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Karyotype differentiation and cytotaxonomic considerations in species of Serrasalmidae (Characiformes) from the Amazon basin

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Six species of Serrasalmidae from the central Amazon, representatives of the genera *Serrasalmus* (*S. elongatus*, *S. maculatus*, *S.* cf. *rhombeus*, and *S. rhombeus*), *Pygocentrus* (*P. nattereri*), and *Colossoma* (*C. macropomum*), were analyzed regarding the distribution of the Ag-NORs, C-positive heterochromatin and 18S and 5S rRNA genes on the chromosomes. All specimens had 2n = 60 chromosomes, except *S.* cf. *rhombeus,* with 2n = 58, and *C. macropomum* with 2n = 54 chromosomes. The Ag-NORs were multiple and located on the short arms of subtelo-acrocentric chromosomes in all *Serrasalmus* species and in *P. nattereri*, but were found on metacentric chromosomes in *C. macropomum*. The 18S rDNA sites were usually coincident with Ag-NORs, although some species had a higher number and/or a distinct localization of these sites. C-positive heterochromatin was preferentially situated in centromeric regions, remarkably on metacentric pair number 7 in all *Serrasalmus* species and number 3 in *P. nattereri*, which beared a conspicuous proximal C-band on the long arms. The 5S rDNA sites were detected in a single chromosomal pair in all species. In *Serrasalmus* and *P. nattereri*, this pair was the number 7 and 3, respectively, thereby revealing its co-localization with the conspicuous heterochromatic band. However, in *C. macropomum*, only one homologue (probably belonging to pair number 12) exhibited 5S rDNA sites on the short arms, close to the centromere. The present data revealed reliable cytotaxonomic markers, enabling the evaluation of karyotype differentiation and interrelationships among Serrasalmidae, as well as the probable occurrence of a species complex in *S. rhombeus*.

Seis espécies de Serrasalmidae da Amazônia central, representantes dos gêneros *Serrasalmus* (*S. elongatus*, *S. maculatus*, *S.* cf. *rhombeus* e *S. rhombeus*), *Pygocentrus* (*P. nattereri*) e *Colossoma* (*C. macropomum*), foram analisadas quanto à distribuição das Ag-RONs, heterocromatina C-positiva e dos genes de RNAr 18S e 5S nos cromossomos. Todos os espécimes apresentaram 2n = 60 cromossomos, exceto *S.* cf. *rhombeus*, com 2n = 58, e *C. macropomum* com 2n = 54 cromossomos. As Ag-RONs foram múltiplas e localizadas nos braços curtos de cromossomos subtelo-acrocêntricos em todas as espécies de *Serrasalmus* e em *P. nattereri*, mas foram encontrados em cromossomos metacêntricos em *C. macropomum*. Os sítios de DNAr 18S, foram geralmente coincidentes com as Ag-RONs, embora algumas espécies tenham apresentado um maior número e/ou uma localização distinta desses sítios. A heterocromatina C-positiva foi preferencialmente encontrada como uma conspícua banda proximal no par metacêntrico número 7 em todas as espécies de *Serrasalmus* e número 3 em *P. nattereri*. Os sítios de DNAr 5S foram detectados em um único par cromossômico nas seis espécies sendo que nas espécies de *Serrasalmus*, este par foi o de número 7 e em *P. nattereri* o de número 3, colocalizados com bandas heterocromáticas conspícuas. No entanto, em *C. macropomum*, apenas um homólogo (provavelmente pertencente ao par número 12) apresentou sítios de DNAr 5S nos braços curtos, próximos ao centrômero. Os dados apresentados revelaram confiáveis marcadores citotaxonômicos, permitindo a avaliação da diferenciação cariotípica, e as inter-relações entre Serrasalmidae, bem como a provável ocorrência de um complexo de espécies em *S. rhombeus.*

Key words: Chromosomal evolution, Fish, Heterochromatin, Piranhas, Ribosomal genes.

Introduction

Chromosome banding techniques have been useful in evolutionary cytogenetics, as they allow the identification of potential chromosome markers related to karyotype interrelationships. The detection of nucleolar organizer regions (NORs) by silver nitrate staining (Ag-NORs) has been one of the most widely employed procedures in fish, especially from 1980s on, providing important information for cytotaxonomic and evolutionary studies (Galetti Jr. *et al.*, 1984; Gold, 1984; Venere & Galetti Jr., 1989; Feldberg *et al.*, 1992, 2003). This tool is usually poorly informative for groups with single NORs on equivalent chromosomes. Moreover, not all the nucleolar organizer regions are necessarily active in species bearing multiple NOR systems and would therefore remain undetectable by silver nitrate staining (Miller *et al.*, 1976).

Since the 1990s, chromosome studies have improved quickly due to the use of base-specific fluorochromes, such as chromomycin A_3 and mithramycin, and the development of fluorescent *in situ* hybridization (FISH). These procedures have

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been successfully applied to NORs and heterochromatin studies in fish metaphase chromosomes, as they are able to detect base richness and both active and inactive nucleolar regions. Fluorescent *in situ* hybridization (FISH) is the most useful method for mapping NOR sites in a species genome.

The aim of the present study was to determine the location of 5S rDNA and 18S rDNA sites in species of Serrasalmidae from the central Amazon and carry out a comparative analysis with Ag-NORs. These data, along with other peculiar karyotype characteristics, proved to be important tools for understanding the interrelationships within this fish group.

Material and Methods

Six species of the family Serrasalmidae were analyzed: *Serrasalmus elongatus* Kner, 1858, *S. maculatus* Kner, 1858, *S. rhombeus* (Linnaeus, 1766), *S.* cf. *rhombeus, Pygocentrus nattereri* Kner, 1858, and *Colossoma macropomum* (Cuvier, 1816) (Fig. 1). The specimens were collected from the central Amazon at Catalão Lake (03º10'45"S 59º54'25.4"W), Marchantaria island (3º11'9.80"S 59º51'42.41"W), Anavilhanas (2°38'58.17"S 60°47'58.97"W) and Uatumã River (01°55'S 59°28'W) in Brazil, identified by Jansen Zuanon (INPA). Voucher specimens were deposited in the fish collection at the Instituto Nacional de Pesquisas da Amazônia (INPA) (*S. elongatus*: INPA 32907 and 32908; *S. maculatus*: INPA 32909 and 32910; *S. rhombeus*: INPA 32911 and 32912. *S.* cf. *rhombeus*: INPA 32913 and 32914; *Pygocentrus nattereri*: INPA 32916 and 32917; *Colossoma macropomum*: INPA 32906 and 32907).

The chromosome preparations were obtained from kidney cells, using the *in vivo* procedure described by Bertollo *et al*. (1978), after mitotic stimulation with biological yeast, using the procedure described by Oliveira *et al.* (1988). The chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), and acrocentric (a) based on the arm ratio (Levan *et al*., 1964) and arranged in decreasing size in the karyotype. The fundamental number (FN) or number of chromosomal arms was determined taking into account that m, sm and st chromosomes bear two chromosomal arms and acrocentric chromosomes bear a single arm.

Constitutive heterochromatin (C-bands) was detected based on the procedure described by Sumner (1972). The nucleolar organizer regions were identified using silver nitrate staining (Ag-NORs), as proposed by Howell & Black (1980), as well as fluorescent *in situ* hybridization (FISH), based on the procedure described by Pinkel *et al.* (1986). The 18S rDNA probe was obtained through polymerase chain reaction (PCR) from the fish *Prochilodus argenteus* (Hatanaka & Galetti Jr., 2004), using the primers NS1 (5'-GTAGTCATATGCTTGTCTC-3') and NS8 (5'-TCCGCAGGTTCACCTACGGA-3'), based on the procedure described by White *et al*. (1990). The 5S rDNA sites were detected using PCR-derived probes from the fish *Leporinus obtusidens* (Martins & Galetti Jr., 1999), using the primers A (5'-TACGCCCGATCTCGTCCGATC-3') and B (5'-CAGGCTGGTATGGCCGTAAGC-3'), as reported by Pendás *et al.* (1994). Both probes were labeled by nick translation (BioNick Labeling System - Invitrogen), following the manufacturer's instructions. The chromosomes were counter-stained with propidium iodide (50µg/ml and 200µl of antifading) and analyzed under an Olympus BX50 epifluorescence microscope. The images were digitized using the CoolSNAP-pro software program (Media Cybernetics).

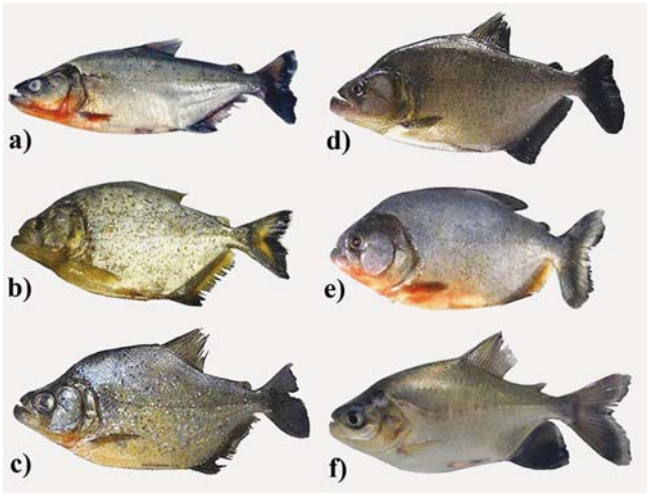

Fig. 1. Analyzed Serrasalmidae species: **a**) *Serrasalmus elongatus* (total length = 19.5 cm); **b**) *Serrasalmus maculatus* (total length = 15.5 cm); **c**) *Serrasalmus* cf. *rhombeus* (total length = 12.5 cm); **d**) *Serrasalmus rhombeus* (total length = 17.5 cm); **e**) *Pygocentrus nattereri* (total length = 15.0 cm); **f**) *Colossoma macropomum* (total length = 19.0 cm).

Results

A diploid number of 60 chromosomes was found in all species, except *S.* cf. *rhombeus,* which had 2n = 58 chromosomes, and *C. macropomum,* which had 2n = 54 chromosomes. The fundamental number ranged from 106 to 110 and no sex-related chromosomes were found (Table 1).

All species exhibited multiple Ag-NORs, located in the terminal position on the short arms of acrocentric and subtelocentric chromosomes, with the exception of *C. macropomum*, in which the Ag-NORs were found on metacentric chromosomes (Fig. 2 a, c, e, g, i, and k). In general, the 18S rDNA sites coincided with Ag-NOR sites. However, in some species, a higher number of 18S rDNA sites were detected (Fig. 2 b, d, f, h, j, and l).

Besides the general location of the C-bands in the centromeric regions (data not shown), a conspicuous heterochromatic block was also evident on the long arms of a metacentric pair close to the centromeres, corresponding to pair number 7 in the species of *Serrasalmus* and to pair number 3 in *P. nattereri* (Fig. 3 a, c, e, g, and i).

The 5S rDNA was located interstitially, close to the centromeres on a single chromosome pair. In *Serrasalmus* and *Pygocentrus,* this site was co-located with the aforementioned interstitial heterochromatic region on the long arms of pairs 7 and 3, respectively. In *C. macropomum*, 5S rDNA signals were

found on the short arms of a single chromosome - probably from pair number 12 (Fig. 3 b, d, f, h, j, and l).

The results are summarized in Table 1. Previous data on another three species of *Serrasalmus* [*S. altispinnis*, *S. gouldingi*, and *S. serrulatus* (Nakayama *et al*., 2008)] were also included in this table to provide a comparative analysis.

Discussion

Ag-NORs in Serrasalmidae have been analyzed since the 1980s and have invariably been visualized as multiple sites usually located in the terminal region on the short arms of subtelo-acrocentric chromosomes, although some species bear terminal Ag-NORs on the long arms (Galetti Jr. *et al.*, 1985; Almeida-Toledo *et al.*, 1987; Cestari & Galetti Jr. 1992a, b; Martins-Santos *et al*., 1994; Nakayama *et al*., 2001, 2002; 2008; Centofante *et al.*, 2002a; Nirchio *et al.*, 2003; Gaviria *et al*., 2005). However, the numerical variation in these regions (in both inter and intra-specific terms) is much more notable than the positional differences, ranging from four sites in *C. macropomum* to 12 sites in *S. rhombeus* (Table 1).

This Ag-NOR variability is also supported by the FISH analysis of 18S rDNA sites, indicating that, alongside with differences in diploid number, fundamental number and karyotype formula, Serrasalmidae species have undergone significant modifications in the chromosomal distribution of ribosomal genes during their evolutionary process. However, full correspondence between 18S rDNA and Ag-NOR sites was not constantly observed, since in some species, the number of 18S rDNA sites was greater than the number of Ag-NORs. Moreover, the location of some 18S rDNA sites was not detected by silver nitrate in *S. rhombeus* and *S. serrulatus* (Nakayama *et al.*, 2008) (Table 1). Two probable reasons may putatively explain these divergences. Firstly,

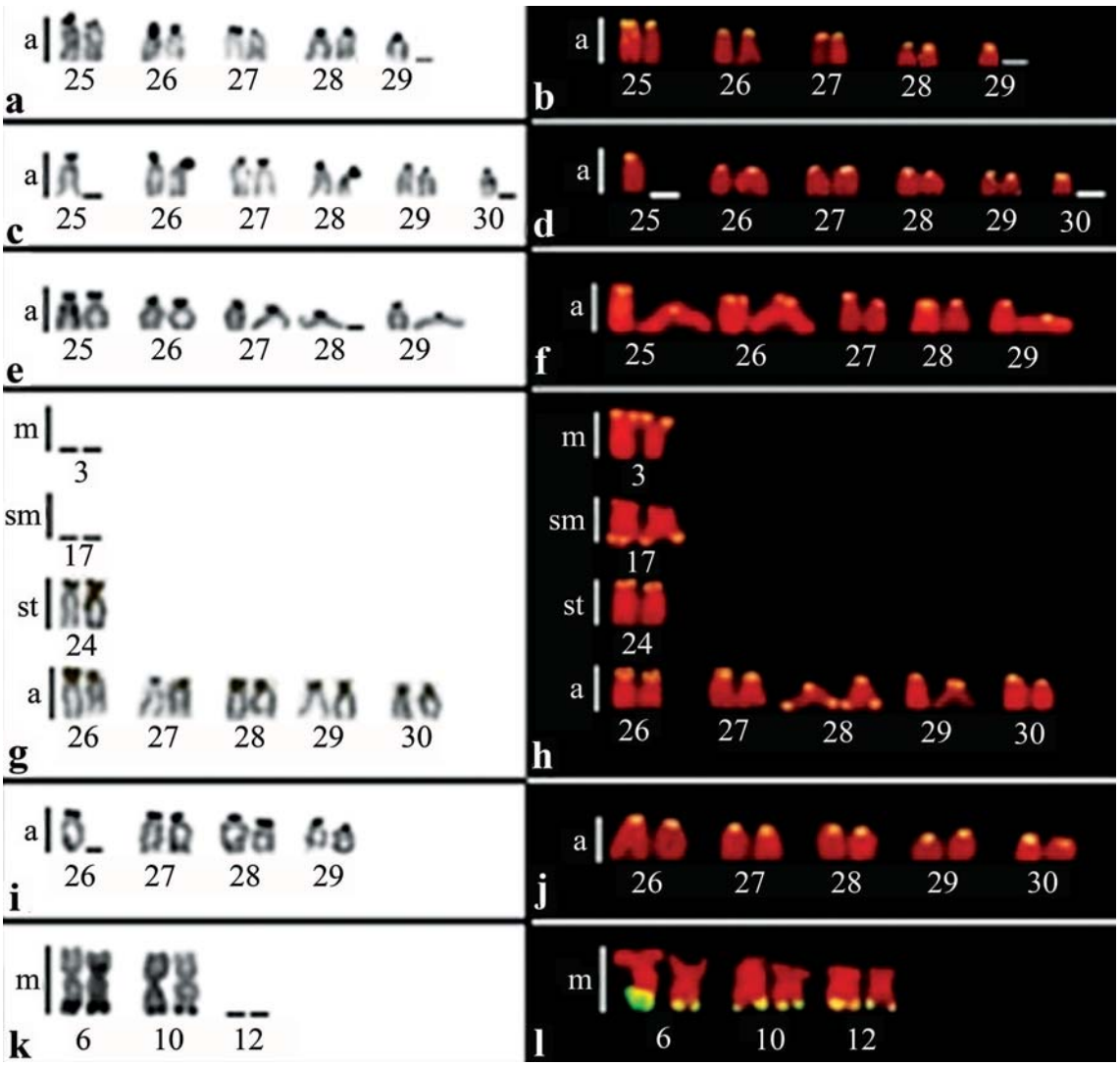

Fig. 2. Partial karyotypes of Serrasalmidae species showing Ag-NORs (left side) and 18S rDNA sites (right side): **a-b**) *Serrasalmus elongatus*; **c-d**) *Serrasalmus maculatus*; **e-f**) *Serrasalmus* cf. *rhombeus*; **g-h**) *Serrasalmus rhombeus*; **i-j**) *Pygocentrus nattereri*; **k-l**) *Colossoma macropomum*. Numbers indicate the corresponding chromosome pairs in the karyotypes of the species.

Table 1. Karyotype characteristics in the studied species of Serrasalmidae. 2n = modal diploid number; m = metacentric, sm = submetacentric, st = subtelocentric, a = acrocentric chromosomes; FN = fundamental number; p = short arm; q = long arm; t = terminal; Ag-NORs = nucleolar organizer regions stained with silver nitrate; 18S rDNA = maximum number of sites detected by FISH; 5S rDNA = number of sites detected by FISH. **S. maculatus* = *S. spilopleura* cytotype A (according to Nakayama *et al.*, 2000; Centofante *et al.*, 2002a).

Species	2n	FN	Karyotype formulae	Ag-NOR	18S rDNA	5S rDNA	Reference
S. altispinnis	60	110	24m+20sm+6st+10a	6-10a, p, t	10a, p, t	2m, 7th pair	Nakayama *et al*. (2008)
S. elongatus	60	108	22m+22sm+4st+12a	6-9a, p, t	9a, p, t	2m, 7 th pair	Present paper
S. gouldingi	60	110	22m+22sm+6st+10a	5-8a, p, t	6a, p, t+2st, p, t	2m, 7 th pair	Nakayama *et al*. (2008)
**S. maculatus*	60	108	24m+20sm+4st+12a	6-10a, p, t	10a, p, t	2m, 7th pair	Present paper
S. rhombeus	60	110	20m+24sm+6st+10a	5-10a, p, t+2st, p, t	10a, p, t+2st, p, t+2sm, q, t+2m, p ,t	2m, 7 th pair	Present paper
S. cf. *rhombeus*	58	106	22m+24sm+2st+10a	5-9a, p, t	10a, p, t	2m, 7th pair	Present paper
S. serrulatus	60	110	20m+22sm+8st+10a	4-12st-a, p, t	8a, p, t+2st, p, t+1m, q, t+1m, p, t	2m, 7 th pair	Nakayama *et al*. (2008)
P.nattereri	60	110	20m+28sm+2st+10a	5-7a, p, t	10a, p, t	2m, 3rd pair	Present paper
C.macropomum	54	108	26m+28sm	4m-sm, q, t	6m-sm, q, t	1m, 12th pair	Present paper

fluorescent *in situ* hybridization identifies ribosomal genes regardless of their activity status, whereas silver nitrate impregnation is only able to detect previously active sites (Miller *et al.*, 1976). Therefore, some 18S rDNA sites that were undetectable by silver nitrate may correspond to inactive genomic regions. Secondly, NORs in Serrasalmidae are usually very small, probably due to a small number of gene copies, which does not allow its detection on the chromosomes. On the other hand, the occurrence of NOR transposition between different chromosomes, facilitated by transposable elements and the consequent diversity in their location within the karyotypes, cannot be excluded (Biémont & Vieira, 2006).

In addition to the standard karyotypes with 2n = 60 chromosomes, previous reports in *S. rhombeus* described a cryptic form (*S.* cf. *rhombeus*) bearing 2n = 58 chromosomes (Nakayama *et al.*, 2001), as well as another karyomorph was identified by chromosomal and molecular analyses (Teixeira *et al.*, 2006), suggesting that *S. rhombeus* is a complex of species.

This hypothesis is strengthened by the present data on 18S rDNA sites, since *S.* cf. *rhombeus* and *S. rhombeus* differed in both number and localization of these sites on the chromosomes (Table 1, Fig. 2 f and h).

In general, the 5S rDNA clusters are interstitially situated on the chromosomes of fish species, usually involving a single pair in the karyotype, which is thought to be a preferential condition for this group (Martins & Wasko, 2004). Similarly, the 5S rDNA in all Serrasalmidae species was interstitially located on the long arms of a metacentric pair, without synteny with NORs. This chromosome corresponds to pair number 7 in *Serrasalmus* and probably pair number 3 in *P. nattereri* and pair number 12 in *C. macropomum* (Fig. 3).

A precise identification of the 5S rDNA-bearing pair in the karyotypes of different species of Serrasalmidae was somewhat difficult to achieve without additional markers, since a great number of chromosomal pairs are morphologically similar. However, based on size, form, 5S rDNA location and conspicuous C-bands, these chromosomes were putatively homeologous between *Serrasalmus* and *Pygocentrus* (Fig. 3). Indeed, 5S rDNA-bearing chromosomes in all *Serrasalmus* species as well as in *P. nattereri* had a peculiar C-positive band close to the centromere on the long arms, associated with the 5S rDNA genes. In some other Neotropical fish groups, such as Anostomidae, Parodontidae, and Prochilodontidae, 5S rDNA-bearing chromosomes also seem to be conserved (Martins & Galetti Jr., 1999, 2000; Vicente *et al.*, 2001; Centofante *et al.*, 2002b; Jesus & Moreira Filho, 2003; Hatanaka & Galetti Jr., 2004). Meanwhile, the same inference is not valid for Serrasalmidae as a whole, once 5S rDNA sites in *C. macropomum* were located on clearly different chromosomes when compared to the other studied species.

Based on inner anatomy, miological, and osteological characters, Machado-Allison (1983) subdivided Serrasalmidae into two distinct groups: "pacus" and "piranhas". Among "pacus", *Colossoma* is thought to represent one of the more derived genera. Among "piranhas", *Catoprion* is thought to be the most basal group, followed by *Pygopristis*, *Pygocentrus, Pristobryconm*, and *Serrasalmus*, the latter two corresponding to the more derived genera. On the other hand, molecular phylogeny based on mtDNA (Ortí *et al.*, 1996) clustered "pacus" and "piranhas", and placed *Colossoma* as the basal genus for the family. Within this phylogenetic analysis, *Catoprion* remains the basal group of piranhas, along with *Pristobrycon striolatus*, followed by other species of *Pristobrycon*, *Serrasalmus*, and *Pygocentrus.*

All these species have a karyotype composed of m, sm, st, and a chromosomes, thus diverging from *C. macropomum*, which has 2n =54 m-sm chromosomes. The location of 5S rDNA sites in similar chromosomes also reinforces a closer relationship between *Serrasalmus* and *Pygocentrus* as well as the separation of *Colossoma*. Therefore, a number of karyotype traits corroborate the distance between *Colossoma* and other species of Serrasalmidae, as proposed by the phylogenetic hypotheses of Machado-Allison (1983) and Ortí *et al.* (1996). Moreover, the evident co-localization of 5S rDNA and C-bands on probable homeologous chromosomes supports the high proximity between the species of *Serrasalmus* and *P. nattereri*, as proposed by Ortí *et al.* (1996).

Considering *C. macropomum* as occupying the most basal position within Serrasalmidae, the karyotype differentiation observed in the other species would be derived from chromosome rearrangements, such as centric fissions,

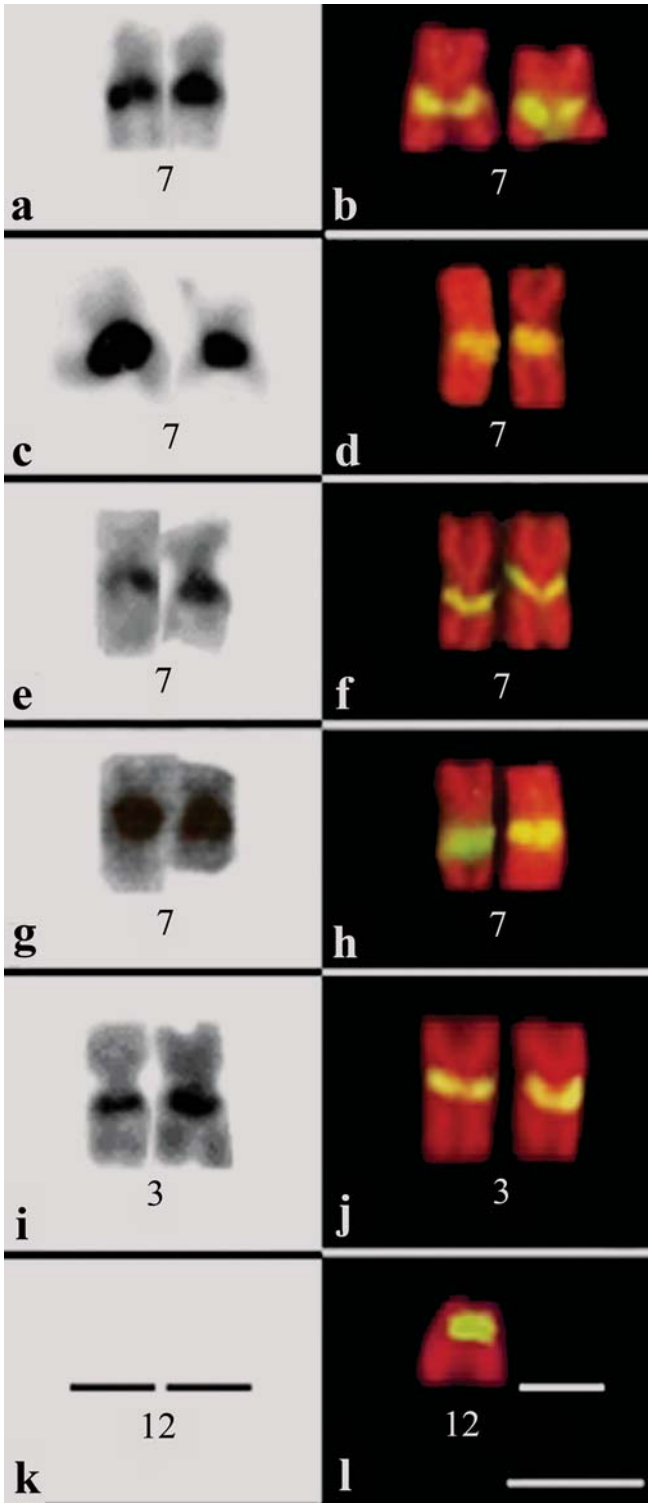

Fig. 3. Chromosome pairs of Serrasalmidae species showing the co-localization of the heterochromatic C-positive band (left side) and 5S rDNA site (right side): **a-b**) *Serrasalmus elongatus*; **c-d**) *Serrasalmus maculatus*; **e-f**) *Serrasalmus* cf. *rhombeus*; **g-h**) *Serrasalmus rhombeus*; **i-j**) *Pygocentrus nattereri*; **k-l**) *Colossoma macropomum*. Numbers indicate the corresponding chromosome pair in the karyotypes of the species. Pair 12 in *Colossoma macropomum* does not exhibit the conspicuous C-band.

increasing the basal diploid number from 2n = 54 meta-submetacentric chromosomes to 2n = 58 and 60 chromosomes, alongside with the formation of acrocentric chromosomes. Similarly, the NORs have also undergone numerical and positional modifications during the karyotype evolution of this fish group, changing from a location on m-sm chromosomes, as seen in *C. macropomum*, to a preferential location on st-a chromosomes, as observed in the other genera.

From this perspective, it is likely that the few m-sm chromosomes bearing 18S rDNA, as detected in *S. rhombeus* and *S. serrulatus*, represent an ancestor feature similar to that found in *C. macropomum.* As for the 5S rDNA clusters, their identical location in all *Serrasalmus* species and in *P. nattereri* suggests that, once established from a putative ancestor condition as that found in *C. macropomum,* their distribution remained conserved. However, while chromosomes bearing 5S rDNA sites are located in pair number 3 in *P. nattereri* karyotype*,* they correspond to pair number 7 in *Serrasalmus*. Therefore, the same location of the 5S rDNA sites on metacentric chromosomes and their co-localization with C-positive heterochromatin *per se* are not a unique feature of the genus *Serrasalmus*, since these aspects are also shared by *P. nattereri*. Instead, the exclusive characteristic of *Serrasalmus* must refer to the position of the chromosomes bearing such markers in the karyotype (pair number 7).

In conclusion, chromosome characteristics, particularly the analysis of ribosomal genes and C-bands, provided reliable cytotaxonomic markers for evaluating karyotype differentiation and evolutionary relationships among species of Serrasalmidae.

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