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Chromosomes of three freshwater stingrays (Rajiformes Potamotrygonidae) from the Rio Negro basin, Amazon, Brazil

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Abstract

Potamotrygonidae is the representative family of South American freshwater elasmobranchs. It is a monophyletic group containing 20 species grouped into three genera. Three species belonging to two genera of this family were collected from the middle Negro River, Amazonas, Brazil, and studied cytogenetically: *Paratrygon aiereba*, *Potamotrygon motoro* and *Potamotrygon orbignyi*. *Paratrygon aiereba* presented $2n = 90$ chromosomes and 4M + 2SM + 10ST + 74A. Both species of *Potamotrygon* presented $2n = 66$ chromosomes and differed in their chromosomal formulas: *P. motoro* had 18M + 12SM + 10ST + 26A and *P. orbignyi* had 22M + 10SM + 8ST + 26A. No sex heteromorphism was detected. The Fundamental Number (FN) was 106 for the three species. A system of multiple NORs was found in the three species, but with interspecific differences in terms of location and position of the active Ag-NORs sites. *Paratrygon aiereba* presented only four sites on the short arms of two chromosomal pairs, both in terminal regions. *Potamotrygon motoro* presented seven sites, on the long and short arms, all in terminal regions of non-homologous chromosomes; *P. orbignyi* presented eight sites on the long arms, all in terminal regions, of non-homologous chromosomes. The constitutive heterochromatin was in pericentromeric regions of all chromosomes, and no significant interspecific difference was found in relation to this marker.

Introduction

Sixty years after the first cytogenetic study on elasmobranchs, the most advanced superorder, the Batoidea, contains the largest number of studied species ($n = 33$), but this represents no more than 2.6% of all living elasmobranchs (Stingo & Rocco, 2001). Only six marine species of Rajiformes, a batoid elasmobranch group that includes both marine and freshwater rays, have been karyotyped, of which four have $2n = 98$, one has $2n = 104$ and one has $2n = 58$ chromosomes (Makino, 1937; Nygren, Nilsson & Jahnke, 1971; Nygren & Jahnke, 1972; Schwartz & Maddock, 1986). Thus, karyological investigations on freshwater rays, especially potamotrygonids, are still lacking.

Potamotrygonidae is a monophyletic rajiform group of South American freshwater stingrays that includes about 20 species distributed in three genera: *Paratrygon*, *Plesiotrygon* and *Potamotrygon*. The first two genera are monospecific and the last one is formed by the remaining species (Carvalho, Lovejoy & Rosa, 2003). The natural distribution of these freshwater stingrays includes the major South American River basins, including the Orinoco, Amazon, Paraná/Paraguai and Essequibo,

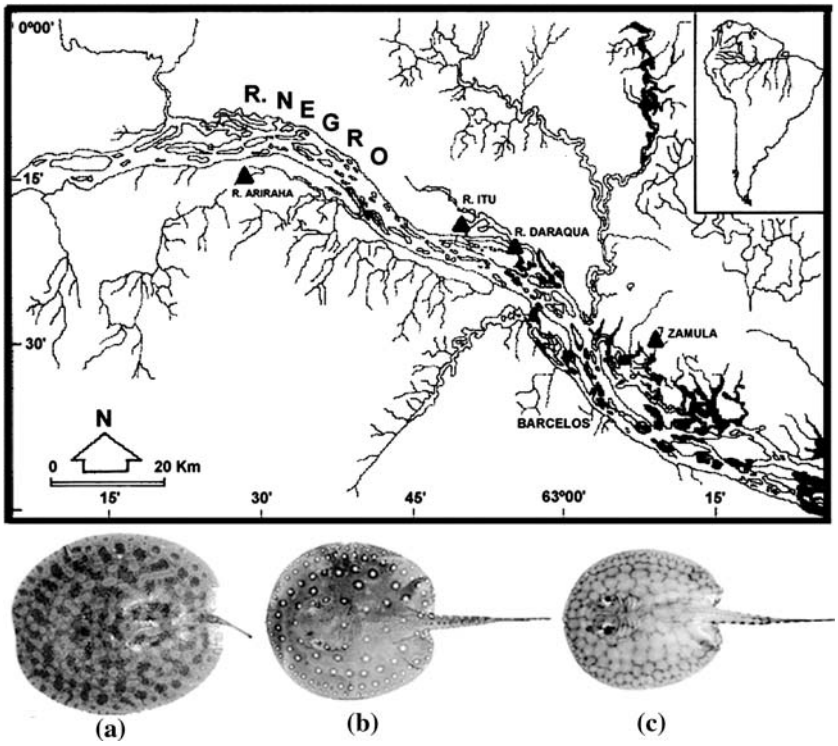

Figure 1. Map of middle Negro River showing the location of stingrays sampling sites and photo of analyzed species: (a) *Paratrygon aiereba*, (b) *Potamotrygon motoro* and (c) *Potamotrygon orbignyi*.

but does not occur on the western side of the Andes, in the São Francisco drainage, or in Brazilian costal rivers (Rosa, 1985; Rosa, Castello & Thorson, 1987; Carvalho, Lovejoy & Rosa, 2003).

Three distinct phylogenetic hypotheses suggest that the freshwater Potamotrygonidae are marine derivatives that colonized the inshore rivers through ancient marine incursions (Brooks, Mayes & Thorson, 1981; Rosa, Castello & Thorson, 1987; Lovejoy, 1996). Brook et al.'s and Lovejoy's hypotheses disagreed about when and from where the invasion occurred. In addition, Rosa et al.'s and Lovejoy's hypotheses disagreed on which genus represents the basal clade. However, they all agreed that *Potamotrygon* is the terminal clade. This paper provides the first description of freshwater stingray karyotypes of Rajiformes to contribute to this discussion.

Materials and methods

Karyotype analyses were performed on three species belonging to two genera: *Paratrygon aiereba* (two males and two females), *Potamotrygon motoro* (seven males and fourteen females) and *Potamotrygon orbignyi* (seven females). The specimens were collected from the Daraquá, Itú, Arirahá (also called Ararirá) rivers and Zamula stream, which are tributaries of the middle Negro River in the Municipality of Barcelos, Amazonas, located about 400 km upriver from the capital, Manaus (Figure 1). Adult specimens (disc of 40 cm or more) were caught with long lines, and the newborn and juveniles were caught with hand-nets, and treated with anaesthetic solution for the removal of the haematopoietic organs. Species were identified following Rosa (1985) and Araújo (1998).

Mitotic activity induction was performed based on the biological yeast induction technique, described by Cole and Leavens (1971). Kidney, epigonal organ and spleen were used for obtaining mitotic chromosomes; the spleen had the highest mitotic index. Mitotic chromosomes were obtained by air-drying, as described by Bertollo, Takahashi and Moreira Filho (1978), but adapted for rays with the following modifications: colchicine concentration was 0.025% at a ratio of 1 ml/100 g of tissue weight and after 90 min of colchicinization the hypotonization process required 35 min at 37°C.

The nucleolar organizing regions (NORs) were identified by silver nitrate staining according to Howell and Black (1980) and Centofante, Porto and Feldberg (2002) for sequential Giemsa-$AgNO_3$ staining. C-banding for locating heterochromatin was performed using barium hydroxide (Sumner, 1972).

Chromosomes were identified by the arm ratio criteria proposed by Levan, Fredga and Sandberg (1964), where the metacentric (M), submetacentric (SM) and subtelocentric (ST) were considered to be bi-armed, and acrocentric (A) one-armed chromosomes.

Results

The diploid chromosome number of *Paratrygon aiereba* was found to be $2n = 90$ chromosomes, with the number of arms (Fundamental Number – FN) equal to 106 and the karyotypic formula 4M + 2SM + 10ST + 74A (Figure 2a). The two species of *Potamotrygon* (*P. motoro* and *P. orbignyi*) showed the same diploid number, $2n = 66$ chromosomes, and the same number of arms, FN = 106. However, the karyotypic formulas were different, 18M + 12SM + 10ST + 26A and 22M + 10SM + 8ST + 26A, respectively (Figure 2b and c). No sex chromosomal heteromorphism was detected.

Multiple NOR-bearing chromosomes characterized the three species. In *Paratrygon aiereba*, up to three NORs sites were detected, on the short arms of two chromosomal pairs, one metacentric and one subtelocentric, both in the terminal region (Figure 2a). In *Potamotrygon motoro*, the NORs were located in terminal regions of up to seven chromosomes: two on long arms of non-homologous metacentric chromosomes, one on long arms of one large submetacentric, two on short arms of a small subtelocentric pair, and two on

Figure 2. Giesma karyotypes and Ag-NOR chromosomes of *Paratrygon aiereba* (a), *Potamotrygon motoro* (b) and *Potamotrygon orbignyi* (c). The Ag-NOR bearing chromosomes are located in the right side and except for *Paratrygon aiereba* the Ag-NOR bearing chromosomes were sequentially stained for Giemsa and silver nitrate. The NORs of *Paratrygon aiereba* are in the box while the NORs of *P. motoro* and *P. orbignyi* are underlined.

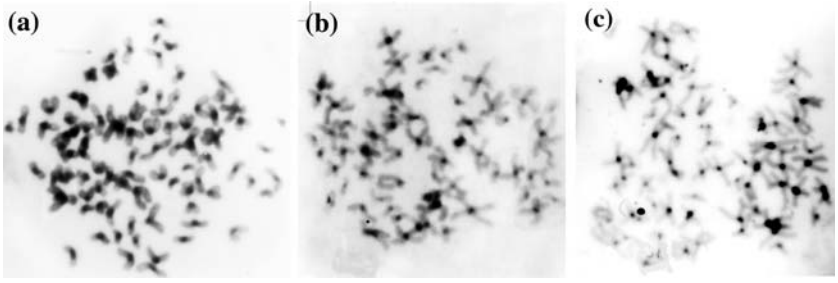

Figure 3. Metaphase of (a) *Paratrygon aiereba*, (b) *Potamotrygon motoro* and (c) *Potamotrygon orbignyi* after C-banding.

long arms of a small acrocentric pair (Figure 2b). In *P. orbignyi*, the NORs were located in terminal regions, on long arms of up to eight chromosomes, five of them non-homologous metacentrics, one large submetacentric, and two small acrocentric pairs (Figure 2c).

A number of interphasic nucleoli stained by silver nitrate were also observed. *Paratrygon aiereba* presented a modal number of four interphasic nucleoli in both sexes, *Potamotrygon motoro* presented a modal number of ten interphasic nucleoli in both sexes, and *P. orbignyi* presented a modal number of eight interphasic nucleoli in females (no males were captured).

C-banding revealed heterochromatin blocks in the pericentromeric region on all chromosomes of the three species, some more conspicuous than others, but no significant differences were observed in this banding type for the species studied (Figure 3a, b, c).

Discussion

Recently, concerns related to conservation of potamotrygonids have emerged on Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES) since tourism development, explotation of stocks (in this case fishery as ornamental fish) and by-catch could affect these not well known freshwater rays (Araujo et al., 2004). Barcelos, the region where was collected the freshwater ray samples, is considered the capital of ornamental fish industry in the Amazon and any genetic information to help plan for mitigate possible adverse impacts of harvesting ornamental fish from this area is mandatory.

Based upon chromosome information we did not recover any difference between populations in each analyzed species. The interspecific chromosomal differences observed lead us to formulate a preliminary karyoevolutionary hypothesis of how chromosomes evolved in these freshwater rays. Before presenting this, however, it is imperative to understand the groups systematics, especially as batoid taxonomy is undergoing major systematic revisions. Some systematists consider them to be a single order, Rajiformes (Carvalho, Lovejoy & Rosa, 2003). Others think that there are five or six orders with the river stingrays – family Potamotrygonidae in the order Myliobatiformes (Compagno & Cook, 1995; Martin, 2004). The taxonomic placement of potamotrygonids we adopted is based primarily on Carvalho, Lovejoy and Rosa (2003), who consider a single order, Rajiformes, with a sub-order Myliobatoidei.

Rosa, Castello and Thorson (1987) proposed a phylogenetic hypothesis for freshwater stingrays in which *Plesiotrygon* is the primitive sister taxon to the clade formed by *Paratrygon* and *Potamotrygon*. However, Lovejoy (1996) proposed another phylogenetic hypothesis for this group, where the genus *Paratrygon* is considered the sister group to the clade formed by the others two genera.

No matter what genus is considered as basal, it is well accepted that potamotrygonids were derived from a marine ancestor during the marine incursions in the Miocene. Two hypotheses are available concerning the marine sister group to Potamotrygonidae. Parasitological evidence (Brooks, Mayes & Thorson, 1981) suggests that potamotrygonids are most closely related to a Pacific urolophid ancestor, while morpho-anatomical and molecular data suggest a Pacific and Caribean dasyatid of the genus *Hymantura*

(Lovejoy, 1996; Lovejoy, Bermingham & Martin, 1998).

The diploid chromosome number of marine rays varies from 28 in *Narcine brasiliensis* to 104 in *Raja meerdervoortii*. However, a closer look at dasyatids and urolophids, hypothesized as putative ancestors to potamotrygonids, reveals in the former a diploid number variation from $2n = 58$ to $2n = 84$ (information available only for *Dasyatis*) and from $2n = 52$ to $2n = 72$ in urolophids (Stingo & Rocco, 2001). Despite the recent advances achieved in fish cytogenetics by the use of high resolution molecular techniques, the investigation of ray karyotypes with chromosomal bandings techniques, such as nucleolus organizing regions (NORs), heterochromatin (C-banding and restriction endonuclease), fluorochromes and FISH, is restricted to a half dozen species belonging to the batoid genera *Raja*, *Torpedo*, and *Taeniura* (Stingo, Rocco & Improta, 1989; Stingo et al., 1995; Rocco et al., 2002; Rocco et al., 2005).

In the potamotrygonid freshwater rays, two diploid numbers were detected: $2n = 90$ for *Paratrygon aiereba* and $2n = 66$ for *Potamotrygon motoro* and *P. orbignyi*. The Fundamental Number (FN) was 106 for the three species. Considering the most recent phylogenetic hypothesis for potamotrygonids (Lovejoy, 1996), it is possible that the most primitive diploid number for South American freshwater stingrays may be $2n = 90$ and subsequent rearrangements (possibly chromosome fusions) reduced the diploid number to $2n = 66$. One can also see that nearly one-third of these chromosomes were almost as small as microchromosomes. Unfortunately we have not yet been able to determine the diploid number of *Plesiotrygon* and this is necessary in order to better understand the chromosome evolution in potamotrygonids.

It has been proposed for Batoids that as the species become more advanced a reduction in the diploid number, an increase in the number of the bi-armed chromosomes and the disappearance of microchromosomes can be observed (Stingo & Rocco, 2001). This phenomenon is evident if the karyotypes of the primitive rays and the advanced ones are compared. This same karyoevolutionary trend was observed on the Potamotrygonidae species studied here, since the diploid number declined from 90 (*Paratrygon*) to 66 (*Potamotrygon*), even while the number of arms (106) remained equal for the three species. The increase of karyotype symmetry apparently occurred by the transformation of the acrocentrics into meta-submetacentrics through robertsonian mechanisms, however still is necessary to analyze more *Potamotrygon* species to confirm this trend.

With respect to the NORs, all the species analyzed in this study presented several ribosomal sites that vary in location, number and size, i.e., up to 3 in *Paratrygon aiereba*, up to 7 in *Potamotrygon motoro* and up to 8 in *P. orbignyi* (Figure 2). Thus, freshwater stingrays may be characterized by a multiple NOR system located in bi-armed and uni-armed chromosomes, which is in accordance with previous studies in the batoids (Stingo et al., 1995; Rocco et al., 2002; Rocco et al., 2005). Chromosomal locations of multiple NORs in potamotrygonids seem to be unstable, with a main constant NOR-bearing pair and some others with variable positions. Low number of NOR sites have been considered basal in some amazonian fish groups that presents multiple NORs, when compared to a high number of NOR sites (Porto et al., 1992). Thus, the number of NOR sites observed here strengthens the hypothesis that *Paratrygon* is basal to *Potamotrygon*.

With respect to the C-banding in the potamotrygonids, the heterochromatin observed was mostly pericentromeric (Figure 3) and the constitutive heterochromatin showed no significant interspecific differences. Studies conducted on localization of constitutive heterochromatin in rays are scarce, a fact that impairs a useful comparative analysis. However, Rocco et al. (2002) revealed that in *Taeniura lymma* the C-banding was located centromerically in 14 pairs of bi-armed chromosomes, as well as in one pair of large acrocentrics, while in *Raja asterias* centromeric blocks of constitutive heterochromatin were detected in all the chromosome pairs except in one of the three bi-armed ones. In *Torpedo ocellata* and T. *marmorata* the heterochromatin localization was distinct: pericentromeric in the bi-armed elements of T. *ocellata* and centromeric in the uni-armed elements of *T. marmorata* (Stingo & Rocco, 2001).

In conclusion, the karyoevolutionary pattern we are postulating for freshwater stingrays encompasses: (1) presence of Robertsonian centric fusion, since the ancestor presumably had a higher

diploid number; (2) multiple NORs, a shared character in potamotrygonids, probably evolved from a low to a high number of NORs sites; and (3) preferential distribution of constitutive heterochromatin in the pericentromeric region that seems to correspond the ancestral state for potamotrygonids.

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