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The species complex *Astyanax fasciatus* Cuvier (Teleostei, Characiformes) – a multidisciplinary approach

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Cytogenetic data have provided important clues that the *Astyanax fasciatus* populations from the Upper Paraná River basin could be a part of a more diverse fish group, usually included on the same taxa. Samples collected in Cachoeira de Emas, SP, in Mogi-Guaçu River basin, show two major cytotypes presenting $2n = 46$ and $2n = 48$ chromosomes, with distinct karyotypic formula, despite the fact that the molecular data suggested some degree of gene flow between these cytotypes. Cytogenetic and morphometric analyses were performed in this species, aiming to contribute to the understanding of the natural history from such fish group. Two allopatric populations with distinct standard cytotypes were analysed, and the data obtained suggest the separation into two groups.
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Key words: cytotaxonomy; karyotype diversification; morphometric analysis.

INTRODUCTION

Different species concepts, based on different premises, have been used in an attempt to recognize the natural boundaries of living organisms. Among them, Mayr's concept, also known as the biological species concept, is the most widely used for living diploid groups having sexual reproduction. Although the description of a new species in practice uses phenotypic characters, increasing the utilization of genetic techniques to access some aspects of the biological species concept, such as the number of migrants per generations (measure of gene flow among populations and/or species) using molecular markers (Ogden & Thorpe, 2002; Hey *et al.*, 2004; Postma & von Noordwijk, 2005; Samonte

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et al., 2007; among others) is also beneficial. Taxonomic revisions on the genus *Astyanax*, for example, have given species status to groups previously classified as sub-species (Melo, 2001) or even sub-groups inside the 'species complexes' (Bertaco & Lucena, 2006).

Especially in the *Astyanax fasciatus* Cuvier, 1819, species, morphological analysis (Melo, 2001) favoured giving species status to the samples that occur in Paraíba do Sul River basin, an important drainage system in Southern Brazil, to *Astyanax parahybae* Eigenmann, 1908 (previously known as *Astyanax fasciatus parahybae*). Cytogenetic analysis has provided clues that the Upper Paraná River basin populations belong to a more diverse group of fish that nowadays integrate the same taxa (Artoni *et al.*, 2006; Pazza *et al.*, 2006; Gross *et al.*, 2004).

Aiming to contribute to the understanding of the '*fasciatus*' group boundaries, samples from Angatuba, SP (Paranapanema River basin) and Araras, SP (Mogi-Guaçu River basin) were analysed. Both of them belong to the Upper Paraná River system. Classic and molecular cytogenetic markers were used, associated with morphometric data analysis. This data integration has provided valuable information, which helps to characterize Neotropical fish species complexes (Moreira-Filho & Bertollo, 1991; Mizoguchi & Martins-Santos, 1998; Maistro *et al.*, 1998; Gross *et al.*, 2006).

MATERIALS AND METHODS

Astyanax fasciatus samples were collected from Araras, SP, Mogi-Guaçu River basin (seven specimens) and Angatuba, SP, Paranapanema River basin (12 specimens) (Fig. 1). Chromosome preparations were made according to Bertollo *et al.* (1978) and Gold *et al.* (1990) using the anterior kidney. The nucleolar organizer regions (NORs) and heterochromatin locations were detected according to Kavalco & Pazza (2004) and Sumner (1972), respectively. Fluorescence *in situ* hybridization (FISH) was performed according to Pazza *et al.* (2006) using 18S rDNA (Hatanaka & Galetti Jr, 2004) and 5S rDNA (Martins & Galetti Jr, 1999) probes. Chromosomes were classified as metacentric (M), submetacentric (SM), subtelocentric (ST) and acrocentric (A) based on the classification of centromere position by Levan *et al.* (1964). Fundamental number (FN) was estimated according to chromosome arm numbers.

All of the individuals were measured using a manual calliper of 0·05 mm precision, following the standard length, head length, head height, body height, tail peduncle height, predorsal distance, pre-anal distance, orbit diameter and interorbital width variables. Discriminant function analysis (Hotelling), neighbour-joining dendrogram based on Bray–Curtis similarity matrix (Bray & Curtis, 1957) and the size-free canonical discriminant analysis (SF-CDA) aiming to remove the effect of within-group ontogenetic variation (Reis *et al.*, 1990) was performed with the Past v1.67b software (Hammer *et al.*, 2001) based on covariance matrix of log-transformed measurements to assess morphometric variation between samples.

RESULTS

The Araras population of fish show a 48 chromosome (8M, 22SM, 12ST, 6A and FN = 90) diploid number [Fig. 1(a)]. Silver nitrate impregnation demonstrated a modal number of two chromosomes bearing Ag-NORs, especially in the distal region of the long arm of acrocentric chromosomes [Fig. 2(a)]. Constitutive heterochromatin was located on the distal regions of the long arms of submetacentric, subtelocentric and acrocentric chromosomes and on

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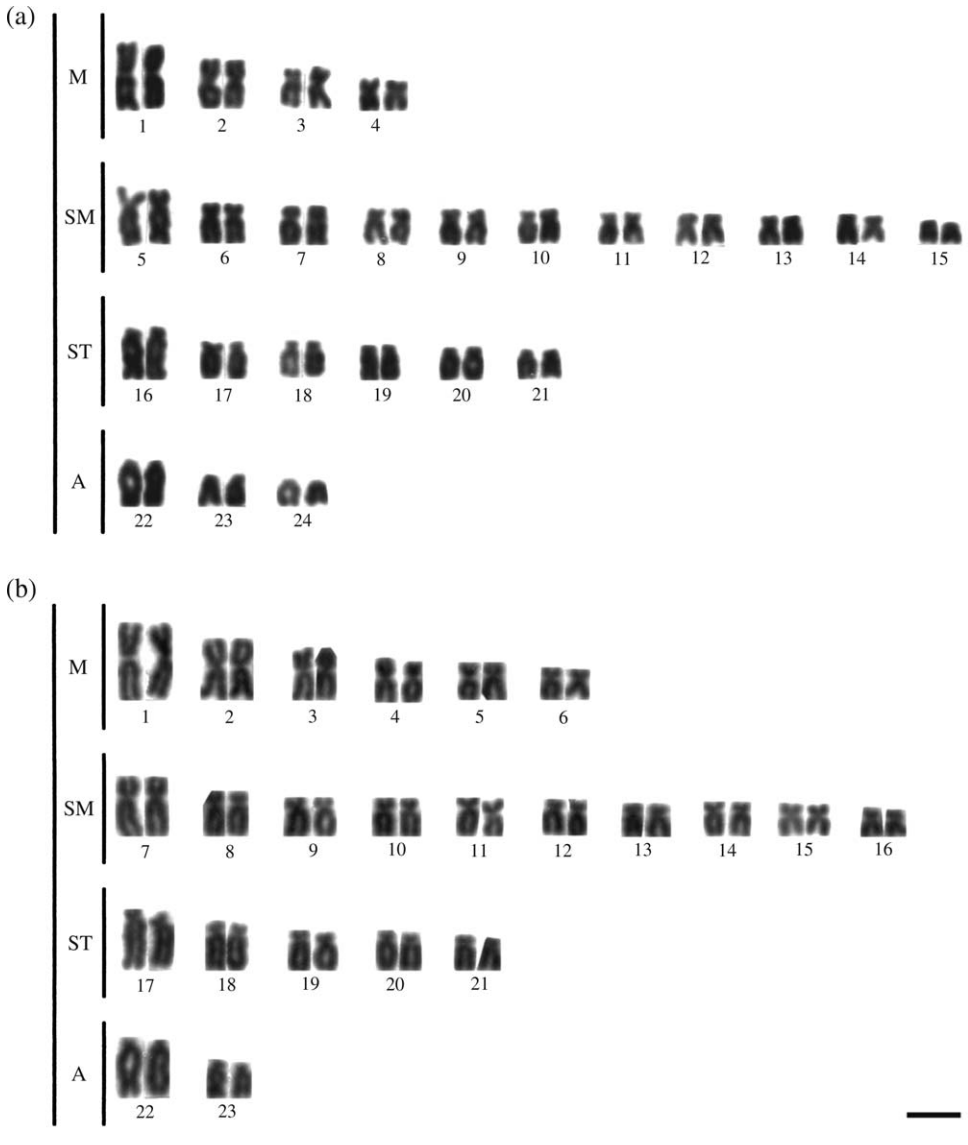

FIG. 1. Karyotypes of *Astyanax fasciatus* from (a) Araras ($2n = 48$) and (b) Angatuba ($2n = 46$) populations. Bar = 10 μm.

the short arm of some chromosomes [Fig. 2(b)]. FISH revealed two chromosome pairs bearing 5S sites, one of them at the pericentromeric region of a metacentric pair and the other one at the proximal region of an acrocentric chromosome [Fig. 2(c)]. In turn, three submetacentric chromosome pairs bearing 18S were found; two of them in the terminal region of the short arm and the other one at the terminal region of the long arm [Fig. 2(d)].

The Angatuba population is characterized by a 46 chromosome (12M, 20SM, 10ST, 4A and FN = 88) diploid number [Fig. 1(b)]. Silver nitrate

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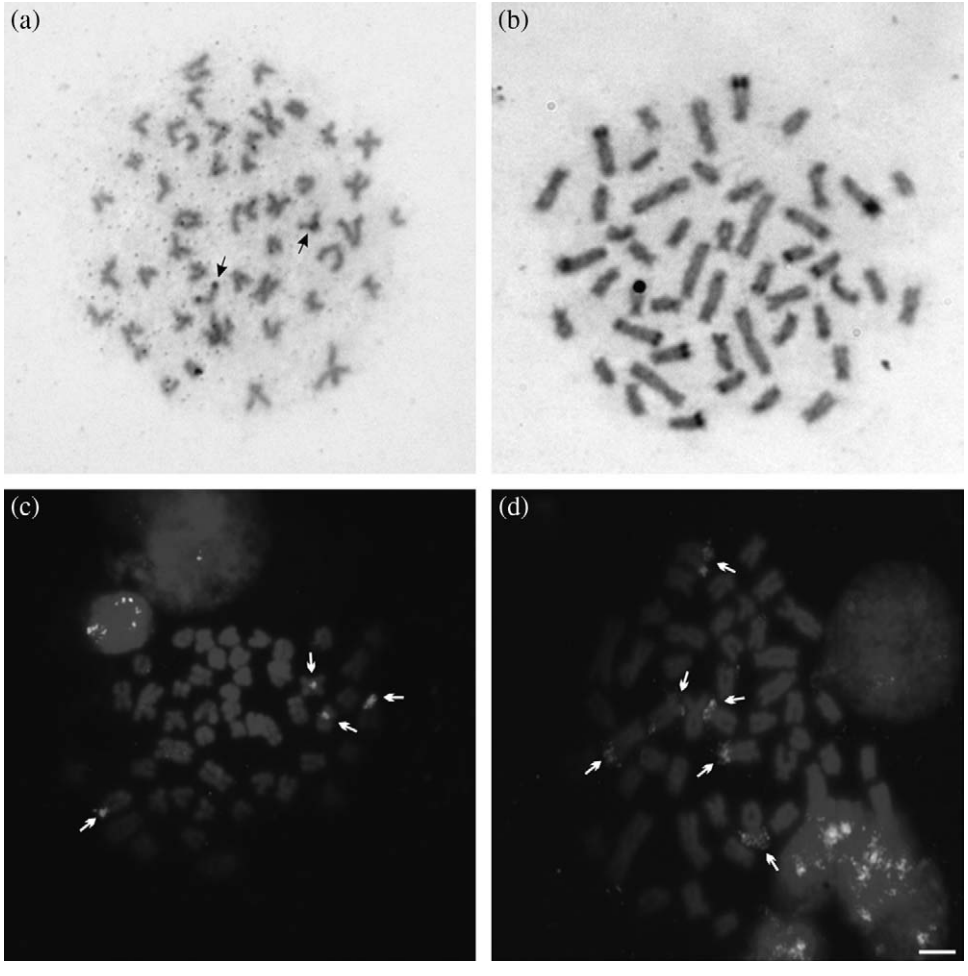

FIG. 2. *Astyanax fasciatus* metaphases from the Araras population. (a) Ag-NORs, (b) C-banding, (c) 5S fluorescence *in situ* hybridization (FISH) and (d) 18S FISH. The markers are shown by arrows. Bar = 10 μm.

impregnation revealed a modal number of four submetacentric chromosomes bearing Ag-NORs in terminal position of the long arms [Fig. 3(a)]. Constitutive heterochromatin was located in the distal region of submetacentric, subtelocentric and acrocentric chromosome long arms [Fig. 3(b)]. FISH revealed two chromosome pairs bearing 5S rDNA sites at the proximal region of a metacentric and an acrocentric chromosome pairs [Fig. 3(c)]. In turn, the 18S rDNA sites were located at the terminal region of long arms of two submetacentric chromosome pairs [Fig. 3(d)].

Discriminant analysis made it possible to test the classification of the data in two groups (populations) (Wilk's lambda = 0·2068; Hoteling's $P = 0{\cdot}01677$). Such analysis shows that one individual from Araras population must belong to the Angatuba population. The withdrawal of this individual permitted an exact classification of individuals according to their populations ($P = 0{\cdot}003$).

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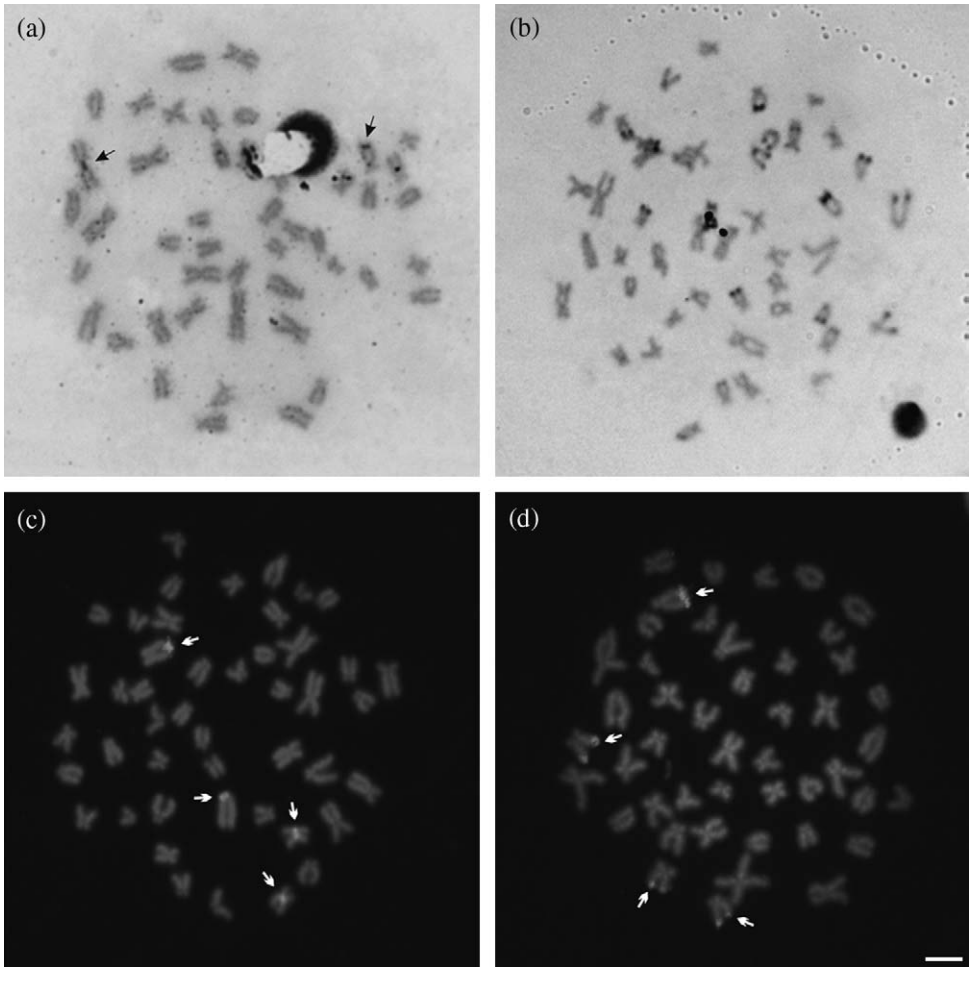

FIG. 3. *Astyanax fasciatus* metaphases from the Angatuba population. (a) Ag-NORs (left arrow showing a nucleolar organizer region association), (b) C-banding, (c) 5S fluorescence *in situ* hybridization (FISH) and (d) 18S FISH. The markers are shown by arrows. Bar = 10 μm.

The neighbour-joining dendrogram based on the Bray–Curtis similarity index shows more similarities within Angatuba population (Fig. 4). The SF-CDA clearly separates the populations even when the discrepant individual was considered (Fig. 5).

DISCUSSION

The karyotypes observed in both populations are quite similar to the previously observed one in the *A. fasciatus* complex, including the positive C-band distribution, the NORs sites evidenced by silver nitrate impregnation and FISH (Pazza *et al.*, 2006).

The cytogenetic markers show that the genus *Astyanax* is greatly diversified. It is interesting that this widespread species shows remarkable variation, such

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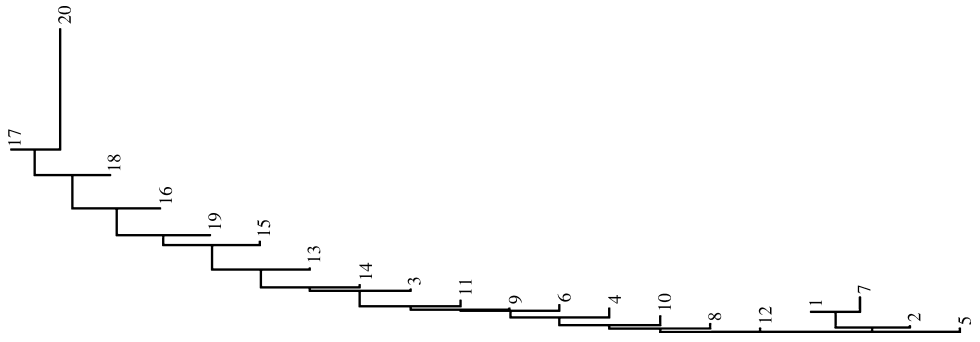

FIG. 4. Neighbour-joining dendrogram based on Bray–Curtis index of similarity. The individuals from Angatuba are represented by 1 to 12 and those from Araras by 13 to 20.

as the C-bands position and the number and location of NORs-bearing chromosomes (Moreira-Filho & Bertollo, 1991; Maistro *et al.*, 1998; Mizoguchi & Martins-Santos, 1998; Pazza *et al.*, 2006; among others). This could be explained by the biological patterns of this group; small fishes with most species inhabitating small streams. Another point is related to the 5S rDNA location, very conserved in most *Astyanax* species (Almeida-Toledo *et al.*, 2002; Kavalco *et al.*, 2004), especially one proximal site in a metacentric chromosome pair. Such conservation contributes to better understanding of the karyotypic evolutionary aspects of the genus since *Astyanax giton* Eigenmann, 1908 and

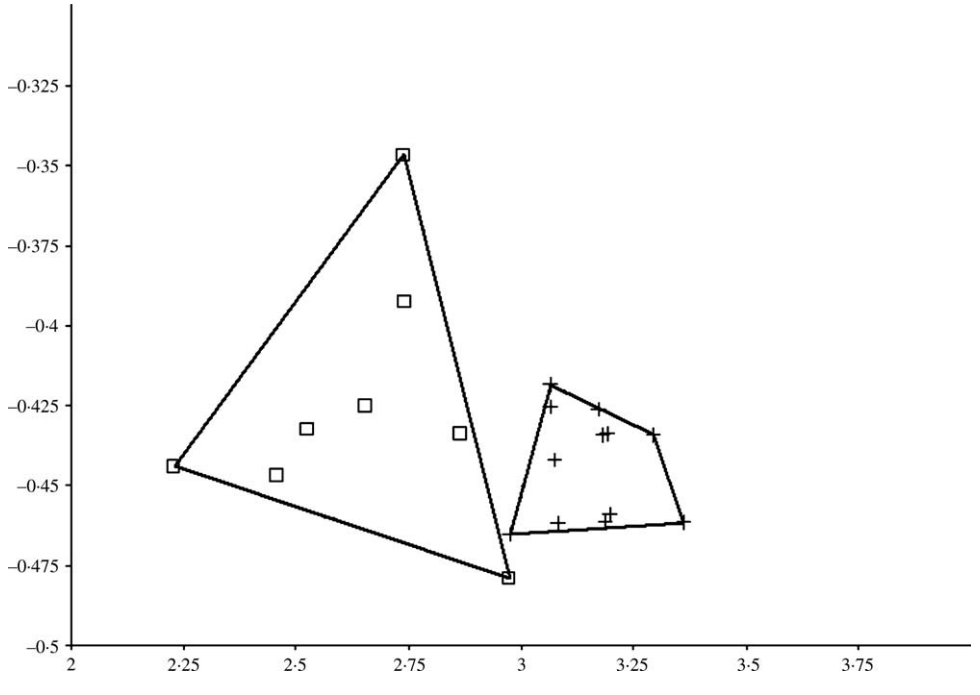

FIG. 5. Size-free canonical discriminant analysis plot showing Angatuba (cross) and Araras (square) populations.

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Astyanax intermedius Eigenmann, 1908 species present a divergent picture, with several 5S rDNA-bearing chromosomes (Kavalco *et al.*, 2004).

In spite of macrokaryotypic differences (chromosome number and type), the analysed populations share characteristics with other *A. fasciatus* populations (Pazza *et al.*, 2006), such as C-band distribution in the terminal region of several chromosomes, submetacentric chromosomes bearing NORs with terminal sites shown by silver nitrate and 18S rDNA FISH and the location of 5S rDNA sites as well.

Considering the *Astyanax* genus karyotypic diversity, such patterns should reflect the evolutionary history of the group and their distribution throughout the Paraná River basin as such basin drains a great area of the Brazilian territory. However, the time of divergence seems to have permitted a certain degree of chromosomal differentiation. Although the role of chromosomal variation in the speciation process is an open question, the correlation between the karyotypic differences and the morphometric analysis between such populations is remarkable. As is observed in some *Astyanax scabripinnis* (Jenyns, 1842) (Moreira-Filho & Bertollo, 1991; Maistro *et al.*, 1998; Mizoguchi & Martins-Santos, 1998) populations, both *A. fasciatus* populations in this study could be discriminated by their chromosome and morphometric patterns.

A similar study was performed with *Astyanax* populations (recently identified as *Astyanax* aff. *fasciatus*) from Tibagi River and the Furnas of Vila Velha State Park (Paraná State) (Artoni *et al.*, 2006). The Furnas are three collapsed wells called 'sinkholes', formed in the Pleistocene. The wells contain water levels reaching the groundwater and no direct communication between them or with other bodies of water, allowing them to be considered as regions of endemism for *Astyanax* (Artoni & Almeida, 2001). The canonical discriminant analysis shows significant differentiation among isolated populations (Shibatta & Artoni, 2005; Artoni *et al.*, 2006). The *Astyanax* populations of these sites show a remarkable karyotypic variability, characterized by at least three cytotypes (Artoni *et al.*, 2006). Unfortunately, the authors did not perform the canonical analysis using the cytotypes as grouping factor since there are some cytotypes distributed among more than one silkhole; meanwhile, there are sympatric cytotypes in others. Indeed, the cytotype identified as 'A' is widespread on Tibagi River and other sites studied by the authors, including the sinkhole 2, which was strongly separated by canonical analysis (Artoni *et al.*, 2006). Nevertheless, the fluctuating asymmetry from sinkhole 2 did not differ from Tibagi River population (Gross *et al.*, 2004).

The cytogenetic data obtained in the present work reinforce the hypothesis previously suggested for the *A. fasciatus* cytotypes distribution in Paraná River basin (Pazza *et al.*, 2006). The authors found a chromosome polymorphism in the Cachoeira de Emas region of the Mogi-Guaçu River, where, besides the standard cytotypes (with correct homologous pairing) with $2n = 46$ and $2n = 48$ chromosomes, one $2n = 45$ variant, another with $2n = 46$ and four $2n = 47$ cytotypes were found in sympatry. Additionally, the frequency of $2n = 46$ chromosomes was higher in this region than that observed in the upper and lower Mogi-Guaçu River, which should suggest an invasive species with the $2n = 46$ cytotypes, $2n = 48$ being the native one (Pazza *et al.*, 2006), although the molecular data suggests a certain degree of gene flow among the cytotypes, possibly

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resulting from a secondary contact (Pazza *et al.*, 2007). The present data reinforces such a hypothesis with the individuals from the Araras site possessing the same karyotypic patterns as observed in the $2n = 48$ cytotype from Mogi-Guaçu River. Additionally, the presence of one individual with divergent morphometric data is notable, and it is possible that this one results from secondary contact introgression as evidenced by molecular markers (Pazza *et al.*, 2007).

The use of the genetic analysis technologies throws light on questions about the natural history of the species. However, traditional analyses such as cytogenetic and morphometric methods can still contribute, especially when it is necessary to identify species in the field.

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