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Comparative Cytogenetics and Molecular Phylogeography in the Group *Astyanax altiparanae – Astyanax* aff. *bimaculatus* (Teleostei, Characidae)

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Key Words
Astyanax altiparanae · *Astyanax bimaculatus* · Molecular cytogenetics · Neotropical fish · Phylogeography

Abstract
The genus *Astyanax* comprises small characin fish of the neotropical region. The so-called ‘yellow-tailed characins’ compose one of the most widely distributed *Astyanax* groups. *A. altiparanae* and *A.* aff. *bimaculatus*, are evolutionarily closely related and commonly found in several Brazilian hydrographic basins. In the present work, chromosomal data of specimens of *A. altiparanae* and *A.* aff. *bimaculatus* from 4 hydrographic basins in the states of São Paulo (Upper Tietê, Paranapanema, Ribeira de Iguape) and Rio de Janeiro (Guapimirim) are shown. All the populations showed 50 chromosomes, with different karyotypic formula. Although only a single Ag-NOR bearing chromosome pair was observed, all populations possess multiple cistrons of 18S rDNA. FISH with the 5S rDNA probe showed single signals at the interstitial position of one metacentric chromosome pair. C-bands are distributed in the terminal and interstitial regions of several chromosomes. However, the As-51 satDNA are frugally located in a few chromosomes of fishes from Upper Tietê, Paranapanema and Guapimirim Rivers, being absent in individuals of *A.* aff. *bimaculatus* from Ribeira de Iguape River basin. Beside these 4 populations, molecular phylogeography studies were also performed in individuals from Middle and Lower Tietê River basin and from 2 additional collection sites in the Paranapanema and Ribeira de Iguape River basins. The phylogeographic analysis using 2 mtDNA regions (totalizing 1.314 bp of *ND2* and *ATPase6/8* genes) of 8 populations of the group of ‘yellow-tailed characins’ from 3 major hydrographic basins showed structuring of populations, suggesting a correlation between chromosomal (nuclear) and molecular (mitochondrial) data.

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The genus *Astyanax*, and particularly the so-called ‘bimaculatus group’ (yellow-tailed characins with a humeral rounded spot), comprises well-adapted species to distinct environmental conditions with a wide array of interactions in both fish assemblage and population density [Orsi et al., 2004]. As a result, this genus is widespread throughout nearly the entire neotropical region [Eigenmann, 1921] where about 100 valid species are currently recognized [Lima et al., 2003]. However, this number is significantly underestimated as several new species have been reported ever since, whether representing previous-

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Table 1. Cytogenetic data from *A. altiparanae* and *A.* aff. *bimaculatus*

Species	Sample localities – Hydrographic basin	2n	Chromosomal types				Reference
			M	SM	ST	A	
A. altiparanae	Meia Ponte River (Goiânia, GO) – Paranaíba River	50	–	26	–	24	Jin and Toledo, 1975[a]
	Mogi-Guaçu River (Pirassununga, SP) – Upper Paraná River	50	10	24	4	12	Morelli et al., 1983[a]
	Claro River (Tamarana, PR) – Paranapanema River	50	10	26	4	10	Pacheco et al., 2001
			10	24	4	12	
			10	22	4	14	
	Paranapanema River (Salto Grande, SP) – Upper Paraná River	50	10	22	6	12	Daniel-Silva and Almeida-Toledo, 2001, 2005
	Mogi-Guaçu River – Upper Paraná River	50	32M+SM		18ST+A		Almeida-Toledo et al., 2002
	Índios' River – Ivaí River	50	6	30	4	10	Fernandes and Martins-Santos, 2004
	Paraná River (Porto Rico, PR) – Upper Paraná River	50	6	26	6	12	
	Paraná River – Upper Paraná River	50	6	26	6	12	Fernandes and Martins-Santos, 2006
	Tatupeba Stream – Upper Paraná River	50	6	26	6	12	
	Keçaba Stream – Upper Paraná river	50	6	26	6	12	
	Maringá Stream – Upper Paraná River	50	6	26	6	12	
	Tarumã Lake (Ponta Grossa, PR) – Tibagi River	50	6	28	8	8	Domingues et al., 2007
	Iraí River (Curitiba, PR) – Iguaçu River	50	6	30	8	6	
	Paraitinga River (Salesópolis, SP) – Upper Tietê River	50	8	20	10	12	Present work
	Pilar do Sul, SP – Paranapanema River	50	8	22	14	6	
A. aff. *bimaculatus*	Sete Barras, SP – Ribeira de Iguape River	50	8	24	12	6	Present work
	Cachoeiras de Macacu, RJ – Guapimirim River/coastal rivers	50	8	24	12	6	
	Contas River (Porto Alegre county, BA) – coastal rivers	50	6	28	12	4	Pamponet et al., 2008
	Contas River (Jequié, BA) – coastal rivers	50	6	28	12	4	
	Mineiro Stream (Itamari, BA) – coastal rivers	50	6	28	12	4	

[a] Originally cited as *A. bimaculatus*.

ly unknown taxa or part of a large group but showing distinctive morphological features.

Wide phylogenetic analyses in *Astyanax* species are not available, but some authors consider some yellow-tailed characins bearing an elliptic humeral spot are likely to be closely related [Garutti, 2003]. *A. altiparanae*, for instance, is a recent denomination for some yellow-tailed *Astyanax* from the Upper Paraná River, formerly considered as *A. bimaculatus* (denomination that actually remains restrict for the Amazonian characins [Garutti and Britski, 2000]). For these reasons, all the fishes of the so-called 'bimaculatus group' distributed in Brazil remain called *A.* aff. *bimaculatus*, until the precise designation. This is a large group, putatively comprising several subspecies and *A. bimaculatus*-like forms [Eigenmann, 1921]. Some of them have already been elevated to a species status, such as *A. altiparanae* [Garutti and Britski, 2000] and *A. rupununi* [Garutti, 2003].

The detection of diverse karyotype formulae within *A. altiparanae* by cytogenetic studies performed in different populations throughout Upper Paraná River suggested this group should represent a species complex [Fernandes and Martins-Santos, 2004]. Although this variation has been observed along a wide basin, the diploid numbers in several populations of *A. altiparanae* remain constant, differently from the pattern frequently reported for *A. fasciatus* and *A. scabripinnis* [for a review, see Pazza and Kavalco, 2007].

Even though the diploid number remains unchanged, such distinct cytotypes might represent operational taxonomic units because hybridization among cytotypes has not been reported so far. Furthermore, the biology of *A. altiparanae*, naturally distributed into structured groups [Garutti and Britski, 2000], favors vicariant processes that are commonly regarded as the major responsible for the diversity of neotropical fishes, particularly of small species inhabiting streams [Castro, 1999] such as *Astyanax* spp. Therefore, the chromosomal peculiarities (table 1) and the genetic differences [Moysés and Almeida-Toledo, 2002; Leuzzi et al., 2004; Paiva et al., 2006; Pamponet et al., 2008], coupled with the structure of natural populations [Garutti and Britski, 2000] and the lack of hybridization among cytotypes, show that *A. altiparanae* populations, although morphologically similar, can be considered a group of cryptic species. A high level of endemism is suggested for *Astyanax* species based on their

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structured distribution because multiple forms with a relatively limited geographical range have been reported in the same hydrographic basin [Garutti and Britski, 2000]. Genetic structuring has been commonly detected in populations of the yellow-tailed *Astyanax* group from different hydrographic basins [Moysés and Almeida-Toledo, 2002; Prioli et al., 2002; Leuzzi et al., 2004; Paiva et al., 2006; Pamponet et al., 2008].

Chromosomal studies have also shown inter-population differences in both karyotype formulae and fundamental numbers, including cases of population-specific karyotypes (table 1). The same diploid number (2n = 50 chromosomes) is observed in all 15 populations of *A. altiparanae* analyzed so far. A slight variation in the chromosomal formulae, albeit a large number of bi-armed chromosomes, particularly submetacentric ones, is usually maintained [Pazza and Kavalco, 2007].

Although previous studies have been performed in several populations of *A. altiparanae* from Upper Paraná River, the specimens from coastal rivers still lack a detailed chromosomal characterization. Phylogeographic approach including distinct hydrographic basins remains overlooked for the entire so-called 'bimaculatus group'. Furthermore, the level of correspondence between chromosomal and molecular data can be evaluated if a data set for the same populations is provided, and this approach was never before applied in the *Astyanax* genus.

In the present work, we show both classical and molecular cytogenetic data of 4 populations of yellow-tailed characins; 2 *A. altiparanae* populations from Tietê and Paranapanema Rivers and 2 *A.* aff. *bimaculatus* populations from Ribeira de Iguape and Guapimirim River basins (belonging to the coastal rivers basin). Additionally, the phylogeographic pattern of these and 4 more other populations was inferred based on the sequencing of 2 regions of the mitochondrial DNA (mtDNA), trying to gain an insight in the direction of chromosomal evolution in the group, their apomorphic and plesiomorphic chromosomal features and their relationships.

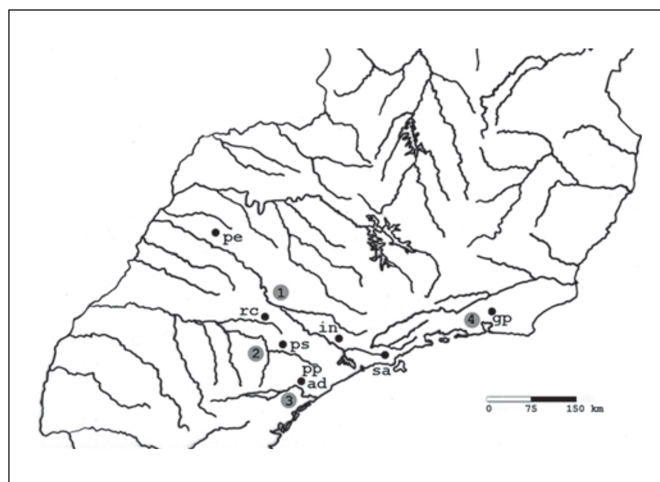

Fig. 1. Map showing the sampling sites of *A. altiparanae* and *A.* aff. *bimaculatus*. The numbers in the circles indicate the main hydrographic basins on Southeastern Brazil: (1) Tietê River; (2) Paranapanema River; (3) Ribeira de Iguape River; (4) Guapimirim River. The letters indicate the collection sites: Penápolis, SP (pe); Indaiatuba, SP (in); Salesópolis, SP (sa); Avaré, SP (rc); Pilar do Sul, SP (ps); Sete Barras, SP (pp, ad); Cachoeiras de Macacu, RJ (gp).

Material and Methods

The analyzed specimens of *A. altiparanae* and *A.* aff. *bimaculatus* were from 8 collection sites (fig. 1 and table 2) from May 2004 to July 2007. After the cytogenetic procedures and tissue sampling for DNA extraction, the individuals were fixed in 10% formaldehyde and stored in 70% ethanol. They were identified and deposited in the fish collection of the UFRGS Museum (Brazil). The tissue samples were deposited in the cell, tissue and DNA bank of the Laboratory of Ecological and Evolutionary Genetics of UFV/Rio Paranaíba (Brazil), under a specific voucher.

Mitotic chromosomes were obtained according to Gold et al. [1990] and the metaphases were colored by Giemsa. The silver nitrate staining (Ag-NOR) was performed according to Kavalco and Pazza [2004]. The C-banding followed the technique described by Sumner [1972]. The direct localization of ribosomal cistrons was performed through fluorescent in situ hybridization (FISH) [Pazza et al., 2006], using 18S rDNA [Hatanaka and Galetti Jr., 2004] and 5S rDNA [Martins and Galetti Jr., 1999] probes. In addition, FISH with a satellite DNA probe (As-51) isolated from *A. scabripinnis* was also carried out [Mestriner et al., 2000].

The chromosomal preparations were analyzed in an optic microscope and digitalized (resolution of 5 Mpx) using a digital camera, the image system CoolSnap Pro, and the software Image Pro Plus (Media Cybernetics). The classification of chromosomal types was based on the arm ratio (AR) [Levan et al., 1964], as follows: metacentric (M: AR = 1.00–1.70), submetacentric (SM: AR = 1.71–3.00), subtelocentric (ST: AR = 3.01–7.00) and acrocentric (A: AR higher than 7.00). Five karyotypes from different specimens were analyzed from each collection site.

The DNA extraction was accomplished using the Wizard® Genomic DNA Purification kit (Promega), following the manufacturer's instructions. The DNA samples were quantified in agarose gel using a Low DNA Mass™ Ladder (Invitrogen) and diluted to a working solution at 10 ng/μl. As outgroup sequences from *A. mexicanus* were used.

Five representatives from each collection site were used, when possible. Two mitochondrial segments were chosen. The following pairs of primers were used in amplification: Met-L4437 (5′-AAG CTT TCG GGC CCA TAC C-3′) + DN2-H4980 (5′-ATT TTTC GTA GTT GGG TTT GRT T-3′) [Macey et al., 1997] that

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Table 2. Species and collection sites sampled in the present work

Species	Locality – Hydrographic basin	Map	Coordinates	Voucher	N	F	M
A. altiparanae	Salesópolis, SP – Upper Tietê River	sa	23°31′23′′S/45°51′42′′W	UFRGS10.277	21/4	16	5
A. altiparanae	Pilar do Sul, SP – Paranapanema	ps	23°48′45′′S/47°42′30′′W	UFRGS10.262	6/4	3	3
A. aff. *bimaculatus*[a]	Sete Barras, SP – Ribeira de Iguape	ad	24°18′39′′S/47°53′30′′W	UFRGS10.273	7/3	4	3
A. aff. *bimaculatus*	Cachoeiras de Macacu, RJ – Guapimirim/coastal rivers	gp	22°38′12′′S/42°42′42′′W	UFRGS10.272	6/5	3	3
A. aff. *bimaculatus*	Registro, SP – Ribeira de Iguape	pp	24°36′01′′S/47°52′32′′W	UFRGS10.268	0/2	–	–
A. altiparanae[a]	Indaiatuba, SP – Middle Tietê River	in	23°05′39′′S/47°15′37′′W	Not deposited	0/1	–	–
A. altiparanae[a]	Penápolis, SP – Lower Tietê River	pe	21°25′11′′S/50°04′39′′W	Not deposited	0/1	–	–
A. altiparanae[a]	Avaré, SP – Paranapanema	rc	23°05′55′′S/48°55′32′′W	Not deposited	0/1	–	–

[a] Samples lacking *ND2* sequences with good quality, being not considered in analysis.
N = Number of analyzed specimens (by cytogenetic/molecular studies); F = female; M = male.

generate fragments of about 550 bp, including the partial sequences of the $tRNA^{Met}$ gene and the subunit 2 of the NADH dehydrogenase gene *(ND2)*; and ATP8.2-L8331 (5′-AAA GCR TYR GCC TTT TAA GC-3′) + CO3.2-H9236 (5′-GTT AGT GGT CAK GGG CTT GGR TC-3′) [Sivasundar et al., 2001] that amplify a segment of nearly 910 bp, comprising the complete sequence of the subunits 6 and 8 of the *ATPase* gene and the partial sequence of the subunit 3 of the cytochrome oxidase gene *(CO3)*.

The amplification reactions were carried out in an Eppendorf Mastercycler® Gradient PCR thermal cycler, totalizing a volume of 25 μl, cointaining 15 ng of template DNA, Tris-KCl (20 mM Tris-HCl pH 8.4 and 50 mM KCl), 1.5 mM $MgCl_2$, 2.5 μM of each primer, 0.1 mM of each dNTP and 2.5 U *Taq*-polymerase. The PCR conditions comprised a first denaturation step at 94°C for 4 min, hybridization at 56°C for 30 s and extension at 72°C for 2 min, followed by 40 cycles of 15 s at 94°C, 30 s at 56°C and 2 min at 72°C and a final extension step for 10 min at 72°C [Prioli et al., 2002]. After checking the amplification results in a 1% agarose gel, the PCR products were purified using the ChargeSwitch® PCR Clean-Up kit (Invitrogen), and another set was directly used as a template in the sequencing reactions, using the BigDye® Terminator v. 3.1 Cycle Sequencing kit (Applied Biosystems), according to the manufacturer's instructions. About 10 ng of template DNA and 5 pmol of each primer were added in each sequencing reaction. The samples were submitted to 25 cycles of 30 s at 96°C, 15 s at 50°C and 4 min at 60°C and precipitated for further sequencing.

The sequences were visualized and edited using the software Chromas Lite v2.01, and compared with the GenBank database (http://www.ncbi.nlm.nih.gov) using BlastN in order to confirm their identity with mitochondrial sequences of other fish species. Afterwards, the DNA sequences were aligned based on pairwise and multiple alignments, with the following penalties: gap opening = 20 and gap extension = 6.66, using the algorithm ClustalW v1.6 [Thompson et al., 1994] available in the software MEGA v4.1 [Tamura et al., 2007].

In order to avoid artifacts in the phylogenetic reconstruction, the low-quality sequences were excluded from the analysis of the concatenated data matrix (table 2).

Xia's test [Xia et al., 2003] was used to assess saturation using DAMBE v 5.0.16. The distances between the sequences were calculated by PAUP* v4.0 [Swofford, 2001] based on γ-distribution with an empirically determined α parameter, using Tamura and Nei's substitution model [Tamura and Nei, 1993].

The phylogenetic analyses were performed with the software MEGA v4.1 [Tamura et al., 2007], using the parsimony method based on the close-neighbor-interchange algorithm [Nei and Kumar, 2000] with calculation of retention, consistence and bootstrap (1.000 replicates) indexes [Felsenstein, 1985]; the neighbor-joining (NJ) distance trees were obtained by maximum composed likelihood (MCL) [Tamura et al., 2004], based on the substitution model of Tamura and Nei [1993], where the rates of both substitution types (between purines and between pyrimidines) are considered separately according to the unequal frequencies of the 4 nucleotides; the nucleotide substitution pattern was estimated by MCL in the software MEGA v4.1 [Tamura et al., 2007]. The p distance was estimated using the software MEGA v4.1 [Tamura et al., 2007] to provide a comparison with the data available in the literature.

The statistical data were obtained using DAMBE v5.0.16, DnaSP v4.50 [Rozas et al., 2003] and MEGA v4.1 [Tamura et al., 2007].

Results

Cytogenetics

All populations presented 2n = 50 for both males and females, with no differences related with the sex of the specimens. Nonetheless, specific cytotypes, distinguishable by the karyotype formula, were observed. In *A. altiparanae*, the individuals from Upper Tietê (Salesópolis, SP) presented 8M + 20SM + 10ST + 12A (fig. 2a), while the population from Paranapanema River presented a karyotype composed of 8M + 22SM + 14ST + 6A (fig. 2b). Both populations of *A.* aff. *bimaculatus* presented a similar karyotype constitution of 8M + 24SM + 12ST + 6A (fig. 2c, d). However, both populations from coastal rivers could be differentiated by slight differences in the karyotype symmetry. Such variation refers to the different size in

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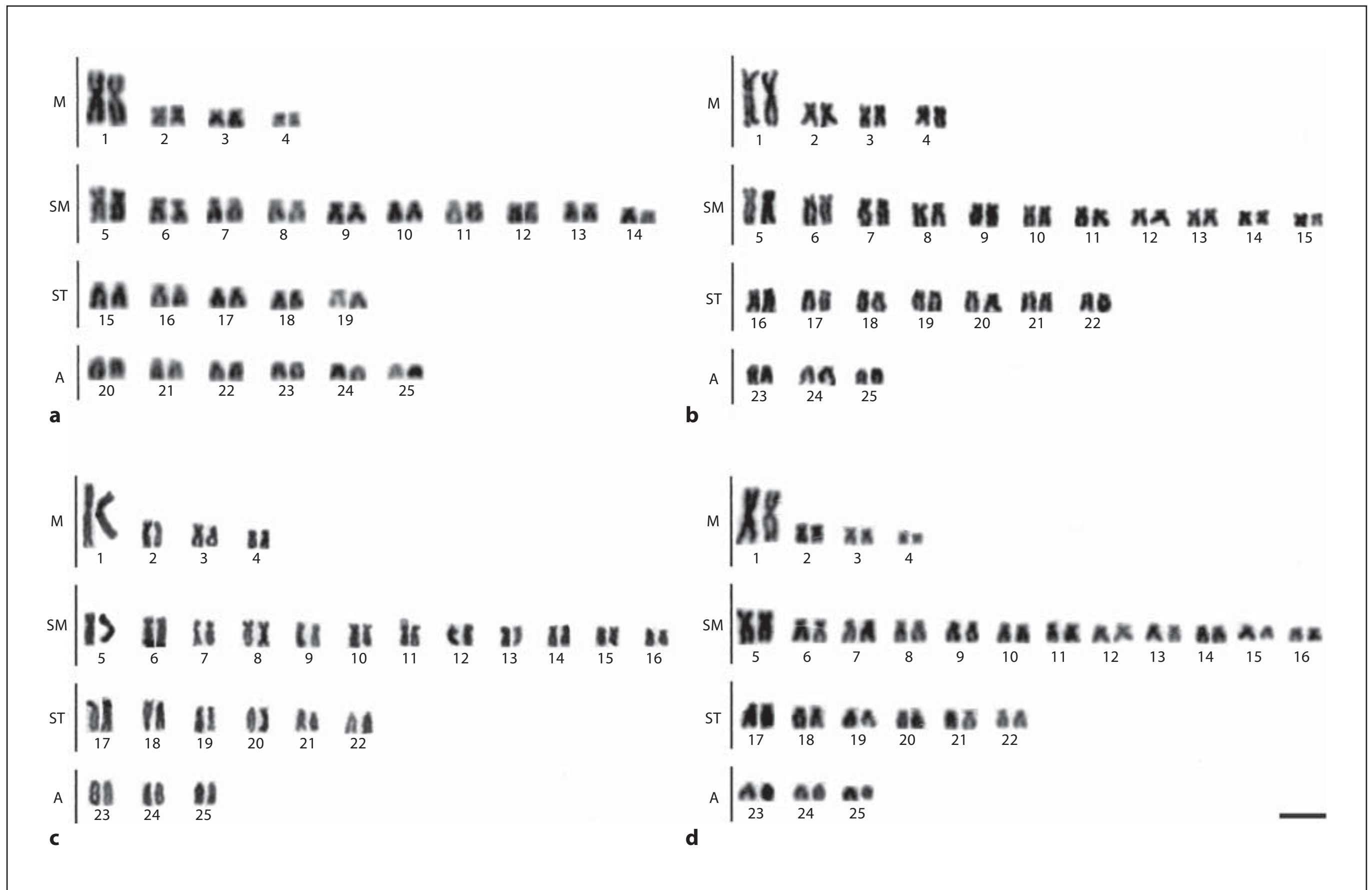

Fig. 2. Karyotypes of *A. altiparanae* from Salesópolis, SP (**a**) and Pilar do Sul, SP (**b**) and karyotypes of *A.* aff. *bimaculatus* from Sete Barras, SP (**c**) and Cachoeiras de Macacu, RJ (**d**) after Giemsa staining. Bar = 5 μm.

morphologically similar chromosomes, thereby supposed to be homeologous. For example, a remarkable size difference was detected in the 23rd, 24th and 25th acrocentric pairs when the populations from Sete Barras (SP) and Cachoeiras de Macacu (RJ) were compared (fig. 2c, d).

The silver nitrate staining revealed single Ag-NORs, located at terminal position on either long or short arms of ST-A chromosomes (fig. 3a, d, g). The positive signals by 18S rDNA-FISH ranged from 2 (fig. 3k) to 4 signals (fig. 3e), all located at terminal position of ST-A chromosomes, except for a site on a single chromosome of the first M pair in the population from Pilar do Sul (SP) (fig. 2e). By 5S rDNA-FISH, we identified 2 interstitial sites on M-SM chromosomes in all analyzed populations (fig. 3c, f, i, l).

The C-banding pattern in the populations from Salesópolis (SP) and Pilar do Sul (SP) comprised few terminal heterochromatic blocks and several interstitial segments (fig. 4a, c). On the other hand, the heterochromatin was preferentially located at pericentromeric region of some chromosomes in *A.* aff. *bimaculatus* from Sete Barras (SP) and Cachoeiras de Macacu (RJ) (fig. 4e, g).

Hybridization experiments using the As-51 satDNA probe revealed homologous chromosomal regions in 3 out of the 4 analyzed populations, being absent in chromosomes of specimens from Sete Barras (SP) (fig. 4f). The specimens from Salesópolis (SP) and Cachoeiras de Macacu (RJ) presented a single SM pair bearing signals on short arms (fig. 4b, h), whereas the samples from Pilar do Sul (SP) showed subtle terminal signals on 5 chromosomes, including one homologous of the largest M pair (fig. 4d).

Molecular Data

Three hundred and seventy-three out of the 1,314 bp analyzed were variable, being 124 informative by parsi-

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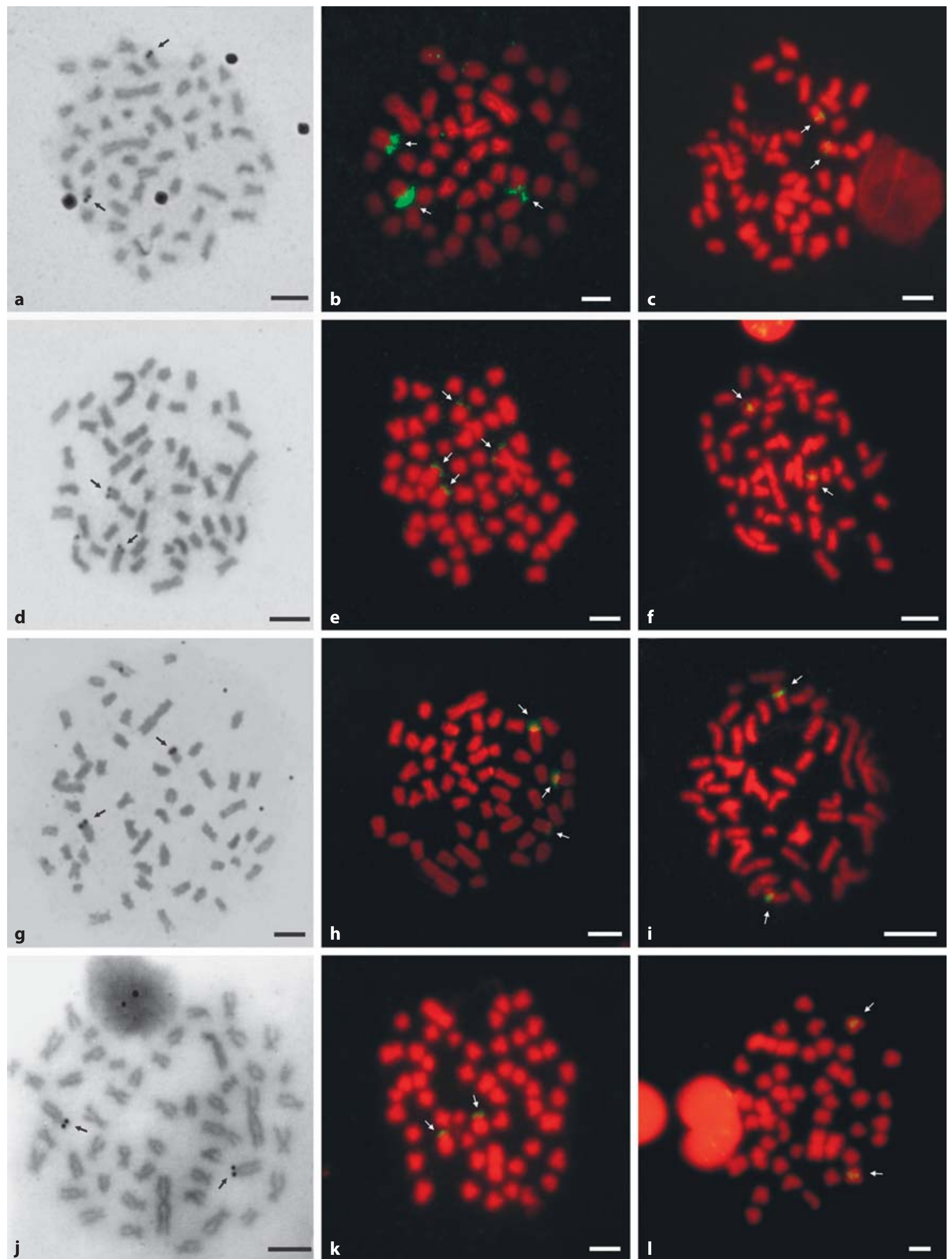

Fig. 3. Metaphases of *A. altiparanae* (**a–f**) and *A.* aff. *bimaculatus* (**g–l**). Ag-NORs (left), FISH-18S (middle) and FISH-5S (right) signals in populations from Salesópolis, SP (**a**, **b**, **c**), Pilar do Sul, SP (**d**, **e**, **f**), Sete Barras, SP (**g**, **h**, **i**) and Cachoeiras de Macacu, RJ (**j**, **k**, **l**). Bar = 5 μm.

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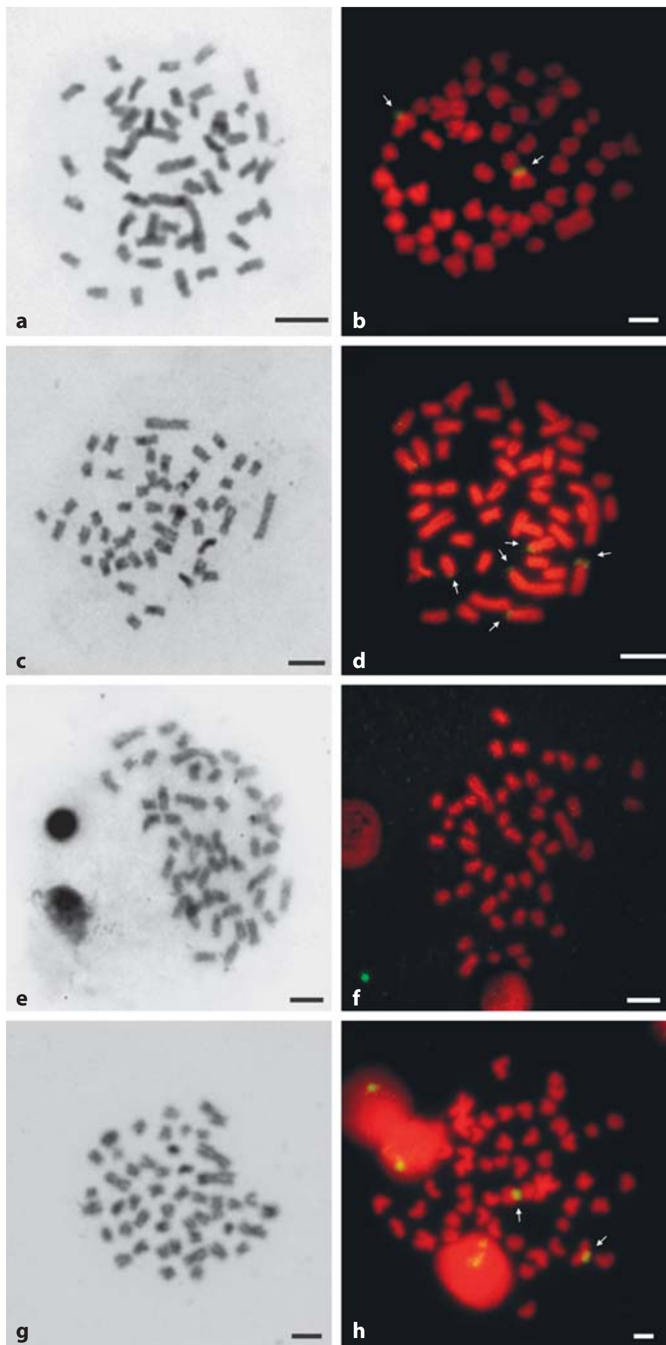

Fig. 4. Metaphases of *A. altiparanae* from Salesópolis, SP (**a**, **b**) and Pilar do Sul, SP (**c**, **d**) and metaphases of *A.* aff. *bimaculatus* from Sete Barras, SP (**e**, **f**) and Cachoeiras de Macacu, RJ (**g**, **h**) after C-banding (left) and FISH with As-51 satDNA probe (right). Bar = 5 μm.

mony. The *ND2* gene contributed with 554 sites, where 161 were variable and 57 informative. The regions from the subunits 6 and 8 of the *ATPase* gene contributed with 854 sites, 218 being variable and 98 informative. In the concatenated data, 36 indels and 15 haplotypes (Hd = 0.99) were observed. The nucleotide frequencies in the concatenated data were: 0.288 (A); 0.289 (T/U); 0.276 (C), and 0.147 (G). The transition/transversion ratio was k_1 = 10.847 (purines) and k_2 = 6.009 (pyrimidines). The total deviation on transition/transversion was R = 3.257. The parameter in the gamma distribution was α = 0.311. The evolutionary distances within and among individuals per hydrographic basin are shown in table 3.

By plotting the number of transitions and transversions versus the genetic distance, the sequence belonging to the *ATPase* gene has proved to be saturated when analyzed separately. The data plotted using the index of substitution saturation and their critical value regarding the *ND2* gene and the concatenated data showed that Iss < Iss.c (low saturation), indicating their usefulness for phylogenetic reconstruction.

All individuals presenting good-quality sequences were included in the analysis because of the existence of haplotypes that did not correspond to clusters per hydrographic basin, i.e., there were closely-related haplotypes shared by different populations depending on the data set analyzed. Moreover, more than 1 haplotype per collection site was observed. One individual of *A. mexicanus* was used as outgroup.

The NJ-MCL trees for each region recovered a similar topology to that from maximum parsimony (MP) trees and, therefore, they were omitted.

Discussion

Comparative Phylogeography

Two fast-evolving genes *(ATPase* and *ND2)* [Chang et al., 1994] were chosen in the present analysis since the *A. altiparanae* – *A.* aff. *bimaculatus* group seems to present a recent divergence as indicated by their evolutionary relationship and the presence of structured populations with distinct cytotypes in different sub-basins. The choice of the region to be sequenced is thought to have more influence on the final topology of the tree than the method of phylogenetic reconstruction itself [Russo, 2009].

Funk and Omland [2003] pointed out that the mtDNA might occasionally fail in presenting a suitable variation rate to reconstruct groups of very recent phylogenetic ra-

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Table 3. Genetic distances between haplotypes of the *A. altiparanae – bimaculatus* group, estimated for each sample locality

	Guapimirim	Tietê	Paranapanema	Ribeira de Iguape	Outgroup	d(TN)	d(p)
Guapimirim	–	0.06467	0.06030	0.02995	0.22263	0.00928	0.00882
Tietê	0.08212	–	0.03182	0.06777	0.21396	0.03006	0.02547
Paranapanema	0.07512	0.03643	–	0.06175	0.21583	0.01226	0.01160
Ribeira de Iguape	0.03499	0.08842	0.07899	–	0.22789	0.04626	0.03923
Outgroup	0.58821	0.55987	0.56895	0.62334	–	–	–

Scores below the diagonal correspond to the distance from Tamura and Nei [1993]. Scores above the diagonal correspond to p distance (uncorrected).

d(TN) = Intrapopulation distance according to the Tamura and Nei [1993] method; d(p) = intrapopulation distance based on p distance.

diation, thereby leading to artifacts. On the other hand, regions that evolve too fast might be saturated and generate trees with a high homoplasy level. According to these authors, depending on the branch-supporting values (bootstrap, for instance), a polyphyletic condition might represent the real tree for the selected mitochondrial gene and not a simple artifact caused by inadequate data. In the present work, the only sequence showing saturation when analyzed separately *(ATPase)* was also the one that originated trees with the lowest bootstrap values (data not shown). The low bootstrap values in the most intern nodes might be related to the high genetic similarity among haplotypes [Prioli et al., 2002], as the genetic distance within the groups was relatively low (table 3).

Nonetheless, inadequate gene regions are not the only reason for polyphyletic trees in molecular evolution analyses. Cryptic or taxonomically controversial species might potentially interfere in the resolution of evolutionary trees, causing polyphyly [Funk and Omland, 2003].

The data from the present work provided mitochondrial evidence to support this hypothesis because a genetic structure was identified comprising basin-specific cytotypes, either within *A. altiparanae* or *A.* aff. *bimaculatus*. However, the latter seems to compose a more homogeneous group in relation to the karyotype formulae of the subspecies analyzed (fig. 2).

The phylogenetic trees indicated that the *Astyanax* group herein analyzed is monophyletic, composed of 2 major clades: *A. altiparanae* and *A.* aff. *bimaculatus* (fig. 5). Furthermore, the yellow-tailed characins share an apomorphic chromosome trait with *A. mexicanus* (outgroup) regarding the presence of a single chromosomal pair bearing 5S rDNA.

Analysis of the *ND2* region indicated a possible paraphyly in the *A. altiparanae* from Tietê River, which shared haplotype groups with those from Paranapanema basin. In the MP tree based on the *ND2* region, a node that could indicate the lack of As-51 satDNA in the population from Sete Barras (SP, pp) is detected (data not shown).

Both *A. altiparanae* and *A.* aff. *bimaculatus* groups were clustered as monophyletic sister-groups when the entire 1,314-bp sequence was analyzed (fig. 5). The concatenated data presented high bootstrap values in the main clades in the MP tree (fig. 5a). The bootstrap was also high in some branches in the concatenated NJ-MCL tree (fig. 5b). Tree analysis and chromosomal data suggest the presence of subclades with shared cytogenetic features per lineage within the same area. However, only the node indicating the increased amount of *As-51* satDNA sequences in the population from Pilar do Sul (SP, Paranapanema River) could be properly defined. The subclade observed in the MP tree based on *ND2* region (indicating the lack of *As-51* satDNA) was absent when the 1,314 characters were taken into account (fig. 5a).

The phylogenetic analysis indicated that these yellow-tailed characins compose a monophyletic group despite of the occurrence of population-specific haplotypes, supported by high bootstrap values. Furthermore, a population structure within the same hydrographic basin was observed in both *A. altiparanae* and *A.* aff. *bimaculatus* groups (fig. 5). Nevertheless, the analyzed 1,314 bp revealed closely related haplotypes in distinct basins but belonging to the same hydrographic system (Upper Paraná or coastal rivers) (fig. 5). The monophyly in this large fish group and the close relationship among specimens from the coastal drainages and among the samples from Upper Paraná River agrees with the nominal redefinition of *Astyanax altiparanae* proposed by Garutti and Britski [2000] to the latter. However, a larger number of populations, comprising the entire Upper Paraná drainage, as well as

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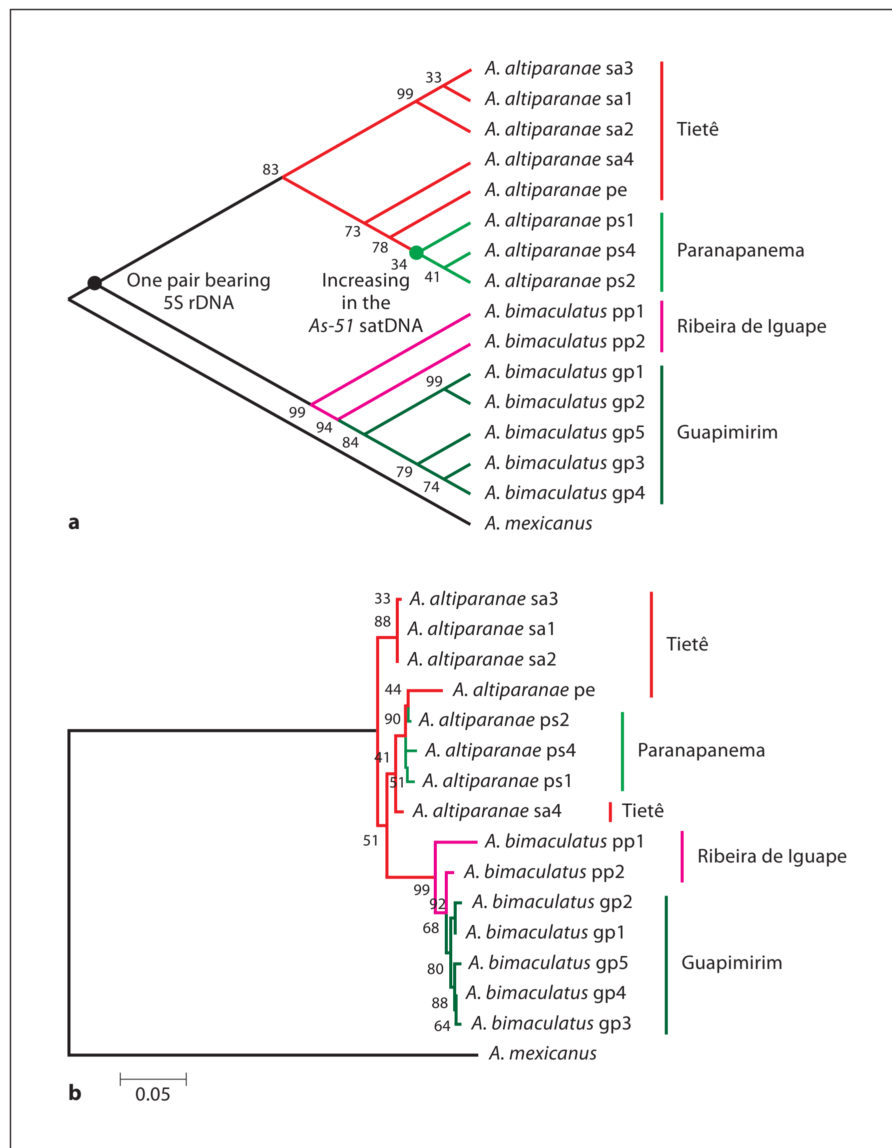

Fig. 5. Tree based on concatenated data of 16 individuals, using the 1,314 positions. The bootstrap values (1,000 replicates) are shown close to the branches. The gaps were recorded as missing data and removed just from pairwise comparisons in the NJ-MCL method (pairwise deletion option). **a** MP tree. Consensus tree based on the 5 most parsimonious ones (number of steps = 589) showing the evolutionary relationships in the *tRNA*^{met}*+ND2* and *ATPase8/6* regions: consistence index (CI) = 0.802410; retention index (RI) = 0.884017. **b** Concatenated tree inferred by NJ, linearized and in scale, where the branch length in the same unit of evolutionary distance was used to deduce the phylogeny, assuming that the evolutionary rates are equal in all lineages. The evolutionary distances were estimated by MCL and shown as units in the number of substitution per sites. The evolutionary rate among sites was modeled using γ-distribution (α = 0.61). The sum of the branch length equals 0.857.

samples from nearby systems should be analyzed to confirm the monophyletic status of *A. altiparanae*.

The studied populations proved to be neither very genetically diverse nor distant (table 3). The observed genetic distance values were relatively similar to those observed in populations from Paranapanema River studied by Leuzzi et al. [2004], but higher than that previously reported for *A. altiparanae* from Paraná and Iguaçu Rivers [Prioli et al., 2002]. The haplotype diversity per collection site was also low (table 3), particularly in specimens of *A.* aff. *bimaculatus*. Although not highly expressive, the mitochondrial diversity herein observed can be related to the hydrographic system, as the populations from coastal drainages were more similar to each other. Actually, the distance between Guapimirim and Ribeira de Iguape populations was lower than within distance of Ribeira de Iguape population.

The presence of closely related haplotypes in individuals from distinct basins (for instance, Paranapanema and Tietê) but possessing differentiated karyotypes does not weaken the hypothesis that these groups should be regarded as different species. According to Funk and Omland [2003], species lacking gene flow might bear differentiated haplotypes after a time period (because of post-introgression mutations) or, instead, they might present similar haplotypes that are clustered in the phylogenetic

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tree. However, those species that have diverged for a long time since the introgression, the persistent allele lineages putatively represent the basal phylogenetic condition. In this case, it is unlikely to find a relationship between haplotypes and geographic distribution due to random events occurred in each species [Funk and Omland, 2003].

Comparative Cytogenetics

The karyotypic studies in the *A. altiparanae* group have been initiated in the 1970s when the research in cytogenetics of Brazilian fish had begun. These former reports referred to the populations from Upper Paraná River as *A.* aff. *bimaculatus* [Jin and Toledo, 1975; Morelli et al., 1983], because the denomination '*A. altiparanae*' was validated several years later [Garutti and Britski, 2000].

The genus *Astyanax* is regarded as a recent group and chromosomal polymorphism occurs in several species [Pazza and Kavalco, 2007]. Although chromosomal differences are able to cause reproductive isolation, they seem to be insufficient to establish effective barriers to gene flow in some species, even when an accentuated polymorphism is detected [Pazza et al., 2007]. Nevertheless, albeit lacking a remarkable polymorphism like the one observed in *A. fasciatus*, the cytogenetic studies have never reported hybrids amongst different populations/cytotypes of *A. altiparanae* (table 1). Therefore, the conservative karyotypic traits in these fishes are not a reliable evidence that they are a single species.

Just like most of *Astyanax* species, the 'bimaculatus group' presents a large metacentric pair and a diploid number of 50 chromosomes, both regarded as symplesiomorphic characters of Characidae. Another common feature in this genus refers to the occurrence of multiple argentophilic sites bearing 18S rDNA (Ag-NORs). Although some 18S rDNA sites are hardly detected in several species of *Astyanax* because of their reduced size [Kavalco and Moreira-Filho, 2003], they usually present at least 2 chromosomes bearing large nucleolar regions [Fernandes and Martins-Santos, 2006; Pazza et al., 2006], which represent the preferential activation sites [Pazza et al., 2006]. Such large NORs were also detected in the specimens from the present work because all Ag-NOR signals were observed in 2 chromosomes (fig. 3).

Two 5S rDNA-bearing pairs represent the most common condition in Characiformes [Martins and Galetti Jr., 2001], including *Astyanax* (*A. bockmanni* [Kavalco et al., 2009b], *A. fasciatus* [Almeida-Toledo et al., 2002; Pazza et al., 2006, 2008b; Kavalco, 2008], *A. parahybae* [Kavalco et al., 2004], *A. scabripinnis* [Almeida-Toledo et al., 2002; Kavalco et al., 2004] and *A. schubarti* [Almeida-Toledo et al., 2002]). These data might suggest that the presence of a single 5S rDNA-bearing pair in *A. altiparanae* and *A.* aff. *bimaculatus* could be regarded as a derived trait. The evolutionary trees showed that the decreasing of 5S rDNA sites of the 'bimaculatus group' occurred in the base of the clade (fig. 5): This feature is shared by the whole group but absent in the outgroup, *A. mexicanus*, which presents 6 chromosomes bearing 5S rRNA genes [Kavalco and Almeida-Toledo, 2007].

The distribution of constitutive heterochromatin in pericentromeric and interstitial blocks is considered a conserved character in *A. altiparanae* [Daniel-Silva and Almeida-Toledo, 2001; Fernandes and Martins-Santos, 2004; Domingues et al., 2007]. Terminal blocks, as observed in the specimens from Salesópolis and Pilar do Sul (fig. 4a, c), are therefore unusual in this fish group. A conserved C-banding pattern was found only in *A.* aff. *bimaculatus* from the coastal drainages (fig. 4e, g).

Differences among the analyzed populations have also involved the distribution of the *As-51* satDNA. Although this marker has never been used to analyze populations in the 'bimaculatus group', a comparative approach can be accomplished based on the results obtained in other species bearing 2n = 50. All previously analyzed species with 50 chromosomes from coastal river basins (*A. giton* [Kavalco et al., 2007], *A. hastatus* [Kavalco et al., 2009a], *A. intermedius* [Kavalco et al., 2007] and *A. ribeirae* [Kavalco, 2008]) lack such satellite DNA sequence. On the other hand, some species from Upper Paraná River basin, especially *A. fasciatus*, have shown a great amount of satDNA *As-51* [Pazza et al., 2008a].

The presence of 5 minor sites bearing *As-51* satDNA in the population from Pilar do Sul seems to represent a unique event (fig. 4d) that has probably arisen in its ancestor, resulting in a structured group in both MP and NJ-MCL trees (fig. 5). Analogously, the lack of *As-51* satDNA in the population from Ribeira de Iguape River basin (fig. 4f) might be an apomorphic trait shared by a subgroup from the coastal drainage, as this event can be detected in the MP tree based on *ND2* sequence (data not shown). This result agrees with the pattern reported for other *Astyanax* species of coastal distribution [Kavalco, 2008].

Conclusions

Few reports in fish have combined chromosomal and phylogeographic studies. One of the few examples refers to the species *Chromaphyosemion bivittatum* (Cyprin-

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odontiformes), in which chromosomal differences are observed in individuals with a same haplotype [Völker et al., 2007]. Likewise, specimens with different karyotypes sharing very similar haplotypes were observed in the studied populations of *A. altiparanae* and *A.* aff. *bimaculatus*. However, the haplotype groups followed the distribution of populations into the major drainage systems (Upper Paraná and coastal rivers) (fig. 5b). The formation of a subclade comprising the coastal populations agrees with their tendency of cytogenetic divergence, being more closely related to each other than to species distributed on inner hydrographic systems [Kavalco et al., 2007; 2009b; Kavalco, 2008].

The data from the present work corroborate previous biological information related to the formation of population aggregates of the yellow-tailed characins along distinct hydrographic basins [Garutti and Britski, 2000], with the advantage of presenting a more reliable phylogeographic hypothesis provided by the phylogenetic trees [Templeton, 2004]. However, bottleneck events followed by geographic dispersal might have influenced the observed structured pattern as well, what is strengthened by the identification of several similar haplotypes in nearby basins. Therefore, both stochastic and selective processes could be determining such population differences, although the concordance between chromosomal and phylogenetic data would favor the selective process as the main force of genetic differentiation in populations of the ‘bimaculatus group’. Although the evolutionary direction in this fish group cannot be tracked yet, the association between nuclear (chromosomal) and mitochondrial markers have been useful to show relevant aspects of their evolutionary biology.

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