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Chromosomal variability in the wild ornamental species of *Symphysodon* (Perciformes: Cichlidae) from Amazon

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Cytogenetic studies were conducted on three discus species which inhabit the Amazon in Brazil: *Symphysodon haraldi* from Manacapuru, *S. aequifasciatus* from Tefé and *S. discus* from Barcelos. All individuals showed $2n=60$ chromosomes, most of them biarmed. No sexual chromosomal heteromorphism was verified. However, different karyotypic formulae, owing to the presence of subtelocentric chromosomes, were verified for *S. aequifasciatus* and *S. discus*. One of the karyotypic formulae from *S. aequifasciatus* (cytotype 2) differs from the others, due to one of the homologues in the first chromosome pair being significantly larger than the other. A large variability was observed toward the nucleolar organizer regions (NOR) of *S. haraldi* and *S. aequifasciatus*. Although the number of silver-stained blocks varied from 2 to 5, confirming different NOR patterns, at least seven homologue pairs were involved with NORs. In *S. discus* only two marks were observed, however two chromosome pairs were involved, characterizing a multiple NOR system for the three species. The heterochromatic blocks were mainly located in the pericentromeric region of all chromosomes but, in some of them, they are also located in the proximal regions, both in the short and long arms. Moreover, in the cytotype 2 from *S. aequifasciatus*, an interstitial heterochromatic block was observed on the long arm of the largest homologue of the first pair. A direct comparison of karyotypes from more related genera (*Heros*, *Uaru*, *Mesonauta* and *Pterophyllum*), makes it clear that a succession of chromosomal rearrangements, mainly pericentric inversions, translocations and fissions/fusions occurred resulting in the present diploid number and intraspecific karyological variability found in *Symphysodon*.

Estudos citogenéticos foram conduzidos em três espécies de acará-disco que habitam a Amazônia no Brasil: *Symphysodon haraldi* coletada em Manacapuru, *S. aequifasciatus* coletada em Tefé e *S. discus* em Barcelos. Todos os indivíduos apresentaram $2n=60$ cromossomos, a maioria deles com dois braços. Heteromorfismo de cromossomos sexuais não foi detectado. Porém, diferentes fórmulas cariotípicas, devido à presença de cromossomos subtelocêntricos, foram verificadas em *S. aequifasciatus* e em *S. discus*. Uma das fórmulas cariotípicas (citótipo 2) de *S. aequifasciatus* difere das outras, devido a um dos homólogos do primeiro par cromossômico ser significativamente maior que o outro. Uma grande variabilidade foi observada em relação à região organizadora do nucléolo (RON) de *S. haraldi* e de *S. aequifasciatus*. Embora, o número de marcações coradas por Nitrato de Prata variou de 2 a 5, confirmando diferentes padrões de RON, pelo menos sete pares de homólogos foram envolvidos com a RON. Em *S. discus* foram observadas apenas duas marcas, envolvendo, contudo, dois pares de cromossomos, caracterizando um sistema de RONS múltiplas, para as três espécies. Os blocos heterocromáticos estão localizados, principalmente, na região pericentromérica de todos os cromossomos, sendo que alguns deles também foram observados em regiões próximas aos centrômeros, tanto nos braços curtos quanto nos braços longos. Além disso, no citótipo 2 de *S. aequifasciatus*, um bloco heterocromático foi observado, intersticialmente no braço longo do maior homólogo do primeiro par. Uma comparação direta dos cariótipos dos gêneros mais relacionados (*Heros*, *Uaru*, *Mesonauta* e *Pterophyllum*), deixa claro que ocorreu uma sucessão de rearranjos cromossômicos, principalmente inversões pericêntricas, translocações e fissões/fusões, que resultou no presente número diplóide e na variabilidade cariotípica encontrada em *Symphysodon*.

Key words: Discus, Cytogenetics, Polymorphism, Microchromosomes, Multiple NORs.

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Introduction

In Brazil, the Amazon basin is responsible for more than 90% of all ornamental fish exported to North American, European and Asian markets and the revenues generated by this trade is valued at approximately 3 million US\$ annually (Chao, 2001). The discus fish is characterized by a colourful and compressed disk-shaped body that captures the attention of aquarium hobbyists worldwide, making it one of the most demanded Amazonian fish in the ornamental fish trade (Goldstein, 1973; Silva & Kotlar, 1980; Axelrod, 1995). Among the top 10 of the main freshwater ornamental fishes certainly the cichlid discus is enrolled on it.

Previously, some authors had suggested that *Symphysodon* contained two species split into five subspecies (Silva & Kotlar, 1980), while others suggested the existence of only a single species of *Symphysodon* (Mazeroll & Weiss (1995). According to recent publications, *Symphysodon* currently includes three species: *S. discus* Heckel, 1840 (Heckel Discus), *S. aequifasciatus* Pellegrin, 1904 (Blue or Brown Discus) and *S. tarzoo* Lyons, 1959 (Green Discus) (Ready *et al.*, 2006) or *S. discus*, *S. haraldi* Schultz, 1960 and *S. aequifasciatus* (Bleher, 2006; Bleher *et al.*, 2007). Ready's and Bleher's publications refer to the Heckel Discus as *S. discus*. However, Ready *et al.* (2006) refer to the Green discus as *S. tarzoo*, while Bleher *et al.* (2007) refer to it as *S. aequifasciatus*. Ready *et al.* refer to the Blue and Brown discus as *S. aequifasciatus*, while Bleher *et al.* recognize them as *S. haraldi*.

Endemic to the Amazon Basin, *Symphysodon* occurs in tributaries of the rio Amazonas. Heckel Discus is found primarily in the tributaries of the rios Negro, Branco, Purus, Abacaxis and Trombetas, whereas Green Discus occurs in the Western Amazon, and Blue and Brown Discus has the widest distribution occurring along almost the entire length of the rio Amazonas from 49° to 70° longitude West (Ready *et al.*, 2006; Bleher *et al.*, 2007).

Despite the large importance of discus fish in the ornamental fish industry, due mainly to a well known breeding program that introduces a half dozen new strains every year, only a handful of genetic studies have been conducted. Over the last 40 years, the chromosomal structure (Ohno & Atkin, 1966; Thompson, 1979; Takai *et al.*, 2002) and cellular DNA content (Ohno & Atkin, 1966) of *S. aequifasciatus* have been described. More recently, the DNA stretches of *S. aequifasciatus* were either studied to analyze the different strains, with the use of RAPD, or to provide the molecular relationship among genera, by using DNA sequencing (Koh *et al.*, 1999; Farias *et al.*, 2000; 2001; Degani, 2003). Currently, analysis of mitochondrial DNA (control region and cytochrome b) has revealed genetically distinct clades/lineages of *Symphysodon* (Ready *et al.*, 2006; Bleher *et al.*, 2007) and evidence of introgressive hybridization. The aim of this work

is to present an evaluation of karyotypic data from "wild discus", and to discuss the cytogenetic data from a karyo-evolutionary perspective.

Material and Methods

Discus specimens were collected from natural habitat in the Amazon Basin and were identified according to diagnostic keys provided by Ready *et al.* (2006), Bleher (2006) and Bleher *et al.* (2007), and we opted to follow Bleher's taxonomy, *i.e.*, *S. aequifasciatus* (Green discus), *S. haraldi* (Brown/Blue discus) and *S. discus* (Heckel discus).

The species collected were: *Symphysodon aequifasciatus* (11 males and five females) from lago Bauana in the rio Tefé system; *S. haraldi* (10 males and 10 females) from the rio Manacapuru; and, *S. discus* (two males, five females and one of indefinite sex) from the Boi-boi Stream, in the middle rio Negro, located directly in front of the town of Barcelos (Fig. 1). The specimens from lago Bauana were identified as belonging to *S. aequifasciatus*, considering the extent of its geographic distribution (Ready *et al.*, 2006; Bleher, 2006; Bleher *et al.*, 2007) and ongoing DNA analysis (Izeni Farias, pers. comun.). However, these specimens, after preservation in formalin, did not present *S. aequifasciatus*'s main diagnostic features, *i.e.*, the presence of red spots on the anal fin and/or on the body.

Voucher specimens were deposited in the Fish Collection at the Instituto Nacional de Pesquisas da Amazônia (INPA) in Manaus, Amazonas State, Brazil: INPA 28582 *Symphysodon aequifasciatus* (Tefé), INPA 28583 *Symphysodon haraldi* (Manacapuru), and INPA 28584 *Symphysodon discus* (Barcelos).

Chromosomal preparations were obtained from kidney cells, following the "air-drying" technique of Bertollo *et al.* (1978). Approximately 30 metaphasic plates were analyzed from each individual. Nucleolar organizer regions (NORs) were identified by silver nitrate according to Howell & Black (1980) and the C bands utilizing the barium hydroxide treatment (Sumner, 1972). Whenever possible, slides were stained sequentially with Giemsa, C-banding and silver nitrate solution according to procedures described in Centofante *et al.* (2002).

The karyotype was determined for each specimen and a total of nine were measured (five from Manacapuru, two from Tefé and two from Barcelos). However, the last ten pairs were measured for all individuals, due to their small size. Chromosome morphology was based on arm ratios (long arm length divided by short arm length) as proposed by Levan *et al.* (1964). The measurements were made with the help of a dry-tip compass and a pachymeter and arranged in a size-decreasing order. Chromosomes were classified in two groups, as proposed by Thompson (1979) to Cichlidae: metacentric-submetacentric (m-sm) and subtelocentric-acrocentric (st-a). For an inter- and intraspecific comparison purpose we opted to organize the karyotype in size decreasing order. The fun-

damental number (FN) was determined by considering m-sm with two arms, st-a and microchromosomes with one arm.

Results

All individuals, male and female, possessed a diploid number equal to 60 chromosomes. There were no differences in chromosome morphology between males and females in the three species. However, different intra-populational karyotypic formulae were found, owing to the presence of subtelocentric chromosomes, indicating the occurrence of chromosome polymorphism among the individuals from *Symphysodon aequifasciatus* and *S. discus*, but not from *S. haraldi*. In *Symphysodon haraldi* a single karyotypic formula was observed since all specimens presented 52 m-sm + 4 st-a (26, 27 pairs) + 4 mi (29, 30 pairs) and NF=112 (Fig. 2A).

In *Symphysodon aequifasciatus* two cytotypes were observed. In the first, seven males and five females presented 48 m-sm + 8 st-a (8, 15, 27, 28 pairs) + 4 mi (29, 30 pairs), NF=108 (Cytotype 1), while in the second, four males presented 50 m-sm + 6 st-a (16, 26, 27 pairs) + 4 mi (29, 30 pairs), NF=110 (Cytotype 2). Besides the distinct karyotypic formula, the differences between both cytotypes were due to one of the homologues in the first chromosome pair being significantly larger than the other (CR% = 8.54/7.73) and the size differences between 3rd and 4th chromosome pairs (Fig. 2B, 2C).

In *Symphysodon discus* two cytotypes were observed. In the first, one male, five females and one undefined sex presented 50 m-sm + 10 st-a (10, 27, 28, 29, 30 pairs), NF=110 (Cytotype1) and one male presented 54 m-sm + 6 st-a (28, 29, 30 pairs), NF=114 (Cytotype2) (Fig. 2D, 2E).

The Ag-NORs revealed an interesting pattern for *Symphysodon* species. *Symphysodon haraldi* presented two active NOR sites in 68% of analyzed cells however, the active NOR sites varied of two to five, while *S. aequifasciatus* presented the modal number of two active NOR sites in 93% of analyzed cells, except for a single specimen that exclusively presented three active NORs. The NORs occupied the whole short arm or its terminal end, and, at times, the terminal portion of the long arms. Despite this, based on the size and morphology of the NOR-bearing chromosome, at least seven homologue pairs were apparently involved in the nucleolar organization of *S. haraldi* (3rd, 5th, 10th, 11th, 21st, 22nd) and *S. aequifasciatus* (5th, 10th, 11th, 15th, 21st, 22nd). Five of these chromosome NOR carriers were shared between *S. haraldi* and *S. aequifasciatus*, and each one had one exclusive pair (Fig. 3A, B, C and Fig. 4A, B). Conversely, *S. discus* presented only two NOR-bearing chromosomes (100% of analyzed cells), being the Ag-NORs always located on the short arms. However, in seven individuals (cytotype 1) the Ag-NORs were assigned to 18th pair (single NOR phenotype) and in one individual (cytotype 2) to the 18th and 24th (multiple NOR phenotype).

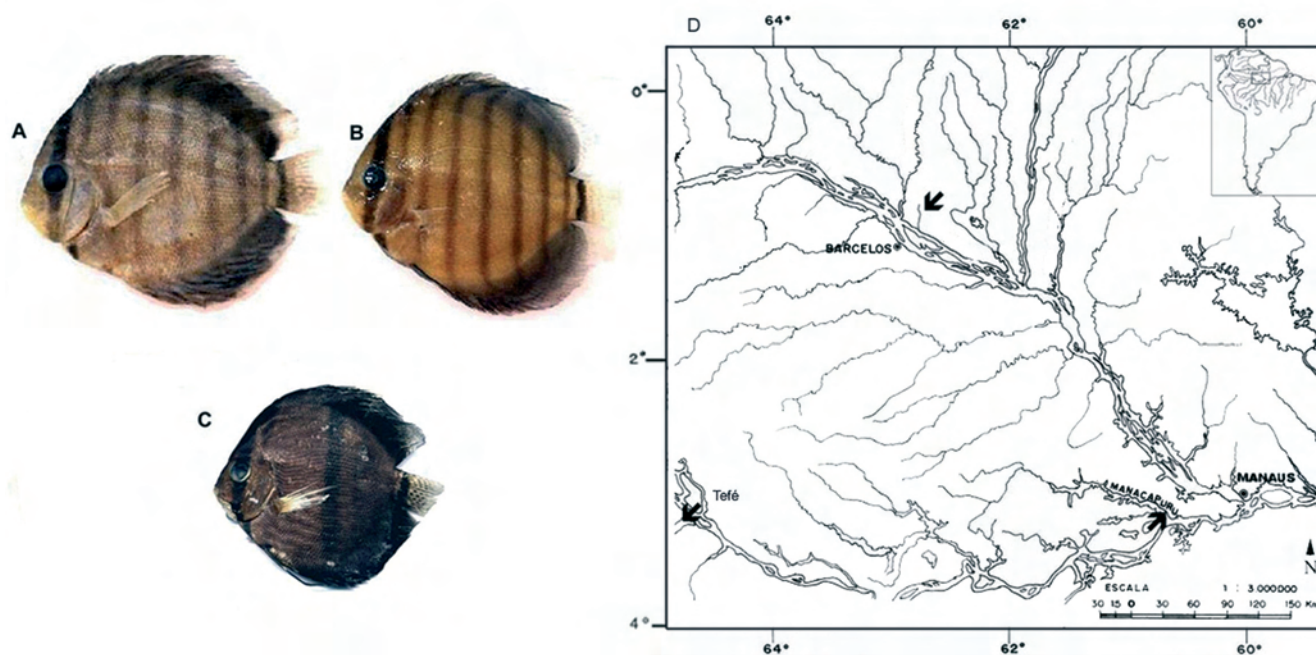


Fig. 1. Photo of analyzed species: (A) *Symphysodon haraldi* from Manacapuru, (B) *S. aequifasciatus* from Tefé, (C) *S. discus* from Barcelos, and map showing the location of discus sampling sites.

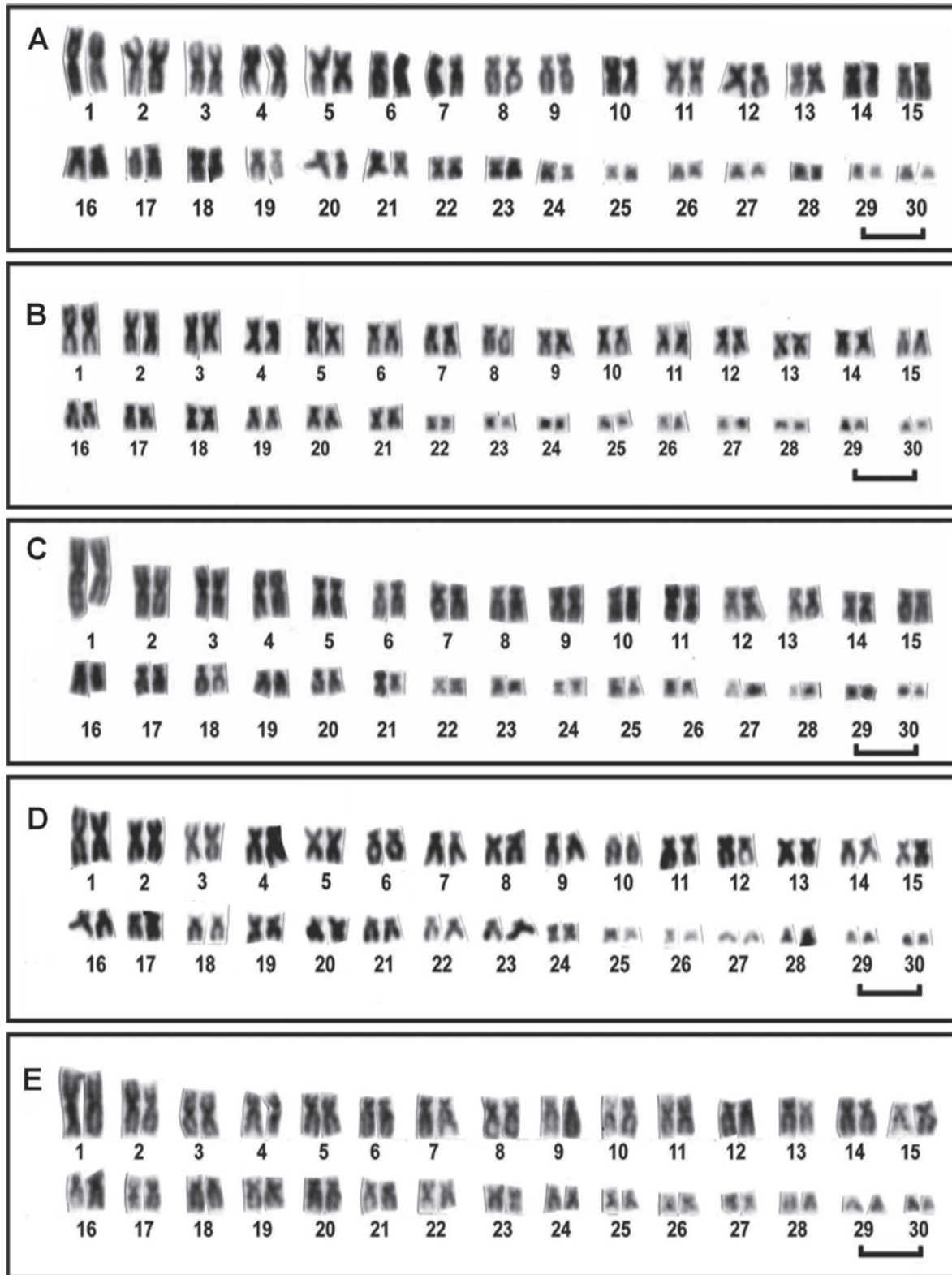


Fig. 2. Conventional Giemsa-stained karyotypes: (A) *Symphysodon haraldi* from Manacapuru, the pairs 26-27 are st-a and 29-30 are microchromosomes; (B) *S. aequifasciatus* from Tefé (cytotype 1), the pairs 8, 15, 26 and 27 are st-a and 29-30 are microchromosomes; (C) *S. aequifasciatus* from Tefé (cytotype 2), the pairs 16, 26 and 27 are st-a and 29-30 are microchromosomes; (D) *S. discus* from Barcelos (cytotype 1), the pairs 10, 27-30 are st-a; (E) *S. discus* from Barcelos (cytotype 2), the pairs 28-30 are st-a. Scale bar represents 5 μ m.

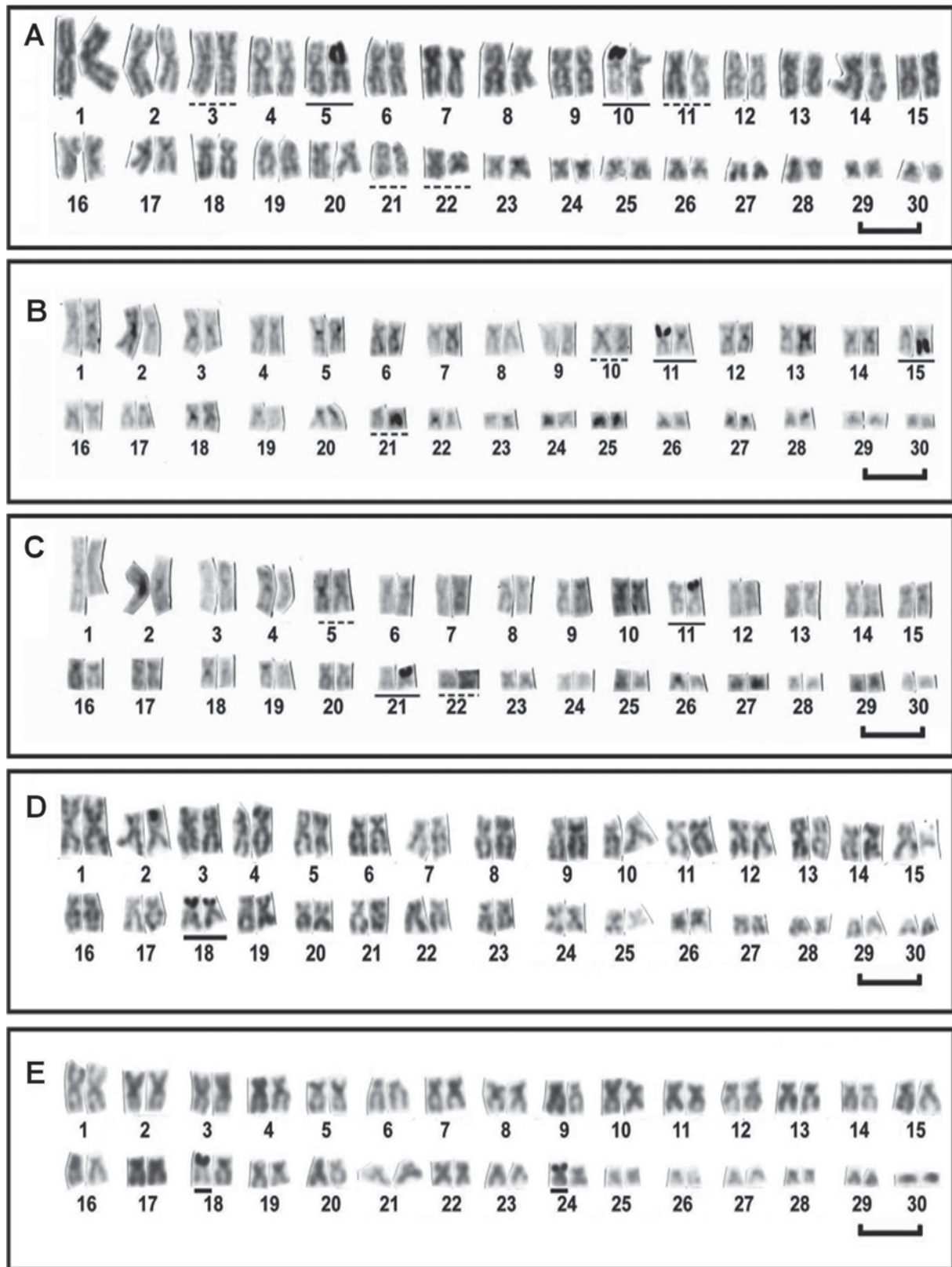


Fig. 3. Karyotypes stained with silver nitrate: (A) *Symphysodon haraldi* from Manacapuru; (B) *S. aequifasciatus* from Tefé (cytotype 1); (C) *S. aequifasciatus* from Tefé (cytotype 2); (D) *S. discus* from Barcelos (cytotype 1); (E) *S. discus* from Barcelos (cytotype 2). The underlined pairs are involved with the NORs. Scale bar represents 5 μ m.

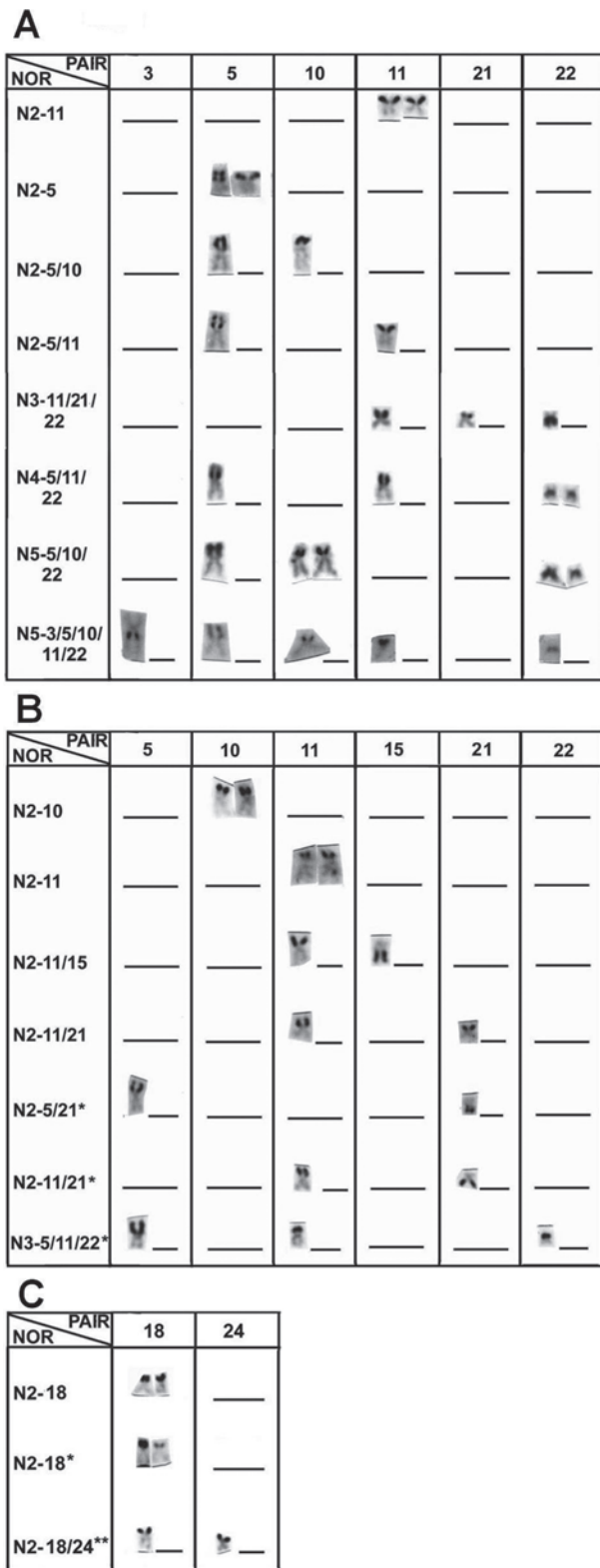


Fig. 4. NORs phenotypes (Nx-xx) corresponding to the numbers of observed NORs and their karyotypic position (pair) seen in: (A) *Symphysodon haraldi* from Manacapuru; (B) *S. aequifasciatus* from Tefé, * represents cytotype 2; (C) *S. discus* from Barcelos, * represents heteromorphic NOR, ** represents cytotype 2.

type). Further, the 18th pair presented a size polymorphism for the NOR (Fig. 3 D, E and Fig. 4C). Considering the number of Ag-NORs phenotypes observed in *Symphysodon haraldi*, *S. aequifasciatus* and *S. discus*, they presented up to eight, seven, and three Ag-NOR phenotypes, respectively (Fig. 4A, B, C).

All individuals have heterochromatic blocks, which were quite evident on the pericentromeric regions of all chromosomes, and on many of them these blocks were also found in the proximal regions of both arms (Fig. 5 A, B, C, D, E). In *S. aequifasciatus*, which showed one of the homologues in the first chromosome pair being significantly larger than the other, presented one interstitial heterochromatic block on the long arm (Fig. 5 C).

Discussion

The cichlids belong to the Perciformes group of fish which present a bimodal diploid number: 48 chromosomes in New World species and 44 in Old World species (reviewed in Feldberg *et al.*, 2003). Morphological and molecular studies of the Cichlidae family place *Symphysodon* in the subfamily Cichlasomatinae, in the Heroini tribe (Kullander, 1998; Farias *et al.*, 2000; 2001). *Symphysodon* is closely related to a group of cichlids which encompasses the genera *Heros*, *Mesonauta*, *Pterophyllum*, and *Uaru*. Except for the last genus in which the species possesses 46 chromosomes, the other ones all possess 48. Surprisingly, the diploid number found in the three species of *Symphysodon* is 60 chromosomes (Ohno & Atkin, 1966; Thompson, 1979; Takai *et al.*, 2002; present work).

In a broad sense, fish cytotaxonomy is the study of the relationships and classification of species using both classical systematic techniques and comparative studies of chromosomes. In the present paper, we show that *Symphysodon aequifasciatus*, *S. haraldi* and *S. discus* present the most derived karyotype amongst all cichlids given the pronounced differences observed in the diploid number ($2n=60$ chromosomes) and fundamental number, when compared to the 135 cichlid species karyotyped so far (Feldberg *et al.*, 2003). By possessing a high diploid number and presenting most chromosomes with two arms, their karyotypic structures differ in many aspects from the presumed ancestral karyotype of all cichlids (*i.e.* $2n=48$ chromosomes, mostly uniarmed) (Thompson, 1979; Feldberg *et al.*, 2003).

Thompson (1976; 1979) was the first to suggest that *S. aequifasciatus* presents the most aberrant karyotype found among all the neotropical cichlids, and that this karyotype probably originated from a succession of events, including polyploidization.

A direct comparison of karyotypes from all related genera (Heroini) makes it clear that the occurrence of a succession of chromosomal rearrangements, mainly pericentric inversions,

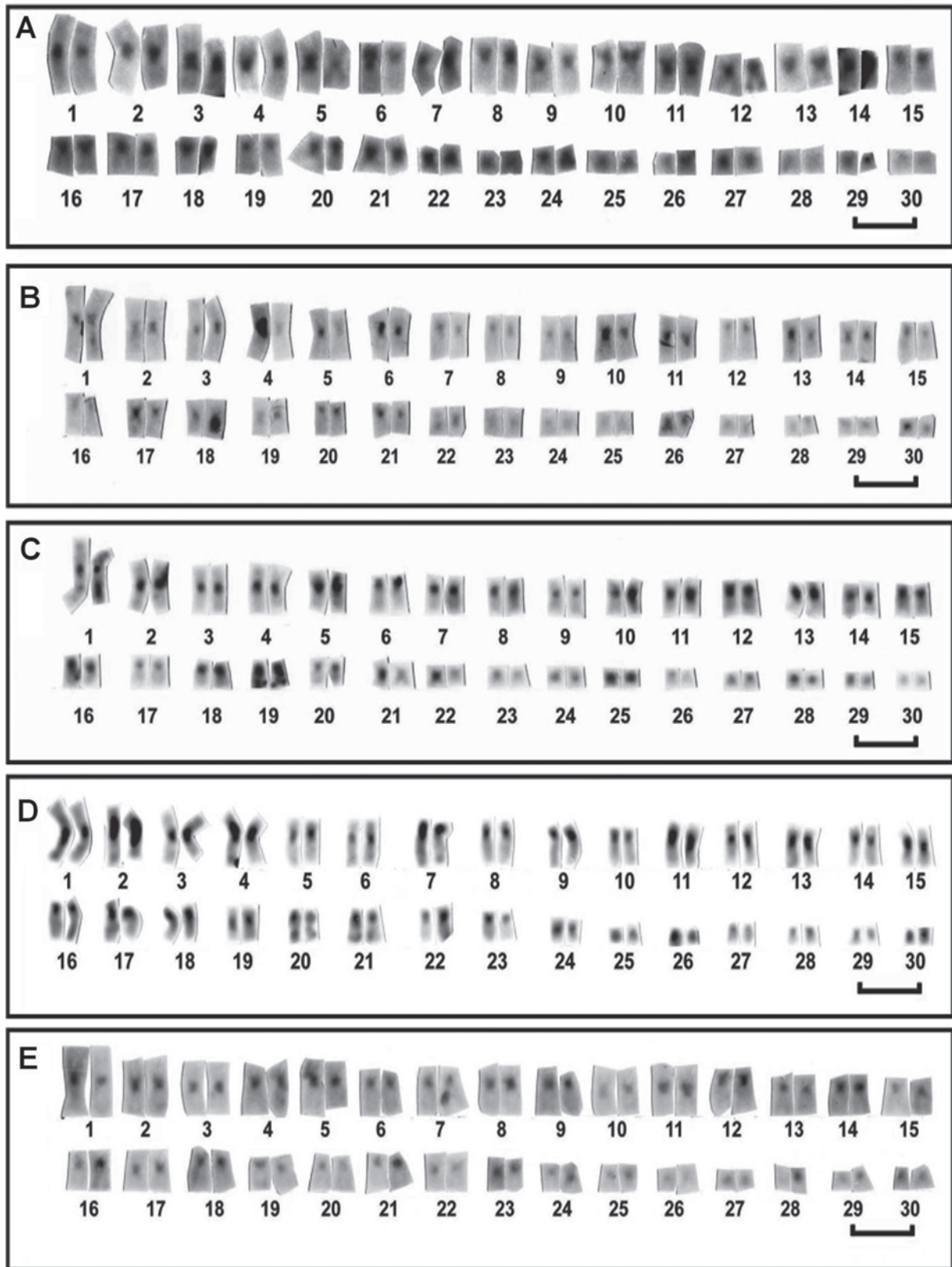


Fig. 5. Karyotypes with C-bands: (A) *Symphysodon haraldi* from Manacapuru; (B) *S. aequifasciatus* from Tefé (cytotype 1); (C) *S. aequifasciatus* from Tefé (cytotype 2); (D) *S. discus* from Barcelos (cytotype 1); (E) *S. discus* from Barcelos (cytotype 2). Scale bar represents 5 μm.

translocations and fission/fusions was necessary in order to create the karyotype found in *Symphysodon*. This sequence of events could account for the appearance of the m-sm chromosomes and to explain the reduced size of some chromosomes.

Ohno & Atkin (1966) and Thompson (1979) noticed that *Symphysodon aequifasciatus* presented some pairs of minute chromosomes. The wild forms of *S. aequifasciatus*, *S. haraldi* and *S. discus* karyotyped in the present paper also presented minute chromosomes. Such asymmetry of macro and microchromosomes was evident in the karyotype of *Symphysodon*, where at least nine last pairs of minute chromosomes in *S. aequifasciatus*, and at least six pairs in both *S. haraldi* and *S. discus* were visible.

Microchromosomes are tiny chromosomes (Denton, 1973) which vary in size between 0.5 and 1.5 μm (Mattey, 1975). They visually resemble a metaphase dot, in which it is not possible to identify the centromere nor assign a chromosome morphology (Tegelström & Rytman, 1981). Microchromosomes can present constitutive heterochromatin and nucleolar organizer regions (Tegelström & Rytman, 1981; Tegelström *et al.*, 1983; De Lucca, 1983; Fillon *et al.*, 1998; Goldschmidt *et al.*, 2000). Considering these descriptions, in *Symphysodon aequifasciatus* and in *S. haraldi* the pairs 29 and 30 were considered microchromosomes, as they did not possess a distinctive morphology in all metaphasic plates. Conversely, in *S. discus*, the size of the minute chromosomes resembled that of a microchromosome. However, it was possible to identify their centromeric position and, consequently, they were not characterized as microchromosomes. This characteristic differentiates *S. discus* from the other two species.

Microchromosomes in vertebrates are rare, being found mainly in reptiles and birds (Gutierrez & Bolanos, 1980; Bull & Legler, 1980; Tegelström & Rytman, 1981). In fish, microchromosomes have been described in such primitive species as *Scaphirhynchus platorhynchus* (Acipenseriformes), *Hydrolagus coliei* (Chimaeriformes) and *Lepisosteus productus* (Lepisosteiformes) (Ohno *et al.*, 1969). The fact that minute chromosomes have been found in *Symphysodon* strongly suggests that rearrangements, namely fission/fusions, pericentric inversions and translocations, were necessary for the derivative karyotype of these species to evolve.

The difference between the cytotypes was related to the number of chromosomes found in m-sm and st-a types, indicating the occurrence of polymorphism within *S. aequifasciatus* and *S. discus*, but not within *S. haraldi*. Also, the cytotype 2 in four individuals of *S. aequifasciatus* differs from the others in that one homologue of the first pair presents a duplication or a translocation on the long arm, con-

firmed by C-band. Interestingly, this chromosome with interstitial C-band has never been seen before in homozygote and was detected in both males and females (unpublished data).

Regarding the NORs, *S. haraldi* and *S. aequifasciatus* presented a high degree of variability relative to the NOR-bearing chromosomes. Intra and inter-individual variation was significantly higher in *Symphysodon haraldi* and, additionally, was also found on the activation of the rDNA sites in *S. aequifasciatus*. Conversely, *S. discus* always presented two NOR-bearing chromosomes. However, on cytotype 1 these were only one pair (18th), and on cytotype 2 the markings were displayed on two non-homologue chromosomes (18th and 24th), thus proving the multiple NOR characteristic in three species. The results of the present study showed clearly that differences among chromosomal NOR patterns in *Symphysodon* species exist, and these NORs patterns are taxonomically informative.

This variability relative to the number of NOR-bearing chromosomes could be due to a difference in activity among the homologues or a difference in the number of ribosomal cistrons, due to an unequal disassociation during cell division or translocations involving these chromosomes. It was still possible to find a difference in the NOR active site position of the same chromosome for the different cytotypes, suggesting the occurrence of pericentric inversions in NOR-bearing chromosomes.

According to Feldberg *et al.* (2003), Cichlidae may be characterised as a family presenting a single NOR system (only one pair of NORs) located on the larger chromosome of the complement. This feature appears to be a plesiomorphic characteristic for cichlids. Therefore, the three species possess a derived chromosomal structure presenting multiple NORs. Further, *Symphysodon haraldi*, *S. aequifasciatus* and *S. discus* were characterised by the presence of eight, seven, and three Ag-NOR phenotypes, respectively (Fig. 4). Thus we considered this NOR polymorphism as a product of distinct activation of presence/absence type. The multiplicity of NORs showing intra- and interindividual differences in the number and localization of the rDNA genes have been observed in many Neotropical fish species, however not in Neotropical cichlids.

Cytogenetic, molecular and morphological data suggest a large variability in the genus *Symphysodon* (Kokoscha & Greven, 1996; Koh *et al.*, 1999; Ready *et al.*, 2006; Bleher *et al.*, 2007). Therefore, studies of *Symphysodon* in its geographic distribution, making use of molecular cytogenetic together with molecular, morphological, taxonomic and systematic studies, could contribute to a better understanding of the complexity existing in this genus.

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