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ORIGINAL ARTICLE

Intriguing evidence of translocations in Discus fish (*Symphysodon*, Cichlidae) and a report of the largest meiotic chromosomal chain observed in vertebrates

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As part of a program to understand the genetics of Amazonian ornamental fish, classical cytogenetics was used to analyze *Symphysodon aequifasciatus*, *S. discus* and *S. haraldi*, popular and expensive aquarium fishes that are endemic to the Amazon basin. Mitotic analyses in *Symphysodon* have shown some odd patterns compared with other Neotropical cichlids. We have confirmed that *Symphysodon* species are characterized by chromosomal diversity and meiotic complexity despite the fact that species share the same diploid number $2n = 60$. An intriguing meiotic chromosomal chain, with up to 20 elements during diplotene/diakinesis, was observed in *S. aequifasciatus* and *S. haraldi*, whereas *S. discus* only contains typical bivalent chromosomes. Such chromosomal chains with a high number of elements have not been observed in any other vertebrates. We showed that the meiotic chromosomal chain was not sex related. This observation is unusual and we propose that the origin of meiotic multiples in males and females is based on a series of translocations that involved heterochromatic regions after hybridization of ancestor wild Discus species.
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Introduction

The Discus fish, genus *Symphysodon* (Heckel, 1840), are the most intriguing and distinctive group among the South American Cichlidae due to their body morphology, beautiful colors and chromosomal features (Feldberg *et al.*, 2003; Ready *et al.*, 2006). Over the last 40 years, a few chromosomal reports, all of which have been restricted to *Symphysodon aequifasciatus*, indicated that *Symphysodon* has the highest diploid number of all cichlids (Ohno and Atkin, 1966; Thompson, 1979; Takai *et al.*, 2002).

Recently, Mesquita *et al.* (2008) indicated that *S. discus* and *S. haraldi* also have a derived karyotype. All of the *Symphysodon* species have a high diploid number ($2n = 60$), with a predominance of meta/submetacentric chromosomes, inter- and intraspecific morphological variation in some chromosomal pairs, and microchromosomes (Table 1). The high diploid number detected in *Symphysodon* was explained as a consequence of polyploidization (Thompson, 1976); it might also have arisen through chromosomal rearrangements, such as pericentric inversions, translocations and fissions/fusions (Mesquita *et al.*, 2008). The different karyotypic formula reported for different *Symphysodon* species may include errors, as the level of chromosome condensation surely interfered in the analysis, and most of authors did not use the terminology 'microchromosomes'.

The karyotypes of the three species contain heterochromatic blocks, which are mainly located in the pericentromeric regions of all chromosomes, and in the proximal regions in the short and long arms of a few chromosomes (Takai *et al.*, 2002; Mesquita *et al.*, 2008). Takai *et al.* (2002) detected a single nucleolar organizer region (NOR) system in *S. aequifasciatus* using silver nitrate staining. However, Mesquita *et al.* (2008) described a multiple NOR system in *S. aequifasciatus* (fifth, tenth, eleventh, fifteenth, twenty-first and twenty-second pairs), *S. discus* (eighteenth and twenty-fourth pairs) and *S. haraldi* (third, fifth, tenth, eleventh, twenty-first and twenty-second pairs).

The analysis of meiotic chromosomal configuration has been very useful for confirming the occurrence of polyploidization, duplication events and chromosomal rearrangements that took place during the chromosomal evolution of the mammals and other vertebrate groups (Villena and Sapienza, 2001; Dumas and Britton-

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Table 1 Karyotype variation described for species of *Symphysodon*

Species	Karyotypic formula	Sample origin	Reference
S. aequifasciatus	48m-sm+8st-a+4mi	Tefé, Amazonas, Brazil	Mesquita *et al.* (2008)
S. aequifasciatus	50m-sm+6st-a+4mi	Tefé, Amazonas, Brazil	Mesquita *et al.* (2008)
S. aequifasciatus	44m-sm+16st-a	—	Ohno and Atkin (1966)
S. aequifasciatus	58m-sm+2st-a	Commercial Source	Thompson (1979)
S. aequifasciatus[a]	52m-sm+8st-a	Commercial Source	Takai *et al.* (2002)
S. discus	54m-sm+6st-a	Barcelos, Amazonas, Brazil	Mesquita *et al.* (2008)
S. discus	50m-sm+10st-a	Barcelos, Amazonas, Brazil	Mesquita *et al.* (2008)
S. haraldi	52m-sm+4st-a+4mi	Manacapuru, Amazonas, Brazil	Mesquita *et al.* (2008)

Abbreviations: a, acrocentric; m, metacentric; mi, microchromosome; sm, submetacentric; st, subtelocentric.
[a]Species described originally as *Symphysodon aequifasciata axelrodi*.

Davidian, 2002; Siqueira Jr *et al.*, 2004; Gallardo *et al.*, 2006). If polyploidization or chromosomal rearrangements have occurred, then the meiotic chromosomal pairing becomes complex and can result in the production of unbalanced or balanced gametes (Sharp and Rowell, 2007).

As in other teleosts, the study of meiotic chromosomes in cichlids has been neglected. The few reports in the literature described remarkable features, such as discordances in chromosome number among the somatic and gonadal tissue cells of *Gymnogeophagus balzanii* (Feldberg and Bertollo, 1984).

In this study, we investigated meiotic chromosomes of wild *Symphysodon* species, with particular focus on the configuration of a complex chromosomal meiotic chain that has not been observed earlier in fish. This information will contribute to understanding the chromosomal rearrangements that have been involved in the karyoevolution of this group of fish.

Materials and methods

Discus specimens were collected from their natural habitat in the Amazon basin, State of Amazonas, Brazil. The fishes were identified according to Bleher's taxonomy, that is, *S. aequifasciatus* (Green Discus), *S. discus* (Heckel Discus) and *S. haraldi* (Brown and Blue Discus) (Bleher, 2006; Bleher *et al.*, 2007).

Thirty-five males and nine females of *Symphysodon* spp. were analyzed in this study, as licensed by the Brazilian Institute of Environment and Renewable Natural Resources—IBAMA (011/2005): eight males and three females of *S. aequifasciatus* from the Tefé river, 15 males and three females of *S. discus* from the Negro river (near the cities of Barcelos and Novo Airão) and 15 males and three females of *S. haraldi* from the Manacapuru river (Figure 1). Voucher specimens were deposited in the Fish Collection at the Instituto Nacional de Pesquisas da Amazônia (INPA) in Manaus, State of Amazonas, Brazil (INPA 28582, INPA 28583 and INPA 25498).

Meiotic chromosomal preparations were obtained using gonadal cells, according to the protocol described by Bertollo *et al.* (1978) with modifications. Uncolchicinized male testicles and immature female ovaries were immersed in a hypotonic solution (KCl 0.075 M) for 30 min and fixed in Carnoy (3 methanol:1 acetic acid) for 20 min, and this step was repeated three times. The material was macerated in 50% acetic acid on a glass slide and dried over a heated plate at 45 °C. All chromosomal preparations were stained with a 5% Giemsa solution for 10 min. The constitutive heterochromatin

was detected according to the method described by Sumner (1972), and NORs were stained with silver nitrate following the protocol described by Howell and Black (1980).

Results

Few females were analyzed because the presence of large amounts of vitello in mature ovaries interferes with the technique used for analysis, rendering it impractical. *S. aequifasciatus*, *S. discus* and *S. haraldi* male and female gonadal cells at interphase and prophase I had no heteropicnotic regions that indicated the presence of sex chromatin (Figures 2a, b and c). The chromosomal behavior in some meiotic phases of *S. aequifasciatus* and *S. haraldi* was similar, but differences were detected among *S. aequifasciatus*/*S. haraldi* and *S. discus*. In diplotene spermatocytes and oocytes, a multivalent chromosomal chain and 20 bivalents were observed in *S. aequifasciatus* and *S. haraldi* (Figures 2d and e), and 30 bivalents were detected in *S. discus* (Figure 2f). In all of the species, most bivalents had two terminal chiasmata. The chromosomal chain (C) reported in diplotene cells of *S. aequifasciatus* and *S. haraldi* was probably composed of 20 chromosomal elements, because up to 20 bivalents (II) were found. Although $2n = 20II + CXX$ represents the highest pairing degree observed in meiotic chromosomes during diplotene of *S. aequifasciatus* and *S. haraldi*, only 2% of analyzed cells have this configuration. In both species, the most frequent configuration was the linear chromosomal chain with a variable number of elements, as well as the numbers of bivalents and univalents (Figure 3). Such variation might be explained by the dynamic cell-division cycle (early, intermediate and later diplotene). Thus, when the number of chain elements and/or bivalents decreased, the number of univalents increased, and a linear chromosomal chain could be detected. Also, in both *S. aequifasciatus* and *S. haraldi*, during diakinesis, a linear chromosomal chain and a variable number of bi- and univalents were visible (Figures 2g–h). In *S. discus*, this stage was characterized by the occurrence of 30 bivalents, some of which exhibited an early chromosomal separation (Figure 2i). *S. aequifasciatus* and *S. haraldi* metaphase I cells had a characteristic zigzag disposition of the chromosomal chain, indicating an alternate orientation of homologous centromeres (Figures 2j and k). *S. discus* metaphase I cells contained typical bivalents aligned in the equatorial plate (Figure 2l). Metaphase II cells from the three species contained $n = 30$ chromosomes, confirming that a regular
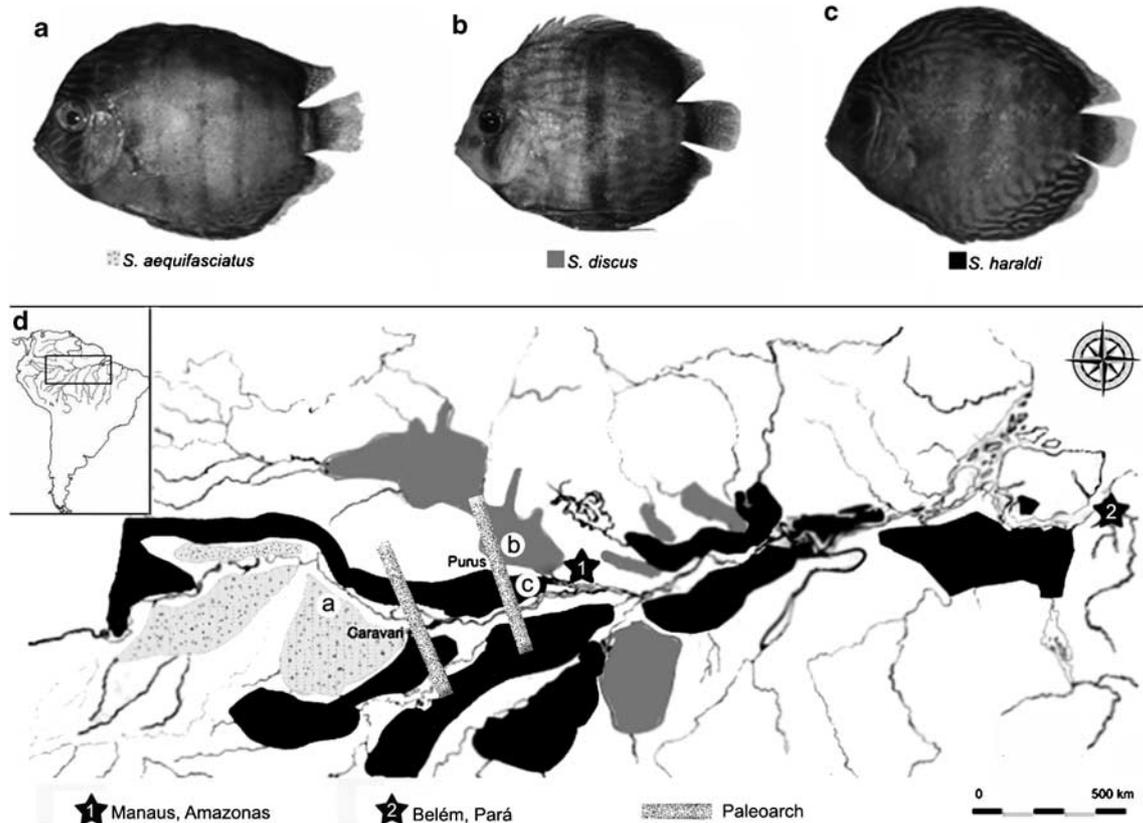
Figure 1 Amazon representatives of the *Symphysodon* species, geographical distribution of *Symphysodon* species (after Bleher (2006), modified) in the Amazon basin and the Purus and Caravari paleoarches. (**a**) *Symphysodon aequifasciatus*. (**b**) *S. discus*. (**c**) *S. haraldi*. (**d**) Geographical map showing the location of Discus sampling sites in Amazonas state, Brazil: a, Tefé; b, Novo Airão; c, Manacapuru.

segregation of all the chromosomes had occurred in the preceding anaphase I (Figures 2m, n and o).

C-banding of diplotene cells in *S. aequifasciatus* and *S. haraldi* indicated that most of the bivalents had two conspicuous heterochromatic blocks, which were probably located in the pericentromeric region, and a variable number of heterochromatic blocks on the chromosomal chain (Figures 4a and b). Positive C-bands were detected in nearly all of the bivalents in *S. discus* diplotene spermatocytes and oocytes, which were similar to the *S. aequifasciatus* and *S. haraldi* bivalents (Figure 4c).

The testicle and ovary cells of the three *Symphysodon* species were positively stained with silver staining in interphase nuclei and prophase I cells. In all the species, the interphase nuclei contained a variable number of silver-stained nucleoli, with up to six nucleolar marks in *S. aequifasciatus* and *S. haraldi*, and four in *S. discus* (Figures 4d, e and f). In *S. aequifasciatus* and *S. haraldi*, up to two NORs were observed at diplotene and diakinesis. In *S. aequifasciatus*, the sites were on the bivalents (Figure 4g), and in *S. haraldi*, the sites were present on one element of the chromosomal chain and on a large bivalent (Figure 4h). *S. discus* cells at late prophase I contained NORs on one or two bivalents (Figure 4i).

Discussion

Despite the lack of a consensus in the taxonomic nomenclature, it is well accepted that there are three distinct *Symphysodon* species based on coloration, morphology, molecular characteristics and geographic distribution throughout the Amazon basin (Ready *et al.*, 2006; Bleher *et al.*, 2007). Discus cytogenetic studies have indicated that a diploid number of $2n = 60$ chromosomes are invariably found in *S. aequifasciatus*, *S. discus* and *S. haraldi*. However, the distinct karyotypic formula, number of NORs and constitutive heterochromatin localization suggested that the karyotypes are species specific (Mesquita *et al.*, 2008).

Both Ready *et al.* (2006) and Bleher *et al.* (2007), by analyzing the mitochondrial cytochrome and D-loop sequences, respectively, suggested that *S. discus* and *S. haraldi* are not genetically different, probably due to introgression events, but both species are genetically distinct from *S. aequifasciatus*. In contrast, Koh *et al.* (1999) reported that there was a clear genetic differentiation between all the three species based upon random amplified polymorphic DNA results and that *S. discus* was the most divergent species relative to the other Discus species.

An analysis of the behavior of meiotic chromosomes in the Discus species has corroborated the mitotic data with regard to the diploid number, heterochromatin and NOR distribution but revealed surprising meiotic features. In *S. aequifasciatus* and *S. haraldi*, 20 bivalents and a large chromosomal chain (up to 20 chromosomal elements) were observed, and in *S. discus*, 30 typical bivalents were observed. It was not possible to affirm which chromosomes form the chromosomal chain using only classical cytogenetic methods. Certainly the chromosomal chains
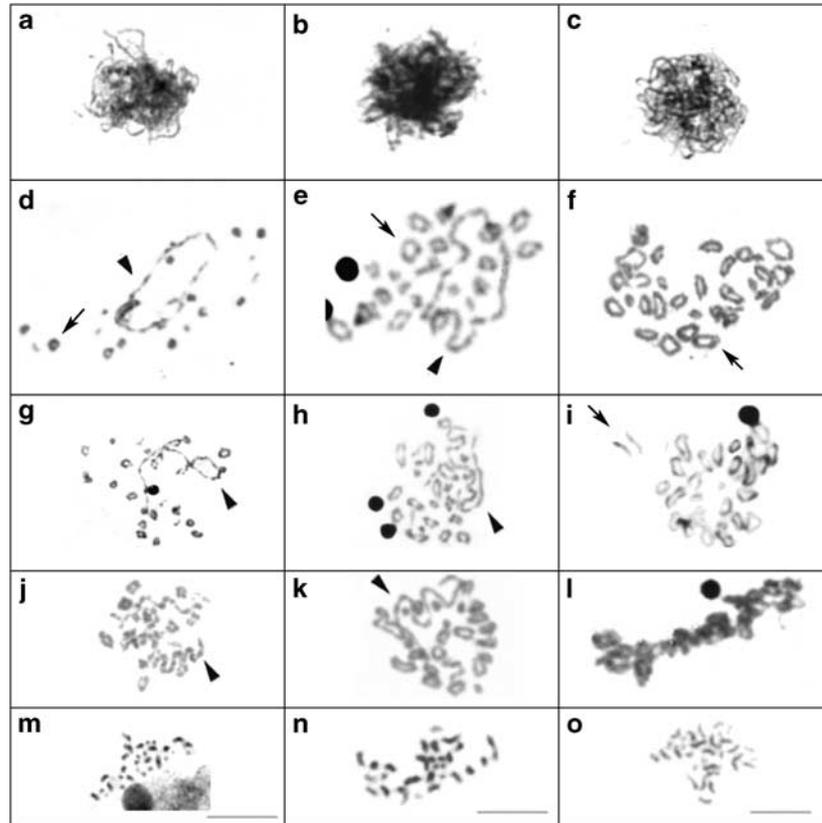
Figure 2 Testicular meiotic cells from *Symphysodon aequifasciatus* (**a, d, g, j, m**), *S. haraldi* (**b, e, h, k, n**) and *S. discus* (**c, f, i, l, o**) after Giemsa staining. (**a–c**) Pachytene. (**d and e**) Diplotene, showing a ring chromosomal chain (arrowhead) and 20 bivalents; the majority of the bivalents had two terminal chiasmata (arrows). (**f**) Diplotene with 30 bivalents, most of them exhibited two terminal chiasmata (arrow). (**g** and **h**) Diakinesis, showing a linear chromosomal chain (arrowheads) and several bivalents and univalents. (**i**) Diakinesis, showing the early separation of the chromosomal elements of a bivalent (arrow). (**j** and **k**) Metaphase I, revealing the zigzag orientation of chromosomes within the chain (arrowheads). (**l**) Metaphase I with bivalents aligned at the equatorial plate. (**m–o**) Metaphase II, with $n = 30$ chromosomes (scale bar: 10 μm).
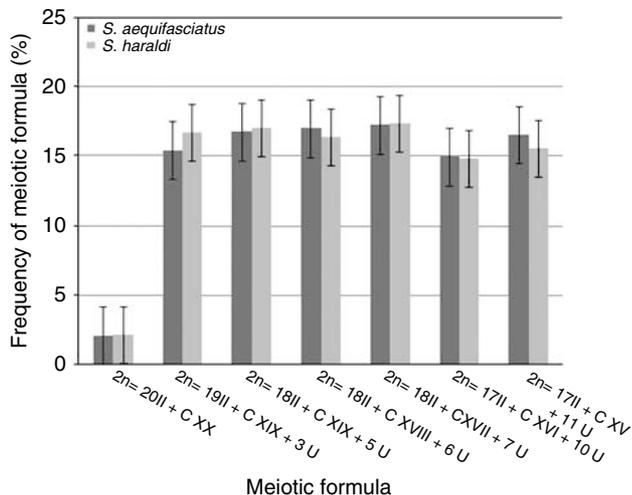

Figure 3 Frequency of meiotic formula in diplotene and diakinesis cells analyzed in *Symphysodon aequifasciatus* and *S. haraldi*, showing standard error bars and a variable number of bivalents, chromosomal elements within the chain and univalents probably due to an early terminalization of chiasmata. II = bivalent; C XX = chromosomal chain with 20 elements; C XIX = chromosomal chain with 19 elements; C XVIII = chromosomal chain with 18 elements; C XVII = chromosomal chain with 17 elements; C XVI = chromosomal chain with 16 elements; C XV = chromosomal chain with 15 elements; U = univalent.

are not composed only of microchromosomes, as a maximum of two such pairs were detected in mitotic analyses. Moreover, one ribosomal site was present in the *S. haraldi* chromosomal chain, and in mitotic chromosomes the third, fifth, tenth, eleventh, twenty-first and twenty-second pairs have Ag-NORs, none of which are microchromosomes (Figure 4h).

Meiotic multiples of more than four chromosomes are rare, and most of these cases are confined to plants and invertebrates, having never been described in fish (for review, see Grützner *et al.*, 2006). Until this study, the biggest chromosomal chains detected in vertebrates were observed in platypus males (*Ornithorhynchus anatinus*) that have five X and five Y chromosomes. These 10 chromosomes form a complex multivalent chain at male meiosis (Grützner *et al.*, 2004). Unlike the platypus, the chromosomal chains observed in the Discus species, *S. aequifasciatus* and *S. haraldi*, are not formed by the sex chromosomes because the chromosomal chains are found in both females and males.

Among fish, multivalents and chromosomal chains not including sex chromosomes have only been reported in a few fish species, such as the salmonids *Salvelinus namaycush* and *Salvelinus fontinalis* (Lee and Wright, 1981; Disney and Wright Jr, 1990), and the gobiid fish *Gobius fallax* (Thode *et al.*, 1988). The origins of multivalent pairings in the above-mentioned species
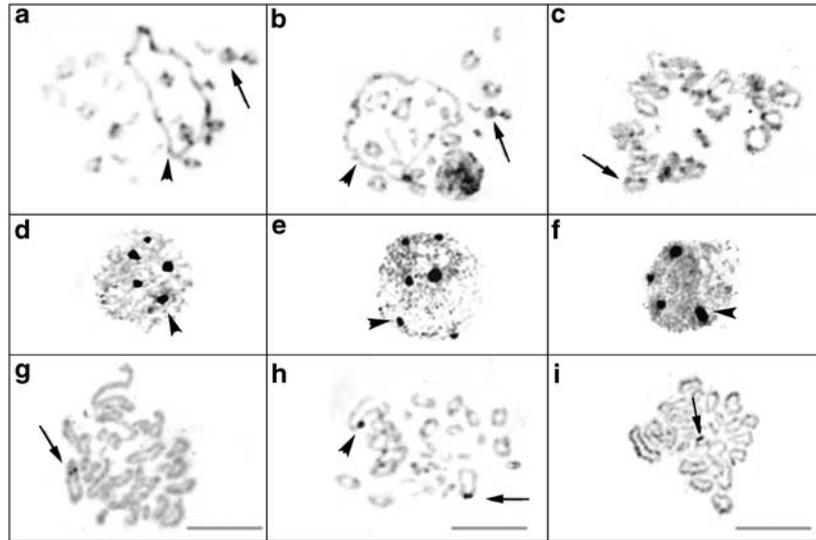
Figure 4 Testicular cells of *Symphysodon aequifasciatus* (**a, d, g**), *S. haraldi* (**b, e, h**) and *S. discus* (**c, f, i**) after C-banding (**a–c**) and silver nitrate staining (**d–i**). (**a** and **b**) Diplotene, showing heterochromatic blocks (arrowheads) in the chromosomal elements of the chain and in nearly all bivalents (arrows). (**c**) Diplotene with positive C-bands (arrows) in most of the bivalents. (**d–f**) Interphase nuclei, revealing five, six and four nucleoli (arrowheads), respectively. (**g**) Diakinesis with an NOR (arrow) on a large-sized bivalent. (**h**) Diakinesis, showing NORs (arrowhead) on an element of the chromosomal chain and on a medium-sized bivalent (arrow). (**i**) Diakinesis with an NOR (arrow) on a small-sized bivalent (scale bar: 10 μm).
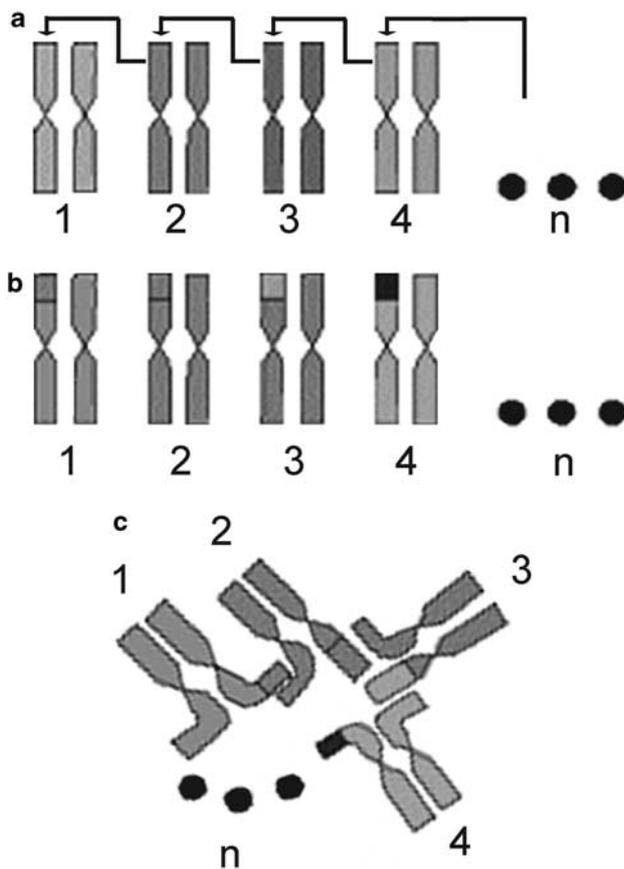

Figure 5 Schematic representation of the possible formation of the chromosomal chain in *Symphysodon aequifasciatus* and *S. haraldi* meiotic cells (*n* represents the other chromosomal pairs evolved in the multiple meiosis). (**a**) Simple and heterozygous translocations involving small segments of some chromosomal pairs. (**b**) Chromosomal elements derived from translocations. (**c**) Meiotic configuration of the chromosomal chain caused by multiple and serial translocations.

have been related to hybridization between species and Robertsonian rearrangements, respectively. In *S. aequifasciatus* and *S. haraldi*, the formation of the chromosomal chains is probably a result of multiple translocations of small terminal segments of chromosomal pairs (Figure 5). Similar mechanisms have been proposed to explain the multivalent configuration in the meiotic cells of the anuran amphibians *Physalaemus petersi* and *Eleutherodactylus binotatus* (Lourenço *et al.*, 2000; Siqueira Jr *et al.*, 2004).

In general, the hybridization and the presence of heterozygous translocations reduce population fitness due to the formation of unbalanced gametes. However, some reports have shown that such translocations may be conserved in populations that have mechanisms that provide an alternate orientation of chromosomes during metaphase I, thus leading to the segregation of translocated chromosomes to the same cell pole (Eichenlaub-Ritter and Winking, 1990; Mirol and Bidau, 1994). As a meiotic chromosomal chain was present in both *S. aequifasciatus* and *S. haraldi* males and females, the heterozygous autosomal translocations seem to be fixed in the population from Manacapuru and Tefé, Amazonas. The zigzag orientation of chromosomes within the chain during metaphase I in *S. aequifasciatus* and *S. haraldi* may be a characteristic of a process that favors the formation of balanced gametes and the fixation of chromosomal rearrangements within such populations. Other animals, including spiders and shrews, presented races in hybridization zones carrying multiple Robertsonian rearrangements with monobranchial homology that results in multivalent chains, and the individuals also are fully fertile (Rowell, 1990; Narain and Fredga, 1997; Sharp and Rowell, 2007).

The additional role of terminal heterochromatic regions (described by Mesquita *et al.*, 2008) in the development of part of the chromosomal chain in *S. aequifasciatus* and *S. haraldi* should be considered. A heterochromatic association of non-homologous

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chromosomes was reported earlier by John and King (1982) in the grasshopper *Heteropternis obscurella* (Orthoptera), which also has a polymorphism of heterochromatin in mitotic karyotypes. Although we did not detect any evidence of intraspecific heterochromatin variation in *S. aequifasciatus* and *S. haraldi*, terminal C-band blocks could be affecting the terminal chiasmata. Thus, the heterochromatin blocks could lead to variation in the number of univalents and bivalents in the different subphases of diplotene and diakinesis as shown in Figure 3. Nevertheless, as only 12 heterochromatic chromosomal arms were present in the karyotypes of these species, the association between heterochromatin blocks in *S. aequifasciatus* and *S. haraldi* could result in a chromosomal chain with 12 elements. This evidence suggests that heterochromatin association is not the only mechanism responsible for the formation of the chromosomal chain, hence there appears to have been multiple translocations.

The heterochromatin in fish is enriched with repetitive sequences, and most of them are transposable elements (for review, see Martins, 2007; Ferreira and Martins, 2008). The transposable elements are considered the major drivers of genomic and biological diversity in vertebrates, and possibly play important roles in speciation and major evolutionary transitions (Bohne *et al.*, 2008). The karyoevolution in *Symphysodon* probably did not occur solely by polyploidy or chromosomal rearrangements, as suggested by Thompson (1976) and Mesquita *et al.* (2008), respectively, but may have been also mediated by transposable element accumulation in terminal heterochromatin and translocations evolving from these regions. Hybridization among Discus species without chromosomal a chain (for example, *S. discus* and an ancestor of *S. aequifasciatus,* and *S. haraldi*) could have triggered transposition and resulted in a population with multiple chromosomal chain in males and females.

Since the 1960s, wild Discus fish have been exported to Japan, Germany and the United States to produce new varieties and to supply to the ornamental fish market. Owing to the trial-and-error breeding programs of the different wild populations to produce novel *Discus* characteristics, today, over 600 different variants now exist, and at least 1.5 million Discus are bred every month worldwide (Chan, 1991; Bleher, 2006).

The artificial selection processes used in Discus breeding have been employed for over 50 years. Selection for strong colors and desirable body and fin shapes has resulted in several cultivated forms (Bleher and Göbel, 1992). Breeders have indicated that most of the color variants are the result of inbreeding and outcrossing *S. aequifasciatus* and *S. haraldi* species. The genetic data found in wild and cultivated Discus fish, as revealed by random amplified polymorphic DNA markers, are consistent with these data (Koh *et al.*, 1999). *S. discus* is not commonly used in outcrosses, and this is probably because the crosses between Discus varieties with a chromosomal chain (those originating from *S. aequifasciatus* and *S. haraldi*) and those without a chromosomal chain (those originating from *S. discus*) have resulted in inviable or infertile offsprings.

From an evolutionary point of view, the chromosomal chains observed in *S. aequifasciatus* and *S. haraldi* appear to be derivative characteristics, and the presence of these chains in both species indicates that they are probably

sister species, with *S. discus* as a sister group or ancestor. However, molecular analyses based on the partial D-loop and cytochrome *b* sequences did not provide unambiguous affirmation that *S. discus* forms the deepest branch within *Symphysodon* (Ready *et al.*, 2006; Bleher *et al.*, 2007).

The tribe heroine, which includes the Discus fish and cichlasomines, is the most recent lineage in the Neotropical radiation, and the estimated age of the emergence of these groups is 12 Mya, based upon molecular analysis of 16S gene sequences. The subsequent diversification of the heroines is estimated to have occurred at 7.5 Mya, in the late Miocene (Farias *et al.*, 1998). It is probable that at this time, an ancestor of the Discus fish existed that had a significantly increased diploid (from 48 to 60 chromosomes) number, due to polyploidy (Thompson, 1976) or chromosomal rearrangements (Mesquita *et al.*, 2008).

Contrary to the hypothesis proposed by Ready *et al.* (2006), we believe that *S. discus* (Heckel Discus) is probably the oldest species. The *S. discus* could have hybridized with an ancestral Discus species that may now be extinct. The hybridization could have triggered the movement of mobile elements, causing multiple chromosomal rearrangements (such as translocations) that led to the original meiotic chromosomal chain. The diversification of *S. aequifasciatus* and *S. haraldi* could have occurred at 5 or 3 Mya when the Caravari and Purus arches were formed, respectively (Hoorn *et al.*, 1995; Irion *et al.*, 1995; Lundberg *et al.*, 1998) (Figure 1). The current subterranean arch (Purus or Caravari) may have acted as a temporary barrier in the ancient populations that would have allowed for species differentiation following the fragmentation of their zones of distribution. Thus, the ancestral *S. aequifasciatus* and *S. haraldi* populations could subsequently have come into secondary contact.

The chromosomal evolution of *Symphysodon* appears to be complex. Further studies should sample new populations and investigate the chromosomal distribution of repetitive elements and include synaptonemal complex analysis. Such information will contribute to a better understanding of the role of chromosomal changes in speciation in these taxa, and will also be applicable to breeding programs for the production of animals of commercial importance.

Acknowledgements

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