAPI (1.2 μg/ml) and the slides were mounted with an antifade solution, as described above.

**Probes for Comparative Genomic Hybridization**

The genomic DNAs (gDNAs) from male and female specimens of *P. marilynae*, *Pyrrhulina aff. marilynae*, *Pyrrhulina* sp., *P. obermulleri*, *Pyrrhulina* cf. *laeta*, *P. australis*, *Pyrrhulina* aff.

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**TABLE 1** | Geographical coordinates, sample size, and diploid number of *Pyrrhulina* (Characiformes, Lebiasinidae) species collected in Brazil.

| Species                  | Locality                                | Sample size | 2n (Sex) | References   |
|--------------------------|-----------------------------------------|-------------|----------|--------------|
| *Pyrrhulina aff. australis* | Rio Sepotuba, Lambiri D’Oeste—MT (15°11’28.0″S 57°41’30.7″W) | 169 229 | 40f9  | de Moraes et al. (2017) |
| *Pyrrhulina aff. marilynae* | Igarapé 12 de Outubro, Comodoro—MT (12°58’41.0″S 59°55’35.0″W) | 147 109 | 40f9  | This study |
| *P. australis*            | Barra do Bugres—MT (15°04’27.5″S 57°11’05.4″W) | 186 309 | 40f9  | de Moraes et al. (2017) |
| *P. brevis*               | Reserva Florestal Adolfo Ducke, Manaus—AM (2°58’20.7″S 59°55’53.0″W) | 174 139 | 42f9  | This study |
| *Pyrrhulina cf. laeta*    | Presidente Figueiredo—AM (1°59’10.8″S 60°03’40.8″W) | 076 059 | 42f9  | This study |
| *P. marilynae*            | Ipiranga do Norte—MT (11°36’02.0″S 55°56’27.0″W) | 146 089 | 32f9  | This study |
| *P. obermulleri*          | Tefé—AM (3°25’50.7″S 64°44’54.8″W) | 216 129 | 42f9  | This study |
| *P. semifasciata*         | Careiro—AM (3°51’00.0″S 60°04’00.0″W) | 126 099 | 41f42 9 | de Moraes et al. (2019) |
| *Pyrrhulina* sp           | Represa, Alto Alegre dos Parecis—RO (12°11’58.0″S 61°46’47.7″W) | 193 29f | 40f9  | This study |
australis, P. brevis, and P. semifasciata were extracted from liver tissue by the standard phenol-chloroform-isoamyl alcohol method (Sambrook and Russell, 2001). For intraspecific comparisons, the male-derived gDNAs of all species were labeled with ATTO550-dUTP and the female gDNAs with ATTO 488-dUTP, by nick translation (Jena Bioscience, Jena, Germany). The repetitive sequences were blocked using unlabelled C<sub>0</sub>-1 DNA in all experiments, according to (Zwick et al., 1997). The final hybridization mixture for each slide (20 μL) was composed of male- and female-derived gDNAs (500 ng each), plus 25 μg of female-derived C<sub>0</sub>-1 DNA from the respective species. The probe was ethanol-precipitated, and the dry pellets were mixed in a hybridization mixture containing 50% formamide, 2× SSC, 10% SDS, 10% dextran sulfate, and Denhardt’s buffer, pH 7.0.

For interspecific comparisons, the gDNA of male specimens of P. australis (Paus), Pyrrhulina aff. australis (Paf), P. semifasciata (Psem), P. brevis (Pbr), P. marilynae (Pmar), Pyrrhulina aff. marilynae (Pafm), Pyrrhulina sp. (Psp), P. obermulleri (Pobe) and Pyrrhulina cf. laeta (Pcf) were hybridized against metaphase chromosomes of P. marilynae. This species was selected since it harbors the lowest 2n = 32 until now register for the genus, coupled with a remarkable karyotype differentiation. For this purpose, male-derived gDNA of P. marilynae was labeled with ATTO 550-dUTP, while the gDNAs of the other species were labeled with ATTO 488-dUTP (P. australis, Pyrrhulina aff. marilynae, P. brevis and P. obermulleri) or ATTO 425-dUTP (Pyrrhulina aff. australis, Pyrrhulina sp., P. semifasciata and Pyrrhulina cf. laeta), both through nick translation (Jena Bioscience, Jena, Germany).

The interspecific comparisons were divided into a set of four slides. In the first slide, the final probe mixture was composed of 500 ng of male-derived gDNA plus 10 μg of male-derived C<sub>0</sub>-1 DNA of each of the following species: P. marilynae, P. australis, and Pyrrhulina aff. australis. In the second slide, the final probe mixture was composed of 500 ng of male-derived gDNA plus 10 μg of male-derived C<sub>0</sub>-1 DNA of each one of the following species: P. marilynae, Pyrrhulina aff. marilynae and Pyrrhulina sp. In the third slide, the final probe mixture was composed of 500 ng of male-derived gDNA plus 10 μg of male-derived C<sub>0</sub>-1 DNA of each one of the following species: P. marilynae, P. brevis, and P. semifasciata. Finally, in the fourth slide, the final probe mixture was composed of 500 ng of male-derived gDNA plus 10 μg of male-derived C<sub>0</sub>-1 DNA of each one of the following species: P. marilynae, P. obermulleri, and Pyrrhulina cf. laeta. The chosen ratio of probe vs. C<sub>0</sub>-1 DNA amount was based on previous experiments performed in our fish studies (de Moraes et al., 2019; Toma et al., 2019; Sassi et al., 2020).

The microscopy images were captured using an Olympus BX50 epifluorescence microscope (Olympus Corporation, Ishikawa, Japan) coupled with a CoolSNAP camera, and the images were processed using Image-Pro Plus 4.1 Software (Media Cybernetics, Silver Spring, MD, United States). Final images were optimized and arranged using Adobe Photoshop, version CC 2020. Chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), or acrocentric (a), according to their arm ratios (Levan, 1964). As the males and females results showed no differences, only male metaphases were represented in the figures.

RESULTS

Karyotypes and Heterochromatin Distribution

The diploid number ranged from 2n = 40 to 42 among the following four species: Pyrrhulina sp. (2n = 40; 2st+38a), Pyrrhulina aff. marilynae (2 = 40; 40a), P. obermulleri (2n = 42; 2m/sm+8st+32a) and Pyrrhulina cf. laeta (2n = 42; 2m/sm+4st+36a), the two latter also sharing a characteristic small metacentric/submetacentric pair. On the other hand, P. marilynae differed by presenting a very distinct karyotype composition (2n = 32; 8m/sm+4st+20a). These results represent the first cytogenetic data for the abovementioned species. The constitutive heterochromatin was distributed at the pericentromeric region of several chromosome pairs in P. marilynae and Pyrrhulina aff. marilynae. In its turn, Pyrrhulina sp., P. obermulleri, and Pyrrhulina cf. laeta presented a remarkable series of interstitial and pericentromeric C-bands, in addition to telomeric ones (Figure 2). In our sampling, we did not observe any karyotype differences between males and females.

Chromosomal Mapping of Repetitive DNA Sequences

All the five species differ by the distribution of the multigene rDNA families. Pyrrhulina sp. and P. marilynae were the only species with only one chromosome pair bearing 18S rDNA sites, found at the telomeric region of acrocentric pairs 4 and 9, respectively. Six to twelve centromeric or telomeric sites occur in the other three species, including bitelomeric sites in Pyrrhulina aff. marilynae (pair 11) and Pyrrhulina cf. laeta (pairs 6 and 13). As for the 5S rDNA, from six to twelve centromeric sites occurred among species, including a syntenic condition for the 5S and 18S rDNA repeats in the chromosome pair 6 of Pyrrhulina cf. laeta, the same pair that displays bitelomeric 18S rDNA signals in this species (Figure 2). The distribution of the microsatellites (CA)<sub>15</sub> (GA)<sub>15</sub>, and (CGG)<sub>16</sub> does not differ significantly among species, having a preferential location in the centromeric and telomeric regions of the chromosomes, in addition to some interstitial sites. However, (CA)<sub>15</sub> differs quantitatively, with a greater number of conspicuous sites compared to the

Microscopy and Images Processing

To confirm the diploid number, karyotype structure and FISH results in at least 30 metaphase spreads were analyzed per individual. The microscopy images were captured using an Olympus BX50 epifluorescence microscope (Olympus Corporation, Ishikawa, Japan) coupled with a CoolSNAP camera, and the images were processed using Image-Pro Plus 4.1 Software (Media Cybernetics, Silver Spring, MD, United States). Final images were optimized and arranged using Adobe Photoshop, version CC 2020. Chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), or acrocentric (a), according to their arm ratios (Levan, 1964). As the males and females results showed no differences, only male metaphases were represented in the figures.

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other microsatellites, especially in *Pyrrhulina aff. marilynae* and *Pyrrhulina cf. laeta*. In the same way, (CGG)_{10} occurs in smaller amounts in the five species (Figure 3). The (TTAGGG)_{n} repeats showed the expected hybridization signals on telomeres of *P. marilynae* (Figure 4F). Whole chromosome painting–WCP.

Two acrocentric chromosome pairs were entirely painted with the PSEMI-Y probe in *Pyrrhulina marilynae*, *P. obermulleri*, *Pyrrhulina sp.*, *Pyrrhulina aff. marilynae* and *Pyrrhulina cf. laeta* (Figures 4A–E).

**Comparative Genomic Hybridization–CGH**

The interspecific genomic comparison among *Pyrrhulina marilynae* and other *Pyrrhulina* species (*P. semifasciata*, *P. australis*, *P. brevis*, *P. obermulleri*, *Pyrrhulina aff. australis*, *Pyrrhulina sp.*, *Pyrrhulina aff. marilynae*, *Pyrrhulina cf. laeta*) revealed a high level of DNA compartmentalization, within all species presenting a distinct composition of repetitive DNA sequences and specific signals. However, *P. marilynae* shows more evident species-specific arrangements when compared to the other species. (Figure 5). Intraspecific genomic hybridization between males and females did not show any clustering for sex-specific sequences in all species (data not shown).

**DISCUSSION**

Overall, two main evolutionary trends are proposed for the karyotypic evolution of the Lebiasinidae: 1) the conservation of a plesiomorphic karyotype in the subfamily Lebiasininae, with *2n* = 36 bi-armed chromosomes, and 2) high variations in diploid numbers and karyotypic structures in the subfamily Pyrrhulininae, with the predominance of acrocentric chromosomes (Sassi et al., 2020). It is noteworthy that the karyotypic structure of Lebiasininae, *2n* = 36 biarmed chromosomes, is similar to that found in the sister family Ctenoluciidae (de Souza e Sousa et al., 2017; Sassi et al., 2019;...
de Souza e Sousa et al., 2021). Therefore, in this scenario, the majority of the acrocentric chromosomes found in the species of the Pyrrhulininae are probably derived from rearrangements such as centric fissions (Sassi et al., 2020).

However, unlike other Pyrrhulina species, *P. marilynae* has the smallest 2n identified in the genus so far, 2n = 32, including four typical meta/submetacentric pairs. Some exceptions within the subfamily showed secondary fusion events of

![Figure 3](imageurl) Male and female metaphase plates of Pyrrhulina marilynae, Pyrrhulina aff. marilynae, Pyrrhulina sp., P. obermulleri and Pyrrhulina cf. laeta shows the general distribution of the microsatellites (GA)$_{10}$, (CA)$_{10}$ and (CGG)$_{10}$ on chromosomes. Bar = 5 μm.

![Figure 4](imageurl) Zoo-FISH with the PSEMI-Y probe on male metaphase plates of *P. marilynae* (A), Pyrrhulina aff. marilynae (B), Pyrrhulina cf. laeta (C), Pyrrhulina sp. (D), and *P. obermulleri* (F) shows the distribution of the telomeric (TTAGGG)$_n$ repeats in *P. marilynae*. Bar = 5 μm.
acrocentric chromosomes giving rise to metacentric chromosomes, reducing the diploid number as observed in Nannostomus unifasciatus (Sember et al., 2020). Biarmed chromosomes could also represent remnants of the ancestral karyotype condition that were maintained during the evolutionary processes. However, no ITS was found in any chromosome of P. marilynae, but such a scenario does not exclude the hypothesis of fusion, given that telomeric regions can be lost after the rearrangement occurs (Bolzán, 2017). Thus, to corroborate such hypotheses and to determine whether the evolutionary trajectory of karyotype change in Pyrrhulina is directed mainly towards centric fusions or fissions, cytogenetic data should be discussed in a larger phylogenetic framework of interspecific and intergeneric relationships of Lebiasinidae.

CGH procedures have greatly assisted cytogenetic studies (Symonová et al., 2013; Gioffi et al., 2017; Gioffi et al., 2019), as among all Pyrrhulina studied so far. In fact, despite showing close genomic similarities, the species also show considerable divergences, in addition to species-specific repetitive DNA and C-band patterns, thus helping to understand their differential evolutionary paths, considering the taxonomic problems still pending in this fish group. In addition, multiple and syntenic ribosomal sites are not frequently observed among fishes, but these chromosomal features are very informative cytotoxic markers regarding Pyrrhulininae species. Comparatively, they

**FIGURE 5** Comparative genomic hybridization (CGH) using male-derived genomic probes from Pyrrhulina species hybridized onto male chromosomes of P. marilynae. The common genomic regions are depicted in the 1st column in each line representing the experiments A-D. Hybridization between the gDNA of P. marilynae (Pmar), P. australis (Paus) and Pyrrhulina aff. australis (Pafa) (A); P. marilynae (Pmar), Pyrrhulina aff. marilynae (Pmar) and Pyrrhulina sp. (Psp) (B); P. marilynae (Pmar), P. brevis (Pbre) and P. semifasciata (Psem) (C); P. marilynae (Pmar), P. obermulleri (Pobe) and Pyrrhulina cf. laeta (Pcfl) (D). Bar = 5 μm.
occur more frequently among Pyrrhulina than in other species of this subfamily (de Moraes et al., 2017; de Moraes et al., 2019; Sassi et al., 2019; Toma et al., 2019; Sember et al., 2020). Like Pyrrhulina aff. australis (de Moraes et al., 2017), Pyrrhulina sp., and P. marilynae present multiple 5S rDNA sites and only one 18S rDNA site, thus differentiating them from Pyrrhulina aff. marilynae, P. obermulleri, and Pyrrhulina cf. laeta, as well as from some other Pyrrhulina species (de Moraes et al., 2017; de Moraes et al., 2019), which have multiple 5S and 18S rDNA sites. Furthermore, the syntenic condition for the 18S/5S rDNAs in Pyrrhulina cf. laeta is shared with P. brevis and P. australis, indicating a high rDNA diversity. (Figure 6). In its turn, the 18S rDNA clusters are distributed on distal chromosome positions for all investigated Pyrrhulina species (de Moraes et al., 2017; de Moraes et al., 2019; this study), so as in the species of the sister family, Ctenoluciidae (de Souza e Sousa et al., 2017; de Souza e Sousa et al., 2021).

Microsatellite distribution patterns have significantly contributed to evolutionary studies in fish species, especially regarding sex chromosome differentiation (Kubat et al., 2008; Cioffi et al., 2012; Terenzi et al., 2012; Kejnovský et al., 2013; Poltronieri et al., 2014; Yano et al., 2014; de Freitas et al., 2018). Among the five Pyrrhulina species now investigated, as well as in other previous analyzed ones (de Moraes et al., 2017; de Moraes et al., 2019), the distribution of the microsatellites did not significantly differ among them, although the (CA)15 repeats present a greater number of more conspicuous sites than the other microsatellites, especially in Pyrrhulina aff. marilynae and Pyrrhulina cf. laeta. It is noteworthy that microsatellites have a preferential location in the telomeric and centromeric regions of fish chromosomes (Cioffi and Bertollo, 2012), as occur with the (CA)15 and (GA)15 motifs in Pyrrhulina, despite some interstitial and pericentromeric signs in Pyrrhulina cf. laeta, P. marilynae, Pyrrhulina aff. marilynae and Pyrrhulina sp., thus differentiating these species from others previously studied (de Moraes et al., 2017; de Moraes et al., 2019). Furthermore, it is also frequent that microsatellites and other repetitive sequences occur in the association among fish (Cioffi and Bertollo, 2012), such as in Hepsetus odoe (Carvalho et al., 2017), Lebiasina bimaculata (Sassi et al., 2019), and Silurichthys phaiosoma (Ditcharoen et al., 2020), for example. This is the scenario that also occurs in Pyrrhulina sp., in which the (CGG)10 microsatellite located in the telomeric region of pair 4 shares the same chromosomal region with 18S rDNA repeats.

Fish, besides presenting high diversity in morphological and genetic characteristics, also have a variety of sex chromosome systems (Sember et al., 2021). About nine differentiated systems, involving the XX/XY and ZZ/ZW sex...
chromosomes and their variations, have been identified among species, including several Neotropical ones (Sember et al., 2021). It is noteworthy that among the multiple systems, the $^0X_X_X_X_Y$, $^0\delta X_X_X_Y$ is the most prevalent one, and commonly originated by centric or tandem fusions of the ancestral $Y$ with an autosomal member of the karyotype, giving rise to neo-$Y$ chromosomes, as identified in a variety of fish species (Sember et al., 2021). That includes P. semifasciata, the only Lebiasinidae representative highlighting heteromorphic sex chromosomes so far (de Moraes et al., 2019), in addition to a putative ZZ/ZW sex system present in P. semifasciata includes Lebiasina bimaculata on the origin of the acrocentric pairs, suggesting that putative proto-XY chromosomes Y-derived probe of speci CGH results in the current analyzed species did not reveal any sex-specific differentiated region, our WCP experiment with the Y-derived probe of P. semifasciata entirely painted two acrocentric pairs, suggesting that putative proto-XY chromosomes may occur in these species. Thus, it supports our previous hypothesis on the origin of the P. semifasciata sex chromosome system through centric fusion between the non-homologous acrocentric, giving rise to the large metacentric Y chromosome. That can be considered as an apomorphy of this species when compared to others of the genus. Furthermore, the experiments also showed that although the karyotype of P. marilynae has large metacentric chromosomes, these do not correspond to the heteromorphic sex chromosome of P. semifasciata (Figure 4).

CONCLUSION

Our data advances the understanding of evolutionary trends of the Lebiasinidae, particularly concerning Pyrrhulina. Karyotypes with $2n = 40–42$, with the predominance of mono-armed chromosomes, are more frequent among its species, except for P. marilynae, which has a smaller diploid number ($2n = 32$), and several atypical biarmed chromosomes, a characteristic that differentiates this species from the others analyzed in the genus. However, we cannot rule out the hypothesis that this karyotypic reduction ($2n = 32$) may have been generated by secondary fusions that allowed the formation of the four meta/submetacentric pairs identified in P. marilynae. The present data also highlighted the putative proto-XY chromosomes that may occur in these species and support the occurrence, through centric fusion, of the multiple sex chromosome system of P. semifasciata as an independent evolutionary event of this Lebiasinidae species. Our results highlight the importance of chromosomal data as valuable markers for understanding the evolutionary relationships among Lebiasinidae species.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Committee on Animal Experimentation of the Universidade Federal de São Carlos (process number CEUA 1853260315).

AUTHOR CONTRIBUTIONS

RM and MC carried out the cytogenetic analysis and drafted the manuscript. TH, AA-R, and PV helped in the cytogenetics analysis, drafted and revised the manuscript. TL, GD, FS, VO, EF and MM drafted and revised the manuscript. All authors read and approved the final version of the manuscript.

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