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Distribution, karyomorphology, and morphology of *Aspidistra subrotata* (Asparagaceae) at different ploidy levels in limestone areas of Asia

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Abstract

Aspidistra subrotata Y. Wan & C.C. Huang, 1987 is considered for the first time as a widespread polyploidy complex in the genus *Aspidistra* Ker Gawler, 1823 from limestone areas of Asia. The chromosome number of the tetraploid is 2n = 76 and the karyotype is formulated as 2n = 44 m + 8 sm + 24 st, while the chromosome number of the diploid is 2n = 38 and the karyotype formula 2n = 22 m + 4 sm + 12 st. In our studies, diploids occupy broader geographical and environmental niche spaces than tetraploids. Although the leaf-shape of *A. subrotata* varies quantitatively between and within diploid and/or tetraploid population(s), no obvious discontinuity in the width of leaf has been observed. The tetraploid plants may be distinguished from the diploid plants by their rigid petioles as well as thick deep green lamina. *A. subrotata* is therefore an interesting material to explore the formation and the evolutionary dynamics of a natural polyploid complex from limestone areas of the tropical regions.

Keywords

Aspidistra, chromosome number, karst plants, karyotype asymmetry, polyploid complex

Introduction

Aspidistra Ker Gawler, 1823 is a large genus including more than 140 species from Asia, belonging to the Asparagacae (Li 2004, Tillich 2014, Zhou et al. 2016, APG IV 2016). Its species diversity center is distributed in southwest China and northern Vietnam. Most of the *Aspidistra* species are diploid, with chromosome numbers of 2n = 36 or 2n = 38, except only two tetraploid species, *A. xilinensis* Y. Wan & X.H. Lu,1987 (2n = 72) and *A. cruciformis* Y. Wan & X.H. Lu, 1987 (2n = 72), both from China, as well as one hexaploid species, *A. sutepensis* K. Larsen, 1961 (2n = 114) from Thailand (Qiao et al. 2008, Meng and Gao 2014, Gao et al. 2015). All of these polyploids are stenochoric species with a chromosome base number of x = 19.

Aspidistra subrotata Y. Wan & C.C. Huang, 1987 was originally found in Guangxi Botanical Garden of Medicinal Plants, Nanning City, Guangxi Province, China. After that, Huang et al. (1997) reported on the chromosome number of *A. subrotata* from Nanning 2n = 38, with a karyotype formula as 2n = 22 m + 2 sm + 14 st (2 sat), while Wang et al. (2000) reported the same chromosome number in plants from Guilin City, Guangxi Province, China, but with different formulae, 2n = 22 m + 6 sm(2 sat) + 10 st. Both of the above plants were cultivated in the Botanical Garden and their wild localities remain unknown. *A. subrotata* subsp. *crassinervis* Tillich, 2005 was found from Vietnam, possibly in Tam Dao, Thai Nguyen Province, which was distinguished by *A. subrotata* subsp. *subrotata* in having its lamina ovate–lanceolate, secondary veins sharply protruding on upper surface of lamina, perigone lobes red–purple, ant the stigma white or with few small red dots on its upper surface. It is noteworthy that Tillich (2005) also pointed out that *A. subrotata* subsp. *subrotata* could be collected from the same locality. When studying the diversity in leaf shape of Thai material of *A. subrotata*, Phonsena and De Wilde (2010) recognized that plants with narrow or broad leaves with a smooth surface or with broad leaves and raised nerves were not found inter-connected with their rhizomes, although they usually grew in the same population. Therefore, three varieties were confirmed: *A. subrotata* var. *subrotata* with leaves 4–7 cm wide and nerves not raised, *A. subrotata* var. *angustifolia* Phonsena, 2010 with lanceolate leaves (1–) 2–2.5 cm wide and nerves not raised, and *A. subrotata* var. *crassinervis* (Tillich, 2005) Phonsena, 2010 with leaves 4–7 cm wide and raised nerves. During our systematic study on *Aspidistra*, we have conducted field work in China and Vietnam, and studied the karyomorphology and external morphology of *A. subrotata*. The tetraploid populations of *A. subrotata* were reported here for the first time, as well as recording widespread polyploidy complex in the genus *Aspidistra* from the limestone areas of Asia. This study is mainly aimed to add our knowledge about cytology of this genus.

Material and methods

The plants were collected from field work in Guangxi Province, China and Hanoi, Vietnam (Table 1) and subsequently cultivated in the experimental garden of Guangxi Institute of Botany, Guilin. Only one sample from Guangxi Botanical Garden of Me-

Table 1. Material examined of *Aspidistra subrotata*.

Sample	Voucher	Location	Latitude Longitude	Altitude	Figure
JL	*Huang Y.S. QG375*	China: Jinlong Town, Longzhou County, Chongzuo City, Guangxi Province	22°26.04'N 107°01.65'E	ca. 300m	
5M	*Huang Y.S. QG378*	China: 5th boundary marker, Nonggang National Nature Reserve, Longzhou County, Chongzuo City, Guangxi Province	22°27.82'N 106°58.03'E	ca. 300m	
NG	*Wu W.H. QG526*	China: Nonggang National Nature Reserve, Longzhou County, Chongzuo City, Guangxi Province†	22°28'N 106°58'E	-	5a
NN	*Gao Q. QG735*	China: cultivated in Guangxi Botanical Garden of Medicinal Plants, Nanning City, Guangxi Province		-	5b
SK	*Anonymous QG766*	China: Shuikou Town, Longzhou County, Chongzuo City, Guangxi Province†	22°28'N 106°35'E	-	
4M1	*Gao Q. QG807*	China: 4th boundary marker, Nonggang National Nature Reserve, Longzhou County, Chongzuo City, Guangxi Province	22°27.61'N 106°57.95'E	ca. 400m	6a
4M2	*Gao Q. QG809*	China: 4th boundary marker, Nonggang National Nature Reserve, Longzhou County, Chongzuo City, Guangxi Province	22°27.58'N 106°57.93'E	ca. 370m	6c
4M3	*Gao Q. QG810*	China: 4th boundary marker, Nonggang National Nature Reserve, Longzhou County, Chongzuo City, Guangxi Province	22°27.57'N 106°57.93'E	ca. 370m	6e
4M4	*Gao Q. QG811*	China: 4th boundary marker, Nonggang National Nature Reserve, Longzhou County, Chongzuo City, Guangxi Province	22°27.53'N 106°57.93'E	ca. 380m	5c, 6h
PM	*Liu Y. QG281*	China: Mt. Poman, Napo Town, Baise City, Guangxi Province†	22°57'N 160°00'E	-	
DQ1	*Liao Y.B. QG662*	China: Mt. Daqing, Pingxiang County-level City, Guangxi Province	22°18.27'N 106°41.93'E	ca. 950m	5f, 7f
DQ2	*Gao Q. QG823*	China: Mt. Daqing, Pingxiang County-level City, Guangxi Province	22°18.06'N 106°42.15'E	ca. 900m	7a
BV	*Ogisu M. QG365*	Vietnam: Mt. Bavi, Hanoi†	21°05'N 105°22'E	ca. 540m	5d, e

† Lack of exact GPS

dicinal Plants may be the clone plants of typical *Aspidistra subrotata*. For chromosome observation, actively growing root tips were pretreated in 0.1% colchicine for 3 h at room temperature and then fixed in Carnoy I (ethanol : glacial acetic acid = 3 : 1). They were macerated in 1 : 1 mixture of 1 M HCL and 45% acetic acid at 60 °C for 4 min, and stained and squashed in 1% aceto-orcein. The karyotype formula was based on measurements of metaphase chromosomes taken from photographs. The symbols used to describe the karyotypes followed by Levan et al. (1964). From the mean values of one to five individual karyotypes, the average chromosome length as well as the karyotype intrachromosomal asymmetry index (A_1) and interchromosomal asymmetry index (A_2) (Romero Zacro 1986) were calculated.

Results

Distribution and karyomorphology

Karyomorphological features were observed in 13 samples of *Aspidistra subrotata* (Table 1). Of these, eight samples of five populations from Longzhou County and one sample from Nanning were tetraploid, while the other four diploid samples were from Mt. Daqing, Pingxiang County-level City, Mt. Poman, Baise City and Mt. Bavi, Hanoi.

Eight samples of five tetrapoloid populations of *Aspidistra subrotata* were all from Longzhou and were located not far away from each other (Map 1). Another single sample from Nanning was cultivated in Guangxi Medicine Botany Garden, which may be

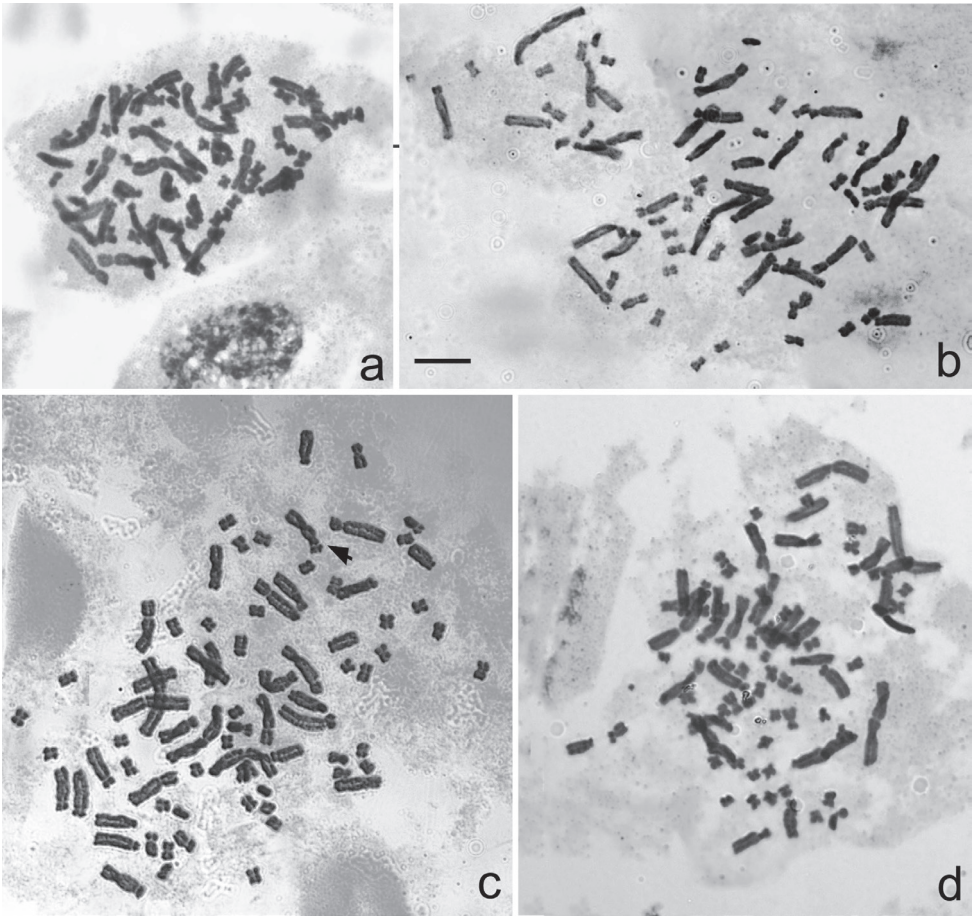


Figure 1. Somatic Chromosome at mitotic metaphase in *Aspidistra subrotata* of 2n = 76. **a** Jilong population **b** 5th boundary marker population **c** Nonggang National Nature Reserve population, and the arrow shows the secondary constriction of the chromosome **d** 4th boundary marker population. Bar = 10 μm.

the clone plants of typical *A. subrotata*. All of them have a chromosome number of 2n = 76, with the karyotype formulated as 2n = 44 m + 8 sm + 24 st (Figure 1, 3). The karyotypes of the tetraploid samples are similar to one another. The first and largest pair was metacentric. The pair II was larger [larger than what?]and submetacentric. Chromosomes from III to VIII pair were larger [again, larger than what? Normally, these chromosome pairs would be expected to be progressively smaller] and submetacentric. The other chromosomes were smaller, of which pair IX was submetacentric and the others were metacentric (Figure 3). It was noted that the secondary constriction occurred in chromosome 12 of the sample NG (Figure 1c and 3c). The average lengths of chromosomes varied from 4.26 to 5.91 μm, while A_1 and A_2 were from 0.33 to 0.39 and 0.55 to 0.64 (Table 2).

Four samples of three populations from Mt. Poman, Mt. Daqing, and Mt. Bavi of *Aspidistra subrotata* have a chromosome number of 2n = 38, uniformly formulated as 2n = 22 m + 4 sm + 12 st (Figures 2 and 4). The first and largest pair was metacentric. The pair II was larger and submedian centromeric. Chromosomes from III to VIII pair were larger [progressively smaller?] and subtelocentric. The other chromosomes were smaller, of which pair IX was submetacentric and the others were metacentric (Figure 4). No satellite was observed. The average lengths of chromosomes varied from 4.30 to 5.61 μm, while A_1 and A_2 were from 0.34 to 0.38 and 0.57 to 0.59 (Table 2).

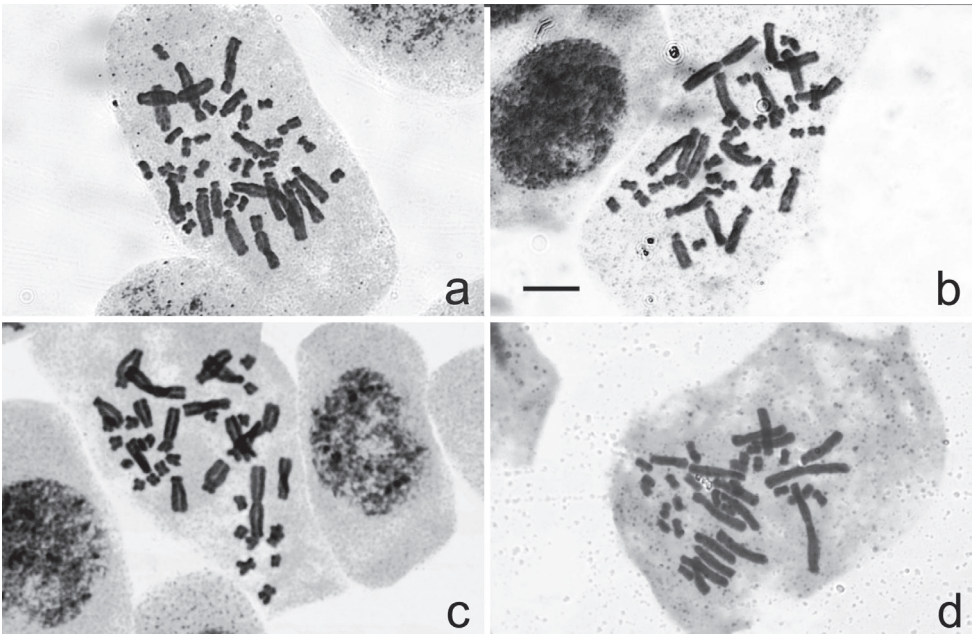


Figure 2. Somatic Chromosome at mitotic metaphase in *Aspidistra subrotata* of 2n = 38. **a** Mt. Poman population **b, c** Mt. Daqing population **d** Mt. Bavi population. Bar = 10 μm.

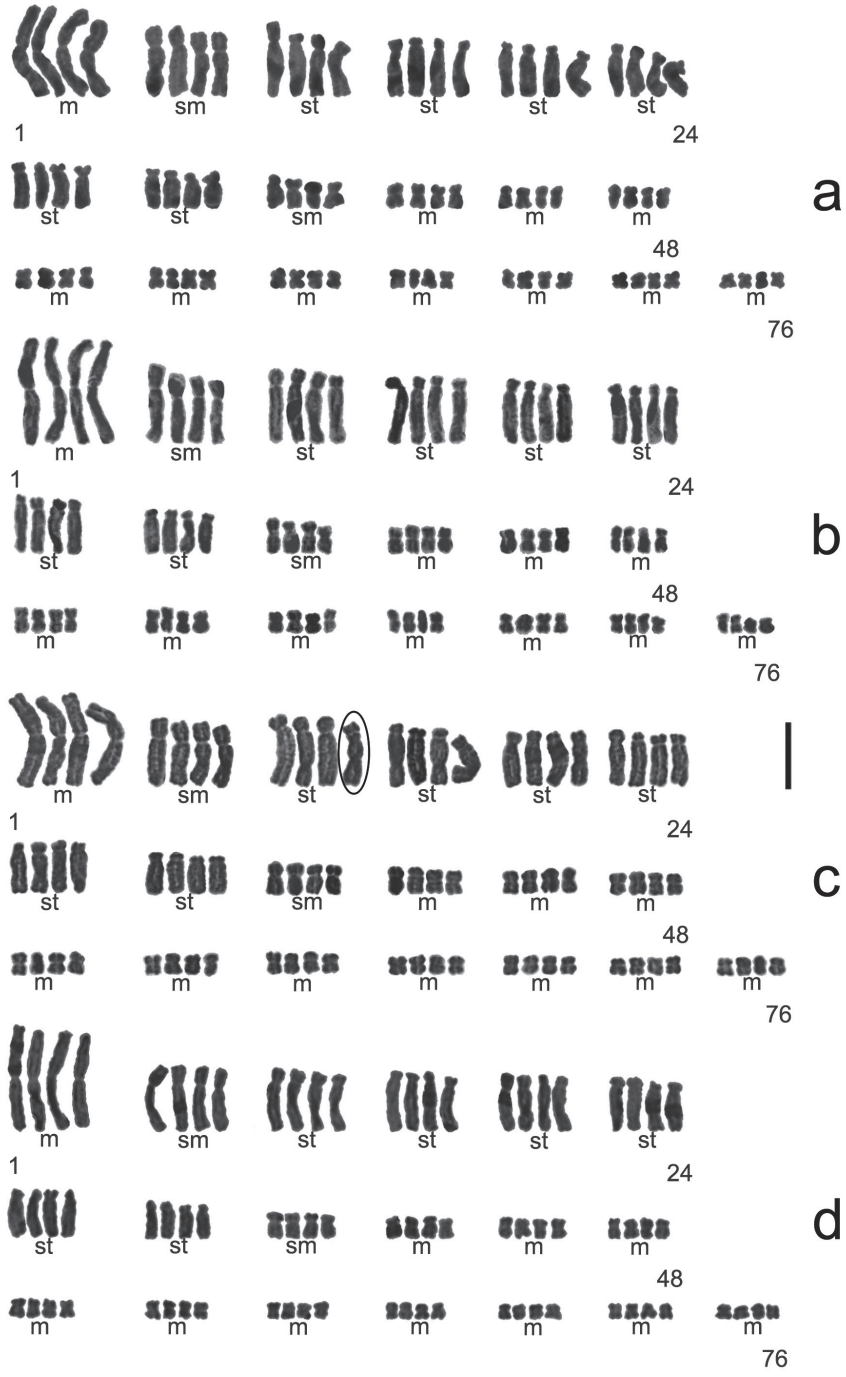


Figure 3. Karyotype of *Aspidistra subrotata* of 2n = 76, formulated as 2n = 44 m + 8 sm + 24 st. **a** Jilong population **b** 5th boundary marker population **c** Nonggang National Nature Reserve population, and the empty ellipse shows the presence of the secondary constriction on the chromosome 12 **d** 4th boundary marker population. Bar = 10 μm.

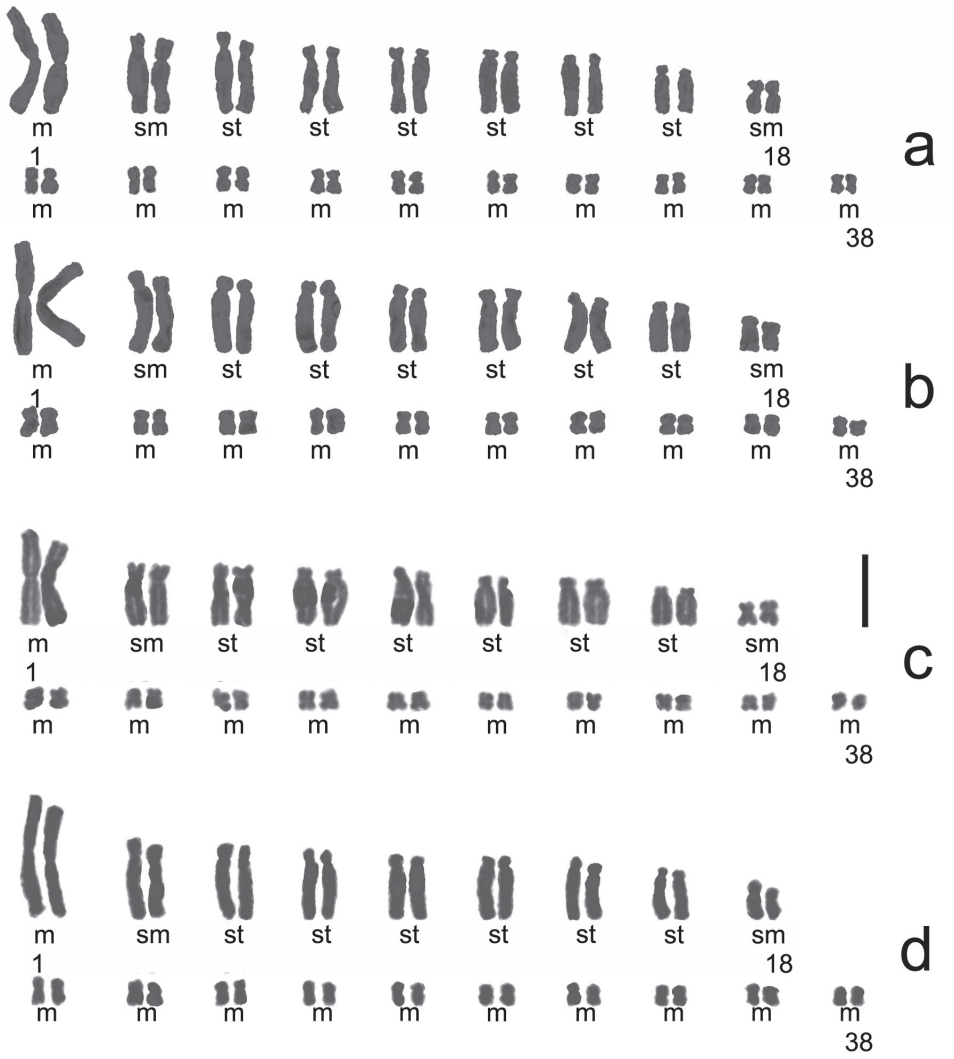


Figure 4. Karyotype of *Aspidistra subrotata* of 2n = 38, formulated as 2n = 22 m + 4 sm + 12 st. **a** Mt. Poman population **b, c** Mt. Daqing population **d** Mt. Bavi population. Bar = 10 μm.

External morphology

Based on the observation in the field, flowers of *Aspidistra subrotata* were commonly found with the perigone lobes red–purple and the stigma white with more or less small red dots on the upper surface (Figure 5). However, some variation occurs in its leaf shape, from sublinear or narrowly elliptical to ovate–lanceolate, from without blotches to with paler, often white blotches, from without raised secondary veins to with raised secondary veins in either the tetraploid population of fourth boundary marker (Figure 6) or the diploid population of Mt. Daqing (Figure 7). There were

Table 2. Karyomorphological characters in *Aspidistra subrotata*. ALC = average length of chromosome, NCC = number of cells calculated, A_1 = karyotype intrachromosomal asymmetry index and A_2 = karyotype interchromosomal asymmetry index.

Sample	Literature	Karyotype formula	ALC(μm)	NCC	A_1	A_2	Figure
JL	this study	2n = 76 = 44 m + 8 sm + 24 st	4.87 ± 0.03	2	0.38 ± 0.01	0.58 ± 0.01	1a, 3a
5M	this study	2n = 76 = 44 m + 8sm + 24 st	4.94 ± 0.51	3	0.35 ± 0.01	0.56 ± 0.01	1b, 3b
NG	this study	2n = 76 = 44m + 8 sm + 24 st	5.7 ± 0.30	3	0.38 ± 0.01	0.55 ± 0.01	1c, 3c
NN	this study	2n = 76 = 44 m + 8 sm + 24 st	5.56	1	0.36	0.60	
SK	this study	2n = 76 = 44 m + 8 sm + 24 st	5.06 ± 0.20	2	0.35 ± 0.00	0.58 ± 0.02	
4M1	this study	2n = 76 = 44 m + 8 sm + 24 st	4.88 ± 0.47	5	0.35 ± 0.02	0.60 ± 0.01	1d, 3d
4M2	this study	2n = 76 = 44 m + 8 sm + 24 st	4.98	1	0.37	0.64	
4M3	this study	2n = 76 = 44 m + 8 sm + 24 st	4.71 ± 0.19	5	0.36 ± 0.01	0.62 ± 0.00	
4M4	this study	2n = 76 = 44 m + 8 sm + 24 st	4.88 ± 0.09	2	0.37 ± 0.01	0.64 ± 0.02	
PM	this study	2n = 38 = 22 m + 4 sm + 12 st	5.71 ± 0.28	4	0.38 ± 0.01	0.60 ± 0.02	2a, 4a
DQ1	this study	2n = 38 = 22 m + 4 sm + 12 st	5.26 ± 0.32	4	0.37 ± 0.01	0.60 ± 0.03	2b, 4b
DQ2	this study	2n = 38 = 22 m + 4 sm + 12 st	4.47 ± 0.17	2	0.33 ± 0.01	0.56 ± 0.00	2c, 4c
BV	this study	2n = 38 = 22 m + 4 sm + 12 st	5.44 ± 0.17	2	0.37 ± 0.01	0.59 ± 0.00	2d, 4d
	Huang et al.1997	2n = 38 = 22 m + 2 sm + 14 st (2sat)	5.26	1	0.41	0.60	
-	Wang et al. 2000	2n = 38 = 22 m + 6 sm (2sat) + 10 st	5.29	1	0.35	0.59	

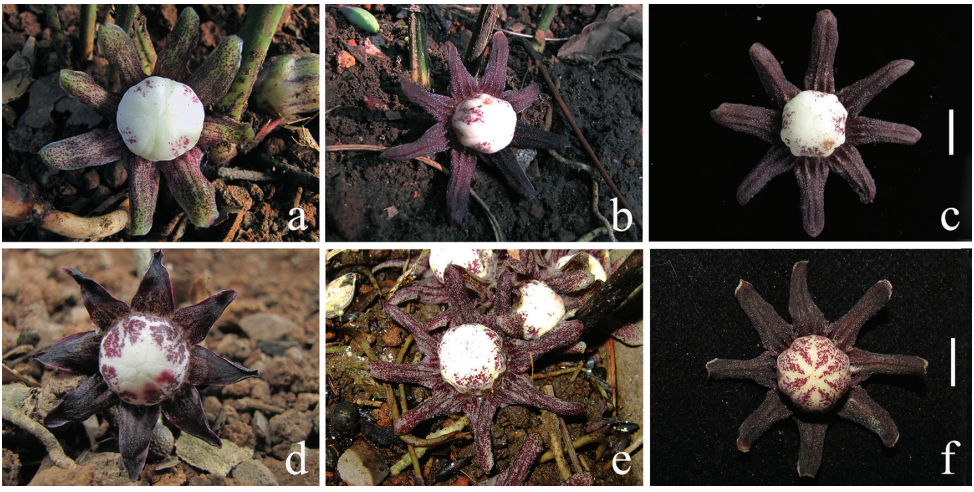


Figure 5. Flower morphology *Aspidistra subrotata*. **a–c** flowers of diploid plants from **a** Nonggang population **b** Nanning population **c** 4th boundary marker population **d–f** flowers of tetraploid plants from **d, e** Mt. Bavi population **f** Mt. Daqing population. Bar = 1 cm.

tiny differences between tetraploids and diploids; the former may be distinguished from the latter by its rigid petiole as well as thick and deep green lamina as well as its ploidy level of chromosome.

Figure 6. Leaf morphology of tetraploid plants of *Aspidistra subrotata.* from 4th boundary marker population. **a–c** sublinear leaves with a. smooth face **b** blotches **c** blotches and raised secondary veins **d–e** narrowly lanceolate leaves with **d** smooth face **e** blotches **f–h** ovate–lanceolate leaves with **f** smooth face **g** blotches **h** blotches and raised secondary veins **i** plants with ovate–lanceolate leaves and ones with sublinear leaves grow together.

Discussion and conclusion

Ploidy and geographic range

The karyotypes of nine samples of six populations of *Aspidistra subrotata* are described here as tetraploid for the first time, with a chromosome number of 2n = 76 and the karyotype formulated as 2n = 44 m + 8 sm + 24 st. Among them, eight samples of five populations are all from Longzhou and are located not far away from each other (Table 1, Map 1). The secondary constriction is occasionally observed (Figure 1c and 3c). The other tetraploid sample is from the plants cultivated in Guangxi Medicine Botany Garden and is possibly the clone plants of typical *A. subrotata*. Unfortunately, the type specimen of *A. subrotata* have not been able to be checked until now. Our results may offer a hint that the plants cultivated in Guangxi Medicine Botany Garden were collected from the above county on the basis that the tetraploids so far have not been observed in any other place, and infer that the type of *A. subrotata* may be tetraploid from Longzhou. Another four samples of three populations from Mt. Poman, Mt. Daqing, and Mt. Bavi were diploid, with a chromosome number of 2n = 38 and the karyotype

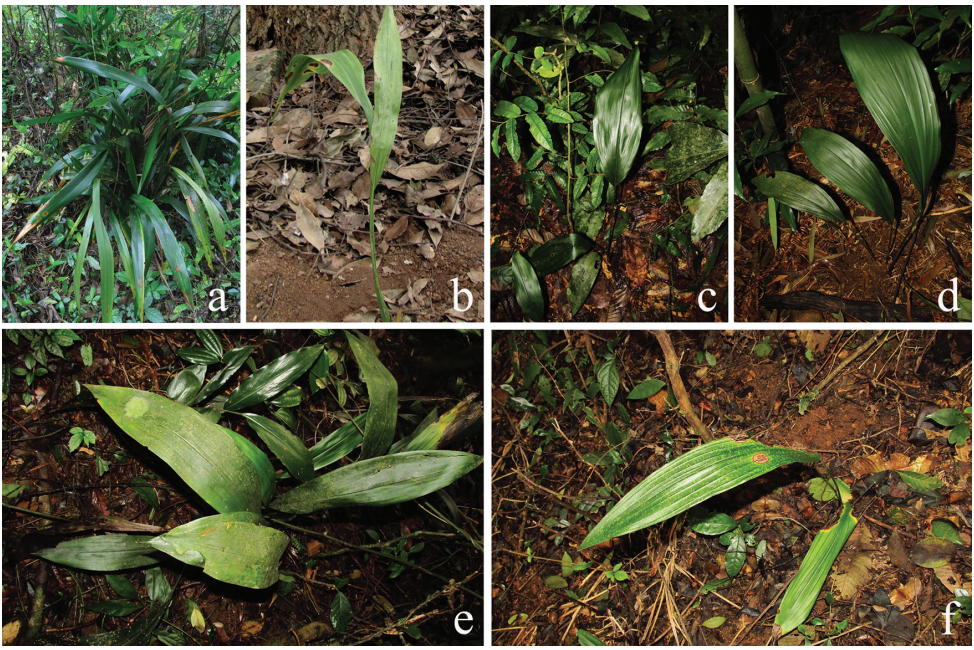
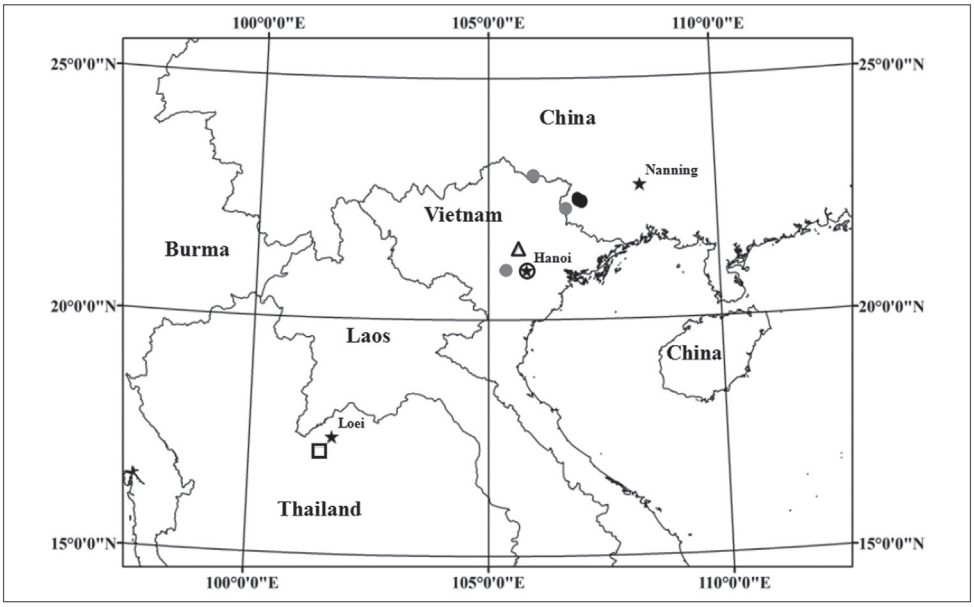


Figure 7. Leaf morphology of diploid plants of *Aspidistra subrotata* from Mt. Daqing population. **a–c** leaves with smooth face. a. sublinear leaves **b** lanceolate leaves **c** ovate–lanceolate leaves **d, e** ovate–lanceolate leaves with inconspicuously raised secondary veins **f** lanceolate leaves with blotches and raised secondary veins.



Map 1. Distribution of *Aspidistra subrotata.* Black circle represents the tetraploid population; gray circle represents the diploid population; empty triangle represents typical *A. subrotata* var. *crassinervis*; empty square represents typical *A. subrotata* var. *angustifolia*.

formulated as 2n = 22 m + 4 sm + 12 st. A difference from the previous report on the diploid states of *A. subrotata* is that no satellite was observed in the chromosomes of IX pair as sm in this study (Wang et al. 2000, Huang et al. (1997). Besides, the pair IX was reported as st in Huang et al. (1997), while one more pair of sm chromosomes occurs in Wang et al. (2000), compared with our results. The material of diploid specimens reported previously is all from cultivated plants and information about their wild population is unknown. Here, three localities are confirmed for the first time where diploid populations are distributed.

There is a long-standing debate on the ecological success of polyploids relative to diploids. Although some studies suggest that polyploids generally have larger ranges (Stebbins and Dawe 1987, Petit and Thompson 1999, Oberprieler et al. 2012), the present studies prefer to support that the correlation between ploidy and range or ecological attributes is inconsistent and appears to be taxon-specific (Martin and Husband 2009, Godsoe et al. 2013, Harbert et al. 2014). In our studies, it seems that the diploids occupy broader geographical and environmental niche spaces than the tetraploids (Map 1), which maybe offer an interesting example with which to explore the formation and the evolutionary dynamics of a new natural polyploidy complex from the limestone area of the tropical regions.

Ploidy and external morphology

Our studies show that leaves of *Aspidistra subrotata* varied in leaf shape, color pattern, and venation in either the tetraploid population of the fourth boundary marker (Figure 6) or the diploid population of Mt. Daqing (Figure 7); the same case occurs in Phu Luang WS, north-eastern Thailand (Phonsena and De Wilde 2010), but unfortunately, the chromosome number of Thai material is unknown. As many polyploid plants are reported to be similar to their diploid parents and hence morphologically cryptic (Tate et al. 2005, Reis et al. 2014, Azizi et al. 2016), there is also tiny difference between diploids and tetraploids of *A. subrotata*. The tetraploid plants may be distinguished from the diploid plants by their rigid petioles as well as thick and deep green lamina. This type of leaf also occurs in the other three polyploid species in the genus *Aspidistra*, i.e. *A. xilinensis*, *A. cruciformis* and *A. sutepensis*, which seemly supports the notion that the polyploidy can exploit newly available habitats (Brochmann et al. 2004).

Although the leaf-shape of *Aspidistra subrotata* varies quantitatively between and within diploid or tetraploid population(s), no obvious discontinuity has been observed. It seems unreasonable to divide it into three varieties on the basis of leaf-shape. According to the independent distribution and external morphology in relation to the ploidal levels of chromosome of *A. subrotata*, two subspecies may be recognized; however, the taxonomic treatment of *A. subrotata* has not been properly dealt with until the types of all three varieties are checked and confirmed, with additional samples, geographical locations of collections, and molecular data analysis together. We hope this study will be helpful not only to better understand the origin and evolution of the species and the

genus but also shed some light on the formation and the evolutionary dynamics of a new natural polyploidy complex in the limestone areas of the tropical regions.

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Cytogenetic characterization of *Hoplias malabaricus* (Bloch, 1794) from the Ctalamochita River (Córdoba, Argentina): first evidence for southernmost populations of this species complex and comments on its biogeography

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Abstract

Hoplias malabaricus (Bloch, 1794), a predatory freshwater fish with a wide distribution throughout South America, represents a species complex with seven well characterized karyomorphs at the cytogenetic level. Although this species has been extensively studied in several Brazilian basins, data are still scarce for hydrographic systems from other South American countries. This study aims to characterize cytogenetically the *Hoplias malabaricus* populations from the Argentinean Central Region, close to the southernmost distribution of this species complex. A total of 32 specimens from the Ctalamochita River, a tributary of Lower Paraná Basin located in the province of Córdoba, were analyzed using cytogenetic techniques (Giemsa staining, C- and Ag-NOR banding and fluorescent in situ hybridization with 18S rDNA). All the specimens showed diploid number 2n=42, chromosomic formula 22m + 20sm and absence of sexual chromo-

somes. Thus, the analyzed populations belong to the karyomorph named A. These populations showed a remarkable degree of divergence in their cytogenetic traits such as karyotypic formula, C-banding, NORs and 18S rDNA patterns for *Hoplias malabaricus* from other populations bearing the same karyomorph in the Middle and Upper Paraná Basin. These findings are consistent with molecular data from a recent study (where specimens collected in the present work were included), which indicate a closer phylogenetic relationship of *Hoplias malabaricus* populations from the Ctalamochita River with those from the Uruguay basin and the coastal regions of South Brazil than with populations from the Middle and Upper Paraná Basin. Overall, these pieces of evidence highlight the distinctive features of *Hoplias malabaricus* from the Ctalamochita River, and also reveal a complex history of dispersion of these populations. The present work is the first to provide cytogenetic information and include some phylogeographic aspects of *Hoplias malabaricus* populations living in close proximity to the southernmost extreme of its distribution area. Therefore, this study expands significantly upon the previously known geographical coverage for karyomorph A and contributes to a better understanding of the karyotypic diversification within this species complex.

Keywords
Biogeography, cytogenetics, *Hoplias malabaricus*, karyomorph A

Introduction

Hoplias malabaricus (Bloch, 1794) is a predatory freshwater fish that belongs to the order Characiformes and family Erythrinidae. It is a widespread species inhabiting 44 out of 52 South American ecoregions proposed by Abell et al. (2008), in most of the hydrographic basins of South America (Oyakawa 2003).

Classically, *Hoplias malabaricus* specimens have been recognized by a few morphological features, such as convergence of dentary bone toward the symphysis, resulting in a "V" shape pattern and tooth plates in the tongue. Although *Hoplias malabaricus* has been considered as a single biological species in reference to its morphological traits, every sampled population shows a bimodal pattern of variation: they are either 2n=42 or 2n=40 with slight modifications. Several cytogenetic studies indicate that *Hoplias malabaricus* represents a species complex, with seven well characterized karyomorphs (or karyotypic variants, also known as cytotypes) nominated with letters A to G, which differ with regards to their diploid numbers, chromosome morphology and the presence of sex chromosome systems (Bertollo et al. 1983, 1997a, 1997b, Dergam and Bertollo 1990, Bertollo and Mestriner 1998, Born and Bertollo 2000, 2001). Some karyomorphs as A, C and F have a wide geographic distribution throughout South America, whereas others are either endemic or restricted to specific drainages in Brazil (Bertollo et al. 2000, Da Rosa et al. 2014). In some cases, two and even more karyomorphs have been found coexisting in sympatric conditions without detection of hybrid forms (Lopes and Fenocchio 1994, Scavonne et al. 1994, Lopes et al. 1998, Lemos et al. 2002, Pazza and Júlio 2003, Born and Bertollo 2006, Da Rosa et al. 2009a, 2009b, 2010).

Frequently, cytogenetic evidence is also supported by, and consistent with, molecular data obtained from analysis of nuclear and mitochondrial DNA sequences

(nuDNA and mtDNA), which reveal a marked divergence and genetic structuration between karyomorphs, suggesting reproductive isolation between the karyomorphs. Additionally, combination of cytogenetic and molecular approaches together with geological information (i.e., estimate data of ancient episodes such as stream piracy between rivers, orogenic activity, etc., involved in vicariance events) has been particularly useful in the study of dispersal events and phylogenetic relationships (Dergam et al. 1998, 2002, Santos et al. 2009, Jacobina et al. 2011, Pereira et al. 2013).

All the aforementioned evidence strongly indicates the existence of a complex of cryptic species grouped into the typical *Hoplias malabaricus* morphotype (Bertollo et al. 1986, 2000), and also shows the relevance of these organisms as an exceptional model for studying karyotypic evolution and biogeography.

It is noteworthy to mention that, although the lack of detailed morphometric information has led to group different species, well defined at the genetic level, in a single one on the basis of a common morphology, some karyomorphs of *H. malabaricus* seem to exhibit subtle morphological differences. For example, in a recent study focused on analysis of morphometric parameters (Azpelicueta et al. 2015) a new species of the genus *Hoplias* has been described. This species inhabiting the lower Paraná River (Misiones, Argentina) has been named *Hoplias mbigua*. However, has been suggested that *H. mbigua* would actually be the karyomorph C of *Hoplias malabaricus* previously described in that region of Paraná Basin.

Despite its wide geographic distribution, most of karyotypic and molecular research in *H. malabaricus* populations has been carried out in Brazil and the few studies conducted in Argentina have been restricted to Mesopotamic region (Lopez and Fenocchio 1994, Bertollo 1996, Dergam et al. 1998, Lopes et al. 1998). In this context, the central region of Argentina, also called Pampa Plain, has never been examined before. Taking into consideration that the ichthyofauna of the Pampa Plain presents the southernmost distribution range of many Neotropical species, increasing the geographical coverage may lead to a better understanding of karyotypic diversification and the historical biogeography of *H. malabaricus* and the regional fauna of the Pampa Plain.

The Pampa Plain is a vast region characterized by gentle slopes, occasionally interrupted by low geomorphological reliefs. The hydrographic systems developed in this area form both endorheic and exorheic basins. Among the latter, the Ctalamochita River (also known as Tercero River) is one of the most important hydrographical systems. This river, located in the province of Córdoba, is a tributary of the Lower Paraná River Basin. Along the Ctalamochita River, there are five reservoirs (artificial lakes), the two most important of them called Embalse Río Tercero (built in 1936) and Piedras Moras (built in 1978). The fish assemblage in the Ctalamochita River is characterized by the presence of members of the families Characidae, Pimelodidae and Erythrinidae, among others. Therefore, *H. malabaricus* is an indigenous species (Haro et al. 1996, Bistoni and Hued 2002).

The aim of the present study was characterizing at the cytogenetic level *Hoplias malabaricus* populations from headwater of the Ctalamochita River using standard and fluorescent in situ hybridization techniques.

Material and methods

Thirty two specimens of *Hoplias malabaricus* consisting of 8 males, 10 females and 14 specimens of undetermined sex were collected in the headwaters of the Ctalamochita River, Córdoba Province, Argentina (Fig. 1a) during December 2004 to March 2005. In order to study the Ctalamochita River headwaters and some of its tributaries, four different sampling sites in the two main reservoirs were chosen (Fig. 1a, black dots, listed from left to right):

- Embalse Río Tercero reservoir and Rio Grande stream
1 (32°13'S, 64°32'W): 4 specimens (2 males, 2 females)
2 (32°13'S, 64°25'W): 14 specimens (14 juvenile stages, undetermined sex)

- Piedras Moras reservoir and Soconcho stream
3 (32°11'S, 64°19'W): 3 specimens (1 male, 2 females)
4 (32°11'S, 64°18'W): 11 specimens (5 males, 6 females)

Specimens (Fig. 1b, right) were identified according to Oyakawa (1990). In all cases the rami of the dentary bone were convergent toward the symphysis, resulting in a ‘‘V’’ shape pattern (Fig. 1b, left).

Mitotic chromosome preparations were obtained according to the technique described by Foresti et al. (1993). The method described by Kligerman and Bloom (1977) was used for the analysis of meiotic chromosomes from male gonads.

Nucleolus organizer regions (Ag-NORs) were visualized with silver staining following Howell and Black (1980). Assays conducted to reveal the pattern of constitutive heterochromatin (C banding) were carried out according to Sumner (1972).

Fluorescent *in situ* hybridization (FISH) experiments were performed using biotinylated 18S rDNA probes (1700 pb long fragments) obtained from the nuclear DNA of the fish *Oreochromis niloticus* (Linnaeus, 1758) labeled with biotin-14-dATP by nick translation (Gibco cat N°18247-015), according to the manufacturer’s instructions. The hybridization technique, post-hybridization washes and visualization were carried out following Swarça et al. (2001).

The preparations were analyzed with an Olympus BX50 microscope, and the best metaphases were captured with a SONY camera, model Exware HAD coupled to the microscope. The FISH slides were observed and the images acquired with a Leica DM 4500 microscope equipped with a DFC 300F9 camera and Leica IM50 4.0 software.

More than thirty metaphases from each specimen were analyzed and the best of them were used to make karyotypes. The chromosomes were arranged in groups classified according to their arm ratios as metacentrics and submetacentrics (Levan et al. 1964, Guerra 1986).

Tissue samples from several specimens were collected for further molecular analysis (study of sequences of mitochondrial and nuclear DNA) and were deposited in the Laboratory of Molecular Systematic Beagle, Universidade Federal of Viçosa, Minas Gerais, Brazil.

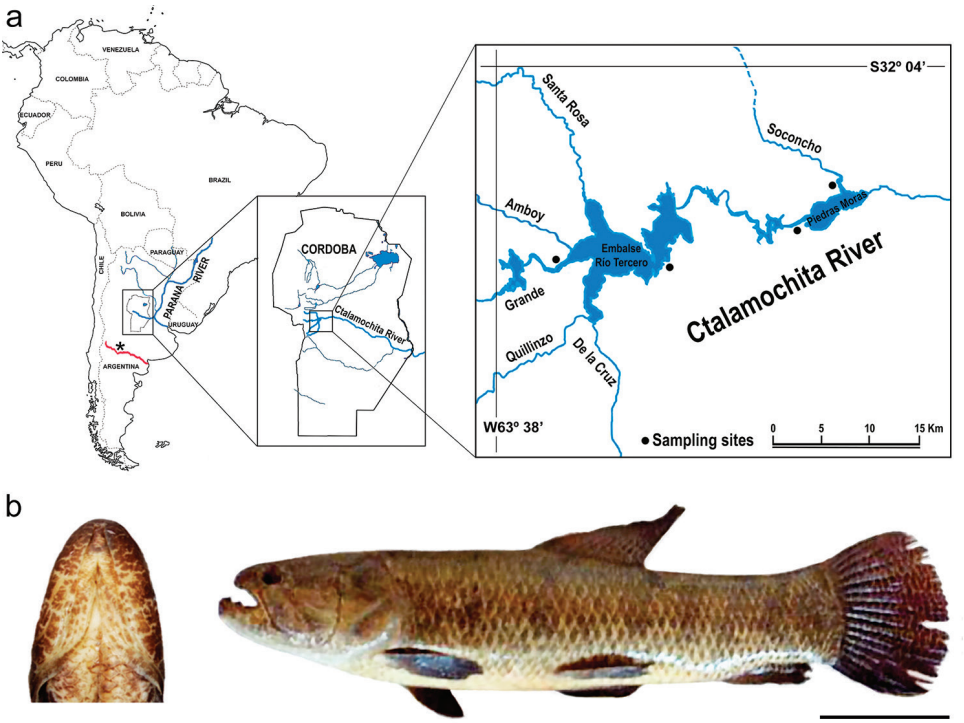

Figure 1. a Ctalamochita River location in Province of Córdoba, Argentina (left and middle images). Asterisk (*) indicate location of the Colorado River in Argentina (in red), the limit of distribution of *Hoplias malabaricus* and most of Neotropical fishes. Distribution of sampling sites is indicated along headwater basin (right image, black dots) **b** Representative specimen of *Hoplias malabaricus* caught in the Ctalamochita River and analyzed in the present study (right picture). All specimens showed the typical morphological feature identifying *Hoplias malabaricus*, the V-shaped gular region (left picture). Bar = 10 cm.

The entire collection was split in two groups, which were deposited at the Museum of Zoology, Universidad Nacional of Córdoba, Argentina (specimens numbered from 1 to 18) and at Fish Cytogenetics Laboratory, Universidad Nacional of Misiones, Argentina (specimens numbered from 19 to 32).

Results

All the specimens of *Hoplias malabaricus* collected from the headwater region of the Ctalamochita River exhibited a diploid number of 2n=42 chromosomes. The karyotype was composed of 22m + 20sm, with a NF=84 (Fig. 2a, b and d).

Analysis of meiotic preparations allowed the identification of 21 bivalents without atypical pairing among them, supporting lack of chromosomal differentiation between sexes (Fig. 2c).

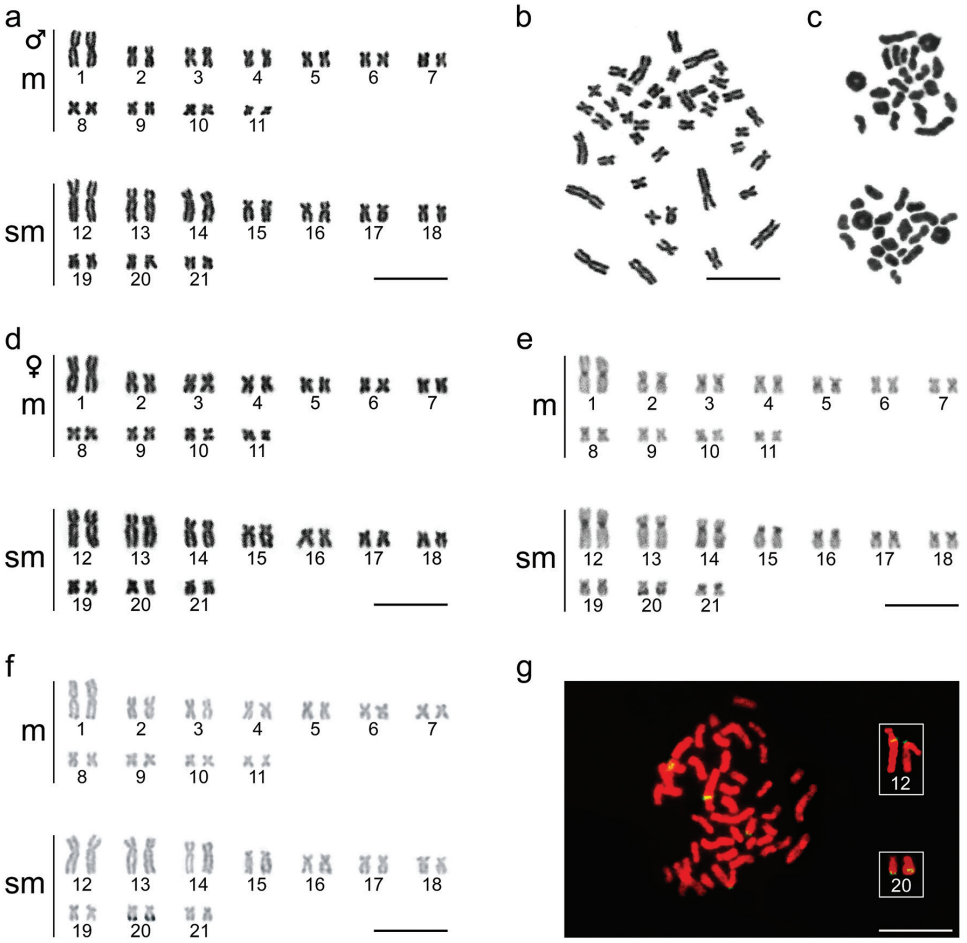

Figure 2. a Male *Hoplias malabaricus* karyotype with conventional Giemsa staining (karyomorph A) **b** Metaphase corresponding to karyotype showed in (**a**) **c** Male meiotic metaphase showing formation of 21 bivalents **d** Female *Hoplias malabaricus* karyotype (karyomorph A) **e** C-banding karyotype exhibiting centromeric staining in most of chromosomes and telomeric signal in pairs number 14 and 20 **f** Ag-NOR banding karyotype displays telomeric signal in chromosome pair 20 **g** FISH with 18S rDNA probes labeling two chromosomic pairs, numbers 12 and 20 (boxed). Bar = 5 μm.

Patterns of heterochromatin distribution revealed by C-banding were mostly associated with centromeric regions of all chromosomes of the complement, as well as in the telomeric region of some pairs (Fig. 2e). Metacentric pair 1 and submetacentric pair 12 showed a conspicuous band in centromeric/pericentromeric position. Additionally, submetacentric pairs 14 and 20 exhibited big heterochromatin blocks in telomeric positions in the long arms, but in the former case the signal was weak and diffuse.

Nucleolus Organizer Regions identified by impregnation by silver nitrate (Ag-NORs) were located in telomeric position on the long arm of a small chromosome

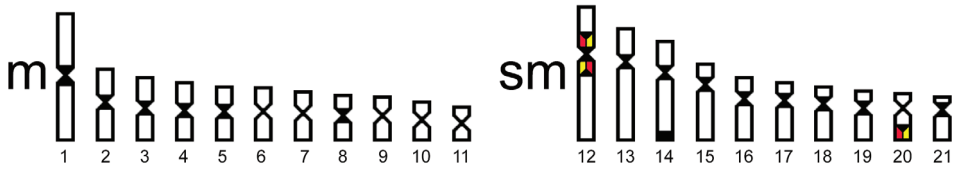

Figure 3. Idiogram referring to the *Hoplias malabaricus* populations (karyomorph A) from the Ctalamochita River, highlighting the chromosome markers. Black: C-positive heterochromatin; red: 18S rDNA sites; yellow: nucleolar organizer regions (NORs). Putative pericentromeric NOR in chromosome pair 12 was also included.

pair number 20 (Fig. 2f). In some cases, weak NOR-positive bands were observed in pericentromeric position of submetacentric pair number 12 (data not shown).

These data coincided with results from FISH technique with 18S rDNA probes, which revealed two positive submetacentric pairs: pair number 12, clearly identified due to its size and exhibiting probe signal in its pericentromeric position, and pair number 20, showing probe signal in its long arm (Fig. 2g).

It is important to note that, concerning all chromosome markers and macrostructural features analyzed in this study, no significant differences were found between populations of Embalse Río Tercero and Piedras Moras reservoir. Thus, it seems these populations are relatively homogeneous (Fig. 3).

Discussion

Hoplias malabaricus populations from the headwater of the Ctalamochita River (Córdoba Province, Argentina) were characterized at the cytogenetic level, providing the first data for this hydrographic system and also for the Argentinean Central Region. These populations exhibited karyotypes with 2n=42 chromosomes composed of 11 metacentric and 10 submetacentric pairs, without apparent heteromorphic sex chromosomes. These results qualify them as belonging to karyomorph A previously described by Bertollo et al. (2000).

Despite sharing some chromosomal similarities with other karyomorph A-bearing populations occurring in the Upper and Middle regions of Paraná Basin, cytogenetic traits of the Ctalamochita population such as karyotypic formula, C-banding, NORs and 18S rDNA patterns were significantly divergent (Figure 3). For example, in the case of *H. malabaricus* populations from Pântano Stream and Cuiabá River described by Cioffi et al. (2009), they exhibited karyotypic formulae 22m + 20sm and multiple and bitelomeric NORs and 18S rDNA sites, located on chromosome pairs 5, 16, 18 and 21. Additionally, in all *H. malabaricus* populations from Tibagi, Ivaí and Iguaçu Rivers described by Vicari et al. (2005) their karyotypes were composed of 24m + 18sm with multiple and bitelomeric NORs and 18S rDNA sites, located on chromosome pairs 10, 16 and 21. In a recent work, Gemi et al. (2014) analyzed *H.*

malabaricus populations from Iguaçu River, whose karyotypes were composed of 24m + 18sm with multiple and bitelomeric NORs located on chromosome pairs 7 and 10. Ribosomal 18S rDNA probes marked chromosome pairs 4, 7, 10, 13, 16, 20 and 21.

Fixation of chromosome rearrangements within major karyomorphs is not completely surprising taking into consideration some biological aspects of *Hoplias malabaricus*, as its wide distribution in most of South America basins, sedentary habits and adaptations to live in small and isolated populations. These features would favor a random fixation of chromosome rearrangements and, thus, certain degree of intra-karyomorph variation would be expected (Sites and Moritz 1987). Indeed, extensive karyotypic variation in karyomorph A has been reported for populations living in adjacent basins (Born and Bertollo 2001, Vicari et al. 2005, Cioffi et al. 2009, Blanco et al. 2010, Da Rosa et al. 2014). In this regards, populations from the Ctalamochita River seem to follow the rule since they display a unique character combination, diverging from other populations of the A karyomorph inhabiting the Paraná Basin and other hydrographical systems.

With respect to distinctive cytogenetic features of these populations, it is important to highlight relevant traits for submetacentric chromosome pairs 12 and 20. In both cases the presence of heterochromatic bands coincident with 18S rDNA sites was demonstrated; in chromosome pair 12 located in pericentromeric position and in chromosome pair 20 in the long arm. With regards to Ag-NORs only chromosome pair 20 had a clear, positive staining for silver nitrate impregnation, showing a conspicuous positive NOR in telomeric position of the long arm. As was mentioned previously, in some cases (approximately 5%) weak NOR-positive staining was observed in pericentromeric position of submetacentric pair number 12, but it was not possible conclude if this variability was caused by limited accessibility of silver nitrate to highly compacted heterochromatic regions or by the occurrence of structural Ag-NOR polymorphisms (Born and Bertollo 2000, Vicari et al. 2005).

Overlapping of C- and Ag-NOR banding suggests the presence of heterochromatic blocks interspersed with ribosomal cistrons in the same region, as previously reported for several chromosomic pairs in karyomorph A (Vicari et al. 2003, 2005, Blanco et al. 2010, Cioffi et al. 2010). A possible role for these NOR / heterochromatin associations in the evolution of sex chromosomes in other *H. malabaricus* karyomorphs has been advanced by Vicari et al. (2003). In fact, recent data indicate that the differentiation of sexual chromosomal pair in karyomorph B, which is clearly derived from the sympatric karyomorph A, could have occurred by accumulation of heterochromatin and 18S rDNA cistrons in the long arms of the submetacentric chromosome pair 21 of some Brazilian populations belonging to karyomorph A (Cioffi et al. 2010, 2011).

These high levels of chromosomal differentiation within the 2n=42 A karyomorphs proposed by Bertollo et al. (2000) has received recent molecular support in a supertree obtained with the ATPAse6 gene (in which specimens collected in the present work were included), where the Ctalamochita populations seem phylogenetically most closely related to 2n=42 A karyomorphs from left bank tributaries of the Uruguay

River and populations from the headwaters of the Uruguay River, and relatively closely to other coastal populations from South of Brazil. In contrast, 2n=42 A karyomorphs of the Upper and Middle Paraná are included in other, distant haplogroups (Santos 2013). Additionally, according to this analysis, it is possible to postulate a direction of dispersal originating from southern coastal basins of Brazil to the Uruguay River and the Ctalamochita hydrographic system.

Although the spread of karyomorph A populations through interaction between coastal drainages and dispersal into continental basins has been previously studied and demonstrated only for restricted Brazilian coastal regions (Dergam et al. 2002, Santos et al. 2009), a possible scenario for larger scale dispersion events involving other, unrelated obligatory freshwater fishes is plausible. In fact, a recent phylogeographic study including four genera of the family Loricariidae suggested that current geographical distribution of these genera is supported by dispersion events from the southern coastal rivers of Brazil to the Uruguay River and from the Uruguay River to the De la Plata River, through stream piracy from coastal Brazilian regions and drainage rearrangements of Paraná basin (Cardoso 2013).

Additionally, fluctuations of sea level during glacial-interglacial cycles and temporary connections between adjacent basins would also have had an important role in spreading karyomorph A *Hoplias malabaricus* populations through coastal regions (Dergam et al. 2002, Jacobina et al. 2011, Pereira et al. 2013). Indeed, the current distribution of several genera of freshwater fishes such as *Australoheros*, *Cnesterodon*, *Jenynsia* and *Corydoras* in Pampa Plain southernmost areas seems to have been strongly influenced by interconnection of coastal rivers during low sea level periods (Bruno et al. 2011, 2016). Emergence of a coastal plain connecting paleo-basins across Brazil, Uruguay and Argentina coastal regions during these periods would have facilitated spread of freshwater species, occurring several times during at least the last 1-2 millions of years (Ponce et al. 2011).

While glacial – interglacial cycles may have promoted dispersal of *H. malabaricus* coastal populations, at the same time they would also have had profound effects on the Pampa Plain ecosystems. The dominant climate characteristics of the Pampa Plain region during these glacial periods were: cold with frequent snowing and windy conditions, with scarce precipitations and frequent decreases of basin water flow. Thus, cycles of expansion of steppe-like climate over the Pampa Plain region occurred during glacial epochs. Constant propagule pressure from the Brazilian region favored exchange and turnover of species in these regions after glacial periods, and this fauna advance over Pampa Plain region would have taken place approximately 15 times during the last one million years (Rabassa et al. 2005).

The chronological frame of these events fits with the estimated dispersal time of the haplogroup including *Hoplias malabaricus* populations from Ctalamochita and Uruguay Rivers and its tributaries (Santos 2013), thus this climate component of glacial-interglacial cycles must have been a powerful modelling force causing a profound impact over the present geographic distribution of *Hoplias malabaricus* populations living in the Argentinean Central Region.

In light of the previously mentioned data, it is evident that the current geographic distribution pattern of *Hoplias malabaricus* in the Ctalamochita River, and probably most of the ichthyofauna in the Pampa Plain region, is a result of the intricate combination of climatic and geomorphological factors. Overall, cytogenetic and molecular evidence presented in this study highlights the distinctive chromosomal features of *Hoplias malabaricus* from the Ctalamochita River, and also reveals a complex history of dispersal of these populations.

Conclusion

Taking into consideration the new findings described previously, the present work is the first to provide cytogenetic information and also including some phylogeographic aspects of *Hoplias malabaricus* populations living in close proximity to the southernmost extreme of its distribution area, in Argentinean Central Region. Therefore, this study expands significantly upon the previously known geographical coverage for karyomorph A and contributes to a better understanding of the karyotypic diversification within this species complex.

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Genomic characterisation of *Arachis porphyrocalyx* (Valls & C.E. Simpson, 2005) (Leguminosae): multiple origin of *Arachis* species with x = 9

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Abstract

The genus *Arachis* Linnaeus, 1753 comprises four species with $x = 9$, three belong to the section Arachis: *Arachis praecox* (Krapov. W.C. Greg. & Valls, 1994), *Arachis palustris* (Krapov. W.C. Greg. & Valls, 1994) and *Arachis decora* (Krapov. W.C. Greg. & Valls, 1994) and only one belongs to the section Erectoides: *Arachis porphyrocalyx* (Valls & C.E. Simpson, 2005). Recently, the $x = 9$ species of section Arachis have been assigned to G genome, the latest described so far. The genomic relationship of *A. porphyrocalyx* with these species is controversial. In the present work, we carried out a karyotypic characterisation of *A. porphyrocalyx* to evaluate its genomic structure and analyse the origin of all $x = 9$ *Arachis* species. *Arachis porphyrocalyx* showed a karyotype formula of 14m+4st, one pair of A chromosomes, satellited chromosomes type 8, one pair of 45S rDNA sites in the SAT chromosomes, one pair of 5S rDNA sites and pericentromeric C-DAPI+ bands in all chromosomes. Karyotype structure indicates that *A. porphyrocalyx* does not share the same genome type with the other three $x = 9$ species and neither with the remaining Erectoides species. Taking into account the geographic distribution, morphological and cytogenetic features, the origin of species with $x = 9$ of the genus *Arachis* cannot be unique; instead, they originated at least twice in the evolutionary history of the genus.

Keywords

Arachis, chromosomes, chromosome evolution, genetic resources

Introduction

The genus *Arachis* Linnaeus, 1753 (Leguminosae) is native to South America and comprises 82 nominal species. These species were assembled into nine sections according to morphology, geographic distribution and cross compatibility (Krapovickas and Gregory 1994, Valls and Simpson 2005, Valls et al. 2013, Santana and Valls 2015). Most of species are diploid with x = 10 (2n = 20); a few (4 species) are diploid with x = 9 (2n = 18) and the rest (5 species) are tetraploid with x =10. Three of the diploid x = 9 species belong to the section Arachis: *A. praecox* (Krapov. W.C. Greg. & Valls, 1994) *A. palustris* (Krapov. W.C. Greg. & Valls, 1994) and *A. decora* (Krapov. W.C. Greg. & Valls, 1994) and one belongs to the section Erectoides: *A. porphyrocalyx* (Valls & C.E. Simpson, 2005).

Recently, a karyotype analysis of the three x = 9 species of the section Arachis revealed that they share a common karyotype structure (Silvestri et al. 2015). This is characterised by having all metacentric chromosomes except for one submetacentric pair; the lack of the small A chromosome pair; the presence of pericentromeric C-DAPI+ bands of the same brightness, position and size in all or almost all chromosome pairs; one pair of 45S rDNA sites on the unique pair of chromosomes with secondary constriction (SAT chromosomes) and one pair of 5S rDNA sites in the chromosome pair 6. This karyotype structure differs from those that characterise the other genomes of section Arachis (A, B, D, F and K genomes), whereby the three x = 9 species of the section Arachis have been assigned to a new genome, designated by the letter G (Silvestri et al. 2015).

The only known population of *A. porphyrocalyx* is located in the state of Minas Gerais (Brazil), near to the Rio Grande, 20 km southeast of Uberaba. Taking into account the geographic areas of the sections described by Krapovickas and Gregory (1994), this location is outside the geographic area of the section Erectoides (Fig. 1).

Arachis porphyrocalyx has thickened secondary roots, flowers mostly at the base of the lateral branches, and presents anthocyanin in the flower calyx, characteristics for which it has been included in the section Erectoides (Valls and Simpson 2005). Also, this species has a perennial life cycle. However, the authors clarify that the above-ground growth of this species resembles that of *Arachis villosa* (Benth, 1841) of the section Arachis (Valls and Simpson 2005). Several molecular analyses have been done to understand the genetic relationships between *Arachis* species but only one includes *A. porphyrocalyx* (Hoshino et al. 2006). This analysis of microsatellite markers placed this species within the cluster of species of section Erectoides but forming a subcluster together with *Arachis vallsii* (Krapov. & W.C. Gregory, 1994) of the section Arachis (Lavia 2001, Lavia et al. 2009), *Arachis subcoriacea* (Krapov. & W.C. Gregory, 1994) of the section Procumbentes and *Arachis dardani* (Krapov. & W.C. Gregory, 1994) of the section Heteranthae (Hoshino et al. 2006). Therefore, the taxonomic position of *A. porphyrocalyx* is not well established.

Moreover, the chromosome data on this species are very peculiar. Peñaloza and Valls (2005) noted that the karyotype of *A. porphyrocalyx* includes subtelocentric chro-

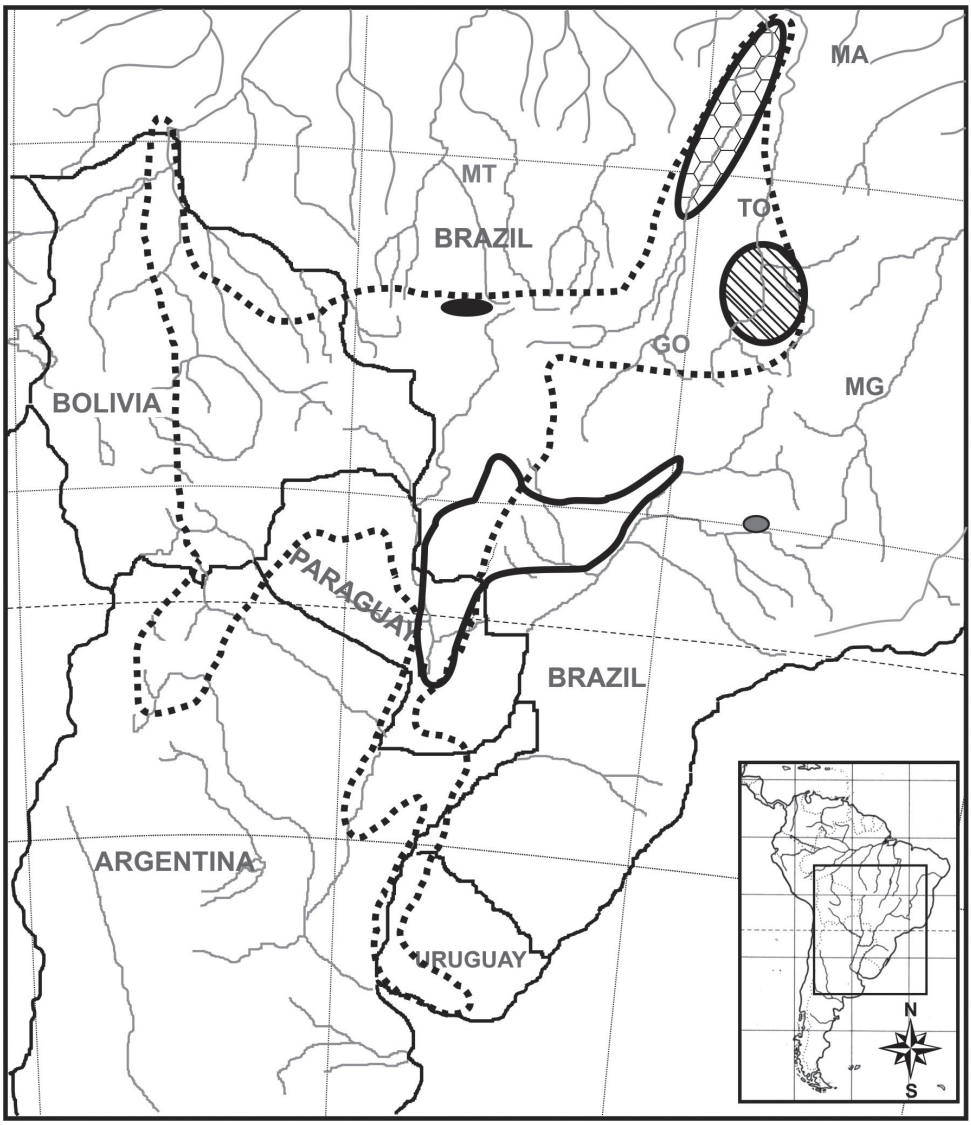

Figure 1. Geographic distribution of *Arachis* species with x=9. *A. decora* – diagonal pattern; *A. palustris* – octagon pattern; *A. praecox* – black field; *A. porphyrocalyx* – grey field. Dashed gray line indicates the distribution of section Arachis and solid gray line the distribution of Erectoides section.

mosomes, which is uncommon in the genus, and it has satellite chromosomes (SAT chromosomes) type 8 based on the appreciation of the metaphases. Futhermore, Lavia (2008) noted that a pair of chromosomes of this species behaves like the 'A' chromosomes, which is a peculiarity of the species with the A genome of section Arachis (Fernández and Krapovickas 1994, Lavia 1996, Robledo et al. 2009). Consequently, the possible presence of this chromosome pair in *A. porphyrocalyx* would be a quite rel-

evant difference from x = 9 species of the section Arachis and raises the question about the relationships of this species with those of section Arachis.

In this context, in the present work, we analysed the presence of 'A' chromosomes using classical cytogenetics on mitotic prometaphases and metaphases, the distribution patterns of C-DAPI+ heterochromatin in the karyotype and the mapping of the ribosomal gene loci by FISH to (i) confirm the presence of 'A' chromosomes in *A. porphyrocalyx*, (ii) build a detailed cytogenetic map, (iii) investigate their karyotype relationships with the x = 9 species of the section Arachis by analysing chromosome homologies and finally (iv) discuss if the origin of all *Arachis* species with x = 9 is single or multiple. The chromosome data provided in this analysis will improve the knowledge of the genome affinities between the wild species, therefore aiding in understanding the variability contained in the secondary gene pool of the most agronomically important species of genus: *Arachis hypogaea* (Linneaus, 1753) (peanut).

Material and methods

Plant material

The material studied of *A. porphyrocalyx* corresponds to accession J.F.M. Valls, J.P. Moss and G.P. Silva 7303, collected in Brazil, Minas Gerais state, municipality of Uberaba, in the gardens of the Uberaba Country Club, on the edge of highway BR-050, next to Río Grande river, 20 km southeast of Uberaba, 19°58'S 47°47'W, in 1983. Germplasm from this original collection has been conserved at the Wild Arachis Genebank of Embrapa, in Brasília, Distrito Federal, and increased seed has been distributed to partner institutions. Seeds used in this study were obtained from the peanut germplasm collections of the Instituto de Botánica del Nordeste in Corrientes, Argentina. The voucher materials of the original accession are deposited in the herbaria CTES and CEN, and are paratypes of the species name. The holotype and isotypes of *A. porphyrocalyx* were collected nine years later from exactly the same site (J.F.M. Valls, C.E. Simpson, R.N. Pittman, D.E. Williams and G.P. Silva 13271).

Chromosome preparations and staining

Feulgen staining

Roots were obtained from seeds germinated in pots under laboratory conditions. Healthy root apices (5–10 mm long) were pretreated with 2 mM 8-hydroxyquinoline for 3 h at room temperature (Fernández and Krapovickas 1994). Subsequently, they were fixed in 3:1 absolute ethanol:glacial acetic acid for 12 h at 4°C and stored at -20°C

until use. For conventional chromosome staining, fixed root apices were washed in distilled water for 5 min, hydrolysed in 1 N HCl for 8 min at 60°C, stained with Schiff's reagent (Feulgen's technique) and then squashed in a drop of 2% acetic orcein. The preparations were made permanent using Euparal as mounting medium.

rDNA detection and DAPI banding

Fixed root apices were digested in 1% (w/v) cellulose (from *Trichoderma viridae*; Onozuka R-10, Serva) plus 10% (v/v) pectinase (from *Aspergillus niger*, Sigma) dissolved in 40% glycerol in 0.01 M citrate buffer (pH 4.8) for 2 h at 37°C. Subsequently, the meristematic cells were removed from the root tip and squashed in 45% acetic acid. After remove of the coverslip with gas carbon dioxide, the slides were air dried, aged for 1–2 days at room temperature and then kept at -20°C until use.

Probe labelling and fluorescence in situ hybridization

The 5S and 45S rDNA loci were localised using probes pA5S, pA18S and pA26S isolated from genomic DNA of *A. hypogaea* (Robledo and Seijo 2008) and labelled by nick translation with digoxigenin-11-dUTP (Roche Diagnostics) or biotin-11-dUTP (Sigma-Aldrich). Pretreatment of slides, chromosome and probe denaturation, conditions for the in situ hybridisation (hybridisation mixes contained DNA probes at a concentration of 2.5–3.5 ng/µl, with a stringency to allow sequences with 80%–85% identity to remain hybridized), post-hybridization washing, blocking and indirect detection with fluorochrome-conjugated antibodies were performed according to Seijo et al. (2004). The first set of antibodies consisted of anti-biotin produced in goat (Sigma-Aldrich) and monoclonal anti-digoxigenin conjugated to fluorescein isothiocyanate (FITC) produced in mouse (Sigma-Aldrich). The second set consisted of anti-goat conjugated to tetramethyl-rodamine isothiocyanate (TRITC) produced in rabbit (Sigma-Aldrich) and anti-mouse conjugated to FITC produced in sheep (Sigma-Aldrich). Preparations were counterstained by mounting them with Vectashield medium (Vector Laboratories) containing 2 mg/ml of 4',6-diamidino-2-phenylindole (DAPI).

Fluorescent microscopy and image acquisition

Chromosomes were viewed with a Leica DMRX fluorescence microscope (Leica) and digitally photographed with a computer-assisted Leica DC 350 digital camera system. Red, green and blue images were captured in black and white using the respective filters for TRITC, FITC and DAPI excitations. Digital images were processed with PHOTOSHOP, version 7.0 (Adobe).

Karyotype analysis

Karyotype measures were obtained by the analysis of five individuals and four Feulgen-stained metaphase plates per individual and using the computer application MICROMEASURE version 3.3 (Reeves and Tear 2000). Karyotype description is based on the nomenclature by Levan et al. (1964). Chromosomes were classified in three categories according to the centromeric index (CI = short arm × 100/total length of chromosome): metacentric (m) when CI = 50–37.5 and submetacentric (sm) when CI = 37.5–25 and subtelocentric when CI = 25–12.5. Classification of SAT chromosomes was performed on the basis of the satellite relative size and position of the centromere (Fernández and Krapovickas 1994). The total chromosome length (TCL) was obtained by summing the average length of each chromosome in the four metaphase samples of each individual, and then the average of the five individuals was performed. The chromosome mean length was calculated by dividing the TCL by the number of chromosomes of the species. The karyotype asymmetry indices were estimated using the intrachromosomal (A_1) and interchromosomal (A_2) indexes by Romero Zarco (1986).

Data from homologous chromosomes were combined first to obtain mean values of different pairs of chromosomes in the same metaphases and, subsequently, of the same chromosome pair in different metaphases. Haploid complements were represented in the ideogram. Chromosomes were ordered first by morphology and then by decreasing size.

Results and discussion

General karyotype features, karyotype formula, presence of 'A' chromosomes, TCL, mean chromosome length, centromeric index, asymmetry indexes, number of chromosomes with heterochromatic DAPI+ bands and number and position of 5S and 45S rDNA loci for *A. porphyrocalyx* are listed in Table 1. To compare with the remaining species with $x = 9$ from the section Arachis, the chromosome data of *A. decora*, *A. palustris* and *A. praecox* (Lavia 1998, Lavia 1999, Silvestri et al. 2015) were included in this table. Representative somatic prometaphases and metaphases of *A. porphyrocalyx* are shown in Figure 2, and the respective ideogram is shown in Figure 3.

General characteristics of karyotypes

The chromosome number of *A. porphyrocalyx*, previously determined by Peñaloza and Valls (2005), was confirmed by the mitotic analysis, $2n = 2x = 18$ (Fig. 2a). Likewise, in all analysed metaphases, a chromosome pair with characteristics of 'A' chromosomes was observed (Fig. 2b). This chromosome pair was easily identified by showing a lower condensation level of the euchromatin regions of their arms in comparison with the same regions in the remaining chromosomes of the complement and by corresponding to the smallest chromosomes of the karyotype. The 'A' chromosome pair corresponds to pair 7 (Fig. 3).

Table 1. Karyotypical features in x = 9 species of the genus *Arachis*.

Species	Karyotype formula	A chromosomes	Total chromosome length (µm)	Chromosome length mean (µm)	CI	Asymmetry indexes		Number of chromosomes with DAPI+ bands	Number and position of rDNA loci	
						A_1	A_2		45S	5S
A. porphyrocalyx	14m + 4st	yes	29.37	1.63	41.60	0.30	0.16	18	one LA pair 1	one LA pair 2
A. decora	16m + 2sm[b]	no	33.66[b]	1.87[b]	45.41[b]	0.22[b]	0.16[b]	18[c]	one LA pair 9[c]	one SA pair 6[c]
A. palustris	16m + 2sm[a]	no	33.23[a]	1.85[a]	43.64[a]	0.22[a]	0.17[a]	16[c]	one LA pair 9[c]	one SA pair 6[c]
A. praecox	16m + 2sm[a]	no	35.28[a]	1.96[a]	43.47[a]	0.23[a]	0.12[a]	18[c]	one LA pair 9[c]	one SA pair 6[c]

Abbreviations: CI = centromeric index; A_1 = Intrachromosomal asymmetry index; A_2 = interchromosomal asymmetry index. m = metacentric, sm = submetacentric, st = subtelocentric, LA = long arm, SA = short arm. Data of *A. decora, A. palustris* and *A. praecox* were taken from Lavia (1998)[a], Lavia (1999)[b] and Silvestri et al. (2015)[c].

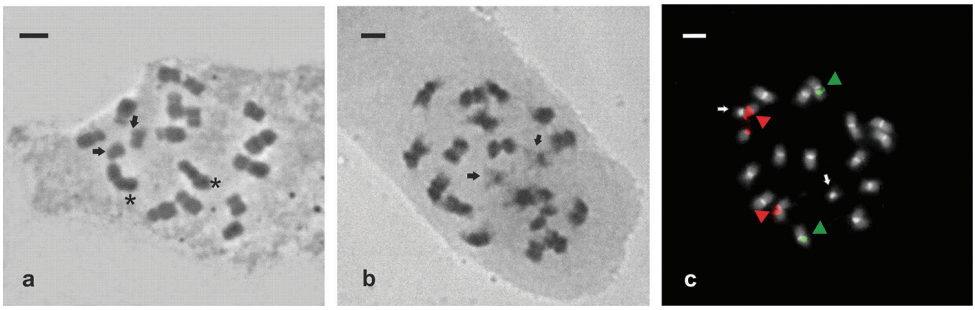

Figure 2. Mitotic chromosomes of *A. porphyrocalyx*. **a–b** Feulgen technique **c** double fluorescent *in situ* hybridization (FISH). **a** Metaphase displaying 2n=18, the starts indicate satellites and the arrows indicate the pair of "A chromosomes" **b** Prometaphase showing the pair of "A" chromosomes indicated by arrows **c** The yellow-green and red signals correspond to the 5S and 45S rDNA loci, and the white correspond to the heterochromatin bands C-DAPI+ after FISH. The arrows indicate the pair of "A chromosomes". Bar = 3 μm.

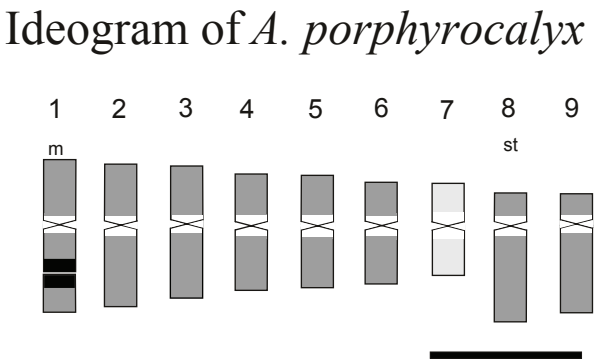

Figure 3. Ideogram of *A. porphyrocalyx* performed with measures of chromosomes obtained by classical technique. The A chromosome is represented with light gray colour. Distribution of 5S rDNA loci is illustrated with a striped signal and 18S-26S rDNA loci with a black signal. Heterochromatic regions counterstained with C-DAPI+ are represented with white bands. Bar = 2 μm.

The karyotype consisted of seven pairs of metacentric chromosomes and two subtelocentric pairs (14m + 4st; Fig. 3). These data do not agree with those reported by Peñaloza and Valls (2005) who observed four pairs of submetacentric chromosomes, but this discrepancy can be due to the fact that the formula published by these authors is based only on a visual analysis. Chromosomal size ranged between 1.24 and 2.08 μm with a mean length of 1.63 μm, belonging to the category of small chromosomes according to Lima de Faría (1980), and the mean length of diploid complement was 29.37 μm (Table 1). The indexes of asymmetry revealed a moderately high degree of intrachromosomal asymmetry (A1 = 0.30) but low interchromosomal asymmetry (A2 = 0.16). Only one pair of SAT chromosomes was found in all analysed metaphases. These chromosomes were the longest metacentric chromosomes of the comple-

ment (pair 1) and, as previously reported by Peñaloza and Valls (2005), correspond to the SAT chromosomes type 8 described by Fernández and Krapovickas (1994).

The metaphases of some individuals showed two or three chromosome pairs with extended primary constrictions (centromeres) and the chromosome arms separated. These chromosomes had the centromere unusually large or stretched during prophase or prometaphase, and consequently the number of chromosomal elements increased up to 25. Similar behaviour has been observed in some chromosomes of other species of the genus *Arachis*, such as *Arachis cardenasii* (Krapov. & W.C. Gregory, 1994), *Arachis helodes* (Mart. ex Krapov. & Rigoni, 1958), *Arachis valida* (Krapov. & W.C. Gregory, 1994), *Arachis duranensis* (Krapov. & W.C. Gregory, 1994) and *Arachis correntina* ((Burkart) Krapov. & W.C. Gregory, 1994) all belonging to the section Arachis (Fernández and Krapovickas 1994), and of other genera such as *Antirrhinum majus* (Linneaus, 1753), *Allium sphaerocephalum* (Crome ex Schltdl, 1824) (Lima De Faria 1956), *Libocedrus chilensis* (Endlicher, 1847) (Hunziker 1961), *Pisum* (Linneaus, 1753) and *Lathyrus* (Linnaeus, 1753) (Neumann et al. 2015). Just recently, Neumann et al. (2015) have classified this type of centromere as 'intermediate' between the two types of centromeres, monocentric and holocentric, and having an organization characterized by multiple Cen-H3 domains.

DAPI staining after FISH revealed C-DAPI+ centromeric bands in all chromosomes of the karyotype (Fig. 2c). These bands had similar sizes along karyotype, except in the 'A' chromosomes, where they were most conspicuous (Fig. 3). The results of in situ hybridisation showed one pair of 45S rDNA loci in proximal position on the long arm of the longest metacentric chromosomes (pair 1) and one pair of 5S rDNA loci in proximal or interstitial positions on the second longest chromosome pair of the karyotype (pair 2; Figs. 2c and 3).

Chromosome homeologies of *A. porphyrocalyx* with the remaining species of the genus, particularly with x = 9 species.

Like most species of the genus *Arachis*, the karyotype of *A. porphyrocalyx* consists of small size chromosomes, mainly metacentric. The smallest chromosome pair showed all features that define the 'A' chromosomes: a chromosome length 54% smaller than the largest chromosomes of karyotype, and showing allocycly in somatic prophases and prometaphases (Fernández and Krapovickas 1994). Chromosomes with these features have not been reported until now for other species not assigned to the A genome of the section Arachis (Fernández and Krapovickas 1994, Robledo et al. 2009). *Arachis porphyrocalyx* has a moderately asymmetric karyotype due to the presence of two pairs of subtelocentric chromosomes. This structure contrasts with the more symmetric karyotypes of the other $x = 9$ species of *Arachis* that are composed by eight metacentric pairs and only one submetacentric (Lavia 1998, 1999). Besides, the presence of subtelocentric chromosomes distinguishes *A. porphyrocalyx* from the other species of the section Erectoides, which have karyotypes formed only by metacentric and submetacentric chromosomes (Fernández and Krapovickas 1994, Lavia 2001, Lavia et al. 2009, Ortiz et al. 2013). Indeed, the

presence of subtelocentric chromosomes in the karyotypes is uncommon within the genus *Arachis*, a feature that *A. porphyrocalyx* only shares with *Arachis batizocoi* (Krapov. & W.C. Gregory, 1974) and *Arachis glandulifera* (Stalker, 1991) (Fernández and Krapovickas 1994), both species of the section Arachis.

Even though *A. porphyrocalyx* owns a unique pair of SAT chromosomes in metaphase, as the other species with $x = 9$, these chromosomes correspond to a different type according to the classification proposed by Fernández and Krapovickas (1994). Thus, *A. porphyrocalyx* has SAT chromosomes type 8, while the remaining $x = 9$ species show type 3 (Lavia 1998, Silvestri et al. 2015). Similarly, it differs from those observed in *Arachis douradiana* (Krapov. & W.C. Gregory, 1994), *Arachis hermannii* (Krapov. & W.C. Gregory, 1994), *Arachis major* (Krapov. & W.C. Gregory, 1994), *Arachis paraguariensis* (Chodat & Hassler, 1904) and *Arachis stenophylla* (Krapov. & W.C. Gregory, 1994) from the section Erectoides that have satellites of type 2, 3A or 4 (Fernández and Krapovickas 1994, Lavia 2001, Lavia et al. 2009, Ortiz et al. 2013).

Until now, four distribution patterns of centromeric C-DAPI+ heterochromatin have been identified in the karyotypes of *Arachis* species (Seijo et al. 2004, Robledo and Seijo 2008, 2010, Robledo et al. 2009, Silvestri et al. 2015, Ortiz, unpublished). One of them, proper to *A. glandulifera* of section Arachis (D genome), is characterised by a markedly heterogeneous distribution, with chromosomes showing large bands, and others with faint bands or devoid of them. The second pattern, with large bands of similar size in all or almost all chromosome pairs, is present in the $x = 9$ species and A and K genome species of the section Arachis. The third pattern, with small blocks at most chromosomes, which are revealed as faint bands or like-dot bands on the flanks of the centromeres, is shown in the F genome species of the section Arachis and in species of sections Erectoides and Procumbentes. And the fourth possibility, with no detectable bands in the entire karyotype, is proper to B genome species of the section Arachis. *Arachis porphyrocalyx* has a pattern that is different from those species of the section Erectoides and is similar to that observed in the $x = 9$ and in some $x = 10$ species of the section Arachis. It even resembles that observed in the A genome species, since the A chromosomes have pericentromeric bands with relative size greater than those in the rest of karyotype; which also in turn strengthens the identity of these chromosomes.

Regarding the number and location of ribosomal loci (45S rDNA and 5S rDNA), *A. porphyrocalyx* has the same number of sites as other $x = 9$ species, that is one pair of each loci (Silvestri et al. 2015). However, the 45S rDNA loci in *A. porphyrocalyx* are located on a metacentric pair as in *A. praecox*, while in *A. palustris* and *A. decora*, they are located on a submetacentric pair. Meanwhile, 5S rDNA loci in *A. porphyrocalyx* are on the long arms of a large-size metacentric pair and in the remaining $x = 9$ species on the short arms of a small-size metacentric pair. Until now, the number of rDNA loci has been characterized for two species of the section Erectoides, *A. stenophylla* and *A. paraguariensis* (Raina and Mukai 1999). The number of ribosomal loci observed in *A. porphyrocalyx* agrees with what has been detected in those species, except that *A. stenophylla* has two pairs of 45S rDNA loci.

The fact that the karyotype of *A. porphyrocalyx* has distinct distribution pattern of heterochromatin, conformed by large bands of the similar size in all chromosome

pairs, and has SAT chromosomes type 8 suggests that it corresponds to a distinct genome from that present in Erectoides species. On the contrary, its banding pattern is most related to that present in $x = 9$ species of the section Arachis. However, due to the presence of a pair of A chromosomes, different SAT chromosomes, different location of the 5S rDNA loci and a more asymmetric karyotype than that of the other $x = 9$ species, it is suggested that *A. porphyrocalyx* also does not have the G genome.

Has the basic chromosome number x = 9 in *Arachis* been originated once or more times in the evolutionary history of the genus?

Although the four $x = 9$ species share the chromosome number, the karyotypic differences between *A. porphyrocalyx* and the remaining three species are evident. Therefore, and as was proposed (Peñaloza and Valls 2005), the reduction in the number of chromosomes might have occurred more than once in the *Arachis* genus.

All *Arachis* species with $x = 9$ are naturally distributed in Brazil (Fig. 1), and their evolutionary history probably is associated with watercourses. The northernward distribution corresponds to *A. palustris* and comprises both sides of Tocantins River in the states of Maranhão and Tocantins, between 7°22'S and 12°33'S. *Arachis decora* is distributed in the northeast of Goiás and in the south of Tocantins state, separated by approximately 150 km from *A. palustris*. In contrast, the only two populations known to *A. praecox* are located in the Mato Grosso state, separated by approximately 900 km of any of the other two $x = 9$ species of the section Arachis. Meanwhile, *A. porphyrocalyx* has been located in Minas Gerais state, municipality of Uberaba, near the Rio Grande, some 20 km southeast of Uberaba (19°58'S, 47°47'W). Thus, the four $x = 9$ species of the genus *Arachis*, at least in the present, belong to three different basins (Fig. 1). That is, *A. decora* and *A. palustris* share the Tocantins River Basin and are the closest species from the geographic standpoint; *A. praecox* belongs to the basin of the Paraguay River, while *A. porphyrocalyx* to the Paraná River. Notoriously the last species is located in the same basin that lodges the species with A chromosomes of section Arachis (Robledo et al. 2009).

The three $x = 9$ species of section Arachis are annuals and morphologically constitute different entities. *Arachis praecox* differs from *A. palustris* and *A. decora* by the short central axis from 2 to 3 cm, while in the other two it has about 15 cm of length. *Arachis decora* and *A. palustris* are morphologically very similar although they are distinguished because the former has bristles in the stipules, while the latter lacks them (Krapovickas and Gregory 1994). Fruit shape also distinguishes these two species. Contrarily, *A. porphyrocalyx* is perennial, has thickened secondary roots, a central axis between 5 and 15 cm of length, flowers concentrated at the base of the plant, lateral branches procumbents, epiphyllum with hairs, petioles and rachis with hairs and bristles (Valls and Simpson 2005), a series of features that lead to its initial allocation in the Erectoides section.

As previously discussed, *A. porphyrocalyx* does not share the same genome of the other $x = 9$ species. The presence of A chromosomes would be a strong reason for the

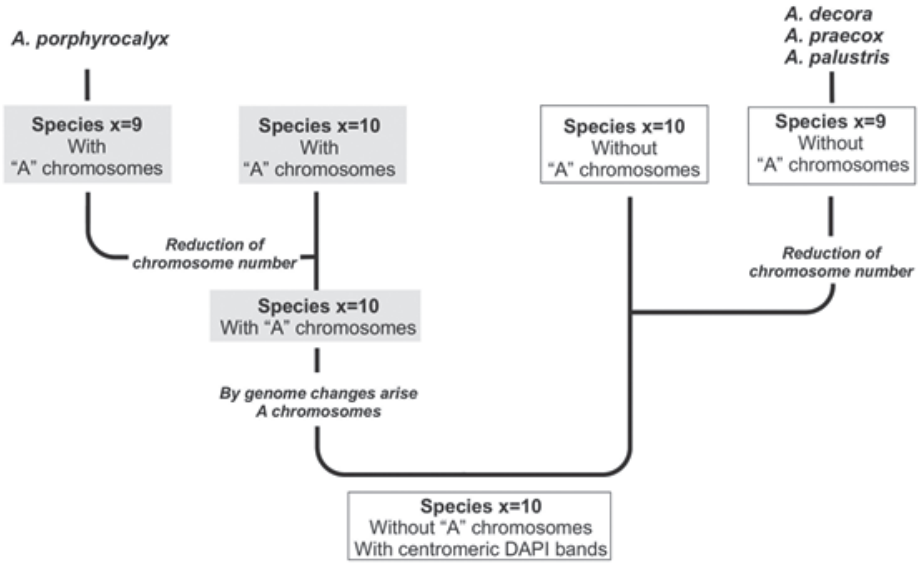

Figure 4. Scheme showing the hypothesis of the multiple origin of x = 9 species with and without A chromosomes in the genus *Arachis*.

assignment of *A. porphyrocalyx* to A genome, but the fact that it has two subtelocentric chromosomes, SAT chromosomes type 8, a single pair of 45S ADNr sites, and as the most significant trait, the basic number *x* = 9 distinguishes it from the three karyotype types established for A genome species (Robledo et al. 2009).

Taking into account the geographic distribution, morphological and cytogenetic features, the hypothesis of Peñaloza and Valls (2005), which suggests the basic chromosome number *x* = 9 would have originated at least twice in the evolutionary history of the genus, becomes relevant.

Some years ago, when the existence of a diploid *x* = 9 species with a pair of A chromosomes was not yet known, it had been proposed that a diploid *x* = 10 species, belonging either to the A genome group (Lavia 1998) or to a non-A genome group (Tallury et al. 2005), was the ancestor of all species with *x* = 9 by reduction of chromosome number. In this work, it has been demonstrated that *A. porphyrocalyx* has not the same genome type of the other *x* = 9 species, suggesting that the reduction of the number of chromosomes must have occurred more than once in the evolution of the genus *Arachis*; therefore, the proposed hypotheses must be updated.

In this sense, we propose that a diploid x = 10 entity, without A chromosomes and with large bands of the similar size in all or almost all, chromosome pairs could be the common ancestor of all x = 9 species as well as the x = 10 species with A and K genome of the section Arachis. The fact that these species share a same type of heterochromatin distribution pattern, different from that observed in the species so far examined of the genus Arachis, would support this proposal. From this ancestor, by chromosomal rearrangements, an entity with A chromosomes has been originated, from which all x =

10 species with A chromosomes (A genome species) derived, and by some cytogenetic phenomenon (probably disploidy), the unique species with x = 9 and A chromosomes (*A. porphyrocalyx*) derived (Fig. 4). Moreover, new molecular data (Silvestri; unpublished) show a minor genetic distance of *A. porphyrocalyx* from the A genome species, compared with any other species of the genus, including the rest of x = 9 species. In parallel, from that same first common ancestor, an evolutionary line of species without A chromosomes continued evolution, and from this, the x = 9 species with the G genome is derived by reducing the number of chromosomes (Fig. 4). Evidence in this direction results in several phylogenetic analyses (Bechara et al. 2010, Friend et al. 2010, Moretzshon et al. 2013), in which the x = 9 species appear as a sister clade to the species without A chromosomes of the section Arachis.

Conclusions

In this work, we confirmed the presence of 'A' chromosomes in the karyotype of *A. porphyrocalyx*. It revealed its particular karyotypic structure, which allows proposing that it does not share the same genome with the remaining *x* = 9 species of *Arachis* and neither with the species so far characterized karyotypically of the section Erectoides. On the contrary, its similarity with karyotypes of species with A chromosomes of the section Arachis suggests that the genome of *A. porphyrocalyx* could be related to the A genome, but molecular studies are needed to confirm this hypothesis. Additionally, considering the morphological and cytogenetic features and the geographic distribution, we propose the existence of two separate events for the origin of species with 18 chromosomes within the genus *Arachis*.

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The authors declare that they have no conflicts of interest.

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RESEARCH ARTICLE

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Cytogenetic data on the agro-predatory ant *Megalomyrmex incisus* Smith, 1947 and its host, *Mycetophylax conformis* (Mayr, 1884) (Hymenoptera, Formicidae)

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Abstract

We provide the first karyotype description of the agro-predatory ant species *Megalomyrmex incisus* Smith, 1947 (Myrmicinae, Formicidae), and chromosome counts of its host *Mycetophylax conformis* (Mayr, 1884) (Myrmicinae, Formicidae) from geographically distinct populations. Colonies of both species were sampled from coastal areas of Ilhéus, Bahia, Brazil, and transferred to the laboratory. Metaphase spreads were prepared from the cerebral ganglia of defecated larvae. The slides were examined and pictures of the best metaphases were taken. The chromosome number for *M. incisus* was 2n=50 and n=25. The karyotype of this species consists of 20 metacentric and 5 submetacentric pairs. Thus, the karyotype formula of the diploid set was 2K=40M + 10SM and a fundamental number FN=100. The host species *M. conformis* has 2n=30 and the karyotype consisting of 11 metacentric and 4 submetacentric pairs. The karyotype formula was 2K=22M + 8SM, and a fundamental number FN=60. *M. incisus* showed a slightly higher chromosome number, placed at the marginal range of the known distribution of haploid karyotypes of the Myrmicinae. The chromosome number and chromosomal morphology of *M. conformis* corresponded to those of previously studied populations, suggesting its karyotype stability.

Keywords
Karyotype, chromosome counts, ants, biodiversity, evolution

Introduction

Chromosomes are the units of inheritance bearing the complete set of information necessary for biological development. In general, species have a fixed number of chromosomes, and closely related species tend to have more similar karyotypes than distantly related ones (Guerra 2013). Changes in karyotype features (e.g., chromosome number and morphology) may have evolved through multiple speciation events, each involving the fixation of particular chromosomal rearrangements (Schubert and Lysak 2011).

Ants are among the insect taxa that exhibit one of the most variable chromosome numbers, ranging from n=1 to 60 (reviewed by Lorite and Palomeque 2010). This high karyotype diversity seems to be correlated to ant diversification, which currently comprises nearly 14,000 described species in 21 subfamilies (Agosti and Johnson 2016). Cytogenetic data are available for about 750 ant species (Lorite and Palomeque 2010), with more data accumulating rapidly; this information has advanced our understanding of ant systematics and evolution (Mariano et al. 2012, Cristiano et al. 2013, Cardoso et al. 2014). However, with the growing number of ant species, more cytogenetic studies are needed to reveal the extent of chromosome diversity.

Myrmicinae is the most diverse subfamily of Formicidae, and it consequently encompasses the majority of species with described karyotypes (Lorite and Palomeque 2010). However, karyotype information is not yet available for some widely distributed genera of this subfamily. For example, *Megalomyrmex* Forel, 1885 comprises 44 described species distributed from Mexico to northern Argentina (Longino 2010), and despite this broad geographic occurrence, cytogenetic data are nonexistent. The genus contains social parasites of fungus-growing ants, with *M. incisus* Smith, 1947 recently described as an agro-predatory ant of *Mycetophylax* Emery, 1913 species (Cardoso et al. 2016). Here, we describe for the first time the karyotype of the agro-predator *M. incisus* as well as its host, the fungus-growing ant *Mycetophylax conformis* (Mayr, 1884).

Material and methods

Colony sampling

Colonies of both species were sampled on the coast of Ilhéus, Bahia, Brazil (14°47'36.61"S, 39°2'46.96"W). A colony of *Megalomyrmex incisus* was collected during excavation of the colony of *Mycetophylax conformis* (see Cardoso et al. 2016). The entrances to the colony are located on the top of a small sand turret and surrounded by a sand crater. Excavation was carried out according to protocols developed by Car-

doso et al. (2011). Thirteen colonies in total were excavated, and *M. incisus* were collected from one putative nest of *M. conformis*. Colonies were collected in their entirety and transported to the laboratory, where they were transferred to rearing systems as described by Cardoso et al. (2011). Species were kept under laboratory conditions (9:15 L:D photoperiod, 25 °C) in order to obtain broods for performing cytogenetic analysis.

Karyotype descriptions

M. conformis was karyotyped to determine whether it has the same chromosome number as populations characterized by Cardoso et al. (2014), as divergent chromosome numbers were already found in some congeneric species. Larvae obtained from the colonies maintained in the laboratory were used for karyotype characterization. One colony of *M. incisus* and eleven out of thirteen colonies of *M. conformis* were evaluated cytogenetically. For *M. incisus* 30 individuals were used in cytogenetic analyses, whereas for *M. conformis* the numbers of individuals analyzed per colony were: ten individuals in five colonies; 8 individuals in two colonies; 6 individuals in three colonies and 4 individuals in one colony. Metaphase spreads were prepared from the cerebral ganglia of prepupae, according to Imai et al. (1988). The cerebral ganglion was dissected in colchicine–hypotonic solution (0.005% w/v colchicine in 1% sodium citrate solution) under a stereoscopic microscope, transferred to a new drop of colchicine–hypotonic solution and incubated in the dark for one hour (see Imai et al. 1988 and Cardoso et al. 2012 for the detailed procedure). The slides were evaluated using a phase contrast microscope. Quality metaphase slides were stained with 4% Giemsa solution in Sørensen's buffer (pH 6.8); the best metaphases were photographed using an Olympus BX51 microscope equipped with a digital camera, and then used for evaluation of the chromosome number and morphology. Chromosomes were classified following the nomenclature proposed by Levan et al. (1964), which is based on centromere positions: acrocentric (A), subtelocentric (ST), submetacentric (SM), and metacentric (M). We measured ten (*M. conformis*) and seven (*M. incisus*) spread metaphases with chromosomal integrity, evident centromeres, and without overlapping during the morphometric karyotype analysis. The following features of chromosomes were evaluated: total length (TL), long arm length (L), short arm length (S), arm ratio between the long and short arms (r=L/S), relative chromosome length (RL) of each chromosome (TL ×100/∑TL) and asymmetry index (∑long arms/∑total length ×100). In order to identify putative cytogenetic markers in *M. incisus*, sequential fluorochrome staining with chromomycin A_3/distamycin A/4', 6–diamidino–2–phenylindole (CMA_3/DA/DAPI) was done according to Schweizer (1980) to characterize CG- and AT-rich regions. These slides were analyzed under an epifluorescence microscope (Zeiss AxioImager Z2) equipped with a digital camera (AxioCam MRc). The fluorescence signals were analyzed using two different filters: a GFP filter (450 to 480 nm) for CMA_3, and a DAPI filter (330 to 385 nm) for DAPI.

Results

All individuals of *M. conformis* from Ilhéus had chromosome counts of 2n=30 (Fig. 1a–b). The karyotype of this species consists of 11 metacentric (M) pairs and 4 submetacentric (SM) pairs ranging in size from large to small. The mean total length of individual chromosomes ranged from 5.49 to 1.59 μM, while the mean total length of all chromosomes was 90.18 μM. The karyotypic formula of the diploid set was 2K=22M + 8SM. Thus, a fundamental number (number of chromosome arms in the diploid karyotype) was FN=60. Morphometric data for chromosomes of *M. conformis* are shown in Table 1.

The chromosome number for *M. incisus* was 2n=50 and n=25 (Fig. 1c–e). The karyotype of this species consists of 20 metacentric (M) pairs and 5 submetacentric (SM) pairs, with less variation in size (Fig. 1c). The mean length of individual chromosomes ranged from 4.65 to 1.85 μM, while the mean total length of all chromosomes was 141.89 μM (Table 2). The karyotypic formula of the diploid set was 2K=40M + 10SM, and a fundamental number was FN=100. Only haploid (male) brood was subjected to sequential fluorochrome staining, which revealed positive GC-rich blocks (CMA_3^+) in a single chromosome at the pericentromeric region (Fig. 1e). AT-rich blocks were not found since the chromosomes were stained uniformly (data not shown).

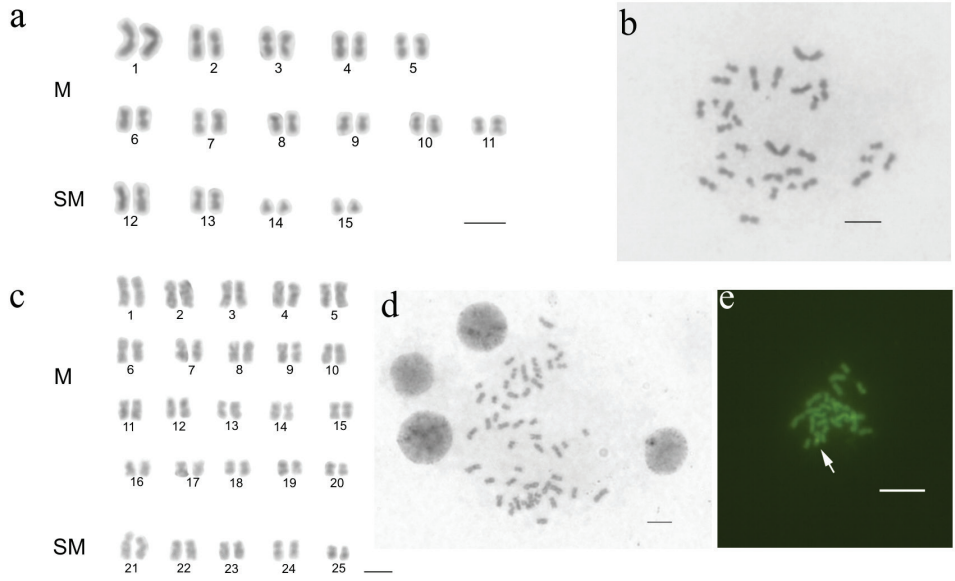

Figure 1. Cytogenetic data of *Megalomyrmex incisus* and its host *Mycetophylax conformis*. **a** *M. conformis* conventional staining of diploid karyotype and **b** metaphase **c** *M. incisus* conventional staining of diploid karyotype and **d** metaphase **e** *M. incisus* metaphase stained with CMA_3, white arrow indicates positive staining for CMA_3. M=metacentric, SM=submetacentric. Bar = 5 μm.

Table 1. Detailed karyotype analysis of *Mycetophylax conformis*.

Chromosome	TL (μM)	L (μM)	S (μM)	RL	*r*	Chromosome classification
1	5.49±0.60	2.99±0.30	2.5±0.32	6.09±0.29	1.19±0.07	Metacentric
1	5.3±0.52	2.9±0.29	2.41±0.25	5.88±0.32	1.19±0.06	Metacentric
2	4.08±0.38	2.18±0.23	1.9±0.17	4.53±0.23	1.16±0.07	Metacentric
2	3.96±0.40	2.06±0.14	1.9±0.26	4.39±0.20	1.09±0.08	Metacentric
3	3.64±0.40	2.01±0.30	1.63±0.15	4.03±0.12	1.22±0.17	Metacentric
3	3.54±0.32	1.95±0.15	1.59±0.19	3.92±0.13	1.22±0.11	Metacentric
4	3.44±0.36	1.83±0.21	1.61±0.23	3.81±0.11	1.09±0.19	Metacentric
4	3.36±0.37	1.82±0.17	1.54±0.24	3.72±0.10	1.19±0.16	Metacentric
5	3.25±0.29	1.74±0.19	1.51±0.14	3.61±0.09	1.13±0.12	Metacentric
5	3.19±0.28	1.75±0.10	1.44±0.24	3.54±0.07	1.17±0.22	Metacentric
6	3.08±0.28	1.7±0.15	1.38±0.19	3.41±0.10	1.24±0.18	Metacentric
6	3.04±0.26	1.66±0.22	1.38±0.16	3.37±0.07	1.1±0.23	Metacentric
7	2.96±0.25	1.64±0.10	1.32±0.21	3.29±0.13	1.25±0.22	Metacentric
7	2.92±0.27	1.73±0.16	1.2±0.12	3.24±0.15	1.47±0.10	Metacentric
8	2.85±0.28	1.6±0.22	1.25±0.12	3.16±0.13	1.3±0.18	Metacentric
8	2.78±0.27	1.57±0.12	1.21±0.17	3.08±0.10	1.34±0.11	Metacentric
9	2.63±0.37	1.56±0.24	1.08±0.15	2.91±0.19	1.42±0.16	Metacentric
9	2.45±0.30	1.38±0.15	1.07±0.18	2.71±0.13	1.31±0.17	Metacentric
10	2.29±0.23	1.29±0.13	1±0.16	2.54±0.15	1.32±0.22	Metacentric
10	2.23±0.18	1.24±0.17	0.99±0.09	2.48±0.13	1.3±0.21	Metacentric
11	2.41±0.64	1.4±0.43	1.01±0.22	2.68±0.71	1.31±0.19	Metacentric
11	2.3±0.60	1.28±0.41	1.01±0.22	2.55±0.67	1.15±0.25	Metacentric
12	3.65±0.75	2.36±0.48	1.29±0.28	4.03±0.64	1.78±0.12	Submetacentric
12	3.52±0.72	2.32±0.41	1.2±0.32	3.89±0.58	1.98±0.23	Submetacentric
13	2.57±0.16	1.65±0.14	1±0.25	2.87±0.36	1.86±0.21	Submetacentric
13	2.49±0.13	1.59±0.10	0.93±0.26	2.78±0.27	2.02±0.22	Submetacentric
14	1.78±0.10	1.33±0.29	0.77±0.56	1.98±0.10	2.32±0.30	Submetacentric
14	1.75±0.15	1.3±0.27	0.73±0.55	1.94±0.10	2.42±0.38	Submetacentric
15	1.65±0.14	1.25±0.36	0.75±0.60	1.83±0.14	2.18±0.35	Submetacentric
15	1.59±0.13	1.28±0.41	0.77±0.62	1.77±0.15	2.22±0.30	Submetacentric
Σ	90.18					

TL (μM) total chromosome length in micrometers; L (μM) long arm length in micrometers; S (μM) short arm length in micrometers; RL relative chromosome length; *r* arm ratio (*r*=L/S).

Discussion

Four distinct karyotypes have been reported for *Mycetophylax,* from three valid species: *M. morschi* (Emery, 1888) harbors two cytotypes, 2n=26 and 2n=30, whereas *M. simplex* (Emery, 1888) and *M. conformis* harbor 2n=36 and 2n=30, respectively (Cardoso et al. 2014). Here we evaluated the chromosome counts of *M. conformis* from Bahia, Brazil, the host of the social parasite *M. incisus* (Cardoso et al. 2016). The studied population is located 1,000 km from the northern population that was cytogenetically

Table 2. Detailed karyotype analysis of *Megalomyrmex incisus*.

Chromosome	TL (μM)	L (μM)	S (μM)	RL	*r*	Chromosome classification
1	4.65±0.92	2.56±0.53	2.09±0.41	3.28±0.20	1.19±0.092	Metacentric
1	4.25±0.85	2.58±0.49	1.71±0.42	3.00±0.10	1.32±0.201	Metacentric
2	4.2±0.84	2.36±0.42	1.84±0.45	2.96±0.13	1.33±0.175	Metacentric
2	4.15±0.76	2.25±0.52	1.88±0.26	2.92±0.13	1.2±0.150	Metacentric
3	3.84±0.80	1.97±0.58	1.88±0.25	2.71±0.08	1.09±0.192	Metacentric
3	3.68±0.85	2.04±0.49	1.63±0.40	2.59±0.13	1.25±0.129	Metacentric
4	3.46±0.83	1.99±0.50	1.6±0.35	2.44±0.11	1.26±0.136	Metacentric
4	3.39±0.82	1.9±0.58	1.61±0.32	2.39±0.12	1.17±0.278	Metacentric
5	3.37±0.80	1.83±0.44	1.55±0.37	2.38±0.12	1.21±0.087	Metacentric
5	3.25±0.75	1.89±0.42	1.36±0.36	2.29±0.11	1.31±0.123	Metacentric
6	3.1±0.74	1.72±0.33	1.43±0.42	2.18±0.11	1.13±0.130	Metacentric
6	3.09±0.69	1.75±0.36	1.35±0.32	2.18±0.08	1.24±0.071	Metacentric
7	3.05±0.69	1.73±0.39	1.41±0.33	2.15±0.09	1.28±0.183	Metacentric
7	3.02±0.67	1.75±0.37	1.36±0.35	2.13±0.08	1.21±0.231	Metacentric
8	2.98±0.67	1.6±0.34	1.39±0.33	2.10±0.08	1.17±0.047	Metacentric
8	2.91±0.65	1.58±0.36	1.33±0.31	2.05±0.07	1.21±0.100	Metacentric
9	2.86±0.62	1.55±0.33	1.34±0.30	2.01±0.05	1.17±0.085	Metacentric
9	2.81±0.63	1.6±0.38	1.29±0.30	1.98±0.06	1.16±0.191	Metacentric
10	2.8±0.59	1.55±0.43	1.29±0.18	1.97±0.04	1.28±0.174	Metacentric
10	2.72±0.60	1.43±0.31	1.3±0.29	1.92±0.05	1.15±0.065	Metacentric
11	2.64±0.60	1.51±0.43	1.2±0.18	1.86±0.07	1.33±0.174	Metacentric
11	2.58±0.62	1.48±0.31	1.2±0.33	1.81±0.09	1.17±0.205	Metacentric
12	2.49±0.64	1.45±0.38	1.18±0.30	1.75±0.11	1.18±0.219	Metacentric
12	2.47±0.61	1.4±0.25	1.12±0.38	1.74±0.10	1.24±0.204	Metacentric
13	2.46±0.57	1.28±0.31	1.22±0.27	1.73±0.08	1.14±0.122	Metacentric
13	2.42±0.55	1.35±0.28	1.13±0.27	1.71±0.07	1.18±0.089	Metacentric
14	2.4±0.52	1.26±0.33	1.18±0.23	1.69±0.07	1.12±0.177	Metacentric
14	2.37±0.50	1.27±0.25	1.12±0.26	1.67±0.07	1.15±0.080	Metacentric
15	2.37±0.50	1.32±0.35	1.12±0.18	1.67±0.08	1.17±0.147	Metacentric
15	2.32±0.49	1.33±0.23	1.06±0.28	1.63±0.06	1.13±0.153	Metacentric
16	2.26±0.48	1.34±0.22	1.04±0.30	1.59±0.08	1.1±0.262	Metacentric
16	2.19±0.48	1.23±0.31	0.97±0.20	1.55±0.08	1.19±0.164	Metacentric
17	2.07±0.53	1.23±0.32	0.89±0.24	1.46±0.11	1.44±0.186	Metacentric
17	2.02±0.43	1.13±0.24	0.92±0.20	1.42±0.07	1.24±0.164	Metacentric
18	2±0.37	1.11±0.17	0.89±0.21	1.41±0.06	1.24±0.117	Metacentric
18	1.94±0.36	1.12±0.20	0.85±0.17	1.37±0.06	1.27±0.120	Metacentric
19	1.88±0.33	1.03±0.19	0.89±0.15	1.33±0.06	1.16±0.097	Metacentric
19	1.85±0.33	1.09±0.13	0.79±0.22	1.30±0.05	1.26±0.176	Metacentric
20	2.05±0.94	1.13±0.70	0.89±0.25	1.44±0.63	1.31±0.343	Metacentric
20	1.98±0.79	1.09±0.64	0.86±0.16	1.39±0.53	1.25±0.392	Metacentric
21	3.45±1.14	2.2±0.86	1.24±0.29	2.43±0.54	1.79±0.321	Submetacentric
21	3.35±0.97	2.18±0.70	1.15±0.30	2.36±0.49	1.76±0.369	Submetacentric
22	3.4±0.37	2.29±0.21	1.13±0.20	2.40±0.35	1.93±0.248	Submetacentric
22	3.13±0.37	1.96±0.36	1.06±0.51	2.21±0.27	1.82±0.292	Submetacentric
23	3±0.37	1.91±0.26	1.02±0.18	2.12±0.22	1.87±0.293	Submetacentric

Chromosome	TL (μM)	L (μM)	S (μM)	RL	*r*	Chromosome classification
23	2.9±0.27	1.86±0.21	1.01±0.10	2.04±0.21	1.83±0.174	Submetacentric
24	2.8±0.28	1.78±0.17	1.02±0.16	1.98±0.21	1.74±0.270	Submetacentric
24	2.62±0.31	1.66±0.26	0.96±0.06	1.86±0.24	1.74±0.174	Submetacentric
25	2.5±0.25	1.64±0.18	0.85±0.09	1.76±0.28	1.89±0.150	Submetacentric
25	2.39±0.22	1.57±0.09	0.82±0.14	1.69±0.27	1.91±0.263	Submetacentric
Σ	141.89					

TL (μM) total chromosome length in micrometers; L (μM) long arm length in micrometers; S (μM) short arm length in micrometers; RL relative chromosome length; *r* arm ratio (*r*=L/S).

analyzed by Cardoso et al. (2014). The diploid number of chromosomes and their morphology did not differ from previously characterized populations, showing 2n=30 with the karyotype comprising metacentric and submetacentric chromosomes. These results suggest that *M. conformis* has a stable karyotype.

Karyotype variation among populations can occur across different species (Lukhtanov et al. 2011). Populations on the edge of distribution ranges of various species were reported to have generally adaptive chromosomal variations (Singh 2015). Clinal variation in ant chromosome structure has also been observed in *Typhlomyrmex rogenhoferi* Mayr, 1862, with populations from Bahia and Pará in Brazil and French Guiana (each 1,000 km apart) showed diploid chromosome numbers of 38, 34 and 36, respectively (Mariano et al. 2006). The population of *M. conformis* studied in the present paper was sampled about 900 km northeast of the population evaluated by Cardoso et al. (2014) in Rio de Janeiro State. It is known that great distances between populations can promote conspicuous genetic variation; however, chromosome counts and karyotype structure of *M. conformis* were identical across the whole distribution range, supporting the proposed chromosomal stability of the species.

M. incisus is the first cytogenetically characterized species of *Megalomyrmex*. It showed a chromosome number of 2n=50 (the haploid number was n=25) with 20 metacentric pairs and five submetacentric pairs. The chromosome number of *M. incisus* is consistent with the range of karyotypic variation in the Formicidae (n=1 to 60; reviewed by Lorite and Palomeque 2010). However, this particular number is rare in the subfamily Myrmicinae, and it thus represents the marginal distribution frequency of chromosome numbers known for this group (Lorite and Palomeque 2010).

The fluorochrome staining confirms a cytological marker that was identified in a number of ant species (Mariano and Delabie 2013), as well in other insects (Cardoso et al. 2012). A single chromosome in haploid males showed a pericentromeric positive GC-rich block (CMA_3^+). Indeed, different ant species show only one pair of chromosomes in diploid females that typically bears a positive GC-rich block. However, these positive GC-rich blocks may differ in the location on the chromosome. For instance, in *M. conformis* the CMA_3^+ block is in the telomeric region, while in *M. simplex* it is pericentromeric (Cardoso et al. 2014). Previous studies have shown that these positive CG-rich blocks are correlated with the location of nucleolus organizer regions

(NORs) and rDNA sites (Cardoso et al. 2012; Barros et al. 2015), indicating that they may represent NORs in this particular case as well.

An accurate karyotype description should take into account physical measurements like length of individual chromosomes, total karyotype length, and arm length ratios. These types of features allow accurate identification of chromosomes, which is critical for robust karyotype analysis. Morphometric data on ant chromosomes are still scarce and exist for only a few species (e.g. Cristiano et al. 2013; Barros et al. 2014), limiting our ability to further extrapolate evolutionary patterns or trajectories. The measurements of chromosomes presented here for *M. incisus* and *M. conformis* allow accurate descriptions of chromosome morphology. As more data became available, general karyotypic patterns can be revealed, increasing our general understanding of chromosome evolution in ants.

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First report of B chromosomes in three neotropical thorny catfishes (Siluriformes, Doradidae)

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Abstract

The family Doradidae (Siluriformes) is an important group of fishes endemic to freshwater ecosystems in South America. Some cytogenetic studies have been conducted focused on the group; however, there are no reports on the occurrence of B chromosomes for the family. In this paper the chromosomal characteristics of *Platydoras armatulus* (Valenciennes, 1840), *Pterodoras granulosus* (Valenciennes, 1821) and *Ossancora punctata* (Kner, 1855) were investigated through classical cytogenetics approaches. The conventional staining reveals 2n=58 in *Platydoras armatulus* and *Pterodoras granulosus*, however with distinct karyotypic formulae, possibly originated by pericentric inversions. In *Ossancora punctata* a derivate karyotype was described with 2n=66 and predominance of acrocentric chromosomes. The C banding pattern was resolutive in discriminating the three species, being considered an important cytotaxonomic marker. All species showed B chromosomes totally heterochromatic with non-Mendelian segregation during meiosis and low

frequencies in mitotic cells. The probably origin of these additional elements was through fragmentations of chromosomes of the standard complement, which occurred recently and independently in these three species. The diploid number observed in *O. punctata* is an evidence of centric fusions and up to the moment it is the highest diploid number reported for Doradidae.

Keywords
Centric fusion, chromosomal rearrangements, diploid number, neotropical fish, pericentric inversions, supernumerary chromosome

Introduction

Cytogenetic studies in Doradidae are scarce and restricted to nine species. Eight of these bear 58 chromosomes and single nucleolus organizer regions (NORs) in terminal positions (Eler et al. 2007, Milhomem et al. 2008). The exception to this pattern is *Trachydoras paraguayensis* (Eigenmann & Ward, 1907) with 56 chromosomes and single NORs in an interstitial position (Fenocchio et al. 1993, Baumgärtner et al. 2016). The members of Doradidae are popularly named thorny catfish and comprise 94 species and 33 genera (Froese and Pauly 2016) endemic to freshwater ecosystems in South America. The family is easily diagnosed among catfishes by the presence of a row of bony midlateral scutes, each usually bearing a single thorn (Birindelli 2014). Phylogenetic studies based on molecular (Moyer et al. 2004, Arce et al. 2013) and morphological (Birindelli 2014) data support the monophyly of Doradidae.

Supernumerary chromosomes have already been reported in several neotropical Siluriformes families, however up to now they have not been observed in Doradidae (Carvalho et al. 2008, Lui et al. 2009). These additional elements were described in different organisms and can originate in two ways: from chromosomal rearrangements in chromosomes from the A complement (the most common), or as a consequence of interspecific crosses. Regardless of their origin, the majority of B chromosomes due to not possess genes and follow an independent evolutionary path characterized by structural differentiation mechanisms, including the accumulation of different repetitive DNA sequences (Camacho et al. 2000).

In most organisms, the B chromosomes are dispensable elements, as their presence is not associated with phenotypic alterations. However, there are exceptions, as described in *Nectria haematococca* Samuels & Rossman, 1999 where the Bs possess resistance genes which grant a better pathogenicity (Coleman et al. 2009), and in *Lithochromis rubripinnis* Seehausen, 1998 in which B chromosomes have a functional effect in sex determination (Yoshida et al. 2011). According to Valente et al. (2016), the recent development of molecular biology associated with the advances in next-generation sequencing technologies have increased knowledge about the biological importance of B chromosomes, revealing that the presence of many genes and other transcriptionally active sequences can modulate the activity of autosomal genes.

In the present study, the karyotypic structure of *Platydoras armatulus*, *Pterodoras granulosus* and *Ossancora punctata* was investigated in mitotic and meiotic cells. This comparative analysis to provide a better understanding of the karyotype diversification in Doradidae, reporting for the first time the occurrence of B chromosomes and discussing the probably origins of this feature in this family.

Material and methods

In the present study, cytogenetic analyses were performed on 9 females and 8 males of *Platydoras armatulus*; 3 females and 6 males of *Ossancora punctata*, all collected in the Miranda river, in Corumbá, Mato Grosso do Sul, in the Brazilian Pantanal (19°31'25"S 57°02'26"W). Additionally, 5 females and 4 males of *Pterodoras granulosus* also collected in the Paraná river, in Pauliceia, São Paulo, Brazil (21°06'10.26"S 51°47'14.1"W), were analyzed. The collection of specimens was authorized by ICMBio (Instituto Chico Mendes de Conservação da Biodiversidade). After processing and subsequent fixation of the material, all specimens were deposited in the Museu de Zoologia da Universidade Estadual de Londrina (data available via SpeciesLink).

Before euthanasia (48 hours), the specimens received an intraperitoneal injection of 2 ml of Broncho-vaxom (bacterial lysate) to trigger an inflammatory process and hence increase the number of kidney cells in mitotic division (Molina et al. 2010). After this, all specimens were anesthetized with clove oil (eugenol) and sacrificed to obtain the mitotic chromosomes from kidney cells (Bertollo et al. 1978) and meiotic chromosomes from testis cells (Kligerman and Bloom 1977). The metaphasic chromosomes were classified as metacentric, submetacentric, subtelocentric and acrocentric according to ratio of arms proposed by Levan et al. (1964). The heterochromatin pattern was determined using the C-banding technique (Sumner 1972) with a modification in staining phase (Lui et al. 2012).

Results

Platydoras armatulus

All specimens analyzed exhibited 58 chromosomes (22m + 14sm + 18st + 4a). Eleven samples showed cells carrying from 1-3 B chromosomes (Fig. 1a) with interindividual frequencies ranging from 5.25% to 61.90% (Table 1). C-banding evidenced heterochromatin blocks in the pericentromeric and terminal regions in the short arm of pairs 3, 5, 10, 12, 14, 15, 18, 19, 24, 26, 28 and in the long arm of pairs 3, 4, 12. Interstitial heterochromatin regions also occurred in pairs 2, 21 and 25. The B chromosomes are totally heterochromatic (Fig. 1b). C-banding applied to meiotic cells confirmed the results observed in mitosis in: spermatogonial metaphase with 58 chromosomes (Fig. 2a); late pachytene (more condensed) (Fig. 2b) and metaphase I, with 29 bivalents (Fig. 2c).

Figure 1. Karyotypes after conventional staining and C-banding – *Platydoras armatulus* **a** Giemsa staining reveals 2n=58 (22m+14sm+18st+4a) and 1-3 B chromosomes **b** C-banding pattern characterized by the many heterochromatin blocks in different positions, including in B chromosomes. *Pterodoras granulosus* **c** Giemsa staining also reveals 2n=58 but with distinct karyotypic formulae: 16m+16sm+14st+12a and 1 B chromosome **d** a few heterochromatin blocks was evidenced after C banding, observe the B chromosome totally heterochromatic. *Ossancora punctata* **e** After Giemsa staining it was observed 2n=66 (12m+8sm+6st+40a), the high number of subtelocentric and acrocentric chromosomes is a remarkable feature of this specie **f** C-banding reveals heterochromatin regions in terminal position and in B chromosomes.

Table 1. Frequencies of supernumerary chromosomes in *Platydoras armatulus*, *Pterodoras granulosus* and *Ossancora punctata*. ♀= female; ♂= male.

Species/Samples	Sex	Number of B/cell				Total of cells	Cells with B
		0	1	2	3		
P. armatulus							
4156	♂	17	6	8	4	35	**51.42%**
4157	♀	29	4	0	0	33	**12.12%**
4158	♂	33	6	3	2	43	**23.25%**
4159	♀	14	0	0	0	14	**0%**
4160	♂	16	8	12	6	42	**61.90%**
4161	♂	17	7	10	5	39	**56.41%**
5032	♂	22	4	1	0	27	**18.51%**
5320	♀	10	0	0	0	10	**0%**
5322	♀	18	1	0	0	19	**5.26%**
5325	♀	15	0	0	0	15	**0%**
7	♂	22	3	2	1	28	**21.42%**
8	♂	31	1	0	2	34	**8.82%**
9	♀	25	0	0	0	25	**0%**
80	♂	11	1	3	0	15	**26.66%**
81	♀	19	0	0	0	19	**0%**
82	♀	21	0	1	1	23	**8.69%**
83	♀	28	0	0	0	28	**0%**
P. granulosus							
601	♀	16	2	0	0	18	**12.5%**
602	♀	15	3	0	0	18	**20%**
603	♂	54	9	0	0	63	**16.6%**
604	♀	63	15	0	0	78	**23.8%**
617	♂	32	4	0	0	36	**12.5%**
618	♀	21	0	0	0	21	**0%**
619	♀	23	0	0	0	23	**0%**
628	♂	16	0	0	0	16	**0%**
631	♂	35	6	0	0	41	**17.1%**
O. punctata							
4561	♀	25	5	3	1	34	**26.47%**
4566	♂	23	7	2	4	36	**36.11%**
5119	♂	21	4	2	3	30	**30%**
5120	♂	32	0	3	2	37	**13.51%**
5692	♂	31	1	0	0	32	**3.12%**
5694	♀	14	0	0	0	14	**0%**
5695	♀	6	0	0	0	6	**0%**
5696	♂	23	1	0	0	24	**4.16%**
5332	♂	32	3	0	4	39	**17.94%**

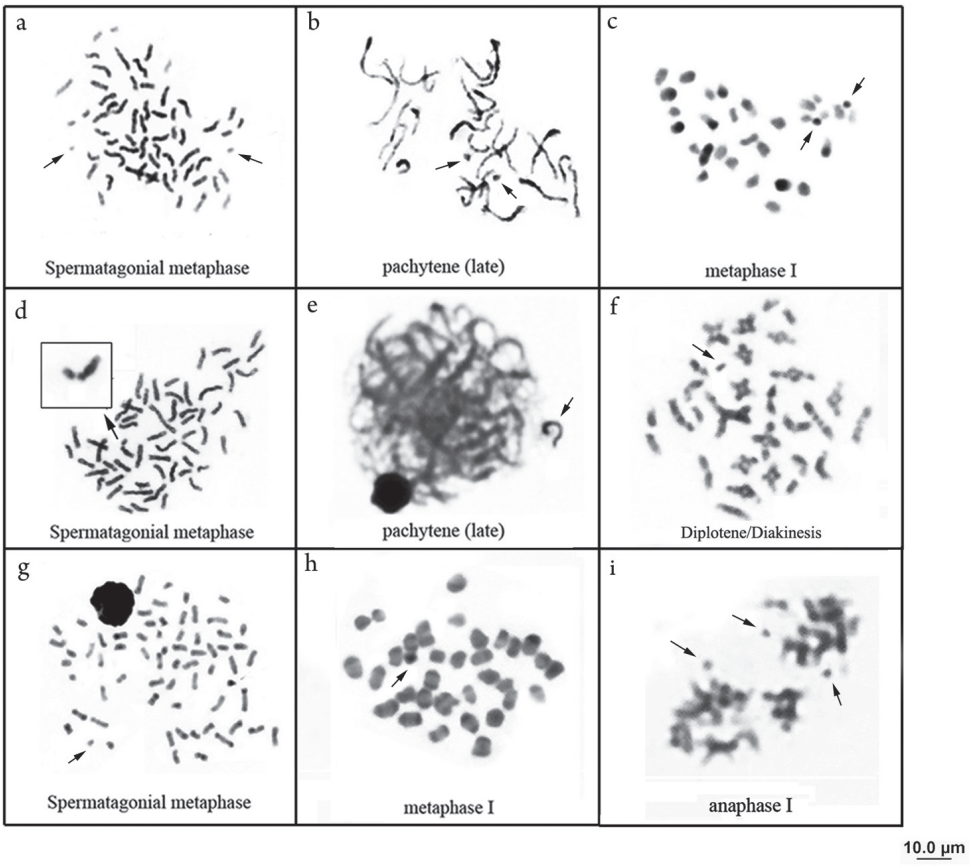

Figure 2. Meiotic cells in different phases with B chromosomes evidenced after C-banding - *Platydoras armatulus* **a** spermatogonial metaphase with 58 chromosomes and 2 B chromosomes **b** late pachytene with bivalents in advanced condensation stage, note two B chromosomes forming univalents without homologies of standard complement **c** metaphase I with 27 bivalents and two B chromosomes. *Pterodoras granulosus* **d** spermatogonial metaphase composed by 58 chromosomes and one B chromosome **e** late pachytene, the isolated univalent probably correspond to B chromosome **f** diplotene/diakinesis with 27 bivalents and one B chromosome, note the high number of chiasms. *Ossancora punctata* **g** spermatogonial metaphase with 66 chromosomes and one B chromosome **h** metaphase I reveals heterochromatic B chromosome and 33 bivalents **i** anaphase I, observe the late segregation of B chromosomes.

Pterodoras granulosus

Conventional staining with Giemsa revealed 58 chromosomes, with a karyotype formula 16m + 16sm + 14st + 12a. In six specimens, one acrocentric supernumerary chromosome was observed (Fig. 1c) with interindividual frequencies ranging from 12.5% to 23.8% (Table 1). C-banding identified few blocks of heterochromatin restricted in some centromeres, short arm of pair 19 and in B chromosome (Fig. 1d). In meiotic

analyses the B chromosomes were observed totally heterochromatic in: spermatogonial metaphase (Fig. 2d), late pachytene with the B chromosome isolated (Fig. 2e) and diplotene/diakinesis (Fig. 2f).

Ossancora punctata

The studied specimens presented 66 chromosomes and a karyotype formula of 12m + 8sm + 6st + 40a. Among all nine specimens analyzed, seven exhibited B chromosomes (Fig. 1e), and the frequencies were considered low, ranging from 3.12 % to 36.11 % of the specimen cells (Table 1). C-banding revealed pericentromeric heterochromatin in most chromosomes, as well as terminal blocks on the long arm of subtelo-acrocentric chromosomes and at the both ends of most metacentric chromosomes (Fig. 1f). The microchromosomes also presented themselves entirely heterochromatic in meiotic analyses in: spermatogonial metaphase (Fig. 2g), metaphase I with 33 bivalents (Fig. 2h) and anaphase (Fig. 2i).

Discussion

Phylogenetic analysis based on morphological and molecular data supports the monophyly of Doradidae that, together with Auchenipteridae, constitutes the superorder Doradoidea (Moyer et al. 2004, Arce et al. 2013, Birindelli 2014). According to some authors, the ancestor of Doradidae had a karyotype composed by 58 chromosomes (Eler et al. 2007, Milhomem et al. 2008, Baumgärtner et al. 2016). In fact, this diploid number is present in *Wertheimeria maculata* Steindachner, 1877 (Eler et al. 2007) the species considered, with *Kalyptodoras bahiensis*, the sister group of this family (Birindelli 2014).

Notwithstanding, not all doradid species have 2n = 58, as is the case of *Trachydoras paraguayensis* with 2n = 56 chromosomes (Fenocchio et al. 1993, Baumgärtner et al. 2016) and *Ossancora punctata* with 2n = 66 (present study). Baumgärtner et al. (2016) identify interstitial telomeric sequences (ITS) in *Trachydoras paraguayensis*, demonstrating the emergence of the 2n = 56 from a karyotype with 58 chromosomes by centric fusion. Diversely, in *Ossancora punctata* the 2n = 66 is the largest diploid number ever reported for the family and probably originated due to centric fissions resulting in a karyotype with many subtelocentric and acrocentric chromosomes. These variations in diploid numbers show that the pericentric inversions are not the only chromosomal rearrangements that generate macro-structural variability (Eler et al. 2007, Milhomem et al. 2008).

The dispersion of heterochromatic regions is a high variable in Doradidae. *Pterodoras granulosus* exhibited few blocks, similarly described for *Platydoras costatus* Linnaeus, 1758 (Milhomem et al. 2008), but distinct from the pattern observed in *Platydoras armatulus* which exhibited many chromosomes bearing heterochromatin blocks in ter-

minal and interstitial positions. This divergence observed in *Platydoras* can be an excellent cytogenetic marker, because these two species have 58 chromosomes and similar karyotypic formulae. The heterochromatin pattern of *Ossancora punctata* is similar to that described in *Hassar wilderi* Kindle, 1895 (Eler et al. 2007), *Hassar orestis* Steindachner, 1875, *Hassar* sp. and *Tenellus ternetzi* Eigenmann, 1925 (Milhomem et al. 2008) with many terminal blocks, some of these located in both chromosome arms. This C-band pattern reinforced the phylogenetic proximity between these three genera, which constitute one of the most derived clades of Doradidae (Birindelli 2014).

Cytogenetic studies in Neotropical Siluriformes revealed the occurrence of B chromosomes in more than 25 species, including representatives of the families Heptapteridae, Callichthyidae, Pimelodidae, Pseudopimelodidae, Auchenipteridae, Tricomycteridae and Loricariidae (Lui et al. 2009). The B chromosomes of *Platydoras armatulus*, *Pterodoras granulosus* and *Ossancora punctata* presented similar structural characteristics, even though the frequencies in mitotic cells were highly variable. This numerical variability is an evidence of the non-Mendelian segregation theory proposed by Jones and Rees (1982) and occurs because the B chromosomes possess a delayed migration during anaphase, as can be observed in some germ cells of *Ossancora punctata* (Fig. 2i). Another feature visualized in some spermatocytes was the presence of B chromosomes forming a univalent isolated of the standard complement. This meiotic behavior suggested a structural differentiation of B chromosomes in relation to the standard complement due to accumulation of different families from repetitive DNA (Camacho et al. 2000).

In Neotropical fish, the mechanisms responsible for the origin and evolution of B chromosomes remain unclear, as several theories were proposed (Lui et al. 2009, Blanco et al. 2012, and others). The B microchromosomes were described in distinct neotropical fishes, including *Schizodon* Agassiz, 1829, *Astyanax* Baird et Girard, 1854, *Moenkhausia* Eigenmann, 1903, *Cyphocharax* Fowler, 1906, *Steindacnerina* Fowler, 1906, *Prochilodus* Agassiz, 1829, *Rhamdia*, Bleeker, 1858 *Iheringichthys* Eigenmann et Norris, 1900, *Callichthys* Scopoli, 1777, *Megalonema* Eigenmann, 1912, *Pimelodella*, Eigenmann et Eigenmann, 1888 and *Loricaria* Linnaeus, 1758 (Carvalho et al. 2008, Lui et al. 2009). An interesting hypothesis to explain the origin of these additional genomic elements is the fragmentation in standard karyotype (Sampaio et al. 2015). Considering the morphological type, non-Mendelian segregation and low frequencies in mitotic cells, it seems likely that the B chromosomes observed in *Platydoras armatulus*, *Pterodoras granulosus* and *Ossancora punctata* have a recent origin from fragmentation in chromosomes from A complement.

This study contributed with relevant information to the better understanding of the karyotype variability in Doradidae. In this family, the 2n=58 is considered a primitive condition, such that the chromosomal diversification is based primarily on pericentric inversions and at lower frequency fissions and fusions. Additionally, the mitotic and meiotic analysis revealed at the first time in Doradidae the occurrence of B chromosomes, which originated recently from fragmentations in chromosomes of standard complement. Additional studies such as the isolation and molecular characterization of these chromosomes can be resolutive in confirming its origin and evolution.

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Chromosome mapping of a Tc1-like transposon in species of the catfish *Ancistrus*

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Abstract

The Tc1 mariner element is widely distributed among organisms and have been already described in different species of fish. The genus *Ancistrus* (Kner, 1854) has 68 nominal species and is part of an interesting taxonomic and cytogenetic group, as well as presenting a variation of chromosome number, ranging from 2n=34 to 54 chromosomes, and the existence of simple and multiple sex chromosome system and the occurrence of chromosomal polymorphisms involving chromosomes that carry the nucleolus organizer region. In this study, a repetitive element by restriction enzyme, from *Ancistrus* sp.1 "Flecha" was isolated, which showed similarity with a transposable element Tc1-mariner. Its chromosomal location is distributed in heterochromatic regions and along the chromosomal arms of all specimens covered in this study, confirming the pattern dispersed of this element found in other studies carried out with other species. Thus, this result reinforces the hypothesis that the sequence AnDraI is really a dispersed element isolated. As this isolated sequence showed the same pattern in all species which have different sex chromosomes systems, including in all sex chromosomes, we could know that it is not involved in sex chromosome differentiation.

Keywords

Repetitive DNA, enzyme digestion, chromosomal mapping, transposable elements, *in situ* fluorescence hybridization

Introduction

The genome of eukaryotes consists mostly of large amounts of repetitive DNA, which has been associated with several functions in the genome, as can be seen in the review carried out by Shapiro (2010). These functions range from important roles in the structure of chromosomes, the telomere and centromere maintenance mechanism (Pardue and Debaryshe 2003, Wong and Choo 2004), involvement in DNA replication process (Li et al. 2002), of recombination (Biet et al. 1999) and gene expression (Liu et al. 2001, Peaston et al. 2004, Han and Boeke 2005, Volff 2006), in origin and evolution of sex and supernumerary chromosomes (Lyon 2000, Steinemann and Steinemann 2005, Parise-Maltempi et al. 2007), besides being used as important markers for cytogenetic studies of evolution, genome organization and identification of chromosomal rearrangements in several groups of organisms (Biémont and Vieira 2006, Martins 2007, Oliveira et al. 2013).

Basically, the repetitive sequences are represented by tandem repeats, as satellite DNA, minisatellite, and microsatellite repeats or dispersed along the DNA as retrotransposons and transposons (Charlesworth et al. 1994). The transposable elements (TEs) are classified according to the type of intermediate transposition, being of class I those that possess RNA intermediates and class II those whose intermediates are DNA molecules (Kidwell 2002). Transposons, belonging to Class II, representing most of the moderately repeated sequences of the eukaryotic genome, can be located in the region of constitutive heterochromatin and / or interspersed through the chromosomes and evolved through the ability to replicate making copies of themselves and moving to other regions of the genome (Capriglione et al. 2002).

When transposed, if the transposition occurs within promoter regions, introns or untranslated regions, it can affect the expression of this gene (Maksakova et al. 2006) and, although most of these mutations are harmful, the transposition of these elements have contributed to diversification of species due to generation of new alleles (Kapitonov and Jurka 2006). Its ability to spread in multiple copies may be regarded as a driving force for the evolution of the genome and, indeed seems to promote the variability of the genome, which may lead to a determination regulatory mutations and chromosomal rearrangements (Syvanen 1984, Charlesworth et al. 1994).

Based on the similarity between the sequences and phylogenetic analysis of the transposase, the transposable elements can be classified in ten families: Tc1/mariner, haT, P element, MuDR/Fokdback, Cacta, PiggyBac, Pif/Harbinger, Merlin, Transib and Banshee (Feschotte and Pritham 2007). Since the discovery of transposable elements in eukaryotes, elements such as Tc1 / mariner have been isolated from different fish species (Radice et al. 1994, Izsvák et al. 1995, Ivics et al. 1996, Capriglione et al. 2002, Krasnov et al. 2005, Pocwierz-Kotus et al. 2007, Liu et al. 2009).

This element, belonging to a superfamily of transposons, presents 1000 up to 2000 bp (Kidwell 2002), characterized by a simple structure with two inverted terminal repeats (TIRs) of approximately 28 bp. Also has an ORF (Opening Read Frame) encoding the transposase (Wallau et al. 2011) and is widely distributed among organisms,

from protozoa to vertebrates. However, due to various events - mutations, deletions and insertions which become permanent component of the genome (Pocwierz et al. 2007), the majority is currently in an inactive form (Miskey et al. 2005).

The genus *Ancistrus* (Kner,1854) is one of the most diverse of tribe Ancistrinae, popularly known as "cascudos", and currently has 68 nominal species (Eschmeyer 2015). Its taxonomy is very confusing and a lot of species already have to be described. Based on chromosomal analysis, (Mariotto et al. 2011) suggested the existence of 13 cytotypes for the *Ancistrus* species found in the basis of rivers Paraguay, Araguaia-Tocantins and the Amazon in the Mato Grosso state (Brazil). They also suggested the existence of possible new species in this region, which show variation in chromosome number diploid ranging from 2n=34 to 54 chromosomes, presence of simple and multiple sex chromosome systems with both heterogametic sex and occurrence of chromosomal polymorphisms involving the chromosomes carrying the nucleolus organizing region for the group (Alves et al. 2003, Alves et al. 2006; Mariotto and Miyazawa 2006, Oliveira et al. 2007, Mariotto et al. 2009). Systems of ZZ/ZW and XX/XY sex chromosomes were found in populations of *Ancistrus* cf. *dubius* and *Ancistrus* sp 08 from the wetland of Mato Grosso state (Brazil) (Mariotto et al. 2004, Mariotto and Miyazawa 2006), the X0 system in *Ancistrus* n. sp.1 from "Rio Vermelho" located in Goiás state (Brazil) (Alves et al. 2006) and multiple systems of XX/XY1Y2 and Z1Z1Z2Z2/Z1Z2W1W2 for the species *Ancistrus* sp.1 "Balbina" and *Ancistrus* sp.2 "Barcelos" from Amazon state (Brazil) (De Oliveira et al. 2008).

Thus, taking into account the karyotype diversity of *Ancistrus*, including different sex chromosome systems, location of nucleolus organizer regions (NOR) and number of chromosomes, this study aimed to isolate repetitive sequences that could help in better understanding of the karyotype organization of the *Ancistrus* species.

Material and methods

Samples

The species of *Ancistrus* covered in this study were collected in the Flecha river, Creek Currupira, Pari and Sangradouro in the Paraguay river basin (Table 1). The collected material was taken to the Animal Genetics Laboratory at the Federal University of Mato Grosso, where 109 chromosome preparations were obtained.

Preparation of mitotic chromosomes

The chromosome preparations were made from the kidney of specimens collected following the methodology described by Foresti et al. (1993). The material was stored in a freezer at -20 °C.

Table 1. Collection site and number of species collected.

Specie	Collection site	Number of collected species
Ancistrus sp1 "Flecha"	15°58'7"S 57°19'7"W	18 F – 6 M
Ancistrus sp "Currupira"	15°7'59"S 56°49'47"W	19 F – 23 M
Ancistrus sp "Pari"	15°36'6"S 56°12'19"W	7 F – 12 M
Ancistrus sp "Sangradouro"	16°4'25"S 57°40'31.1"W	5 F – 4 M

Characterization of karyotypes

C-bands were detected according to Sumner (1972) to assemble the karyotypes.

Obtaining repetitive sequences

The extraction of genomic DNA was performed from liver and fin of the specimens collected, basically following the protocol phenol / chloroform / isoamyl alcohol by Sambrook and Russel (2001). The extracted genomic DNA was digested with various restriction enzymes to isolation of repetitive sequences in a proportion of 30 µl DNA (100 ng) in 3 µl of enzyme. This solution was left at 37 °C (temperature according to the enzyme used) overnight and after 7 hours of digestion was further added 3 µl of enzyme. For precipitation and purification of the digested DNA 2 µl of 5M NaCl and 200 µL of ice cold 100% ethanol was added. This solution was stored for two hours at -80 °C and centrifuged after two hours. Digested DNA was eluted in 10 µl of distilled water and analyzed in agarose gel 1% stored in the freezer for later use. The bands of potential repetitive sequences were then purified according the QIAquick PCR Purification Kit protocol (Qiagen). To perform the cloning, competent bacteria were prepared in the laboratory according to chemical transformation with $CaCl_2$ (Mandel and Higa 1970). The DNA fragments were inserted into plasmid vectors with pMOS Blue Kit (Amersham Biosciences) following the manufacturer's specifications.

DNA sequencing

The amplified and purified DNA by treatment with ExoSAP enzyme (USB) was sequenced by the method of Sanger et al. (1977) through outsourcing of services by MacroGen company (Korea). The editing of the sequences was performed on the program BioEdit sequence alignment editor v7.0.5.3 (Hall 1999) using the Clustal W tool for performing alignment of the sequences. For the characterization of the tools sequences were used: BLAST - Basic Local Alignment Search Tool at National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/blast);

CENSOR; RepeatMasker, website (http://www.repeatmasker.org/cgi-bin/WEBRepeatMasker) and ORF Finder, the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/ gorf / gorf.html).

DNA amplification via PCR

The recombinant clones were subjected to PCR (Polymerase Chain Reaction) for amplification using the universal primers M13 F - GTA AAA CGA CGG CCA G and M13 R - CAG GAA ACA GCT ATG AC under the following conditions: denaturation at 95 °C for 3 minutes, 34 cycles of denaturation at 95 °C for 30 seconds, annealing at 50 for 1 minute, 72 °C extension for 2 minutes and elongation at 72 °C for 5 minutes.

After sequencing, the divergent primers were designed KD7116F – TCA CAA CAC ACG TTT GTG GA and KD7116R – AGA GCA GGC TTT GAA TCG G manually, which was synthesized by SIGMA company. Subsequently, the amplification of the sequence with the primer KD7116-1 also in other possible different species from other populations was performed following conditions: denaturation at 94 for 5 minutes, 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 58 °C for 1 minute 72 °C extension for 1 min and elongation at 72 °C for 7 minutes.

Fluorescence *in situ* hybridization

In situ hybridization was performed following the protocol by Pinkel et al. (1986) with some modifications. The fluorescent probes were labeled with digoxigenin by nick translation. The slides were mounted with antifading solution containing DAPI and chromosomes observed using an Olympus BX51 microscope and digital camera model D. The images were captured using the DP Controller software.

Results

Karyotype analysis

The analysis of constitutive heterochromatin by C-banding was performed to characterize all species karyotypes covered in this study. *Ancistrus* sp.1 "Flecha" has 2n=44 chromosomes and no heterochromatin block or sex chromosome system was shown (Fig. 1). *Ancistrus* sp. "Currupira" has 2n=44 chromosomes and showed heterochromatin mainly at pericentromeric regions and a block in pair 13 (Fig. 2A), *Ancistrus* sp. "Pari" has 2n=42 chromosomes with heterochromatin along pericentromeric regions and a block in pair 15 (Fig. 2B) and *Ancistrus* sp."Sangradouro" has 2n=42 chromosomes, a karyotype similar to *Ancistrus* sp. "Pari" but its heterochromatin block is in pair 6 (Fig. 2C).

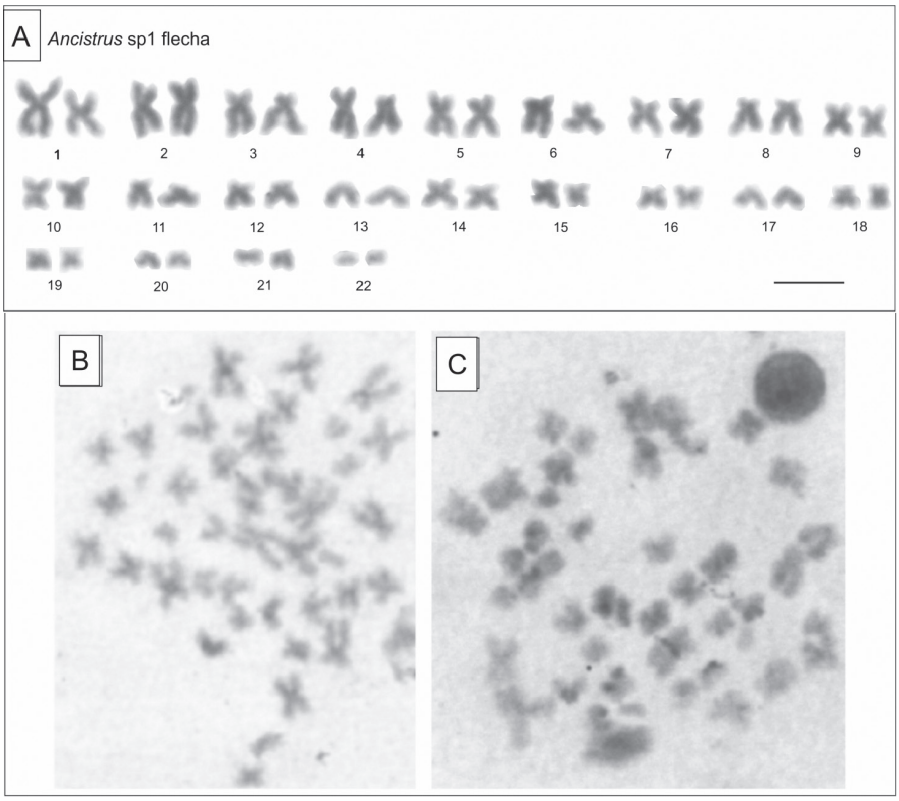

Figure 1. Karyotype of *Ancistrus* sp.1 "Flecha" using C-banding. **A** Conventional staining- showing the karyotype of *Ancistrus* sp.1 "Flecha" with 2n = 44 chromosomes, including a pair of microchromosomes **B, C** is C-band showing that the chromosomes of *Ancistrus* is not rich in heterochromatic regions in female and male. Bar = 10 μm.

Analysis of AnDraI sequence

After isolation of repetitive sequences with restriction enzymes using *Ancistrus* sp.1 "Flecha" DNA, it was possible to observe the formation of a band of approximately 700 bp. The product from this band was then cloned, resulting in 34 recombinant clones, from which one, named AnDraI was used in this study. The sequence of this clone had 618 bp and 44.34% of GC base pair. According with the databases consulted: Blast 2.0 RepeatMasker and Censor, the sequence obtained showed greater than 86% identity with the type of transposon Mariner/Tc1 of *Xenopus tropicalis* (Gray, 1864). In the analysis performed for possible coding regions, an ORF region of frame 3+ 188 bp (87-275) was found. As conserved domain, a region of approximately 180 nucleotides which corresponds to HTH_Tnp_Tc3_2_Transposase was found. By submitting this sequence in the protein data bank (Blastx), similarities were found with transposases of several species, including, *Rana pipiens* (Schreber, 1782), *Xenopus tropicalis*, *Dicentrarchus labrax* (Linnaeus, 1758), *Salmo salar* (Linnaeus, 1758), and *Cyprinus carpio* (Linnaeus, 1758) (Fig. 3).

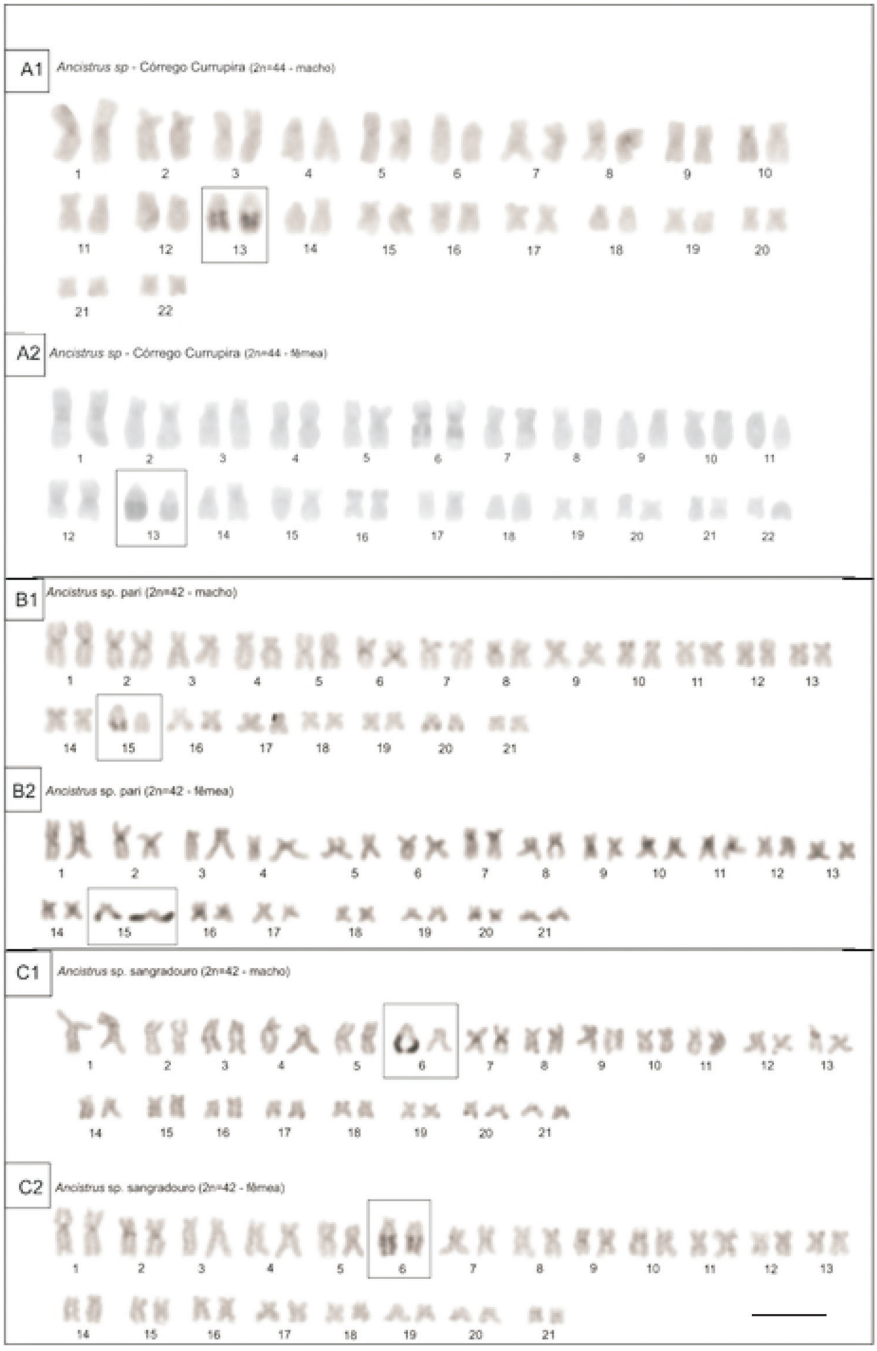

Figure 2. Karyotype after C band. *Ancistrus* sp. "Currupira" has 2n=44 chromosomes and showed heterochromatin blocks mainly at centromeric regions and pair 13 (**A**), *Ancistrus* sp. "Pari" has 2n=42 chromosomes with heterochromatin blocks along centromeric regions and a big block in pair 15 (**B**) and *Ancistrus* sp. "Sangradouro" has 2n=42 chromosomes, a karyotype similar to *Ancistrus* sp. "Pari" but the big heterochromatin block is in pair 6 (**C**). Bar = 10 μm.

Figure 3. Comparison of the amino acid sequence of Transposase expected of AnDraI with Tc1-like transposase of other species. **X. t.** *Xenopus tropicalis* **S. s.** *Salmo salar* **C. c.** *Cyprinus carpio* **D. l.** *Dicentrarchus labrax*.

PCR analysis of the sequence Tc1-like in specimens from other populations

It was realized an amplification of AnDraI element by polymerase chain reaction in the genome of other specimens of other populations: *Ancistrus* sp. "Currupira" from Currupira Creek, *Ancistrus* sp. "Pari" from Pari Creek and *Ancistrus* sp. "Sangradouro" from Sangradouro Creek. Both, males and females, of this species showed the same length of fragments (not shown).

Chromosomal mapping of the transposon

Chromosomal *in situ* hybridization performed on *Ancistrus* sp.1 "Flecha" revealed signals throughout all the chromosomes preferentially located in pericentromeric regions, with no difference between males and females (Fig. 4).

Cross-Fish conducted in individuals from other localities also showed results similar to that found in *Ancistrus* sp.1 "Flecha" (Fig. 5).

Discussion

The transposable element Tc1, first identified in invertebrates as *Caenorhabditis elegans* (Maupas, 1900), have around 1600 bp and share similar structures, known as Terminal inversed regions (TIRs) that show a sequence of 5 to 6 bp identical in/or near the highly conserved ends (CAGTG/CAGTC) (Brezinsky et al. 1990, Avancini et al. 1996). However, there is a great variation between the number of copies, distribution and types of transposable elements, between different species arising from factors such as intrinsic characteristics of the transposable elements (TEs) and the different evolutionary forces that act on these (Capy et al. 1998).

The Tc1-like element isolated in this work from *Ancistrus* sp.1 "Flecha" appears to share a very similar structure to TcMar-Tc1 of *Xenopus* (Siluriana) *tropicalis*, which is 532 bp, equivalent to 86.1%. This element appears widely distributed throughout all

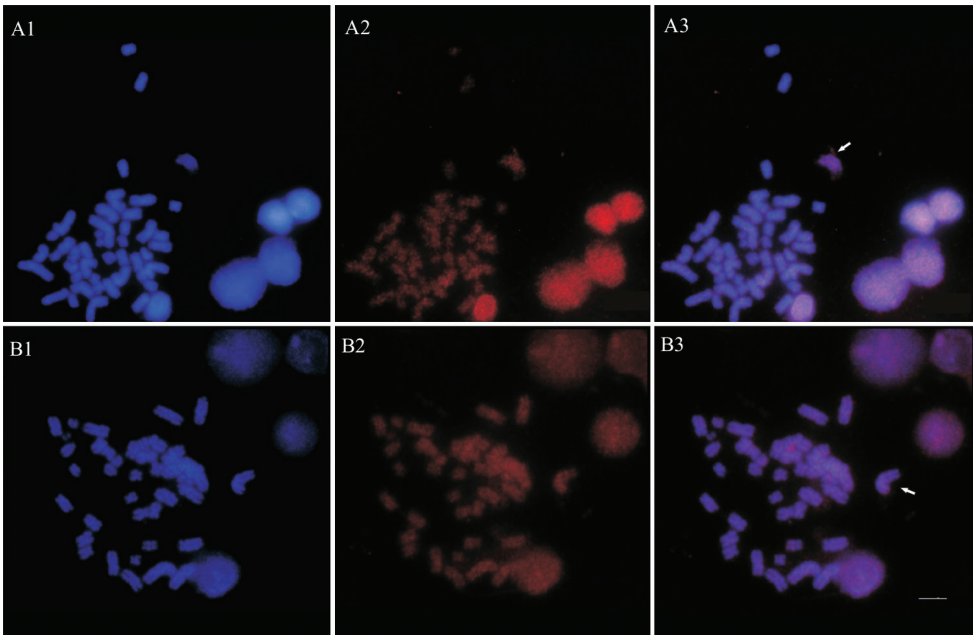

Figure 4. *In situ* fluorescence hybridization using AnDraI as probe in *Ancistrus* sp.1 "Flecha": **A** female **B** male. The arrows show some examples of pericentromeric and spread signals. Bar = 10 μm.

the chromosomes of all specimens of *Ancistrus* covered in this study, with some specific concentrations in pericentromeric regions. These specimens belong to distinct localities with a high geographic distance and isolated among themselves, with different karyotypes which show number chromosomes ranging from 2n=42 to 44 and species with no sex chromosomes system and others with XX/XY or ZZ/ZW sex chromosomes. Despite of the known cytogenetic differences among the *Ancistrus* group it can be said that, at least among the specimens analyzed, the AnDraI element presents the same homogeneous pattern of distribution and no correlation can be made regarding the karyotypes differences neither the origin and differentiation of sex chromosomes of this group involving its isolated sequence. However, it can be inferred that, despite the low number of populations analyzed it is possible to consider that the AnDra I element is present in the genome of the *Ancistrus* genus. Although the Siluriformes group have a scientific and economic importance, their systematic and taxonomy are still highly problematic and, in this context, studies involving repetitive sequences, which are showed to be important cytogenetic markers, may help uncover the evolutionary history of the group.

Repetitive sequences may be present in centromeres and telomeres of eukaryotic chromosomes which are rich regions in heterochromatin, as well as regions over the interstitial chromosomal arms (Csink and Henikoff 1998). The *in situ* hybridization experiments in the chromosomes of *Ancistrus* sp. showed that the Tc1-like element is located throughout all the chromosomes with preferential markings in heterochro-

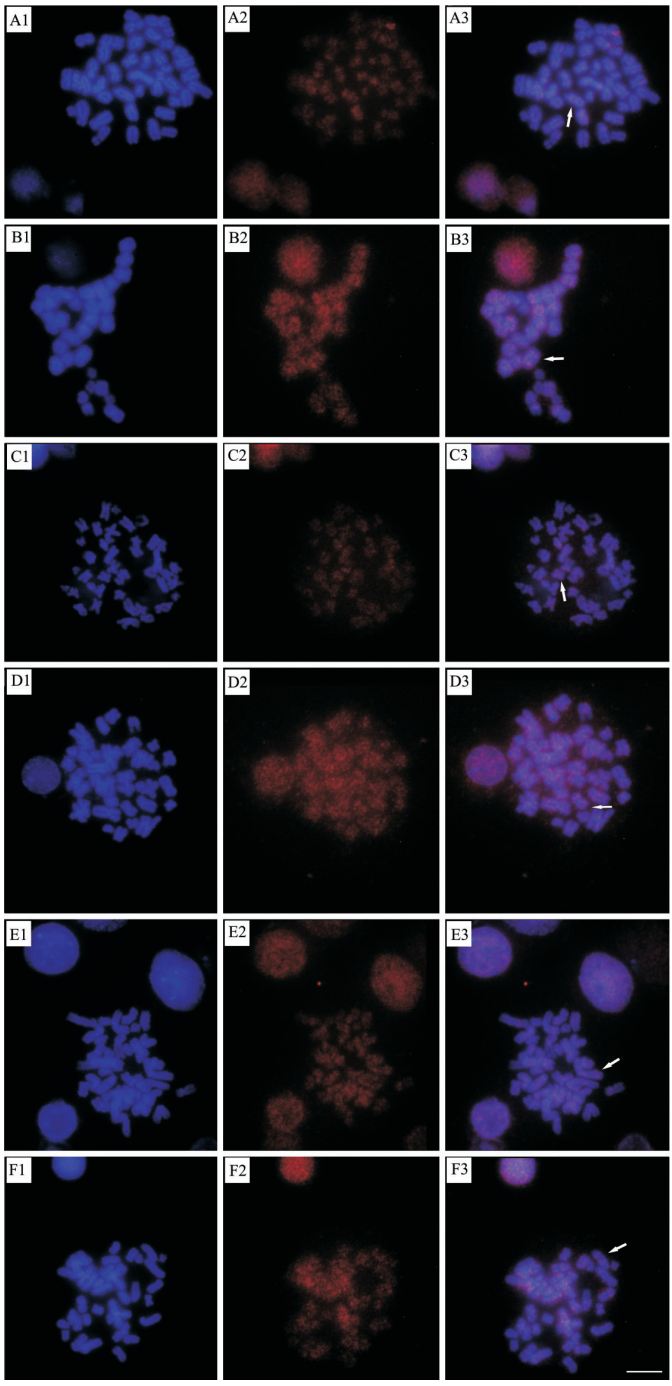

Figure 5. *In situ* fluorescence hybridization using AnDraI as probe in *Ancistrus* chromosomes: **A** Female of Currupira creek **B** Male of Currupira creek **C** Female of Pari creek **D** Male of Pari creek **E** Female of Sangradouro creek e **F** Male of Sangradouro creek. The arrows show some examples of pericentromeric and spread signals. Bar = 10 μm.

matic regions along the chromosomal arms, corroborating the results found in other studies carried out in other fish species, evidencing a pattern to disperse these elements (Capriglione et al. 2002, Ozouf-Costaz et al. 2004, Schemberger et al. 2014).

Results found in the literature about the genomic organization of transposons suggest that these elements are differently distributed in distinct groups of fish (Ferreira et al. 2011), as for example in the *Oreochromis niloticus* (Linnaeus, 1758) (Charlesworth et al. 1994, Martins 2007, Oliveira et al. 2013), Antartic Perciformes (Ozouf-Costaz et al. 2004) and in species of the subfamily Hypoptopomatinae (Dasilva et al. 2002), in which these elements can be found scattered throughout the genome. However, in species such as *Tetraodon nigroviridis* (Marion de Procé, 1822) (Dasilva et al. 2002, Fischer et al. 2004) and in most of the species of Cichlidae (Gross et al. 2009, Valente et al. 2011) they can be found accumulated in chromosomal regions rich in constitutive heterochromatin. Among the Siluriformes, the elements Rex1 and Rex3, in *Hisonotus leucofrenatu* (Ribeiro, 1908), *Pseudotocinclus tietensis* (Ihering, 1907) and *Parotocinclus maculicauda* (Steindachner, 1877), presented dispersed distributions patterns in the genome, similar to the pattern found for the transposable element AnDraI (Ferreira et al. 2011a), as well as a new dispersed element, BamHI, isolated by Ferreira et al. (2011b), in the genome of *Hisonotus leucofrenatus*. The genomic organization result of the Tc1-like element obtained in this work reinforces the hypothesis that the sequence AnDraI isolated is a dispersed element, and reinforces the hypothesis proposed by Ferreira et al. (2011) in which all transposable elements behave similarly inside of a family or subfamily. Also the study and characterization of these sequences can start to help to understand the evolutionary dynamics of *Ancistrus* genome, as well as the great karyotypic and chromosomal variability of this group, especially in the Paraguay river basin.

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RESEARCH ARTICLE

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Origin of B chromosomes in *Characidium alipioi* (Characiformes, Crenuchidae) and its relationship with supernumerary chromosomes in other *Characidium* species

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Abstract

B chromosomes are apparently dispensable components found in the genomes of many species that are mainly composed of repetitive DNA sequences. Among the numerous questions concerning B chromosomes, the origin of these elements has been widely studied. To date, supernumerary chromosomes have been identified in approximately 60 species of fish, including species of the genus *Characidium* Reinhardt, 1867 in which these elements appear to have independently originated. In this study, we used molecular cytogenetic techniques to investigate the origin of B chromosomes in a population of *Characidium alipioi* Travassos, 1955 and determine their relationship with the extra chromosomes of other species of the genus. The results showed that the B chromosomes of *C. alipioi* had an intraspecific origin, apparently originated independently in relation to the B chromosomes of *C. gomesi* Travassos, 1956 *C. pterostictum* Gomes, 1947 and *C. oiticicai* Travassos, 1967, since they do not share specific DNA sequences, as well as their possible ancestral chromosomes and belong to different phylogenetic clades. The shared sequences between the supernumerary chromosomes and the autosommal sm pair indicate the origin of these chromosomes.

Keywords

Microdissection, Chromosome painting, FISH, B chromosomes

Introduction

B or supernumerary chromosomes are extra genomic elements in addition to the standard chromosomal set (A) and are found in approximately 15% of eukaryotic organisms (Camacho 2005). In general, B chromosomes are derived from the A chromosomes of their own species (intraspecific origin) or closely related species (interspecific origin) (Banaei-Moghaddam et al. 2015). The intraspecific origin of B chromosomes was demonstrated in maize (Lamb et al. 2005; Peng et al. 2011), locusts (Teruel et al. 2010), rye (Martis et al. 2012) and fishes (Mestriner et al. 2000; Silva et al. 2013; Valente et al. 2014; Utsunomia et al. 2016). On the other hand, there are also cases in which B chromosomes may have arisen spontaneously in response to new genomic conditions such as interspecific hybridization, which has been observed in grasses of the genus *Coix* Linné, 1753 (Sapre and Deshepande 1987), the fish *Poecilia formosa* Girard, 1859 (Schartl et al. 1995) and the wasp *Nasonia vitripennis* Walker, 1836 (McAllister and Werren 1997).

A significant evolutionary feature of B chromosomes is the accumulation of repetitive DNA sequences (Camacho 2005; Houben et al. 2014; Banaei-Moghaddam et al. 2015). The cytogenetic and/or cytogenomic data about this type of DNA have been very informative for understanding the origin and evolution of B chromosomes. The findings using fluorescence *in situ* hybridization (FISH) techniques and genomic sequences analysis showed the intraspecific origin of B chromosomes and the presence and expression of intact genes in these elements in fishes (Silva et al. 2013, 2016; Utsunomia et al. 2016; Valente et al. 2014), as also observed in rye (Banei-Moghaddam et al. 2015).

Among genomes of fish species bearing B chromosomes, the genus *Characidium* Reinhardt, 1867 exhibits interesting cytogenetic features; the B chromosomes probably originated independently in the different species of this group, whereas the heteromorphic ZZ/ZW sex chromosomes seem to have originated once in the genus. Therefore, the B chromosomes of *C. oiticicai* Travassos, 1967 originated interspecifically, whereas in *C. gomesi* Travassos, 1956 and *C. pterostictum* Gomes, 1947, these elements have an intraspecific origin from the sex chromosomes (Pansonato-Alves et al. 2014, Serrano et al. 2016). In the present study, a novel occurrence of B chromosomes is described for *C. alipioi*, a species in which these elements were not found until now. In addition, the origin and evolution of these elements were studied using conventional cytogenetic techniques, including C-banding, microdissection, chromosome painting and fluorescence *in situ* hybridization with repetitive DNA probes.

Material and methods

Origin of the fishes/individuals, karyotype analysis and DNA extraction

A total of 19 *C. alipioi* samples were analyzed (9 females and 10 males) from the Ribeirao Grande river, Paraíba do Sul River Basin, Pindamonhangaba, São Paulo (22°49'00.3"S 45°25'23.7") (Table 1). The animals were collected in accordance with Brazilian envi-

Table 1. B chromosome polymorphisms in *Characidium alipioi*. Samples: number of males (M) and females (F) analyzed. Prevalence: the total and sex-specific percentage of individuals carrying B chromosomes.

Samples	Samples number with up to:					Prevalence
	50	**51**	**52**	**53**	**54**	
9 F	5	2	0	1	1	44%
10 M	5	3	1	0	1	50%
19 Total	**10**	**5**	**1**	**1**	**2**	**45%**

ronmental laws for the permission to collect issued by MMA/IBAMA/SISBIO, number 3245. The collection procedures, maintenance and analysis of the animals were performed in accordance with the international regulations for animal experiments, followed by the Universidade Estadual Paulista (CEEAA/IBB/UNESP protocol number 304). The samples were identified and deposited into the fish collection of the Biology and Genetics Laboratory of Fish at Botucatu, São Paulo, Brazil, under number 22287.

To perform the cytogenetic preparations, the animals were anesthetized and dissected, and mitotic chromosome preparations were obtained following the protocol of Foresti et al. (1981). C-banding was performed according to the protocol described by Sumner (1972). Chromosome morphology was determined according to Levan et al. (1964), and the chromosomes were classified as metacentric (m), submetacentrics (sm) and subtelocentric (st) and organized in the karyotype by descending size.

DNA extraction was performed using the Wizard Genomic DNA Purification Kit (Promega) according to the manufacturer's instructions.

Mitochondrial DNA analysis

Amplification and partial sequencing of cytochrome oxidase c subunit 1 (COI) and cytochrome b (Cyt B) was performed to identify the specimens. The primers used were as follows: Cyt BL 14841 (5'-CCA TCC CAA ATC ACT GCA TGA TGA AA-3 ') and Cyt BH 15915b (5'-AAC CTC TCT CGA GCT GAT TACAAG AC -3') (Kocher et al. 1989) for Cyt B and COI L6252-Asn (5'-AAG GCG GGG AAA GCC GCC GCA G -3 ') and H7271-COXI (5'-TCC TAT GCC GAA GTA TGG TTC TTT T 3') for COI (Melo et al. 2011). The sequences were analyzed using Geneious Pro v8.05 software, and the alignment was performed with the algorithm MUSCLE (Edgar 2004). The average distances between the sequences were calculated using the "pairwise deletion" option in MEGA 4.0 software (Tamura et al. 2007).

Microdissection and preparation of chromosomal probe

Chromosome microdissection was performed in an Eppendorf TransferMan NK2 micromanipulator coupled with a Zeiss Axiovert 100 microscope. For chromosome

painting, ten B chromosomes were microdissected from the cytogenetic preparations of the samples from each species (*C. alipioi*, *C. gomesi* and *C. oiticicai)* carrying one extra chromosome. The probes for *C. alipioi*, *C. gomesi* and *C. oiticicai* denoted CaB, CgB and CoB, respectively.

Microdissected DNA from each species was placed into a tube containing 9 μL of DNase-free ultrapure water and amplified using the GenomePlex Single Cell Whole Genome Amplification Kit (wga4 Sigma) (Gribble et al. 2004). After the initial amplification, the DNA probes CaB, CgB and CoB were generated and were then labeled with Digoxigenin-11-dUTP (Roche Applied Science) using the GenomePlex Whole Genome Amplification Reamplification Kit (wga3 Sigma) according to the manufacturer's protocol.

Repetitive DNA probe

Probes for 18S and 5S rDNA, U2 snDNA and histone H3 genes were obtained using PCR from the *C. alipioi* genome with previously described primers. Sequences for 5S and 18S rDNA were amplified using the *primers* 5SA (5'-TCA ACC AAC CAC AAA GAC ATT GGC AC-3') and 5SB (5'-TAG ACT TCT GGG TGG CCA AAG GAA TCA-3') (Pendás et al. 1995), 18S6F (5'-CTC TTT CGA GGC CCT GTA AT-3') and 18S6R (5'-CAG CTT TGC AAC CAT ACT CC-3') (Utsunomia et al. 2016). To amplify the H3 histone gene were utilized the following *primers*, H3F (5'-ATG GCT CGT ACC AAG CAG ACV GC-3') and H3R (5'-ATA TCC TTR GGC ATR ATR GTG AC-3') (Colgan et al. 1998). The U2 snDNA was amplified by the *primers* U2F (5'-ATC GCT TCT CGG CCT TAT G-3') and U2R (5'-TCC CGG CGG TAC AAT TGC A-3') (Silva et al. 2015).. The 18S rDNA, U2 snDNA and histone H3 probes were labeled with Digoxigenin-11-dUTP (Roche), and the 5S rDNA probe was labeled with biotin-16-dUTP (Roche). Oligonucleotide probes sequences containing microsatellite $(CA)_{15}$, $(GA)_{15}$ and $(GAG)_{10}$ were labeled directly with TAMRA during the synthesis process by Sigma according to methods described by Kubat et al. (2008).

Fluorescent in situ hybridization (FISH)

For the FISH experiments, the prehybridization conditions were performed according to procedures described by Pinkel et al. (1986). Posthybridization washes were performed according to the applied probe: (i) the slides probed with rDNAs, snDNA, histone and B chromosome probes were washed in 0.2× SSC/15% formamide for 20 min at 42°C, followed by a second wash in 0.1× SSC for 15 min at 60°C and a final wash at room temperature in 4× SSC/0.5% Tween for 10 min; probe detection was performed using avidin-FITC and anti-digoxigenin-rhodamine; and (ii) the slides probed with oligonucleotides were washed in 2× SSC for 5 min, followed by a second wash in 1×

PBS for 1 min. Chromosomes were counterstained with DAPI (Vector Laboratories, Burlingame, Calif., USA). The images were digitally captured using Image Pro Plus 6.0 software (Media Cybernetics) with the appropriate filters on an epifluorescence microscope (Olympus BX61) equipped with an Olympus DP70 camera. The final composition of the images was performed using Adobe Photoshop CS6 image editor software with the image and uniform size scales.

Results

Analysis of mitochondrial DNA

Analysis of mitochondrial DNA was performed in order to make a correct identification of the specimens, besides that, the position of the clades could be informative to discuss aspects of the origin of the B chromosomes in the genus. The average distance analysis of the COI and CytB sequences obtained in this study and other species taken from GenBank showed high similarity between the *C. alipioi* sequences of Santa Bárbara do Tugúrio-MG and the specimens analyzed here (Suppl. materials 1, 2), which confirmed the taxonomic status of the analyzed samples (Figure 1).

Chromosomal analysis

The analyzed *C. alipioi* individuals showed diploid chromosome number 2n = 50 and karyotype composed of 32 m + 18 sm with heteromorphic ZZ/ZW sex chromosomes, which was similar to the findings in other species of the genus (Figure 2). In addition, cells bearing 0-4 B chromosomes were observed in 45% of the individuals (Table 1). The B chromosomes were mitotically unstable, once the number of these chromosomes can variete between cells of the same individual. C-banding showed constitutive heterochromatin blocks in the pericentromeric region of all chromosomes and a conspicuous distal block in the longer arm of the Z chromosome. The W and B chromosomes were entirely heterochromatic (Figure 2b).

Chromosome painting

Chromosome painting performed in the *C. alipioi* chromosomes with the CaB probe showed signals on the entire length of the B chromosomes and the pericentromeric region of the sm chromosome pair nº 19 of the A complement (Figure 3a). On the other hand, this same probe did not reveal any signals on the *C. gomesi* and *C. oiticicai* chromosomes (Figure 3b and c). Similar findings were observed by hybridization with the CoB probe in *C. alipioi* chromosomes (Figure 3e). Conversely, the CgB probe showed hybridization signals on the W chromosome of *C. alipioi* (Figure 3d).

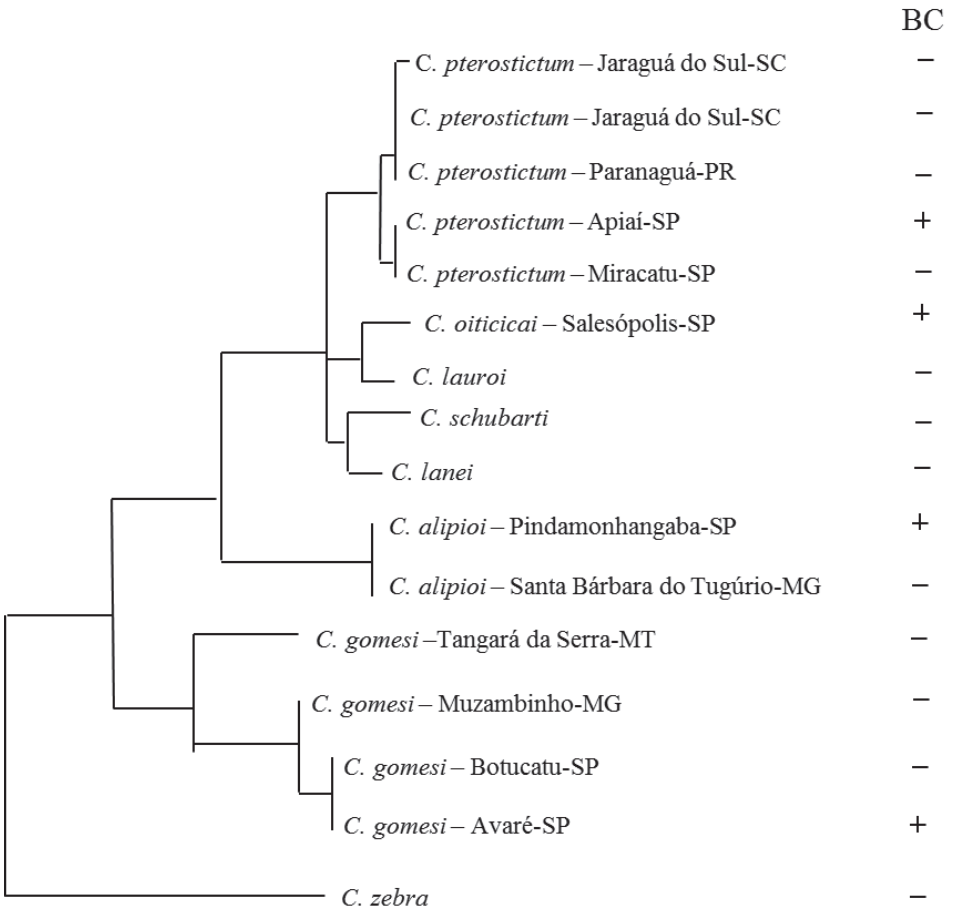

Figure 1. *Characidium* phylogeny adapted from Pansonato-Alves et al. (2014) and positioned for the population analyzed here. BC: presence or absence of B chromosomes.

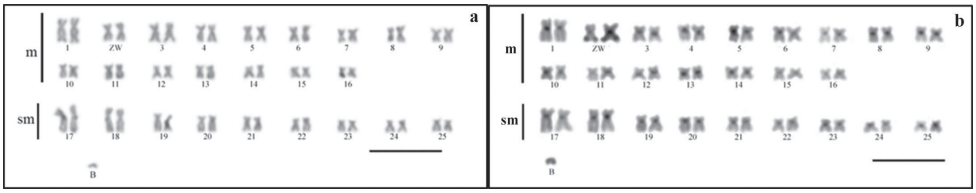

Figure 2. *Characidium alipioi* karyotypes arranged from mitotic metaphases after to conventional Giemsa-staining (**a**) and C-banding (**b**). Bar = 10 μm.

Distribution of repetitive DNA probes

FISH experiments on the *C. alipioi* chromosome preparations using a 18S rDNA probe revealed sites of this gene in the terminal position of autosomal pair No. 18,

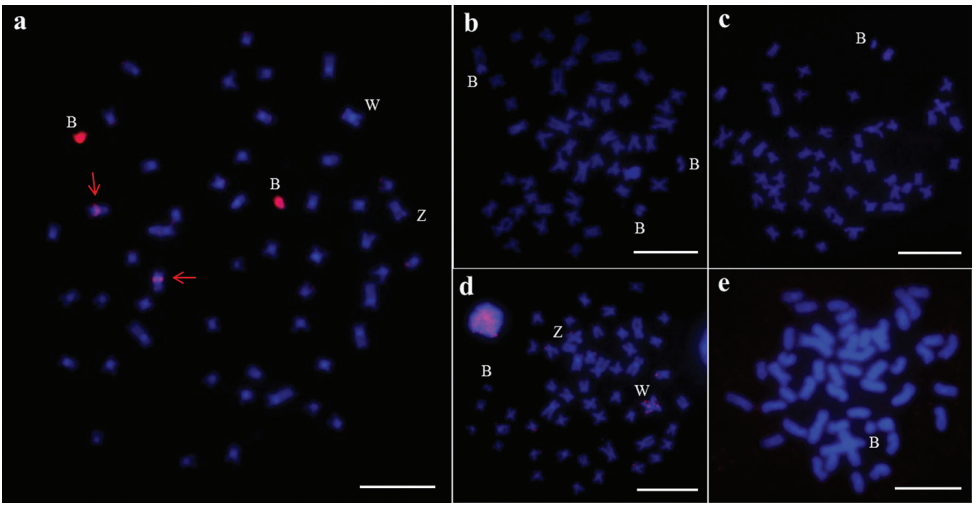

Figure 3. Cross-species chromosome painting. CaB probe in *Characidium alipioi* (**a**), *C. gomesi* (**b**) and *C. oiticicai* (**c**) and with the CgB (**d**) and CoB (**e**) probes in *C. alipioi*.

whereas the 5S rDNA was mapped in the pericentromeric region of chromosome pair No. 20 (Figure 4a). Histone H3 sites were found in the m chromosome pair No. 10 (Figure 4b), whereas the U2 snRNA gene was located on the sm chromosomes pair No. 17 (Figure 4c).

FISH with probes containing the microsatellite sequences $(CA)_{15}$ and $(GA)_{15}$ in the *C. alipioi* chromosomal preparations showed similar patterns of distribution with preferential accumulation in the terminal regions of the chromosomes, except for the W and B chromosomes (Figure 5a and b), which had lower abundance and weak hybridization signals. Furthermore, the intense signals of the microsatellite $(GAG)_{10}$ showed preferential accumulation on the W and B chromosomes of this species (Figure 5c).

Discussion

The occurrence of B chromosomes in *Characidium alipioi* genome was revealed for the first time in this study, particularly since the population of *C. alipioi* of Santa Bárbara do Tugúrio-MG analyzed by Pansonato-Alves et al. (2014) showed no extra chromosomes. The presence of these elements in only certain populations was observed in other species of the genus *Characidium* such as *C. zebra*, *C. pterostictum* and *C. gomesi* (Pansonato-Alves et al. 2014). Our data does not allow to conclude about the dynamics or relationship of these chromosomes between populations; however, at least two mechanisms, which are based on the findings of two model organisms have been proposed to be involved in the absence of B chromosomes in certain populations: geographical barriers that limit the spread of individuals with these elements in

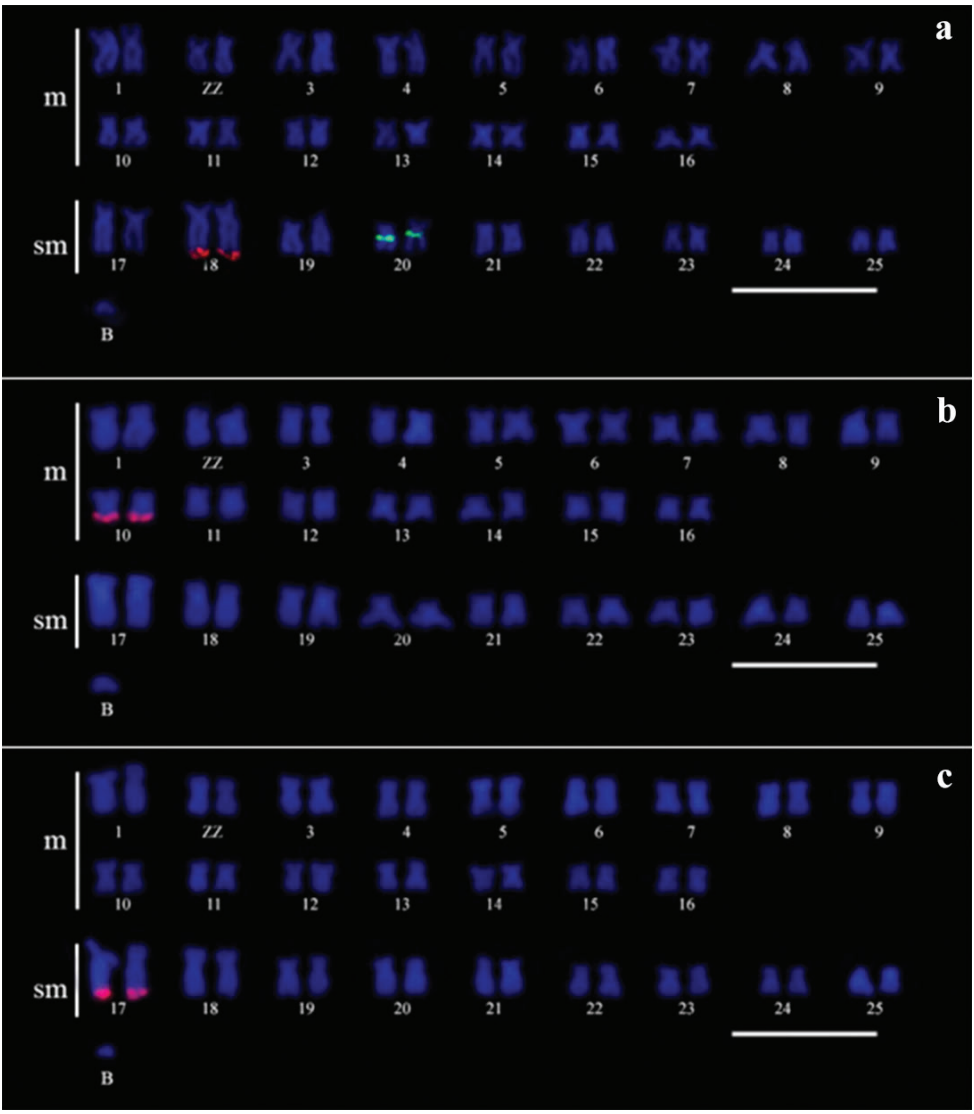

Figure 4. Karyotypes of *Characidium alipioi* arranged from mitotic metaphases after FISH with repetitive DNA probes. **a** 18S and 5S rDNA probes **b** histone H3 probe, and **c** U2 snRNA probe. Bar =10 μm.

Eyprepocnemis plorans Charpentier, 1825 (Cabrero et al. 1997, Manrique-Poyato et al. 2015) and the close relationship between temperature and rainfall factors that could influence the variations in the presence of B chromosomes, as observed in *Myrmeleotettix maculatus* Thunberg, 1815 (Hewitt and John 1967; Hewitt and Ruscoe 1971).

The individuals of the same species and location included in this study were analyzed in a previous study by Centofante et al. (2003); however, these authors did not detect B chromosomes in their analysis. Although cytogenetic studies in *Characidium* have revealed the occurrence of supernumerary chromosomes restricted to certain

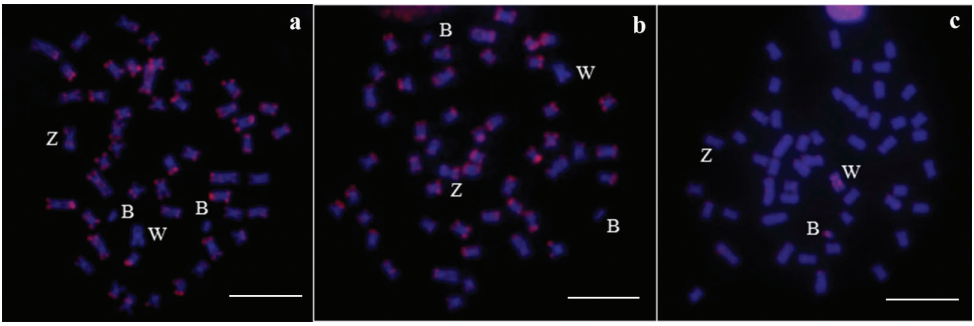

Figure 5. Mitotic metaphases after FISH with the microsatellites. **a** $(GA)_{15}$ **b**) $(CA)_{15}$ and **c**) $(GAG)_{10}$. Note the accumulation of (GAG) 10 in the B and W chromosomes.

populations of some species (Pansonato-Alves et al. 2014), it should be noted that the geographical proximity of the sampling sites and the time between the present analysis and the analysis by Centofante et al. (2003) suggest that the two samples do not belong to different populations. Similarly, it can't be stated that the B chromosomes have arisen in this population after the first study. However, because some individuals with no B chromosomes were also identified in the present study, it is likely that the samples analyzed in the 2003 study were formed by individuals who do not carry these extra elements.

Chromosome painting using the B chromosomes of *C. alipioi* (CAB) as the probe indicated shared sequences between the A and B complements and, more specifically, the pericentromeric region of a sm chromosome pair (pair No. 19). This result probably reflects a relationship between these chromosomes and intraspecific origin of the supernumerary chromosomes in genome of this species and that pair No. 19 is likely the chromosome of its origin. Previous studies have identified the origin of B chromosomes from the A complement of the host species (Bugrov et al. 2003; De Jesus et al. 2003; Lamb et al. 2005; Teruel et al. 2010; Peng et al. 2011; Silva et al. 2014; Valente et al. 2014; Utsunomia et al. 2016); however, in a few cases it was possible to identify the ancestral chromosome, such as in the grasshopper (Teruel et al. 2010; Bueno et al. 2013) and in two fish species *Astyanax paranae* Eigenmann, 1914 (Silva et al. 2014) and *Moenkhausia sanctaefilomenae* Steindachner, 1907 (Utsunomia et al. 2016).

The intraspecific origin of B chromosomes has been reported in other species of the genus *Characidium*, namely *C. pterostictum*, *C. gomesi* and *Characidium* sp. aff. *C. vidalli* (Pansonato-Alves et al. 2014; Schacchetti et al. 2015). However, because in these species this chromosome originated from the Z and W chromosomes and the CaB probe in the present study showed no signals on these chromosomes, these supernumerary elements do not seem to share the same ancestral supernumerary chromosomes that are present in *C. alipioi*. In addition, the *C. alipioi* B chromosomes showed no homology with the supernumeraries of *C. gomesi* and *C. oiticicai*, which are two species whose B chromosomes are apparently composed of different types of repetitive DNA and have different origins

(Pansonato-Alves et al. 2014). Therefore, it possible that the supernumerary elements in *C. alipioi* originated independently from the other types of B chromosomes reported in the representatives of this genus, and the phylogenetic position of these species at different clades (Figure 1) supports this assumption. However, these conclusions should be made with caution because the probes obtained by microdissection are composed of anonymous sequences, and the amplification method (GenomePlex) may favor specific sequences of repetitive DNA that are present in the B chromosomes and absent on other A chromosomes. In this context, it cannot be ruled out that other sequences shared between A and B chromosomes are not represented in the probes used in this study, which was previously noted by Pansonato-Alves et al. (2014).

Hybridizations with microsatellite DNA sequences demonstrated the presence of these repetitive elements in B chromosomes. $(GA)_{15}$ and $(CA)_{15}$ are both dispersed with conspicuous blocks in the terminal regions of the A chromosomes and are less abundant in the supernumerary chromosomes. Moreover, a clear accumulation was observed with respect to the $(GAG)_{10}$ sequence. Notably, the hybridization with the $(GAG)_{10}$ probe revealed a preferential accumulation in B and W chromosomes in *C. alipioi*. Similarly, microsatellites were detected in the B chromosomes of maize (Ananiev et al. 2005), rye (Langdon et al. 2000) and locusts (Milani and Cabral-de-Mello 2014; Ruiz-Ruano et al. 2015). Given that the CaB probe did not paint the *C. alipioi* sex chromosomes, a possible explanation for the accumulation of $(GAG)_{10}$ in the B and W chromosomes would be that the CaB probe does not contain this microsatellite, which probably occurred due to the amplification method used in this study, as mentioned above. However, if it is considered that the accumulation mechanisms of this type of repetitive DNA permit its fixation on certain chromosomes due to its non-recombinant nature and preferential accumulation in heterochromatic regions, as observed in other studies (Cuadrado and Jouve 2011, Lohe et al. 1993, Scacchetti et al. 2015), then the distribution of microsatellites in these chromosomes may not reflect the aspects of its origin, but its accumulation after the appearance of the B chromosomes.

Our present results extend the knowledge of the structure and composition of B chromosomes between representatives of the *Characidium* genus, particularly in *C. alipioi*. In addition, the shared sequences between the A and B chromosomes of this species suggests an intraspecific origin of these chromosomes that is independent from the B chromosomes of other congeneric species. These observations reinforce the idea that this fish group is an interesting model to study the origin and structure of B chromosomes.

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Supplementary material 1

Table S1

Authors: Érica Alves Serrano, Ricardo Utsunomia, Patrícia Sobrinho Scudeller, Claudio Oliveira, Fausto Foresti

Data type: molecular data

Explanation note: Average distance between the CytB sequences of the *Characidium* species.

Supplementary material 2

Table S2

Authors: Érica Alves Serrano, Ricardo Utsunomia, Patrícia Sobrinho Scudeller, Claudio Oliveira, Fausto Foresti

Data type: molecular data

Explanation note: Average distance between the COI sequences of the *Characidium* species.

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RESEARCH ARTICLE

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Variation in genome size and karyotype among closely related aphid parasitoids (Hymenoptera, Aphelinidae)

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Abstract

Genome sizes were measured and determined for the karyotypes of nine species of aphid parasitoids in the genus *Aphelinus* Dalman,1820. Large differences in genome size and karyotype were found between *Aphelinus* species, which is surprising given the similarity in their morphology and life history. Genome sizes estimated from flow cytometry were larger for species in the *A. mali* (Haldeman, 1851) complex than those for the species in the *A. daucicola* Kurdjumov, 1913 and *A. varipes* (Förster,1841) complexes. Haploid karyotypes of the *A. daucicola* and *A. mali* complexes comprised five metacentric chromosomes of similar size, whereas those of the *A. varipes* complex had four chromosomes, including a larger and a smaller metacentric chromosome and two small acrocentric chromosomes or a large metacentric and three smaller acrocentric chromosomes. Total lengths of female haploid chromosome sets correlated with genome sizes estimated from flow cytometry. Phylogenetic analysis of karyotypic variation revealed a chromosomal fusion together with pericentric inversions in the common ancestor of the *A. varipes* complex and further pericentric inversions in the clade comprising *Aphelinus kurdjumovi* Mercet, 1930 and *Aphelinus hordei* Kurdjumov, 1913. Fluorescence *in situ* hybridization with a 28S ribosomal DNA probe revealed a single site on chromosomes of the haploid karyotype of *Aphelinus coreae* Hopper & Woolley, 2012. The differences in genome size and total chromosome length between species complexes matched the phylogenetic divergence between them.

Keywords

Aphelinidae, *Aphelinus*, parasitoid, genome size, flow cytometry, karyotype

Introduction

Genome size estimates and karyotypic studies provide data for comparative research at various taxonomic levels and allow evaluation of phylogenetic associations (Gokhman 2009, Hanrahan and Johnston 2011, Lopes et al. 2009). The completeness of genome assemblies can be difficult to assess, and independent estimates of genome size can aid in the assessment of completeness of genome assemblies (Gregory et al. 2013). Flow cytometry and Feulgen densitometry have been used to accurately measure genome size, and both methods have been extensively validated and various sources of error have been minimized through best-practice protocols (Gregory et al. 2013, Hare and Johnston 2011). Karyotypes can further help in assessing genetic linkage maps, and thus aid in mapping quantitative trait loci (Gokhman and Kuznetsova 2006). To visualize karyotypic features, various techniques of conventional and differential staining of chromosomes have been used, including fluorescence *in situ* hybridization (FISH), which allows physical mapping of DNA sequences onto chromosomes (Gadau et al. 2014, Macgregor and Varley 1988). Increasing the numbers of genome size estimates and karyotypes across the tree of life provides resources for the advancement of evolutionary genomics (Jacobson et al. 2013, Sharakhova et al. 2014). Furthermore, both flow cytometry and karyotypes can be used to detect cryptic species (Baur et al. 2014, Vergilino et al. 2012).

Genome size estimates have been published for more than 13,000 species of animals and plants (Animal Genome Size Database, http://www.genomesize.com; Plant DNA C-values Database, http://data.kew.org/cvalues; accessed 29 August 2014). There are currently 930 estimates of insect genome size in the Animal Genome Size Database, 152 of which are for species of Hymenoptera, and these genome sizes range from 98 to 1115 Mb. Genome size is usually considered constant within species, and limited intraspecific variation is a standard assumption in measurement and comparison of genome sizes. However, genome size can vary widely between closely related species (Gregory and Johnston 2008) and even within species (Biemont 2008, Bosco et al. 2007). The most common source of inter- and intraspecific genome size variation is differing amounts of repetitive DNA (Biemont 2008, Bosco et al. 2007). Differences in chromosome size can result from differences in heterochromatin content and amount of repetitive DNA in euchromatin, and differences in both chromosome size and number can result from fissions and fusions (Gokhman 2009, White 1973). Chromosome numbers and other karyotypic features have been published for about 70,000 species of plants and animals (Rice et al. 2014, White 1973; Tree of Sex: A database of sexual systems, doi: 10.1038/sdata.2014.15), including more than 1,500 species of Hymenoptera, whose haploid chromosome numbers range from 1 to 60 (Gokhman 2009, Ross et al. 2015).

Here we report genome size estimates and karyotypes for males and females in nine species of *Aphelinus* Dalman, 1820 (Hymenoptera: Chalcidoidea: Aphelinidae) all of which are parasitoids of aphids. Parasitoids are free-living as adults, but are

parasitic as larvae, and represent one of the most species-rich groups of insects, constituting more than 10% of all described insect species (Eggleton and Belshaw 1992, Heraty et al. 2007). Parasitoids are important regulators of arthropod populations, including major agricultural pests (Godfray 1994). The genus *Aphelinus* comprises more than 90 recognized species (Hopper et al. 2012; Universal Chalcidoidea Database, www.nhm.ac.uk/entomology/chalcidoids/index.html, accessed 10 October 2016). Within *Aphelinus*, several complexes of closely related species provide excellent opportunities to explore genetic differentiation, speciation, and the evolution of reproductive compatibility, host use, and morphology (Heraty et al. 2007, Hopper et al. 2012). We studied species in three complexes of *Aphelinus*: (1) five species in the *A. varipes* (Förster,1841) complex from throughout Eurasia; (2) three species in the *A. mali* (Haldeman, 1851) complex from eastern Asia; (3) one species in the *A. daucicola* Kurdjumov, 1913 complex from North America. The *A. varipes* complex comprises 12 described species (Förster 1841, Hayat 1972, 1994, Hayat and Fatima 1992, Howard 1908, Kurdjumov 1913, Nikol'skaya and Yasnosh 1966, Pan 1992, Yasnosh 1963). The monophyly of the *A. varipes* complex is well supported by a combination of morphological and genetic characters (Heraty et al. 2007). However, some species within the complex show little morphological divergence, making identification difficult. The *A. mali* complex comprises 14 recognized species, some of which also show little morphological divergence (Ashmead 1888, Evans et al. 1995, Gahan 1924, Girault 1913, Haldeman 1851, Hayat 1998, Hopper et al. 2012, Prinsloo and Neser 1994, Timberlake 1924, Yasnosh 1963, Zehavi and Rosen 1988). The *A. daucicola* species complex comprises three species that differ from the members of the *A. mali* complex in several traits (Hopper et al. 2012). Using flow cytometry, we estimated the genome sizes of species in these complexes. We also made and examined chromosomal preparations to determine their karyotypes. We found consistent differences in genome size between complexes, and these differences correlated with differences in relative sizes estimated from karyotypes. We detected chromosomal rearrangements as well as karyotypic synapomorphies.

Materials and methods

Specimens

The parasitoid species studied, the sources of the colonies, and the permit and voucher numbers are listed in Table 1. These colonies were reared on aphids at the USDA-ARS, Beneficial Insect Introductions Research Unit, in Newark, Delaware, USA. Vouchers for these populations are maintained at -20 °C in 100% molecular grade ethanol at the Beneficial Insect Introduction Research Unit, Newark, Delaware. Females of the yellow-white strain of *Drosophila melanogaster* (Meigen, 1830) (stock number 1495, obtained from the Bloomington Drosophila Stock Center at

Table 1. The nine *Aphelinus* species studied, the year and country of their collection, permit and voucher numbers.

Species complex	Species	Authority	Year	Country	Permit and voucher
A. varipes	*A. atriplicis*	Kurdjumov, 1913	2000	Georgia	P526P-15-04274, VGg00_Dn
	A. varipes	(Förster, 1841)	2009	France	P526P-13-02503, VFr09_Rp
	A. certus	Yasnosh, 1963	2001	Japan	P526P-01-53096, VJp01_TU
	A. kurdjumovi	Mercet, 1930	2000	Georgia	P526P-13-02503, VGg00_Rp
	A. hordei	Kurdjumov, 1913	2011	France	P526P-15-04274, VFr11_Dn
A. daucicola	*A. daucicola*	Kurdjumov, 1913	2013	USA	P526P-15-04274, DUSA12_UD
A. mali	*A. glycinis*	Hopper et Woolley, 2012	2007	China	P526P-08-02142, MKor09_M
	A. coreae	Hopper et Woolley, 2012	2009	Korea	P526P-01-72318, MCh04_Bj
	A. rhamni	Hopper et Woolley, 2012	2005	China	P526P-01-53096, MCh05_Bj

Indiana University, http://flystocks.bio.indiana.edu) were used as internal controls for flow cytometry. All institutional and national guidelines for the care and use of laboratory animals were followed.

Flow cytometry

Live *Aphelinus* were sexed, flash frozen in liquid nitrogen, and stored at –80°C. To estimate genome sizes, we used the flow cytometry protocol described by Hanrahan and Johnston (2011) and Hare and Johnston (2011). We dissected heads from both males and females of the *Aphelinus* species in cold Galbraith buffer (Galbraith et al. 1983). Heads of female *D. melanogaster* were used as internal standards (1C = 175 Mb or 0.17 pg). To release the nuclei from cells, heads from 15 female *Aphelinus* and one female *Drosophila* Fallén, 1823 for each replicate were ground together in one milliliter of cold Galbraith buffer using 15 strokes of the "A" pestle in a 2-ml Kontes Dounce tissue grinder. As with other Hymenoptera, *Aphelinus* species have haplodiploid sex determination, with males coming from unfertilized eggs and females from fertilized eggs. Thus males carried half as much DNA per cell as females, which made male genome sizes too close to that of *D. melanogaster*. Thus when processed the heads of 15 males per replicate as described above, we included the heads from 15 females of the same parasitoid species as internal standards. The samples were passed through a 35 micron filter and then stained with 40 parts per million of propidium iodide in the dark for 3–5 hours at 4°C. Samples were analyzed with laser excitation at 488 nm on a Becton Dickinson FACSCalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA) at CTCR Core Facility, University of Delaware. Red fluorescence from the propidium iodide was detected using an FL2 filter. Three to six replicates were measured for females and males of each species.

The haploid content of DNA in megabases (Mb) was calculated for each *Aphelinus* sample from the ratio of mean fluorescence of the sample to mean fluorescence of the

standard times the genome size of the standard. We report genome size estimates in megabases, but also give estimates in picograms (pg) calculated by dividing the amount of DNA in Mb by the standard 1C value of 978 Mb.

Karyotypes

Chromosome preparations were made from cerebral ganglia of prepupae using a modified version of the technique in Imai et al. (1988). Wasps were dissected in 0.5% hypotonic sodium citrate solution containing 0.005% colchicine, and the tissues were incubated in fresh solution for ~30 minutes at room temperature. The material was transferred to a pre-cleaned microscope slide using a Pasteur pipette and gently flushed with Fixative I (glacial acetic acid: absolute ethanol: distilled water 3:3:4). Tissues were disrupted in an additional drop of Fixative I using dissecting needles. Another drop of Fixative II (glacial acetic acid: absolute ethanol 1:1) was then applied to the center of the area and blotted off the edges of the slide. The slide was air dried for ~30 minutes at room temperature. For conventional staining, preparations were stained with freshly prepared 3% Giemsa solution in 0.05M Sørensen's phosphate buffer (Na_2HPO_4 + KH_2PO_4, pH 6.8). Mitotic divisions were studied and photographed using an optic microscope Zeiss Axioskop 40 FL fitted with a digital camera AxioCam MRc (Carl Zeiss, Oberkochen, Germany). To obtain karyograms, the resulting images were processed with image analysis programs: Zeiss AxioVision version 3.1 and Adobe Photoshop version 8.0. Mitotic chromosomes were measured for 5–19 cells in 1–6 wasps per species using Adobe Photoshop. We report total length (μm) of all chromosomes in each karyotype for males and females; for diploid sets, we divided total length by two to make the values comparable to haploid sets. We also report relative lengths (RL: 100 × length of each chromosome divided by total length of the set) and centromeric indices (CI: 100 × length of shorter arm divided by total length of a chromosome) for females of each species. Chromosomes were classified into metacentric (M) or acrocentric (A) according to the guidelines in Levan et al. (1964).

Fluorescence *in situ* hybridization

A custom biotinylated fragment from the 28S rDNA gene was used to probe *A. coreae* chromosomes with fluorescence *in situ* hybridization (FISH). To prepare the probe, we extracted DNA from ~50 adult parasitoids using a Qiagen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). From this DNA, we amplified a ~650 nt fragment of the 28S rDNA gene using the following primers and PCR protocol: reaction mix - 5 μl NEB PCR buffer and 0.5 μl *Taq* polymerase (New England Biolabs, Ipswich, MA, USA), 4 μl each of 2.5 mM dATP, dCTP, dGTP, 4 μl 0.25 mM dTTP plus 1 μl 1mM biotinylated-11-dUTP, 1 μl 10 μM forward primer (5'-cgt gtt gct tga tag tgc agc) and 1 μl 10 μM reverse primer (5'-tca aga cgg gtc ctg aaa gt), 4 μl genomic DNA (50 ng/μl),

21.5 µl ultrapure H_2O; cycling - 3 min at 95 °C, then 35 cycles 95 °C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec, and a final extension at 72 °C for 3 min. Unincorporated dNTPs, primers, and other unwanted components were removed from the PCR product using precipitation with sodium acetate and ethanol, and the resulting pellet was resuspended in 50 µl ultrapure H_2O, yielding a solution of probe at 300 ng/µl.

Chromosomes were prepared for probing using the protocol described above for karyotyping. To probe the chromosomes, a protocol modified from Matsumoto et al. (2002) was used. Chromosomes were baked onto slides at 65°C and UV crosslinked in a Spectrolinker XL-1000 UV Crosslinker (Spectronics, Westbury, NY, USA) twice at 120 mJ/cm^2. The slides were treated with RNase A, dehydrated with ethanol, denatured in formamide, and then dehydrated again with ethanol. The slides were then treated with proteinase K and dehydrated a third time with ethanol. Hybridization solution was prepared from the biotinylated probe (7 µg in 23 µl), formamide (90 µl), 30% dextran sulfate solution (60 µl), 10 mg/ml salmon sperm DNA (5 µl), and 20x SSC (22 µl). This solution was denatured at 95°C, placed on ice, and then 50 µl was applied to each slide, which were then covered with parafilm and left in a moist chamber at 37°C for 12–16 h. The slides were washed twice in formamide (50% in 2x SSC) and twice in 2x SSC with gentle shaking, and transferred to BN buffer (100 mM $NaHCO_3$, 0.1% Nonidet P-40) for 10 min at room temperature. After this, blocking buffer (100 mM $NaHCO_3$, 0.05% Nonidet P-40, 0.02% NaN_3, 5% non-fat dry milk) was applied, and the slides were covered with parafilm and incubated for 10 min at room temperature. The buffer was removed, streptavidin-Alexa fluor 568 conjugate (Thermo Fisher Scientific, Waltham, MA, USA), diluted 1/50 in blocking buffer, was added, and the slides were covered with parafilm and incubated at 37°C for 1 h. The slides were washed with three changes of BN buffer in a light-tight chamber with gentle shaking. Signal enhancement was done following Pinkel et al. (1986). Fifty microliters of biotinylated goat anti-Avidin D (Vector Laboratories, Burlingame, CA, USA), diluted 1/50 in the blocking buffer, was applied to each slide, which were then covered with parafilm and incubated at 37°C for 1 hour. The slides were then washed with BN buffer, more streptavidin-labeled fluor was added, the slides were washed again with BN buffer, and then air-dried in the dark at room temperature. Anti-fade medium (ProLong Gold Antifade Reagent with DAPI, Cell Signaling Technology, Danvers, MA, USA) was added and a glass coverslip was placed over the chromosomal preparation. The chromosomes were imaged using a Zeiss 510 NLO Multiphoton microscope and a Zeiss Elyra PS 1 microscope (Carl Zeiss, Pleasanton, CA, USA) with confocal microscopy at the Bio-Imaging Center, Delaware Biotechnology Institute, Newark, DE, USA.

Data analysis

Genome sizes and total lengths of chromosome sets were compared among species and between sexes in generalized linear models with species and sex as fixed main-effects

and Poisson error distributions using the glm function in R (R Core Team 2014). The set of relative lengths among species in a multivariate analysis of variance was compared with the Pillai–Bartlett statistic and the manova function in R. Centromeric indexes among species in generalized linear models were compared for each chromosome with species as a fixed effect and Poisson error distributions using the glm function in R. Because chromosomal formulae were different for the *A. varipes* complex *versus* the *A. mali* and *A. daucicola* complexes, we analyzed the effects of species on relative lengths and centromeric indexes separately within these groups. For genome size, the experimental unit was either 15 heads of female parasitoids and one *D. melanogaster* head pooled or 15 heads of male parasitoids and 15 heads of female parasitoids pooled. For total lengths of chromosome sets and relative lengths and centromeric indexes of chromosomes, the experimental unit was an individual mitotic cell. Post-hoc comparisons of means were done using the glht and cld functions in the multcomp package in R. We tested the relationships between genome sizes from flow cytometry and total lengths of chromosome sets with linear regression using the lm function in R. Data are archived on the Ag Data Commons website (data.nal.usda.gov; DOI 10.15482/USDA.ADC/1329930).

Results

Genome sizes from flow cytometry

Haploid genome sizes of *Aphelinus* differed among species (model deviance = 444.0; residual deviance = 5.4; df = 6, 60; $P < 0.0001$). Female genome sizes ranged from 330 to 483 Mb so the largest was 1.5 times the smallest (Table 2, which also shows results of multiple comparisons among means of each species). Female and male *Aphelinus* had similar haploid genome sizes, with female and male sizes within 2–13 Mb (1–4 percent) of one another, so the sexes did not differ significantly (model deviance = 1.1; residual deviance = 4.3; df = 1, 59; $P = 0.30$). Genomes (averaged across sexes) in the *A. mali* complex were significantly larger (37–148 Mb or 9–44 percent) than those *A. varipes* complex, and genomes in the *A. varipes* complex were significantly larger (1–59 Mb or 1–18 percent) than those in the *A. daucicola* complex (model deviance = 378.10; residual deviance = 73.3; df = 6, 60; $P < 0.0001$). The genome of *A. rhamni* was significantly larger (43–53 Mb or 10–12 percent) than the genomes of the other species in the *A. mali* complex. The genome of *A. hordei* was significantly larger (40–58 Mb or 9–17 percent) than the genomes of the other species in the *A. varipes* complex.

Karyotypes

Species in the *A. varipes* complex had four chromosomes in haploid males and thus eight chromosomes in diploid females, whereas species in the *A. mali* and *A. daucicola*

Table 2. Haploid genome sizes of nine *Aphelinus* species estimated from flow cytometry. Shared letters after means indicates that they do not differ significantly.

Species complex	Species	Sex	n replicates	Genome size		95% CI
				(pg)	(Mb)	(Mb)
A. varipes	*A. atriplicis*	female	6	0.361	353a	338–368
		male	3	0.366	358a	337–380
	A. varipes	female	3	0.340	333a	313–354
		male	3	0.348	340a	320–362
	A. certus	female	6	0.369	361a	347–377
		male	3	0.375	367a	346–390
	A. kurdjumovi	female	4	0.356	348a	331–367
		male	3	0.363	355a	334–377
	A. hordei	female	4	0.402	393b	374–412
		male	3	0.406	397b	375–421
A. daucicola	*A. daucicola*	female	4	0.337	330a	313–348
		male	3	0.351	343a	322–364
A. mali	*A. glycinis*	female	6	0.442	432c	416–449
		male	5	0.441	431c	413–450
	A. coreae	female	3	0.449	439c	416–464
		male	3	0.454	444c	421–468
	A. rhamni	female	6	0.494	483d	466–501
		male	3	0.498	487d	463–513

complexes had five chromosomes in haploids and thus ten chromosomes in diploids (Table 3; Figs 1–2). Karyotypes of the *A. varipes* complex usually included a large metacentric chromosome 1 and a small metacentric chromosome 2 and small acrocentric chromosomes 3 and 4, except for *A. kurdjumovi*, in which the small metacentric chromosome 2 appears to have been replaced by an acrocentric chromosome 2 of similar size; whereas species in the *A. mali* and *A. daucicola* complexes had metacentric chromosomes only, and their chromosomes showed a continuous gradation in length (Tables 4 and 5). Relative lengths of chromosome sets differed significantly among species in the *A. varipes* complex ($F = 3.2$; df = 16, 540; $P < 0.0001$), but did not quite differ significantly among species in the *A. mali* and *A. daucicola* complexes ($F = 1.7$; df = 12, 255; $P = 0.07$).

Centromeric indexes for chromosome 1 did not differ among species, and centromeric indexes for chromosome 2 did not differ among species in the *A. mali* and *A. daucicola* complexes. However, in the *A. varipes* complex, the centromeric index of chromosome 2 in *A. hordei* was significantly lower than in other members of the *A. varipes* complex. Centromeric indexes for chromosomes 3 and 4 in *A. daucicola* were significantly lower than those for *A. rhamni*, and the centromeric index for chromosome 5 in *A. daucicola* was significantly lower than those for *A. coreae* and *A. rhamni* (Table 6). Total lengths of chromosome sets differed among species

Table 3. Karyotypic features of nine *Aphelinus* species. Shared letters after means indicate that they do not differ significantly within each sex.

	species complex	species	number nuclei measured	number chromosomes	chromosomal formula	total length of chromosome set (μm)	
						mean	95% confidence interval
female	*A. varipes*	*A. atriplicis*	14	8	4M + 4A	15.6ac	13.7–17.9
		A. varipes	16	8	4M + 4A	15.1ab	13.3–17.1
		A. certus	19	8	4M + 4A	14.0a	12.4–15.8
		A. kurdjumovi	13	8	2M + 6A	16.8ac	14.8–19.2
		A. hordei	8	8	4M + 4A	14.3ab	11.9–17.1
	A. daucicola	*A. daucicola*	15	10	10M	16.8ac	14.8–19.0
	A. mali	*A. glycinis*	5	10	10M	19.6ac	16.1–23.9
		A. coreae	6	10	10M	21.3c	17.9–25.4
		A. rhamni	19	10	10M	18.4bc	16.5–20.4
male	*A. varipes*	*A. atriplicis*	3	4	2M + 2A	16.3ab	12.3–21.6
		A. certus	3	4	2M + 2A	25.0bc	19.9–31.3
		A. hordei	21	4	2M + 2A	17.8b	16.1–19.7
	A. daucicola	*A. daucicola*	7	5	5M	23.7ac	20.4–27.6
	A. mali	*A. coreae*	6	5	5M	29.8c	25.8–34.5
		A. rhamni	4	5	5M	22.5bc	18.3–27.7

M = metacentric; A = acrocentric.

Table 4. Relative lengths of chromosomes in *Aphelinus* species**.** Means with 95% confidence intervals in parentheses.

Species complex	Species	Chromosome				
		1	2	3	4	5
		Relative length				
A. varipes	*A. atriplicis*	40	26	18	16	
		(38–42)	(24–28)	(17–20)	(14–17)	
	A. varipes	41	26	18	15	
		(39–43)	(24–27)	(17–19)	(14–17)	
	A. certus	40	26	18	16	
		(38–43)	(24–28)	(17–20)	(14–17)	
	A. kurdjumovi	43	24	18	15	
		(40–45)	(22–26)	(17–20)	(14–17)	
	A. hordei	41	25	19	16	
		(37–44)	(23–27)	(17–21)	(14–18)	
A. daucicola	*A. daucicola*	24	22	19	18	18
		(23–26)	(20–24)	(18–21)	(17–20)	(17–20)
A. mali	*A. glycinis*	24	22	21	18	18
		(21–27)	(19–25)	(18–24)	(16–21)	(16–21)
	A. coreae	24	22	20	18	18
		(21–27)	(20–25)	(18–23)	(16–21)	(16–21)
	A. rhamni	24	22	20	18	18
		(23–26)	(21–24)	(19–22)	(17–19)	(17–19)

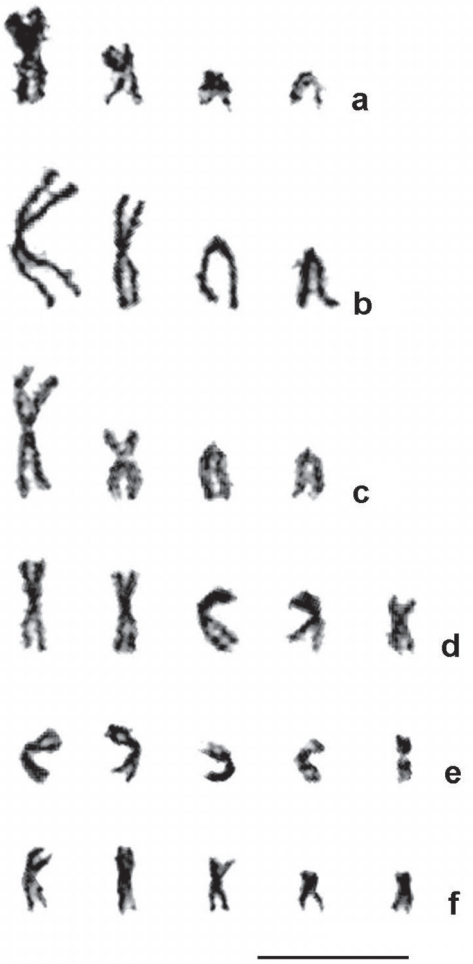

Figure 1. Haploid mitotic karyograms of six *Aphelinus* species. **a** *A. atriplicis* **b** *A. certus* **c** *A. hordei* **d** *A. coreae* **e** *A. rhamni* **f** *A. daucicola*. Species in the *A. varipes* complex have n = 4 *versus* n = 5 in the *A. mali* and *A. daucicola* complexes. Scale bar: 10 μm.

(model deviance = 65.1; residual deviance = 153.2; df = 8, 150; $P < 0.0001$) and between sexes (model deviance = 34.3; residual deviance = 118.9; df = 1, 149; $P < 0.0001$). Total lengths ranged from 14 to 21 μm so the longest set was 1.5 times the shortest (Table 3). Total lengths were significantly greater in the *A. mali* complex than in the *A. varipes* complex for both males and females, with the values in *A. daucicola* complex intermediate between these extremes (females: model deviance = 20.5; residual deviance = 92.8; df = 2, 112; < 0.0001; males: model deviance = 26.2; residual deviance = 38.8; df = 2, 41; $P < 0.0001$). Mean total chromosome length correlated with mean genome size estimated from flow cytometry ($F = 6.3$;

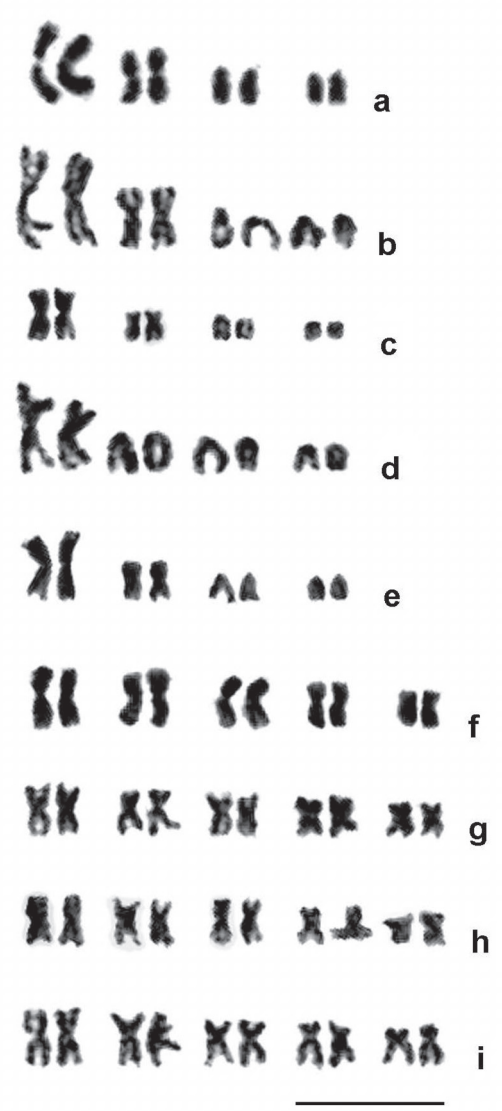

Figure 2. Diploid mitotic karyograms of nine *Aphelinus* species. **a** *A. atriplicis* **b** *A. certus* **c** *A. hordei* **d** *A. kurdjumovi* **e** *A. varipes* **f** *A. coreae* **g** *A. glycinis* **h** *A. rhamni*, **i** *A. daucicola*. Species in the *A. varipes* complex have 2n = 8 *versus* 2n = 10 in the *A. mali* and *A. daucicola* complexes. Scale bar: 10 μm.

df = 1, 7; $P = 0.04$; $r^2 = 0.47$; Fig. 3), primarily because of the difference in total chromosome length between the *A. mali* and *A. varipes* complexes.

Hybridization with a 28S rDNA probe revealed a single rDNA cluster on chromosomes of the haploid set and two rDNA clusters in the diploid set (Fig. 4). These clusters were near the centromere on a medium-sized metacentric chromosome.

Table 5. Centromeric indexes of chromosomes in *Aphelinus* species**.** Means (95% confidence intervals); shared letters within a species complex and chromosome indicate means that are not significantly different.

complex	species	Chromosome				
		1	2	3	4	5
		Centromeric index				
A. varipes	*A. atriplicis*	46a	47b	0	0	
		(44–49)	(45–50)			
	A. varipes	46a	47b	0	0	
		(44–49)	(45–49)			
	A. certus	46a	46ab	0	0	
		(44–49)	(44–48)			
	A. kurdjumovi	47a	0	0	0	
		(44–49)				
	A. hordei	47a	41a	0	0	
		(44–51)	(38–45)			
A. mali and *A. daucicola*	*A. daucicola*	45a	45a	41a	39a	38a
		(43–48)	(43–48)	(38–43)	(37–41)	(36–40)
	A. glycinis	48a	44a	44ab	44ab	42ab
		(43–52)	(40–48)	(40–48)	(40–49)	(39–47)
	A. coreae	44a	47a	43ab	44ab	44b
		(41–48)	(43–51)	(40–47)	(40–47)	(40–47)
	A. rhamni	46a	46a	45b	45b	43b
		(44–49)	(44–48)	(43–47)	(43–47)	(41–45)

Table 6. Analysis of deviance for differences in centromeric indexes among species of *Aphelinus*; acrocentric chromosomes were not included in these analyses.

complex	chromosome	model		residual		
		df	deviance	df	deviance	P
A varipes	1	4	0.2	135	23.1	1.00
	2	3	9.3	110	22.3	0.03
A. mali and *A. daucicola*	1	3	1.8	86	20.7	0.62
	2	3	1.2	86	23.0	0.74
	3	3	7.2	86	33.2	0.07
	4	3	16.6	86	29.0	0.0008
	5	3	12.7	86	52.8	0.005

Discussion

The large genome size differences between the *A. varipes* complex *versus* the *A. mali* complex matched the phylogenetic divergence between these complexes (Heraty et al. 2007). The difference between the complexes is also supported by the difference in karyotypes: four chromosomes with one-two metacentrics and two-three acrocentrics in the *A. varipes* complex *versus* five metacentric chromosomes in the *A. mali* complex.

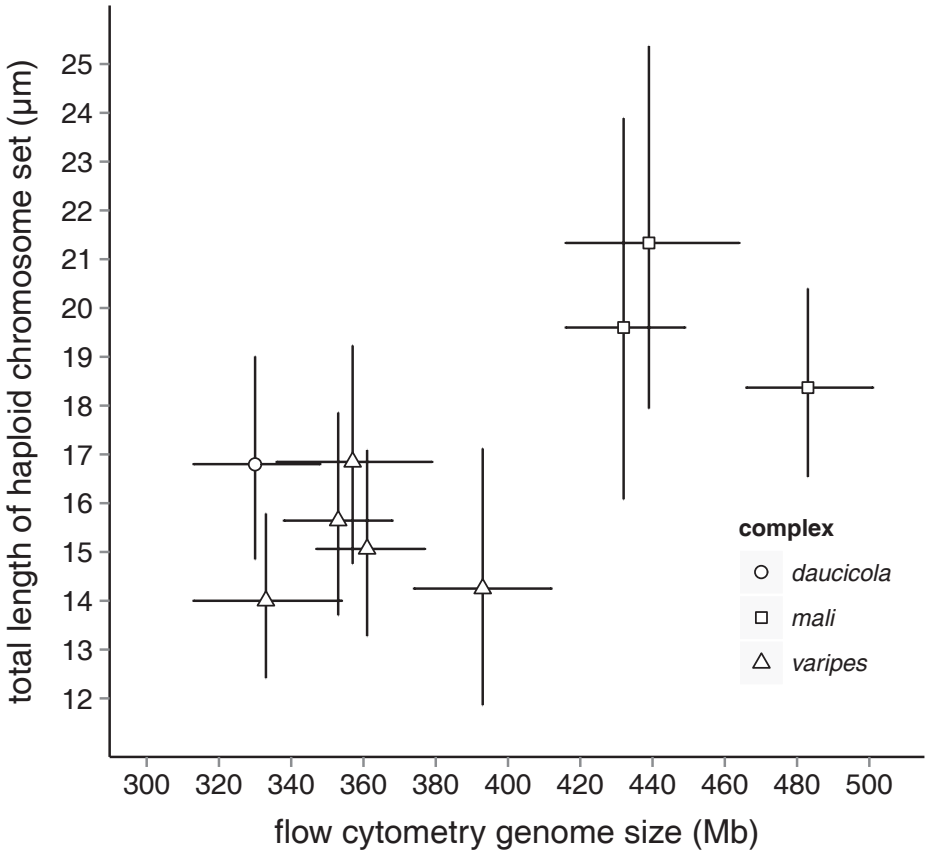

Figure 3. Total length of chromosome set (µm) *versus* flow cytometry genome size (Mb) for nine *Aphelinus* species. Error bars are 95% confidence intervals.

However, the additional chromosome in the *A. mali* complex does not necessarily account for a corresponding increase in genome size, because *A. daucicola* also has five chromosomes and yet has the smallest genome we observed. Four haploid genome sizes have been published for the family Aphelinidae, including an estimate of 635 Mb (=0.65 pg) for *Aphelinus abdominalis* (Dalman, 1820) (Ardila-Garcia et al. 2010) which is much larger than our estimates for other species of *Aphelinus*. However, beyond *Aphelinus*, other Hymenoptera genera have genome sizes spanning wide ranges (e.g., some ants, *Tapinoma* Förster, 1850, 362–597 Mb; *Solenopsis* Westwood, 1840, 372–753 Mb; http://www.genomesize.com). This also applies to some genera of parasitoids, e.g. *Leptopilina* Förster, 1869 (Figitidae) with genome sizes ranging from 363 to 520 Mb (Gokhman et al. 2011).

Genome size has been hypothesized to depend on several factors, including eusociality, parasitism, and developmental biology in insects (Ardila-Garcia and Gregory 2009, Gregory 2002, Johnston et al. 2004). These hypotheses come from the asso-

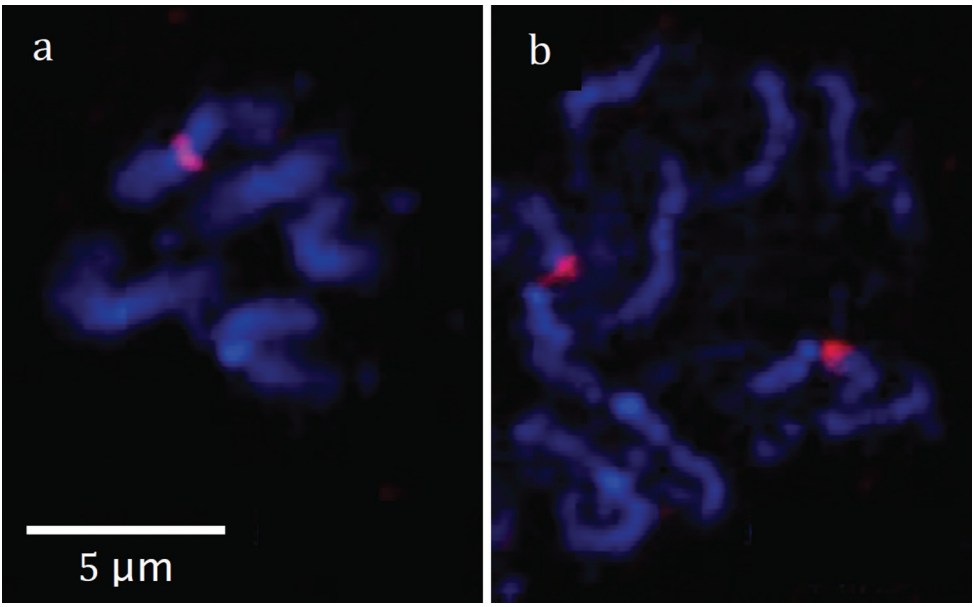

Figure 4. Fluorescence *in situ* hybridization with 28S rDNA probe. **a** metaphase chromosomes of the haploid karyotype and **b** prometaphase chromosomes of the diploid karyotype of *A. coreae*. Red = hybridization signal (a single rDNA cluster in the haploid set and paired clusters in the diploid set), blue = counterstaining of chromosomes with DAPI.

ciations between genome size, cell size, and cell division rates found in many taxa (Ardila-Garcia and Gregory 2009, Gregory 2005). The mean genome size for species of parasitic Hymenoptera (293 Mb) does not differ greatly from the mean genome size for species of eusocial Hymenoptera (333 Mb), but the genomes for both groups are significantly smaller than those for species of non-parasitoid solitary Hymenoptera (469 Mb) (Ardila-Garcia and Gregory 2009). However, it is unclear why there should be so much variation in genome size among species of *Leptopilina* or *Aphelinus*, given the very similar biologies within each genus.

Mapping karyotypic data on a molecular phylogeny of *Aphelinus* and two outgroup species allowed reconstruction of karyotype evolution in the species we studied (Fig. 5). The phylogeny was modified from Heraty et al. (2007) with results from Heraty et al. (2013), Kim and Heraty (2012), and unpublished data. Chromosomal formulae were mapped on the phylogeny using Mesquite (Maddison and Maddison 2016). Chromosomal formulae for *Aphytis mytilaspidis* (Le Baron, 1870) and *Encarsia formosa* Gahan, 1924 are from (Gokhman 2003). Concerning chromosome number, *Aphelinus asychis* Walker, 1839, has four chromosomes (Gokhman 2003), which is what we found for all the species we studied in the *A. varipes* complex. On the other hand, we found five chromosomes for *A. daucicola* and three species in the *A. mali* complex, which is also the chromosome number reported for *Aphelinus mali* (Haldeman, 1851) (Viggiani 1967).

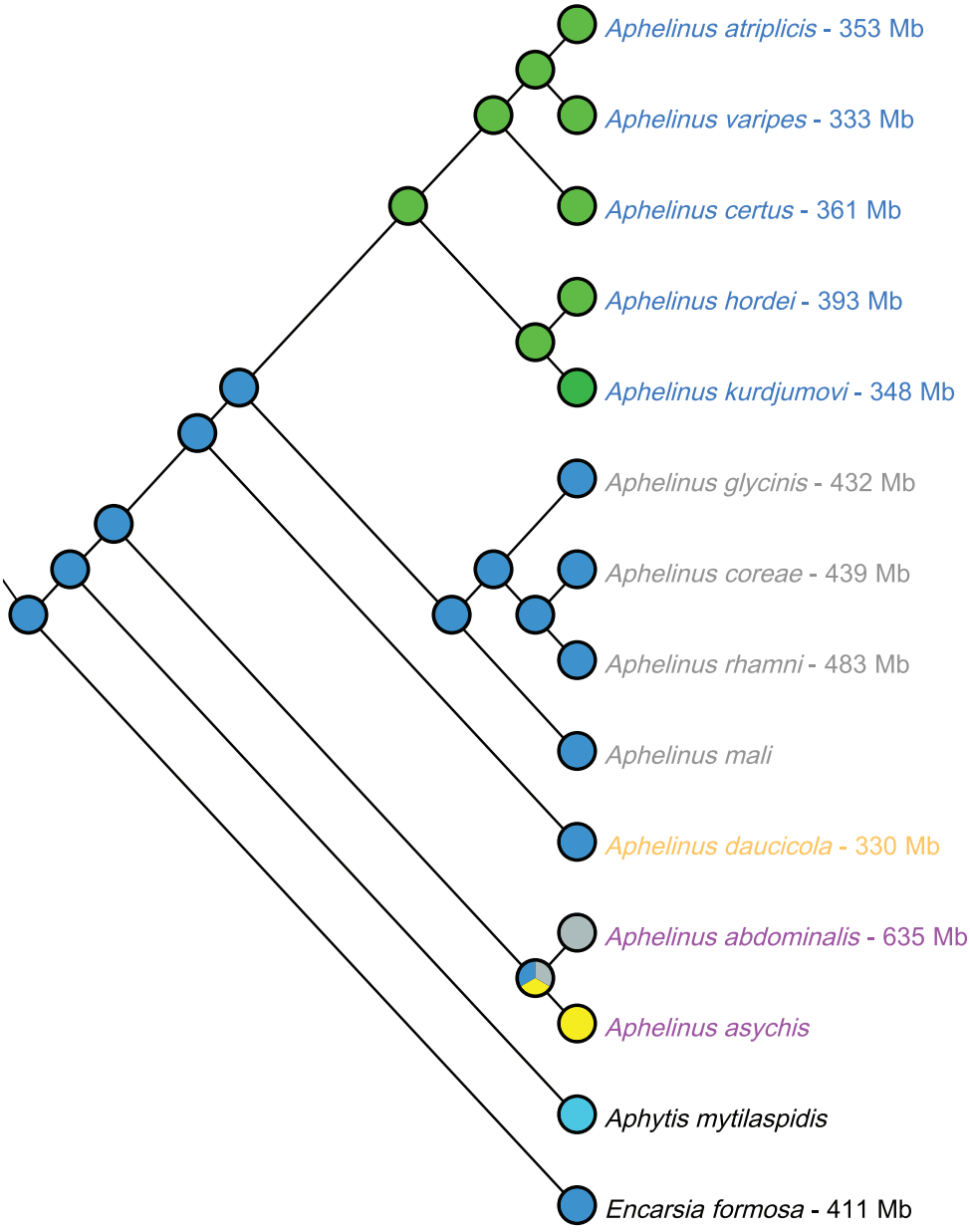

Figure 5. Chromosomal formulae and genome sizes on phylogeny of *Aphelinus* species and several outgroups. Blue = 5 metacentric chromosomes; aqua = 1 metacentric and 4 acrocentric chromosomes; green = 2 metacentric and 2 acrocentric chromosomes; dark green = 1 metacentric and 3 acrocentric chromosomes; yellow = 2 metacentric, 1 subtelocentric, and 1 acrocentric chromosome; grey = unknown. Numbers after species names are genome sizes estimated from flow cytometry; values for *Aphelinus abdominalis* and *E. formosa* are from (Ardila-Garcia et al. 2010). The different colors for species of *Aphelinus* indicate membership in the four species complexes for which data are available.

The *A. varipes* and *A. mali* complexes are sister clades, but *A. asychis* is the most basal species in the phylogeny of the genus, at least for the species for which there are phylogenetic data. This information alone would suggest four chromosomes is the ancestral state. However, five or six chromosomes are the numbers most frequently reported in chalcidoids (although it has been hypothesized that 9–10 is the ancestral state) (Gokhman 2009). Furthermore, five chromosomes has been reported for *Aphytis mytilaspidis* (Rössler and DeBach 1973), a species in the genus most closely related to *Aphelinus* for which chromosome number has been reported, and five chromosomes have been reported for many species of *Encarsia* Förster, 1878, a genus of aphelinids in another subfamily of Aphelinidae (Gokhman 2009). Chromosomal fusion (and hence decreased chromosome number) is a trend of karyotype evolution in many groups of organisms, including parasitic Hymenoptera (Gokhman 2009). Chromosomal fissions are also possible, but they are substantially less frequent, probably because they break existing linkage groups and therefore can decrease fitness. Thus, the reduced chromosome number in *A. asychis* has probably arisen independently of that in the *A. varipes* complex, because these two groups also have different karyotype structures. *Aphelinus asychis* has a haploid karyotype with two metacentric chromosomes, a subtelocentric chromosome and an acrocentric chromosome, with the three smallest chromosomes being similar in size (Gokhman 2009). The same chromosome number in *A. asychis* and in the *A. varipes* complex could be an example of karyotypic orthoselection (White 1973), i.e. similar karyotypes with independent origins. However, the hypothesis of chromosomal fusion giving rise to four chromosomes in the *A. varipes* complex cannot explain the significant and substantially smaller genome sizes in this complex compared to those in the *A. mali* complex.

Chromosomes in the *A. varipes* complex differ from those in the *A. mali* complex in relative length and centromeric indices. The longest metacentric chromosome in species in the *A. varipes* complex is much longer than the other chromosomes. We suggest that this metacentric chromosome resulted from a fusion of two smaller chromosomes from an ancestral karyotype with five chromosomes. Species in the *A. varipes* complex have two smaller acrocentrics that, in turn, could originate from metacentric chromosomes of the ancestral karyotype via pericentric inversions. Moreover, the position of the centromere of the second largest chromosome underwent further changes in two sister species in the *A. varipes* complex, *A. kurdjumovi* and *A. hordei*. The centromere is significantly shifted in *A. hordei* (CI= 41 *versus* 46 in *A. certus*, and 47 in *A. atriplicis* and *A. varipes*), and is further moved to a terminal position in *A. kurdjumovi* (CI= 0). We propose that consecutive pericentric inversions in *A. hordei* and *A. kurdjumovi* would be the most parsimonious explanation. These chromosomal rearrangements in the *A. varipes* complex are an example of a general trend in karyotype evolution in parasitic Hymenoptera, namely, karyotypic dissymmetrization, which involves an increase in size differentiation between chromosomes and an increase in the proportion of acrocentric chromosomes (Gokhman 2009).

A recent review of the distribution of rDNA sites on chromosomes of parasitic Hymenoptera showed that the number of these sites correlates with chromosome number (Gokhman et al. 2014). We found that this is also true for at least one *Aphelinus* species: haploid males of *A. coreae,* with their low number of chromosomes (n = 5), had a single

rDNA site, while diploid females of *A. coreae* had two rDNA sites. Fluorescence *in situ* hybridization is especially useful for studying karyotypes with morphologically similar chromosomes that are difficult to recognize with conventional staining, like the chromosomes of *A. coreae* and other species in the *A. mali* and *A. daucicola* complexes.

Total chromosomal length was correlated with genome size in *Aphelinus*, but this was because of the difference in chromosome length between the *A. mali* and *A. varipes* complexes. Although a similar correlation was found for species in the family Figitidae (Gokhman et al. 2014), only large differences in chromosome length were distinguished in both cases, probably because of intraspecific variation in chromosomal condensation. Total lengths of male chromosomes exceeded those of females, although male and female genome sizes did not differ, and indeed it would be surprising if they did, given that males inherit their chromosomes from their mothers. The difference in the chromosome length between males and females may have resulted from differences in chromosomal condensation between the sexes, and this could compensate for the differences in chromatin available for transcription in the haploid and diploid genomes.

Conclusions

Differences as large as 44% were found in genome size between *Aphelinus* species, which is surprising given the similarity in their morphology and life history. Mean total chromosome length correlated with mean genome size. The differences in genome size and total chromosome length between species complexes matched the phylogenetic divergence between species complexes. Chromosomal rearrangements in the *A. varipes* complex are an example of karyotypic dissymmetrization, which involves an increase in size differentiation between chromosomes and an increase in the proportion of acrocentric chromosomes, which is a general trend in karyotype evolution in parasitic Hymenoptera.

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RESEARCH ARTICLE

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Cytogenetic studies in the redtail catfish, *Phractocephalus hemioliopterus* (Bloch & Schneider, 1801) (Siluriformes, Pimelodidae) a giant fish from Amazon basin

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Abstract

The objective of this study was to cytogenetically analyze *Phractocephalus hemioliopterus* comparing the findings with other data to infer relationships among Pimelodidae species. The results revealed a diploid number of 2n = 56 and the karyotype composed of 16 metacentric, 20 submetacentric, 6 subtelocentric and 14 acrocentric chromosomes (FN = 98). The Ag-NORs, 18S rDNA and CMA_3 signals were coincident in location occupying the short arm of an acrocentric chromosome pair (23^{th}), in a secondary constriction. The 5S rDNA genes were localized near the centromere on the short arms of one submetacentric chromosome pair. C-bands were localized predominantly in the terminal regions of chromosomes, including the AgNORs and a small metacentric pair with a conspicuous positive band on interstitial region. This chromosome pair could be considered a species-specific cytogenetic marker.

Keywords

Neotropical fish, Parrot catfish, karyotype, Ag-NORs, 18S rDNA, CMA_3, C-banding

Introduction

The genus *Phractocephalus* Agassiz, 1829 belongs to Pimelodidae family and contains three species, one extant, *Phractocephalus hemioliopterus* (Bloch & Schneider, 1801) and two extinct species recently described, *P. nassi* (Lundberg and Aguilera, 2003) and *P. acreornatus* (Aguilera et al. 2008). According to Lundberg et al. (1998) "isolation of peripheral drainage system south, west and north of the Paraná, Amazonas and Orinoco systems provided opportunity for allopatric divergence, and also was accompanied by much extirpation of once more widespread tropical fish species".

The large catfish, *P. hemioliopterus*, has a wide distribution in the lowland, meandering rivers and lagoons of the Orinoco, Amazon and Essequibo (Lundberg and Aguilera 2003) and as a monotypic taxon possesses several uniquely derived characteristics (de Pinna 1998). This catfish is known as "cajaro" in Venezuela and Colombia and in Brazil as "pirarara" (parrot – fish) because of its red or orange caudal fin (Lundberg and Aguilera 2003). In the areas of occurrence, the species has economic importance and is much appreciated by fishermen. However, in spite of its large size, *Phractocephalus* is also a common aquarium fish (Carvalho and Medeiros 2005).

From a systematic point of view, Pimelodidae remains as a controversial group, possessing some internal inconsistencies, represented by the "*Pimelodus* group", "*Calophysus* group" and a basal branch including three genera *Phractocephalus*, *Leiarius* Bleeker, 1862 and *Perrunichthys* Schultz, 1944 (Lundberg and Littman 2003).

Available cytogenetic data partially support this hypothesis because several chromosomal studies on Pimelodidae have revealed that the species of this family have a predominant diploid number of 56 (Table 1) with a few exceptions, such as species included in the "*Calophysus* group" that show some characteristic cytogenetic features as 2n = 50, reported in *Calophysus* Müller & Trosche, 1843, *Luciopimelodus* Eigenmann & Eigenmann, 1888 and *Pinirampus* Bleeker, 1858 (Ramirez-Gil et al. 1998, Swarça et al. 1999, Sanchez et al. 2010) and *Megalonema platanum* (Günther, 1880) with 2n = 54 (Carvalho et al. 2011). According to phylogenetic tree of Lundberg and Littman (2003) the branch that includes *Leiarius*, *Perrunichthys* and *Phractocephalus* has been never studied cytogenetically; this fact demonstrates that more species must be chromosomally studied to increase the number of cytogenetic data to better understand the species relationships and the karyotypic evolution in this fish group. The present work aims to report for the first time the cytogenetic study of *P. hemioliopterus*, a unique extant species of the genus *Phractocephalus* from the Amazon Basin.

Material and methods

Six specimens of *P. hemioliopterus* from Amazon Basin/Brazil maintained in the fishing farm of the Universidade Estadual de Londrina were studied cytogenetically. The chromosome preparations were obtained from lymphocyte culture according to Fenocchio and Bertollo (1988), avoiding the sacrifice of specimens. Silver staining of NORs

Table I. Cytogenetic data on the family Pimelodidae. 2n = diploid number. Only published data were used.

	2n	References
"*Pimelodus* group"		
Bergiaria Eigenmann & Norris, 1901	56	Dias and Foresti (1993)
Iheringichthys Eigenmann & Norris, 1900	56	Carvalho et al. (2004); Carvalho and Dias (2005); Carvalho et al. (2010); Vissotto et al. (1999); Ribeiro et al. (2008); Sanchez et al. (2014)
Parapimelodus La Monte, 1933	56	Treco et al., 2008.
Pimelodus Lacepède, 1803	Predominant 56	Schell (1973); Toledo and Ferrari (1976); Dias and Foresti (1993); Vissotto et al. (1999); Swarça et al. (2001b); Borin and Martins-Santos (2002); Souza et al. (2003); Borin and Martins-Santos (2004); Souza et al. (2004a, b); Garcia and Moreira Filho (2005); Treco and Dias (2009); Moraes-Neto et al. (2011)
"*Calophysus* group"		
Calophysus Müller & Troschel, 1843	50	Ramirez-Gil et al. (1998)
Pinirampus Bleeker 1858	50	Swarça et al. (1999); Sanchez et al. (2010)
Luciopimelodus Eigenmann & Eigenmann, 1888	50	Sanchez et al. (2010)
"*Megalonema* group"		
Megalonema Eigenmann, 1912	54	Carvalho et al. (2011)
"*Sorubiminae* group"		
Hemisorubim Bleeker, 1862	56	Martins-Santos et al. (1996); Swarça et al. (2013)
Pseudoplatystoma Bleeker, 1862	56	Fenocchio and Bertollo (1992); Martins-Santos et al. (1996); Swarça et al. (2005b), Moraes-Neto et al. (2011); Nirchio el al. (2013)
Zungaro Bleeker, 1858	56	Martins-Santos et al. (1996); Swarça et al. (2001c)
Sorubim Cuvier, 1829	56	Fenocchio and Bertollo (1992); Martins-Santos et al. (1996); Moraes-Neto et al. 2011
Brachyplatystoma Bleeker, 1862	56	Gonçalves et al. (2014)
Steindachneridion Eigenmann & Eigenmann, 1919	56	Swarça et al. (2005a); Swarça et al. (2006); Moraes-Neto et al. (2011)
Phractocephalus Agassiz, 1829	56	Present data

(AgNORs) was performed using the method of Howell and Black (1980). C banding and Chromomycin A_3 (CMA_3) staining were carried out using the methods of Sumner (1972) and Verma and Babu (1995), respectively. Fluorescence *in situ* hybridization (FISH) experiments were performed using biotinylated 18S rDNA probes (1700 bp fragments) obtained from the nuclear DNA of the fish *Oreochromis niloticus* (Linnaeus, 1758) labeled with biotin-14-dATP by nick translation (Gibco cat Nº 18247-015), according to the manufacturer's instructions. The hybridization technique, post-hybridization washes and visualization were carried out following Swarça et al. (2001c). The preparations were analyzed in an Olympus BX50 microscope, and the best metaphases were captured with a SONY camera, model Exware HAD coupled to the microscope. The FISH slides were observed and the images acquired with a Leica DM 4500 mi-

croscope equipped with a DFC 300F9 camera and Leica IM50 4.0 software. Chromosome morphology was determined on the basis of Levan et al. (1964) and Guerra (1986) with some modifications and chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a). NF (chromosome arm number) was determined considering m/sm/st chromosomes having two arms and acrocentric chromosomes having one arm.

Results and discussion

The family Pimelodidae is composed of 109 valid species (Eschmeyer and Fong 2016), but only 27 species have been analyzed cytogenetically (Swarça et al. 2007). *Phractocephalus* is a monotypic genus. The only species of the genus, *P. hemioliopterus*, is widely distributed in the rivers of the Orinoco, Amazon and Essequibo basins (Lundberg and Aguilera 2003). The extinction of the other two species (*P. nassi* and *P. acreornatus*) was hypothetically explained by Lundberg et al. (1998).

The diploid number (2n = 56) and karyotype constitution, 16m, 20sm, 6st, 14a (FN = 98) of *P. hemioliopterus* is reported for the first time (Fig. 1). According to Swarça et al. (2000) the chromosome number is identical to other large species that belong to the "*Pimelodus* group" comprising at least *Hemisorubim* Bleeker, 1862, *Zungaro* Bleeker, 1858, *Sorubim* Cuvier, 1829, *Pseudoplatystoma* Bleeker, 1862 that could be called informally "Sorubiminae group" and includes the largest catfishes from South America (de Pinna 1998, Lundberg and Littman 2003) (Table 1). Although *P. hemioliopterus* does not belong to these systematic and/or taxonomic groups, this species shares many cytogentic traits, such as the chromosome shape, size and staining patterns, with the species included in "Sorubiminae".

As stated below, diploid number 56 with high fundamental number, NORs located at the terminal position on the short arm of an acrocentric chromosome pair (23^{th}), coincident with positive C-bands (Fig. 2a) represent common features in almost all pimelodid species analyzed so far, suggesting that these cytogenetic traits were conserved during the karyotype evolution and may have an ancient common origin (Sanchez et al. 2010). The presence of ribosomal genes on the short arm of one st/a chromosome pair is coincident with the location observed in the "*Calophysus* group" (Sánchez et al. 2010) and "Sorubiminae group" (Swarça et al. 2008) and differs from the "*Pimelodus* group", where the ribosomal genes are located almost exclusively on the long arm of m/sm chromosome pairs (Swarça et al. 2007).

The data obtained with CMA_3 indicate that the Ag-NORs of *P. hemioliopterus* are rich in GC pairs (Fig. 2b), a general pattern also found in the family Pimelodidae by several authors (Swarça et al. 2001a, b, c, Garcia and Moreira-Filho 2005, Swarça et al. 2005b, Nirchio et al. 2013, among others). However, the exact location of ribosomal genes on chromosomes could be revealed exclusively by means of *in situ* hybridization using 18S and 5S rDNA probes. After application of this procedure these regions showed bright signals on short arms of one subtelocentric pair (18S rDNA

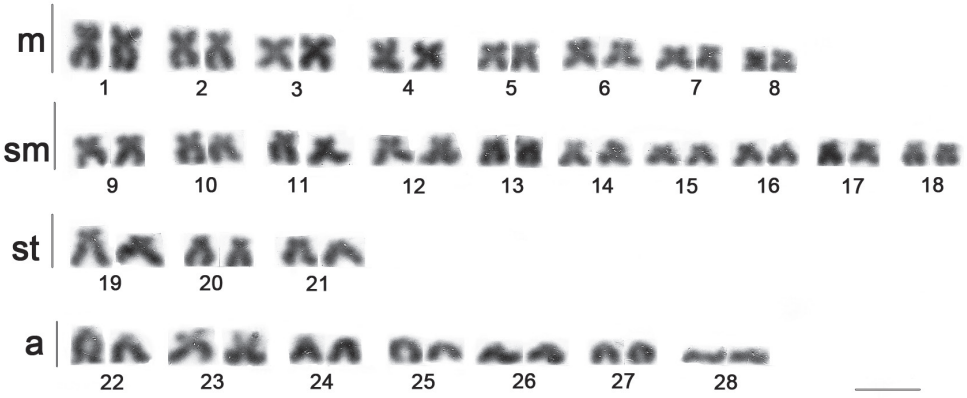

Figure 1. Karyotype of *Phractocephalus hemioliopterus.* Conventional Giemsa staining. Scale bar: 5 μm.

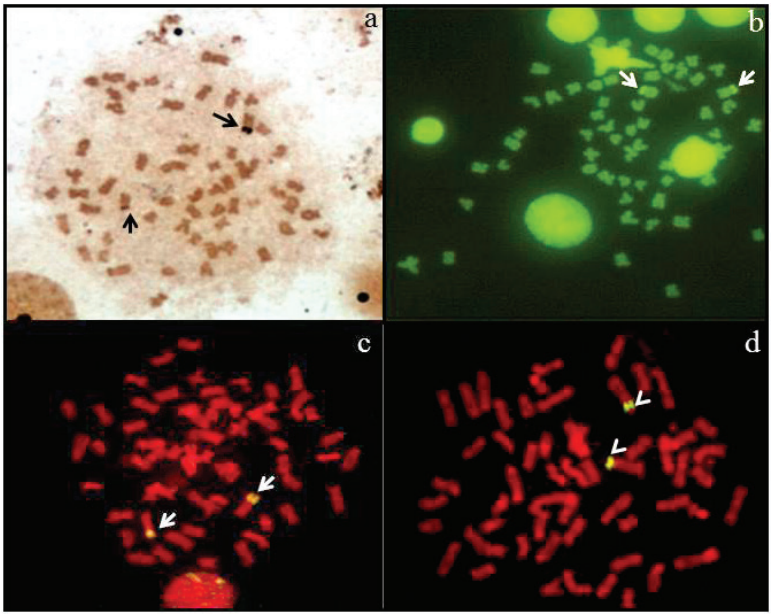

Figure 2. Metaphases of *Phractocephalus hemioliopterus*. **a** $AgNO_3$ staining **b** CMA_3 banding **c** FISH with 18S rDNA probe and **d** FISH with 5S rDNA probe. Arrows indicate the NOR-bearing chromosomes and arrowheads indicate the chromosome pair with 5S rDNA.

probe) and on short arms of another submetacentric chromosome pair (5S rDNA probe) (Fig. 2c, d). In general, the 18S and 5S rDNA sites are not syntenic but located on different chromosome pairs, this feature being the most frequent patter in several Pimelodidae species (Carvalho et al. 2010, Swarça et al. 2008, 2009). However, recently syntenic localization of the major rDNA clusters and the 5S sites were reported in other species (Ziemniczak et al. 2012, Konerat et al. 2014, da Rocha et al. 2016). So far, both patterns of rDNA and 5S rDNA localization, syntenic and not syntenic,

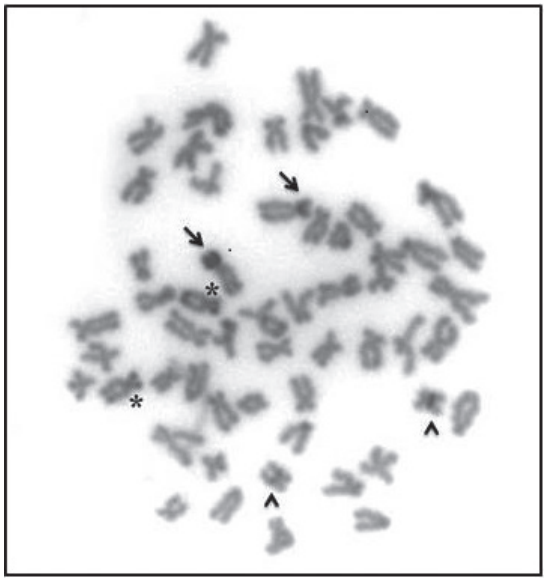

Figure 3. Somatic metaphase of *Phractocephalus hemioliopterus* after C-banding. Arrowheads indicate the chromosome pair with interstitial heterochromatin; arrows indicate the NOR-bearing chromosome and asterisks indicate the chromosomes with heterochromatin blocks in both terminal regions.

have been described in Pimelodidae. Still, the evolutionary trend of ribosomal genes chromosome distribution has not been yet outlined.

Heterochromatin distribution revealed by C-banding was evidenced on telomeric regions of some chromosomes, one pair with bitelomeric bands and in the secondary constriction on the short arm of NOR bearing pair (Fig.3). This last feature represents a common trait shared by most pimelodids. Another interesting cytogenetic characteristic is the presence of a small metacentric pair that shows a conspicuous heterochromatic block in interstitial region (Fig. 3). Heterochromatin interstitially located has been reported in some species of the family Pimelodidae, such as *Pseudoplatystoma tigrinum* (Valenciennes, 1840) (Fenocchio and Bertollo 1992), *Hemisorubim platyrhynchos* (Valenciennes, 1840) (Martins-Santos et al. 1996), *Iheringichthys labrosus* (Lütken, 1874) (Vissotto et al. 1999) and also in species of the genus *Pimelodus* Lacépède, 1803 (Treco et al. 2008). The interstitial localization of a strong C-band in *P. hemioliopterus* on a small metacentric chromosome can be a species-specific cytogenetic marker and could be useful for future studies on the internal relationships of the species included in this group.

Taking into consideration the findings described previously, the present work is the first to provide cytogenetic information about *P. hemioliopterus*.

The cytogenetic description of *P. hemioliopterus* allowed the karyotypic characterization and the comparison of certain cytogenetic features shared in general with other Pimelodidae, however, some of these traits distinguish the "Sorubiminae group", suggesting that this species could be integrated into the branch of the great catfishes.

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RESEARCH ARTICLE

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Immunocytological analysis of meiotic recombination in two anole lizards (Squamata, Dactyloidae)

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Abstract

Although the evolutionary importance of meiotic recombination is not disputed, the significance of interspecies differences in the recombination rates and recombination landscapes remains under-appreciated. Recombination rates and distribution of chiasmata have been examined cytologically in many mammalian species, whereas data on other vertebrates are scarce. Immunolocalization of the protein of the synaptonemal complex (SYCP3), centromere proteins and the mismatch-repair protein MLH1 was used, which is associated with the most common type of recombination nodules, to analyze the pattern of meiotic recombination in the male of two species of iguanian lizards, *Anolis carolinensis* Voigt, 1832 and *Deiroptyx coelestinus* (Cope, 1862). These species are separated by a relatively long evolutionary history although they retain the ancestral iguanian karyotype. In both species similar and extremely uneven distributions of MLH1 foci along the macrochromosome bivalents were detected: approximately 90% of crossovers were located at the distal 20% of the chromosome arm length. Almost total suppression of recombination in the intermediate and proximal regions of the chromosome arms contradicts the hypothesis that "homogenous recombination" is responsible for the low variation in GC content across the anole genome. It also leads to strong linkage disequilibrium between the genes located in these regions, which may benefit conservation of co-adaptive gene arrays responsible for the ecological adaptations of the anoles.

Keywords
Synaptonemal complex, chromosomes, crossing over, *Anolis*, *Deiroptyx*, lizard, Reptilia

Introduction

Meiotic recombination (crossing over) plays a dual role in sexually reproducing organisms. At least one crossover per chromosome is necessary and sufficient to secure orderly segregation of homologous chromosomes during the first meiotic division. Crossing over shuffles allele combinations between homologous chromosomes, increasing the genetic variation in the progeny, on the one hand, and shaping local patterns of GC-content (i.e., creating or modifying isochores) along the chromosome length, on the other hand (Eyre-Walker and Hurst 2001).

The number and distribution of the crossovers along a chromosome depends on its length, chromatin composition, genetic content and crossover interference (Lynn et al. 2002, Pardo-Manuel de Villena and Sapienza 2001). The longer the chromosome, the more crossovers it may accommodate. Euchromatic regions show higher recombination rate than heterochromatic regions. At the DNA sequence level, recombination rate at hotspots can be hundreds of times higher than in the adjacent regions (Zickler and Kleckner 2016). The occurrence of a crossover usually reduces the probability of another crossover close by. This phenomenon, which is called crossover interference, also makes a substantial contribution to the number and distribution of crossovers along the chromosome (Moens 2006).

The patterns of crossover distribution have been studied across a variety of vertebrates such as fish (Moens 2006, Lisachov et al. 2015), birds (Pigozzi 2001, Calderon and Pigozzi 2006) and mammals (Anderson et al. 1999, Borodin et al. 2008, Basheva et al. 2008). Usually vertebrate chromosomes show an uneven crossover pattern with more or less pronounced recombination hotspots and low-recombining regions.

There are several hypotheses which connect the recombination landscape with species' ecology and speciation (Barton and Otto 2005, Ortiz-Barrientos et al. 2016). Lower numbers of crossovers and their uneven distribution, which creates recombination "valleys", is thought to be beneficial for preserving adaptive allele combinations in ecologically specialized species living in stable conditions, and for suppressing interspecies gene flow in hybrid zones. Higher recombination, which creates more diverse offspring, is beneficial in unstable and diverse conditions (Burt and Bell 1987, Otto and Barton 2001).

Reptiles are particularly interesting organisms in which to study the evolution of recombination because they show a wide array of karyotypes and ecological specializations, and extensive homology and synteny between reptilian and avian chromosomes has been demonstrated (Pokorná et al. 2012). Data on recombination patterns in reptiles remain scarce. Most studies used chiasma counts and distribution at diakinesis-metaphase I (Cobror et al. 1986, Lamborot et al. 2012, Reed et al. 1992). However, the resolution of chiasmata analysis is rather poor, because the chromosomes at metaphase

I are condensed, making precise chiasmata localization along the chromosome difficult. Recently recombination in one reptile species (*Crocodylus porosus* Schneider, 1801) was estimated via linkage analysis of microsatellite markers (Miles et al. 2009). However, the resolution of linkage analysis depends on the number and distribution of available markers. Low density of mapping leads to underestimation of the recombination rate.

The most widespread technique for studying recombination rate and localization is by immunofluorescent mapping of MLH1 (the mismatch repair protein associated with mature recombination nodules) along the synaptonemal complexes (SCs) at prophase (Moens 2006, Pigozzi 2001, Borodin et al. 2008). MLH1 marks about 90–95 % of all recombination events in mouse (Guillon et al. 2005), thus providing reliable estimates of the total recombination rate, as well as the frequency and distribution of recombination events in individual chromosomes (Froenicke et al. 2002).

One of the most species-rich and diverse reptilian clades are iguanians (infraorder Iguania), which include nearly 30% of all lizard species (Uetz and Hošek 2005). Iguanians are further subdivided into pleurodonts (Pleurodonta) and acrodonts (Acrodonta). The former clade includes New World and Madagascan species (former family Iguanidae *sensu lato*), and the latter includes chameleons (Chamaeleonidae Rafinesque, 1815) and Old World and Australian dragon lizards (Agamidae Gray, 1827). Many of them have a conservative karyotype with 2n = 36, including 12 submetacentric and metacentric macrochromosomes and 24 microchromosomes. This karyotype is presumed to be ancestral for Iguania (Deakin et al. 2016).

Among iguanians, anoles (Dactyloidae Fitzinger, 1843, Pleurodonta) are one of the best studied lineages. They are the classical model organisms in studies of reptilian ecology, evolution, biogeography, karyology and genetics (Hertz et al. 2013, Fleishman and Pallus 2010, Giovannotti et al. 2016). One of their representative, *Anolis carolinensis* Voigt, 1832, is the first reptile whose genome was almost fully sequenced (Alföldi et al. 2011).

In this study, we assessed the pattern of meiotic recombination in two anole species, *A. carolinensis* and *Deiroptyx coelestinus* (Cope, 1862). Although these species are separated by a relatively long evolutionary history (Nicholson et al. 2012), they both possess the ancestral iguanian karyotype (Gorman 1973). We examined the number and distribution of crossovers along their macrochromosomes using immunofluorescent localization of MLH1 at SC spreads.

Materials and methods

Specimens

The specimens, two male *A. carolinensis* and one male *D. coelestinus*, were purchased from commercial breeders. Handling and euthanasia of the animals were performed according to the protocols approved by the Animal Care and Use Committee at the Institute of Cytology and Genetics. The specimens were deposited in the research collections of the institute.

Chromosome preparation and immunostaining

The spreads of meiotic cells were prepared according to the protocol of Peters et al. (1997). Immunostaining was performed according to the protocol described by Anderson et al. (1999) using rabbit polyclonal anti-SYCP3 (1:500, Abcam), mouse monoclonal anti-MLH1 (1:50, Abcam), and human anticentromere (ACA) (1:100, Antibodies Inc) primary antibodies. As secondary antibodies Cy3-conjugated goat anti-rabbit (1:500, Jackson ImmunoResearch), FITC-conjugated goat anti-mouse (1:50, Jackson ImmunoResearch), FITC-conjugated donkey anti-human (1:100, Vector Laboratories) were used. All antibodies were diluted in PBT (3% bovine serum albumin and 0.05% Tween 20 in 1xPBS). A solution of 10% PBT was used for blocking non-specific antibody binding. Primary antibody incubation was performed overnight in a humid chamber at 37°C, and secondary antibody incubation was performed for 1 h at 37°C. Finally, slides were mounted in Vectashield with DAPI (Vector Laboratories) to stain DNA and reduce fluorescence fading. After image acquisition of the immunofluorescent signals, the slides were subjected to FISH.

The preparations were visualized with an Axioplan 2 Imaging microscope (Carl Zeiss) equipped with a CCD camera (CV M300, JAI), CHROMA filter sets, and ISIS4 image processing package (MetaSystems GmbH).

Image processing and analysis

Brightness and contrast of all images were enhanced using Corel PaintShop Photo Pro X6 (Corel Corp). The centromeres were identified by the ACA foci. The MLH1 signals were scored only if they were localized on SCs. The length of the SC of each chromosome arm was measured in micrometers and the positions of centromeres and MLH1 foci in relation to the centromeres were recorded using MicroMeasure 3.3 software (Reeves 2001). Relative distances between the MLH1 foci and between the MLH1 foci and centromeres were calculated as fractions of the SC and arm length respectively.

To map the MLH1 foci distribution along the macroSCs we calculated the absolute position of each MLH1 focus multiplying the relative position of each focus by the average absolute length for the corresponding chromosome arm. These data were pooled for each arm and plotted to represent a recombination map.

Statistica 6.0 software package (StatSoft) was used for descriptive statistics. MLH1 foci distribution along the SCs was analyzed using CODA v.1.1 software (Gauthier et al. 2011). To estimate the strength of crossover interference we used the shape parameter (ν) of the gamma distribution. This distribution describes the probability of the distances between MLH1 foci under the assumption that their precursors are randomly placed along the bivalent and every *ν*-th precursor would result in a focus. The ν-value varies from 1 (every precursor results in a focus, i.e. no interference) to 20 (high interference).

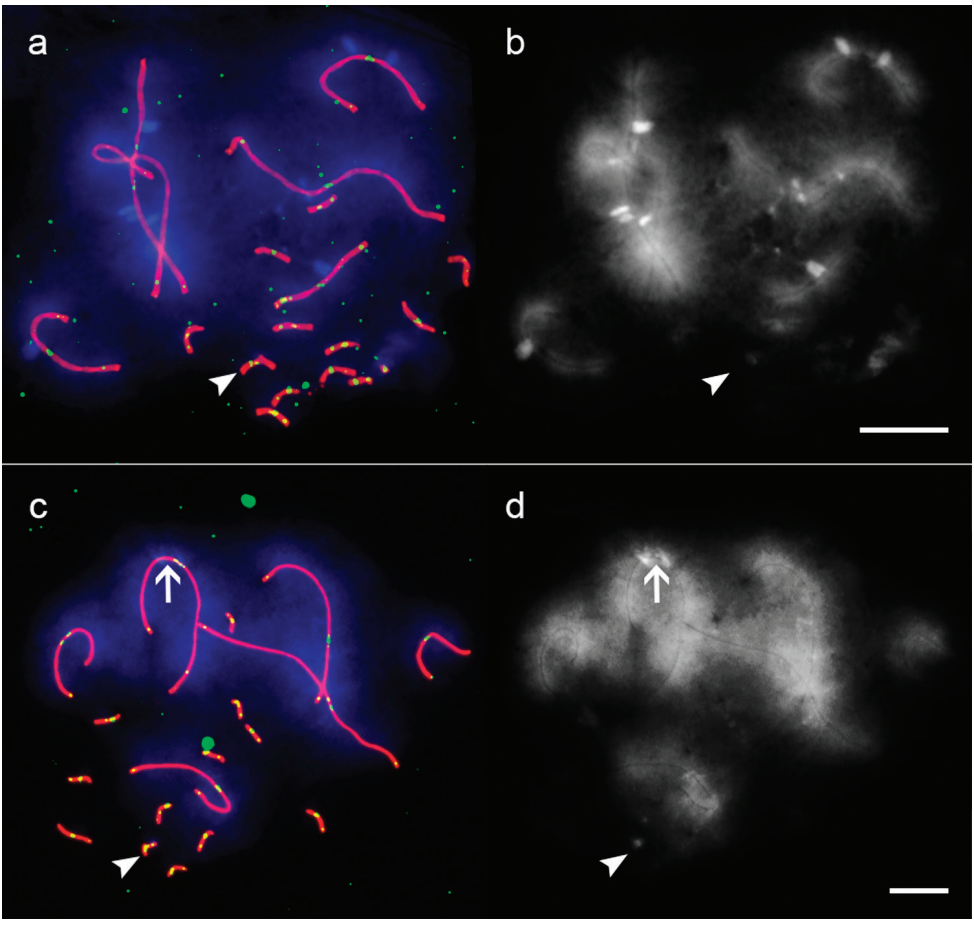

Figure 1. The SC spreads of *A. carolinensis* (**a, b**) and *D. coelestinus* (**c, d**). **a, c** immunofluorescence and DAPI. Red: SYCP3, green: centromere and MLH1, blue: DAPI **b, d** DAPI channel separately. Arrowheads show the XY bivalent (Lisachov et al. in press). Arrow shows the DAPI+ band on the SC2 of *D. coelestinus*. Scales bars: 5 μm.

Results

Figure 1 shows the microphotographs of the surface spreads of the pachytene spermatocyte nuclei of *A. carolinensis* and *D. coelestinus.* Each SC spread contains 6 macroSCs and 12 microSCs (2n = 36). We analyzed 96 pachytene nuclei of *A. carolinensis* and 100 nuclei of *D. coelestinus.*

All the macroSCs of *A. carolinensis* show DAPI-positive bands in their pericentromeric regions. In *D. coelestinus* such bands were detected only at SC2 and at one microSC. Such bands are observed in many species and generally correspond to C-heterochromatin. They mainly contain satellite repeats (Charlesworth et al. 1994). The centromeric indices of the macrochromosomes of both species were around 0.45–0.5,

Table 1. Average SC length (μm) and of MLH1 foci number (±S.D.) in macroSCs in two anole species.

	A. carolinensis		*D. coelestinus*	
SC rank	**SC length (μm)**	**No. of MLH1 foci**	**SC length (μm)**	**No. of MLH1 foci**
1	28.8±5.1	1.90±0.47	25.7±5.2	1.98±0.34
2	25.5±4.3	1.88±0.45	24.5±4.7	1.90±0.30
3	20.1±3.0	1.92±0.43	18.5±3.6	1.69±0.53
4	18.0±2.7	1.89±0.40	16.9±2.9	1.68±0.51
5	14.3±2.0	1.80±0.43	12.6±2.1	1.34±0.48
6	10.9±1.3	1.45±0.50	10.2±1.8	1.11±0.31

except for chromosome 3 in *D. coelestinus* which had average centromeric index of 0.35. Thus in *D. coelestinus*, we were able to identify SC2 by its DAPI-positive band and SC3 by its centromeric index. The SCs 1, 4, 5, and 6 of *D. coelestinus* and all macroSC of *A. carolinensis* were identified by their length (Table 1).

The mean number of MLH1 foci on each of the macrochromosomal bivalents was calculated (Table 1). We used only the SCs which contained at least one MLH1 focus.

The distribution of MLH1 foci along all the macroSCs in both species was extremely uneven (Fig. 2). The prominent peaks of the foci occurred near the telomeres of both arms, and the distal 20% of the arm length contained more than 90% of all foci. The bivalents with MLH1 foci located beyond the terminal regions usually carried three foci, and two of them were always located near the telomeres. Thus, the intermediate MLH1 foci may be considered as the second crossovers pushed proximally by crossover interference (Fig. 2). We detected a moderate interference in anoles. In *A. carolinensis* the ν-value was estimated as 5.6 (95% CI 5.0–6.7), and in *D. coelestinus* it was estimated as 5.0 (95% CI 4.5–5.5).

Discussion

The most interesting feature of the recombination pattern of *A. carolinensis* and *D. coelestinus* macrochromosomes is an extreme polarization of the recombination events. Similar terminal localization of crossovers was previously observed in several anole species, including *A. carolinensis*, using chiasmata analysis at diakinesis-metaphase I (Gorman and Atkins 1968, Gorman and Atkins 1966, Beçak et al. 1964).

Subtelomeric peaks in the distribution of crossovers are common for most vertebrates. This phenomenon is explained by the fact that meiotic pairing of the homologs is usually initiated at the telomeres (Zickler and Kleckner 2016). Whereas this general pattern is common for most species, the numbers of crossovers and their exact distribution varies greatly between taxa. However, such an extreme pattern of crossovers, which we observe in the anoles, is unusual.

Crossover interference is unlikely to be the cause of almost complete suppression of recombination beyond the subtelomeric regions, because the macrochromosomes of

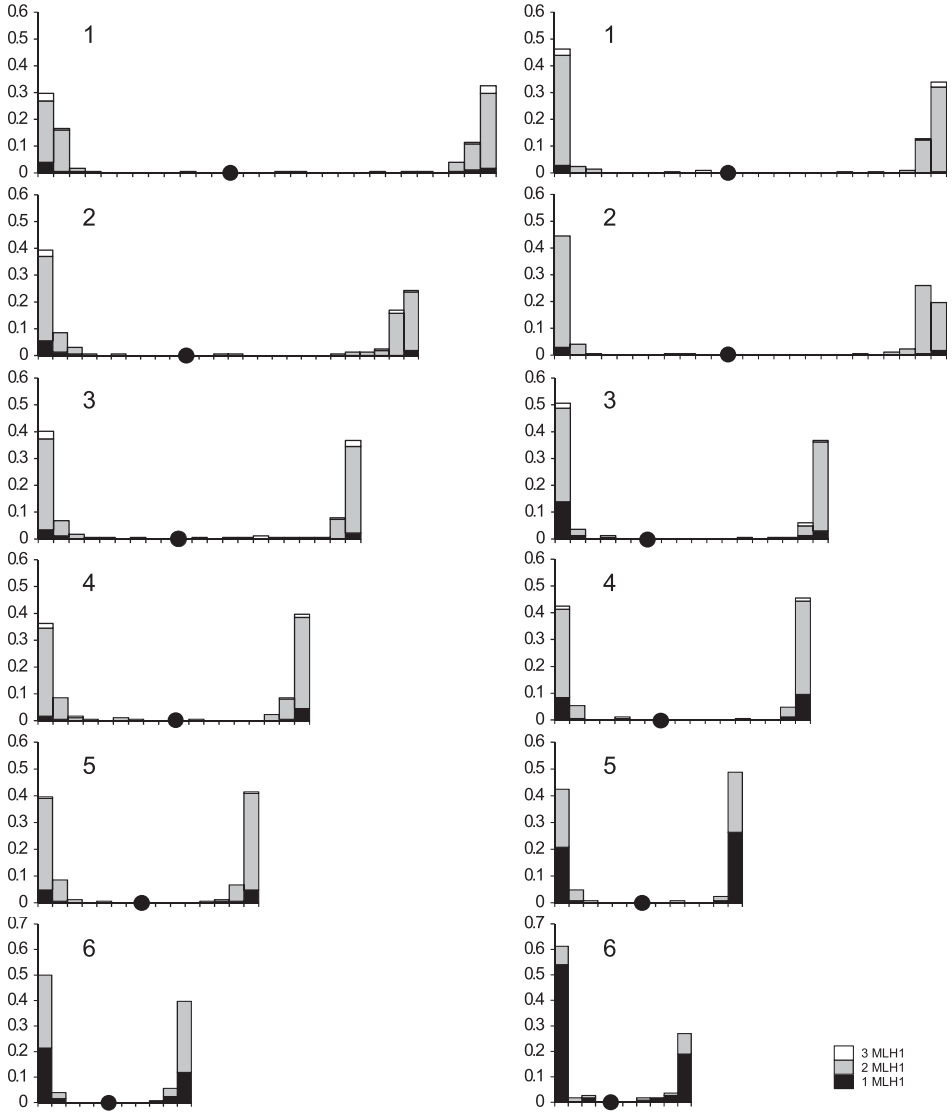

Figure 2. The distribution of MLH1 foci along the macrochromosomes of *A. carolinensis* and *D. coelestinus.* The X-axis shows the position of MLH1 foci, the marks on this axis are separated by 1 μm. Black dots indicate centromeres. The Y-axis indicates the frequency of MLH1 foci in each 1 μm – interval. Stacked columns show the frequency for the SCs containing MLH1 foci at each interval.

both species demonstrate a rather moderate degree of interference. Our estimate of the ν-value (approximately 5.0) is the first estimate of this parameter in reptiles, so we can only compare it with estimates obtained for mammalian chromosomes of similar size. It was substantially lower than the values detected in the largest chromosomes of common shrews (11.1: Borodin et al. 2008) and mouse (13.7−14.4: De Boer et al. 2006).

Additional evidence against crossover interference as the cause of the extreme distal location of the crossovers is the fact that single crossovers are also located distally. Single crossovers tend to be located in the middle of mammalian chromosomes, because they suppress the occurrence of other crossovers at both chromosome ends (Anderson et al. 1999, Borodin et al. 2008). The distribution observed in the anole macrochromosomes may be determined by a very early and short time window for the initiation of homologous pairing and recombination.

Almost total suppression of recombination in the intermediate and proximal regions of chromosome arms would lead to strong linkage disequilibrium between the genes located in these regions. This may benefit the conservation of co-adaptive gene arrays or "supergenes" (Pál and Hurst 2003, Thompson and Jiggins 2014, Charlesworth 2016). According to the Red Queen theory, low recombination is favored under stable environmental conditions and stabilizing selection (Otto and Michalakis 1998). Indeed, the ecology and morphology of anoles have remained unchanged for tens of millions of years, which is supported by molecular phylogeny (Nicholson et al. 2012) and also by the remarkable finds of fossil anoles preserved in the Dominican amber (Sherratt et al. 2015). Perhaps, the recombination suppression serves to keep the "supergenes" which are responsible for their lifestyle adaptations.

The divergence between *Anolis* and *Deiroptyx* is one of the basal radiations among Dactyloidae (Nicholson et al. 2012). Therefore, this recombination pattern is probably ancestral for the whole family. It remains unknown if a similar recombination pattern is conserved in the anole lineages (e. g. *Norops* Wagler, 1830, *Ctenonotus* Fitzinger, 1843) which underwent a series of chromosome fusions and fissions (2n = 28–30, 2n = 40 in comparison with the ancestral 2n = 36), which led to the appearance of new macrochromosomes (Castiglia et al. 2013).

The results of our analysis of crossover distribution along anole macrochromosomes might shed light on a peculiarity of their genome organization. One of the specific characters of the genomes of cold-blooded vertebrates is weak regional variation in GC-content (i.e. less prominent isochore structure) in comparison with birds and mammals (Costantini et al. 2009). Until recently (Figuet et al. 2014, Costantini et al. 2016) it was even thought (Fujita et al. 2011, Alföldi et al. 2011) that in the genome of *A. carolinensis* there is no isochore structure at all. In mammals, GC-rich isochores are known to be located at recombination hotspots, and it is suggested that they are formed by recombination via GC-biased gene conversion (gBGC) (Eyre-Walker and Hurst 2001). Considering this fact, Olmo (2008) postulated that the relatively homogenous distribution of the GC-content in reptiles probably reflects the relatively homogenous distribution of crossovers along reptilian chromosomes. According to Olmo, this should produce more points for chromosomal rearrangements (since they originate as recombination errors), which should reinforce karyotypic evolution and therefore speciation.

The extremely distal localization of crossovers in the males of both anole species here analyzed might be considered as evidence against this hypothesis. The weak prominence of isochores in reptiles is apparently produced by some forces other than gBGC and does not reflect the distribution of recombination hotspots. Moreover, intense

and homogenous recombination, which is known for example for birds, is apparently not enough to drive intense karyotypic evolution, since bird karyotypes are the most conservative and archaic among all vertebrates (Uno et al. 2012).

There are two additional important points to be considered in the discussion. In some reptile species chiasma number and localization depend on environmental conditions (Cobror et al. 1986, King and Hayman 1978). It remains possible that the median regions of the anole chromosomes could recombine in other conditions. However, we consider this possibility unlikely since the chiasma distributions found in previous studies in anoles from wild populations agree with the MLH1 distribution found in our study (Gorman and Atkins 1968, Gorman and Atkins 1966, Beçak et al. 1964). Further studies of recombination under alternative controlled conditions are necessary to clarify this point.

Sex difference in recombination rate and distribution should also be taken into account. Females tend to have higher recombination rates than males and more even distribution of crossovers along the chromosomes (Burt et al. 1991, Mank 2009). We cannot exclude that the median regions recombine in female meiosis of anoles. In mammals and birds, newborn females or even female embryos are used to obtain the female SC spreads. We did not detect any meiotic divisions in the gonads of newborn and juvenile lizards. In our opinion, lampbrush chromosome analysis may help to solve this question.

Conclusions

For the first time we directly assessed meiotic recombination in reptilian species using MLH1 mapping in SCs. We found that, in male anole lizards *Anolis carolinensis* and *Deiroptyx coelestinus*, MLH1 foci are mainly located in the terminal parts of the chromosome arms, whereas recombination intensity in the median parts of the chromosomes is extremely low. This result disagrees with the hypothesis of "homogenous recombination" as the cause of low isochore prominence in the genome of anoles. However, recombination in females has to be studied before drawing any final conclusions about overall recombination rate and distribution in anoles.

Competing interests

The authors declare that they have no competing interests.

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RESEARCH ARTICLE

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Variability and evolutionary implications of repetitive DNA dynamics in genome of *Astyanax scabripinnis* (Teleostei, Characidae)

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Abstract

DNA sequences of multiple copies help in understanding evolutionary mechanisms, genomic structures and karyotype differentiation. The current study investigates the organization and distribution of different repetitive DNA in the standard complement and B chromosomes in *Astyanax scabripinnis* (Jenyns, 1842) chromosomes from three allopatric populations in Campos do Jordão region, São Paulo State, Brazil. The location of microsatellite sequences showed different chromosome distribution between Lavrinha Farm Stream (LFS) and Lake of Pedalinho (LP) populations. However, the karyotype of these populations basically followed the pattern of dispersed distribution in the A complement, conspicuous in telomeric/interstitial regions and preferential accumulation in the B chromosome. The B chromosome showed heterogeneous location of microsatellite probes CA, CAC and GA. The H3 and H4 histone genes were isolated from the total genome of the species and then the chromosomal mapping was performed by fluorescence *in situ* hybridization (FISH). The FISH signals showed high similarity for the probes H3 and H4 mapping in genomes of the populations analyzed. The sequences $(GATA)_n$ revealed a sex-specific trend between the chromosomal location in males and females at (LFS) and (LP) populations. Although species that comprise the *Astyanax scabripinnis* complex do not have morphologically differentiated sex chromosomes, the preferential GATA location – sex-associated – may represent a sex chromosome in differentiation.

Keywords
Microsatellites chromosomic mapping, sex-specific GATAn sequence location, B chromosomes microsatellites accumulation

Introduction

DNA sequences of multiple copies help in understanding evolutionary mechanisms, genomic structures and karyotype differentiation, including in fishes. *Astyanax scabripinnis* (Jenyns, 1842) is a freshwater fish species of the family Characidae (Lima et al. 2003). It is widely distributed and recognized as a complex of cryptic species (Moreira-Filho and Bertollo 1991).

Astyanax scabripinnis is a model in evolutionary studies due to the frequent presence of B chromosomes in some populations (see Moreira-Filho et al. 2004 for review). Differences in the C-band pattern of this extra genome chromosome may be found and they vary from fully (Vicente et al. 1996) to partially heterochromatic (Mizoguchi and Martins-Santos 1997). Although a fully heterochromatic B chromosome comparable in morphology and size was found in the species *A. fasciatus* (Cuvier, 1819), *A. schubarti* (Britski, 1964) and *A. scabripinnis* (Jenyns, 1842) (Moreira-Filho et al. 2001), the origin of these chromosomes is still a question that deserves attention. The occurrence of B microchromosomes, e.g. in *A. goyacensis* (Santos et al. 2013), suggests the recurrent origins of these supernumerary chromosomes in *Astyanax* Baird & Girard, 1854. The most accepted hypothesis is that the B chromosome emerged in *A. scabripinnis* from standard karyotype (subtelocentric or acrocentric chromosome) and it formed an isochromosome followed by heterochromatinization (Vicente et al. 1996, Mestriner et al. 2000, Vicari et al. 2011). However, there is no evidence that B chromosome is functional in this species, although recent studies show the *in situ* location of possible genes (sequences/probes) such as 18S rDNA and H1 histone in the B chromosome of *A. paranae* – a species that belongs to complex *A. scabripinnis* (Silva et al. 2014). The absence of activity in the 18S sequences, located in B chromosome, suggests that these genes are possible pseudogenes (Ferreira-Neto et al. 2012). However, the occurrence of functional genes in B chromosomes has been evidenced, mainly in plants (see Banaei-Moghaddam et al. 2015 for review).

Some functional genes found in the DNA of eukaryotes may have simple copies and unique sequences, whereas other genes have repetitive nature when they are found in more than one copy (Hardman 1986) such as in the case of multigenic families encoding the histones (Nei and Rooney 2005) and the rRNA (Martins 2007). These sequences are broadly conserved among the organisms and became important tools for evolutionary studies. The H3 and H4 histones are some of the most conserved proteins in the genome of eukaryotes, wherein the chromosomal location of H3 sequences varied among the organisms and it was already identified as: dispersed or tandem (Rooney et al. 2002). The rRNA may be classified as minor rDNA (5S), which is transcribed to rRNA 5S; and major rDNA (45S), which encodes the 28S, 5.8S and 18S rRNA (Eickbush and Eickbush 2007).

The sequences of repetitive DNA with tandem distribution are classified as satellites, microsatellites and minisatellites according to the degree of repetition (Jurka et al. 2005). The short in tandem repeated sequences of telomeric DNA $(TTAGGG)_n$ vary in number of copies in different organisms and contribute for the stability and replication of the chromosome (Blackburn and Szostak 1984).

Other repetitive DNAs with great evolutionary and functional interest are the *Bkm* (banded krait minor satellite) satellite sequences, which are found in the heterogametic sex of most vertebrates (Nanda et al. 1992). It consists of a simple GATA sequence repetition, which is a conserved tetranucleotide related to sex differentiation (Singh et al. 1984). Subramanian et al. (2003) assumed that the $(GATA)_n$ repetition plays a functional role in human sex chromosomes.

The microsatellite chromosomic mapping is widely used in evolutionary studies, including in *Astyanax* (Piscor and Parise-Maltempi 2016). The $(GATA)_n$ repetitive sequence, for example, allowed to verify differences in the chromosome structure and possible relation with the sexual differentiation in two species of lizards. (Pokorná et al. 2011).

The aim of this study is to investigate patterns of organization and distribution of different repetitive DNAs, in the standard complement and B chromosomes in *Astyanax scabripinnis*.

Material and methods

Animals and chromosome preparation

Fifty six specimens of *Astyanax scabripinnis* (18 females and 38 males) were analyzed from three different locations in the Campos do Jordão region, State of São Paulo, Brazil, collected with permission from Ministério do Meio Ambiente, Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis, Instituto Chico Mendes de Conservação da Biodiversidade – ICMBio MMA / IBAMA / SISBIO, number 15115-1: Lavrinha Farm Stream (LFS) (22°40'49.5"S; 45°23'31.9"W), Lake of Pedalinho (LP) (22°43'02.8"S; 45°33'91.9"W) and Ribeirão das Perdizes (RP) (22°44'35.3"S; 45°34'11.6"W). For cytogenetic analyses, the specimens were anesthetized with benzocain 0.01% and dissected, and the mitotic chromosomes were obtained from kidney tissue using the technique described by Bertollo et al. (1978) with modifications (Blanco et al. 2012). The C-banding technique was performed following the protocol described by Sumner (1972) and nucleolar organizing regions (Ag-NORs) was employed according to Howell and Black (1980). The chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a), based on the classification proposed by Levan et al. (1964). All procedures were made according to international protocols for animal testing and authorized by the Ethic Committee in Animal Experimentation (protocol number 4509/08) of Universidade Estadual de Ponta Grossa. The specimens were identified and received

a deposit number from Coleção Ictiológica do NUPELIA (Núcleo de Pesquisas em Limnologia, Ictiologia e Aquicultura) of Universidade Estadual de Maringá (NUP number 17482, 17484, 17486).

DNA amplification and sequencing

The genomic DNA extraction was carried out by the cetyl trimethyl ammonium bromide method (Murray and Thompson 1980) with modifications. The primers used in PCR are described in Table 1. To obtain the 18S rDNA probe, 18S primers isolated from *Prochilodus argenteus* Spix & Agassiz, 1829 were used according to Hatanaka and Galetti Jr. (2004). For the 5S rDNA, primers isolated from the rainbow trout were used according to Martins and Galetti Jr (1999). The *As*51 satellite DNA described by Mestriner et al. (1999) was obtained from the nuclear DNA of *A. scabripinnis*. The $(TTAGGG)_n$ and $(GATA)_n$ probes amplification followed Ijdo et al. (1991). The H3 histone gene amplification followed Colgan et al. (1998) and H4 histone gene amplification followed Pineau et al. (2005). The PCR assay were performed using 100 ng DNA template in a final volume of 25 µl. Each reaction contained 1 X PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.1 µM of each primer (10 pmol), and 0,5 U *Taq* DNA polymerase (Invitrogen). PCR of histone sequences was performed in the Eppendorf Mastercycler (30 cycles of 5 min at 95°C, 30 s at 95°C, 45 s at 52°C, 1 min 20 s at 72°C, and 7 min at 72°C). The product purification of the target DNA was performed using the High PCR Cleanup Micro Kit (GE Healthcare Amersham Biosciences™), following manufacturer instructions.

The nucleotide sequencing of the clones and DNA fragments was performed in an automatic sequencer ABI 3130x1, using o Kit Big Dye (Applied Biosystems) following manufacturer instructions. The sequences were aligned in Clustal W program (Thompson et al. 1994), using the BioEdit 7.0 editor (Hall 1999). To verify the identity, the

Table 1. Primer sequences employed.

Chromosomal markers	Primer sequence (5'-3')	Reference
18S rDNA F	GTAGTCATATGCTTGTCTC	Hatanaka and Galetti 2004
18S rDNA R	TCCGCAGGTTCACCTACGGA	Hatanaka and Galetti 2004
5S rDNA F	TACGCCCGATCTCGTCCGATC	Martins and Galetti 1999
5S rDNA R	CAGGCTGGTATGGCCGTAAGC	Martins and Galetti 1999
***As*51 F**	GGTCAAAAAGTCGAAAAA	Mestriner et al. 1999
***As*51 R**	GTACCAATGGTAGACCAA	Mestriner et al. 1999
H3 F	ATGGCTCGTACCAAGCAGACVGC	Colgan et al. 1998
H3 R	ATATCCTTRGGCAT RATRGTGAC	Colgan et al. 1998
H4 F	TSCGIGAYAACATYCAGGGIATCAC	Pineau et al. 2005
H4 R	CKYTTIAGIGCRTAIACCACRTCCAT	Pineau et al., 2005

sequences were subjected to search in BLASTn (http://www.ncbi.nlm.nih.gov/blast) and deposited in the GenBank (http://www.ncbi.nlm.nih.gov/genbank/) with the following accession number: H3 histone gene (LFS – KT633503, LP – KT633504, RP – KT633505), H4 histone gene (LFS – KT633506, LP – KT633507, RP – KT633508).

Chromosome probe

Seven different types of repetitive probes were used as chromosomal markers: 18S rDNA; 5S rDNA; *As*51 satellite DNA; H3 and H4 histone genes; the general vertebrate telomere sequence minisatellite $(TTAGGG)_n$; and the $(GATA)_n$ sequence. The *As*51 satellite DNA, 5S rDNA and H4 histone gene probes was labelled with Biotin-11-dUTP (Roche Applied Science), whereas 18S rDNA, H3 histone gene, $(GATA)_n$, and $(TTAGGG)_n$ probes were labelled with digoxigenin by *nick translation*.

Oligonucleotide probes containing microsatellite sequences $(CA)_{15}$, $(CAC)_{10}$, $(CAG)_{10}$, $(CAT)_{10}$, $(GA)_{15}$, $(GAA)_{10}$, $(GAG)_{10}$ and $(GC)_{15}$ were directly labeled with Cy5 during synthesis by Sigma (St. Louis, MO, USA), as described by Kubat et al. (2008).

Fluorescence in situ hybridization (FISH)

For each FISH assay 30 cells were analyzed. The FISH was performed using the protocol of Pinkel et al. (1986) with adaptations. The protocol was adjusted to high stringency (2.5 ng/mL probe, 50% deionized formamide, 10% dextran sulphate, 2 × SSC at 37°C overnight). After hybridization, the slides were washed in 15% formamide/0.2 × SSC at 42°C for 20 min and 4 × SSC/0.05% Tween at room temperature for 10 min. The signal detection was performed using alexa fluor 488 streptavidin (Molecular Probes™) for 5S rDNA and H4 histone gene, whereas the anti-digoxigenin-rhodamin (Roche™) was used for 18S rDNA, H3 histone gene, $(GATA)_n$ and $(TTAGGG)_n$ probes detection. The chromosomes were counterstained with DAPI (0.2 µg/mL) diluted in antifade solution (Fluka™). Chromosomes were analyzed under epifluorescence microscopy Zeiss AxioCam MRm and software ZEN pro 2011 (Carl Zeiss®).

Results

Chromosomal analysis and rDNA

The diploid number verified for *Astyanax scabripinnis* was 2n = 50 chromosomes in the three analyzed populations. The fundamental number (FN) was equal to 88 and

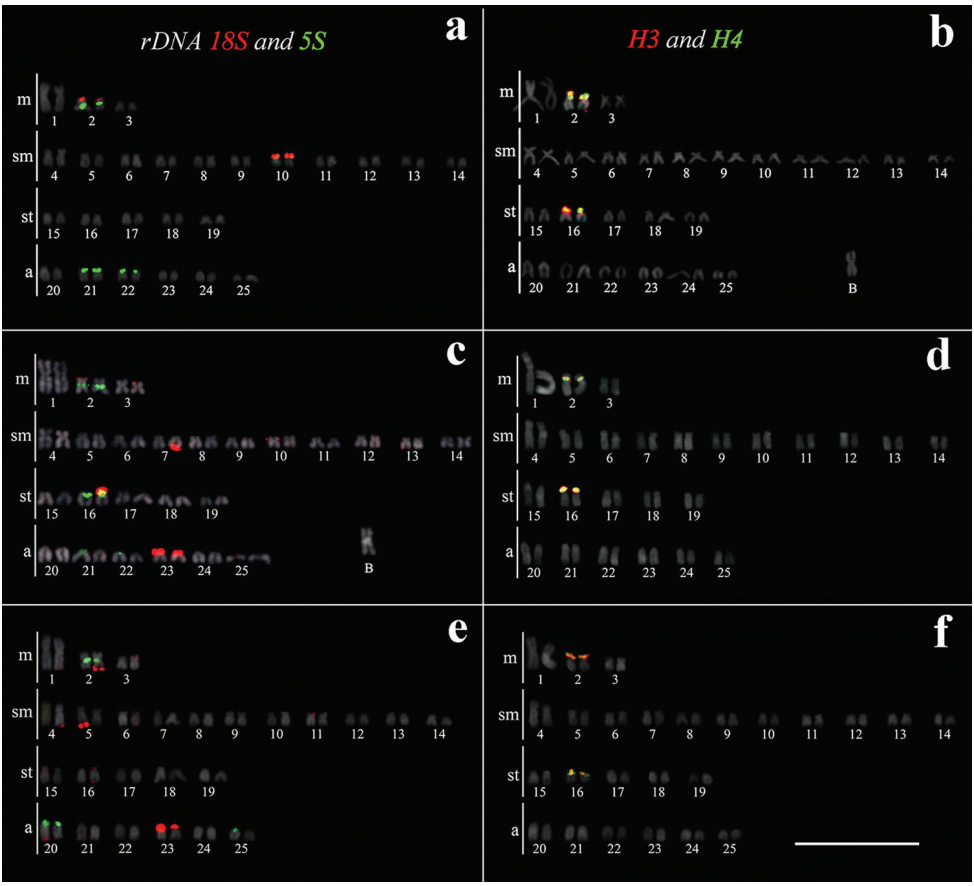

Figure 1. Karyotype of the three *Astyanax scabripinnis* populations (**a**, **b** Lavrinha Farm Stream – LSF); (**c**, **d** Lake of Pedalinho – LP); (**e**, **f** Ribeirão das Perdizes – RP). In **a**, **c**, **e** are visualized signals of 18S (red) and 5S (green) with rDNA probes and in **b**, **d**, **f** H3 (red) and H4 (green) with histones probes, after double Fluorescence *in situ* Hybridization (FISH), respectively. Scales bar: 10 μm.

karyotype formula composed by 6 m + 22 sm + 10 st + 12 a. Some specimens showed B chromosomes in 100% of the analyzed cells (2n = 51) (Suppl. material 1). These data corroborated previous studies in this species complex (see Moreira-Filho et al. 2004 for review). The (LFS) population showed 18S rDNA sites in the metacentric chromosome pair No. 2 and submetacentric pair No. 10 (carrier of Ag-NOR), both in short arm in terminal position The 5S rDNA sites was syntenic with 18S rDNA was located in the chromosome pair No. 2 in proximal region. In addition, plus sites were found in the acrocentric chromosomic pairs No. 21 and 22 (Fig. 1a). In the (LP) population the 18S rDNA was in terminal regions of pairs 7 sm, 16 st (carrier of Ag-NOR) and 23 a, since the location occurs in only one of the homologues in the pairs 7 and 16 (Fig. 1c). The 5S rDNA showed signal in the pair 2 m in proximal region and in the pair 16 st in terminal region, co-located with 18S rDNA

(Fig. 1c). The 18S rDNA in the (RP) population was also found in only one of the homologues in the pairs 2 m and 5 sm (carrier of Ag-NOR) and pair 23 a, whereas 5S rDNA was located in the pair 2, in proximal region and syntenic to 18S rDNA and in the pair 20 a (Fig. 1e). The B chromosome did not evidence signal with the ribosomal probes used (Fig. 1c).

H3 and H4 histone genes and sequence analysis

The amplification of the H3 and H4 histone genes from the total genome of the specimens was performed. The product of the PCR generated bands with approximately 400 base pair (bp), when it was analyzed in gel electrophoresis (Suppl. material 2). This product was submitted to nucleotide sequencing and it was used as FISH probe. The sequence alignment of H3 and H4 histones with other sequences of *Astyanax* deposited in GenBank resulted in 97% minimum similarity.

The H3 and H4 histones were co-located in two chromosomal pairs: metacentric pair No. 2 and subtelocentric pair No. 16 in the three populations (LFS, LP, RP). The sites of chromosome metacentric pair 2 were proximal, whereas the signal were terminal in other chromosomal pairs (Figs 1b, d, f). The B chromosome did not show any signal with the used probes H3 and H4 (Fig 1b).

$(TTAGGG)_n$ sequence, $(GATA)_n$ sequence and heterochromatin localization

The probe of the minisatellite sequence (TTAGGG)*n* was uniformly located in the telomeres of all chromosomes at the studied populations, including B chromosome. No interstitial signal (ITS) was found (data not shown) (Suppl. material 3).

The $(GATA)_n$ sequence was hybridized in males and females of the three populations (Fig. 2), and it was followed by C-banding for the analysis of heterochromatic regions (data not shown) (Suppl. material 4). The acrocentric chromosome pairs 20 and 21 showed signal in centromeric regions of male cells in (LFS) (Fig. 3a). The non-homologous chromosomes 21 and 23 bear (GATA)n signal in centromeric region of females, as well as in terminal long arm region of a chromosome of subtelocentric pair No. 15 (Fig. 3b). All the regions hybridized with the $(GATA)_n$ probe overlapped the positive C-band regions.

Males and females from the (LP) population did not show significant differences regarding the signal of the $(GATA)_n$ probe. Females showed three main signals: the homologues of metacentric chromosome pair No. 2, in the proximal region, without heterochromatin; and one of the homologues of pair 24 a in terminal region of the long arm, with evidences of heterochromatin (Fig. 2d). Males showed the same signal, with an additional signal in one of the homologues of pair 24 a (Fig. 2c), without heterochromatin accumulation.

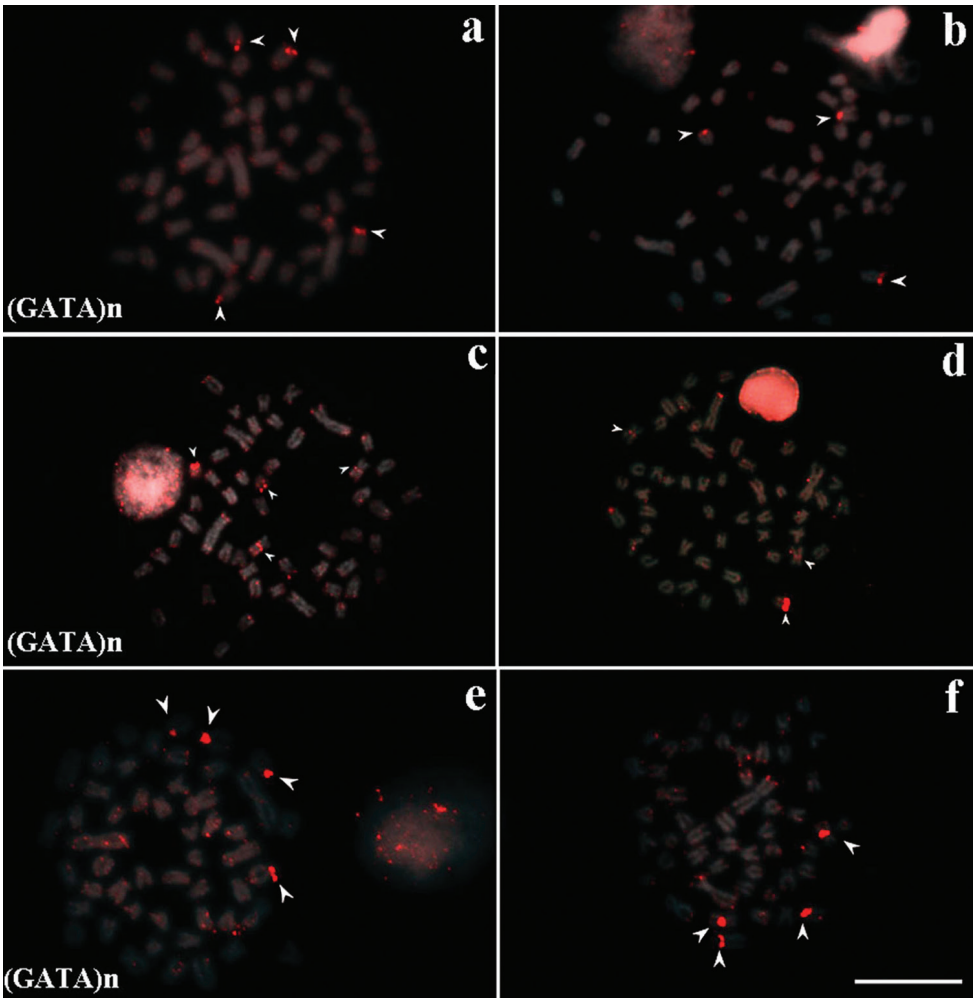

Figure 2. Fluorescence *in situ* Hybridization in metaphases of the three *Astyanax scabripinnis* populations (**a**, **b** Lavrinha Farm Stream – LSF); (**c**, **d** Lake of Pedalinho – LP); (**e**, **f** Ribeirão das Perdizes – RP) with (GATA)n probe. **a**, **c**, **e** males and **b**, **d**, **f** females. Scales bar: 10 μm

The male specimens from the (RP) population showed the pair of subtelocentric chromosomes 15 and 16 as their main signal (Fig. 2e), whereas the $(GATA)_n$ signals on females is in the pair 2 m in proximal region and in the pair 20 a in terminal region (Fig. 2f) – heterochromatin places are associated with the probe in both sexes. The $(GATA)_n$ probe did not evidence signal in the B chromosomes (Fig. 2a).

As51 satellite DNA and / microsatellite sequences probes

The *As*51 satellite DNA occurs in acrocentric chromosomes of the three populations: pairs 22 and 23 in the (LFS) and (LP) populations, pair 20 in the (RP) population and

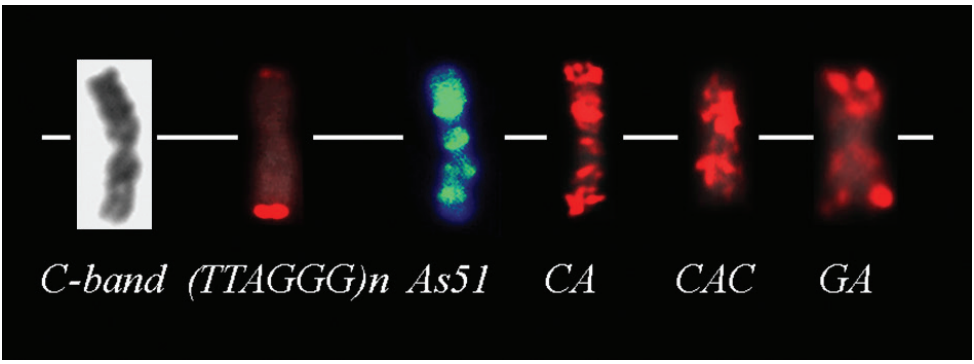

Figure 3. B chromosome of *Astyanax scabripinnis* showing the chromatin and localization of DNA sequences by FISH.

also in the chromosome B (Fig. 3). According to Barbosa et al. (2015) (Fig. 5), additional signals are observed in pairs 4 and 6 sm of the (LFS) population.

The oligonucleotide probes $(CAC)_{10}$, $(CA)_{15}$ and $(GA)_{15}$ microsatellite sequences showed dispersed signal among the chromosomes of complement A in the three populations. In addition, the probes showed an accumulation in B chromosomes of individuals of the (LFS) population (Fig. 3 and Fig. 4a, b, c). However, the $(CA)_{15}$ sequence revealed preferential localization on telomeric regions and on some interstitial sites in the chromosomes of the (LP) population, although this distribution was not homogeneous among the chromosomes (Fig. 4d, e, f). In contrast, the others tested microsatellites probes: $(CAG)_{10}$, $(CAT)_{10}$, $(GAA)_{10}$, $(GAG)_{10}$ and $(GC)_{15}$ appeared to be dispersed and poorly represented in the karyotypes of *A. scabripinnis* (data not shown).

Discussion

Although the diploid number (2n) verified for *Astyanax scabripinnis* species complex indicates a variation of 46, 48 or 50 chromosomes (Moreira-Filho and Bertollo 1991), data found for Campos do Jordão region showed a common feature regarding the diploid and fundamental numbers (2n = 50; FN = 88). These fishes keep a polymorphic B chromosome in some populations (Vicente et al. 1996, Ferro et al. 2003, Vicari et al. 2011, present study). The results obtained herein for the karyotype of the (LFS) population corroborated the findings by Ferro et al. (2001), but differentiated by the verification of a single NOR without the occurrence of multiple sites. It may represent the fixation of only one chromosomal pair carrying 18S ribosomal cistrons in this isolated population.

The karyotype description for the (LP) population was performed here for the first time. The population showed standard diploid number and karyotype formula, with multiple NORs. The karyotype of the (RP) population corroborates data obtained by

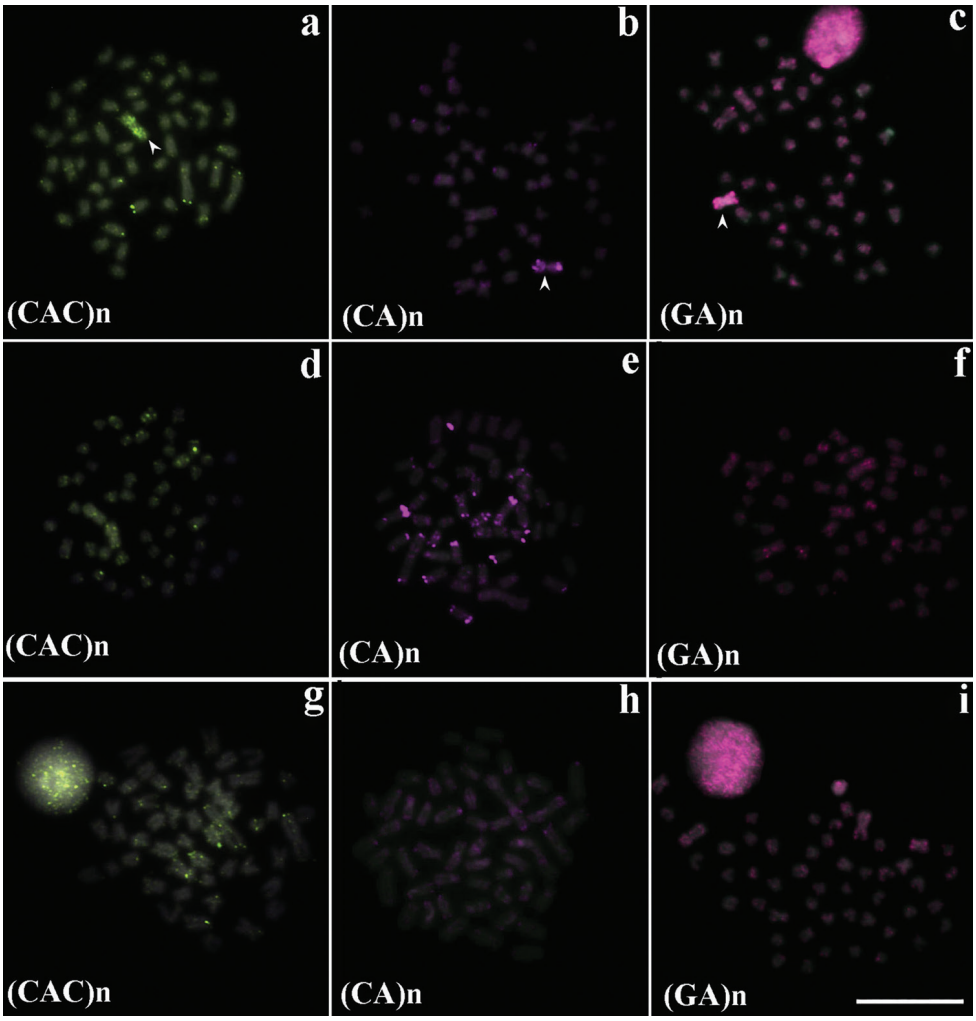

Figure 4. Fluorescence *in situ* Hybridization in metaphases of the three *Astyanax scabripinnis* populations (**a, b, c** Lavrinha Farm Stream – LSF); (**d**, **e**, **f** Lake of Pedalinho – LP); (**g, h, i** Ribeirão das Perdizes - RP) with microsatellites probes. Scales bar: 10 μm.

Vicente et al. (1996) regarding the presence of B chromosome. However, differences in the location of NORs within this population were also observed, since they were exclusively located in the short arm of pair 10 sm in previous studies. Probably, these trends are mediated by the association of additional sites of NORs with *LINE* retrotransposons – among other possible transposable elements (TEs) – and with repetitive sequences such as *As*51 (Barbosa et al. 2015).

Astyanax scabripinnis forms small isolated populations in streams and it facilitates the establishment of chromosomal changes that lead to polymorphism which may explain karyotype variability among individuals and populations (see Moreira-Filho et

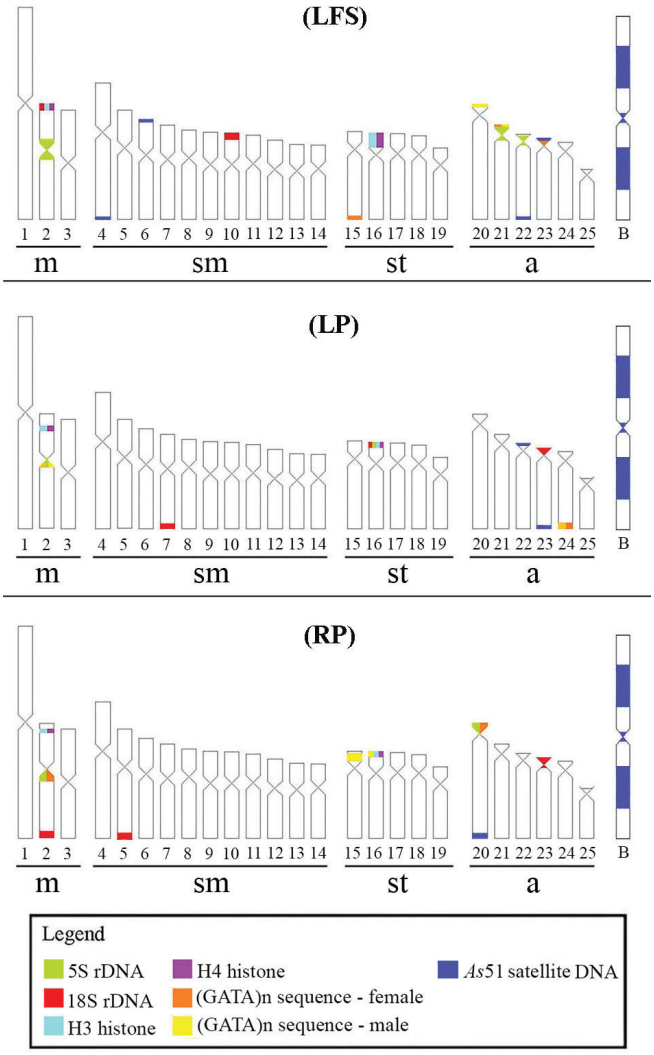

Figure 5. Idiograms of the three *Astyanax scabripinnis* populations (LFS: Lavrinha Farm Stream); (LP: Lake of Pedalinho); (RP: Ribeirão das Perdizes) highlighting all markers of molecular cytogenetics used in this study.

al. 2004 for review). The higher amount in the number of ribosomal gene copies and in the transposition may originate natural polymorphisms, e.g. in *Gymnotus carapo* Linnaeus, 1758, *Apteronotus albifrons* (Linnaeus, 1766), *Sternopygus macrurus* (Bloch & Schneider, 1801), *Eigenmannia virescens* (Valenciennes, 1836) and *Eigenmannia* sp., in which only one of the homologues carries the NOR (Foresti et al. 1981), as it occurs in the specie *A. scabripinnis*. Variations in NORs location may be also due to structural chromosomal rearrangements, such as translocation of ribosomal sites and/or inversions (Gornung 2013) or by association with TEs (reviewed in Reed and

Philips 2000). Data available for the detection and location of major ribosomal DNA genes in different populations of *A. scabripinnis* make the evolutionary forces possible to generate, fix and diversify the NORs. One hypothesis is that the balance would happen due to evolutionary processes – opposite from 'birth-and-death' – *versus* the concerted evolution.

The number of 5S rDNA sites varied between 4 and 5 in the populations, wherein the proximal signal located in pair 2 m is a feature conserved among them. Similar results were described by Ferro et al. (2001) and Mantovani et al. (2005) in a study involving *A. scabripinnis* and by Silva et al. (2014) regarding *A. paranae*. The conserved location of 5S rDNA proximal to the centromere seems to provide protection against rearrangements and dispersions in chromosomes (Martins and Wasko 2004). However, the increase in the number of sites may be associated to TEs, as it occurs in *Gymnotus paraguensis* Albert & Crampton, 2003 (Silva et al. 2011), or to pseudogenes, as suggested for *Semaprochilodus taeniurus* (Valenciennes, 1821) (Terencio et al. 2012), fact that is frequently associated to the weaknesses of the genome rich in repetitive sequences (Rebordinos et al. 2013).

Data on the location of histone genes are still rare. According to Pendás et al. (1994), these genes are arranged in tandem in a unique chromosomal pair. The chromosome mapping of H3 and H4 probes sequences in *A. scabripinnis* was similar to verified by Silva et al. (2013) in *A. bockmanni* and Silva et al. (2014) in A. paranae, which are in syntenic organization in the short arm of metacentric chromosome pair No. 2. Thus, we suggest a conserved location among the species of the genus. The colocation of these clusters is also found in other evolutionarily distant organisms, such as in Acrididae grasshoppers (Cabrero et al. 2009) and it increases the evidences regarding the syntenic positional conservation of these sequences. However, exceptions must be highlighted, as the multiple sites of H2B-H2A and H3 histones found in the marine perciform *Rachycentron canadum* (Linnaeus, 1766) (Costa et al. 2014). Thus, it is still necessary to expand the studies on the physical location and organization of histone genes, in order to better understand this evolutionary scenario.

The location of interstitial telomeric sites (ITS) is an important tool that helps to tell the evolutionary history of a group (Meyne et al. 1990) and it indicates the occurrence of possible chromosomal rearrangements (Ashley and Ward 1993). Centric fusions in chromosomes are described in fish (see Ocalewics 2013 for review), for example, in the Loricariidae family, *Rineloricaria lima* (Rosa et al. 2012) and *Hypostomus iheringii* (Traldi et al. 2013). So far, there is no evidence of ITS in *A. scabripinnis* using telomeric probe. According to the study, the signal was homogeneous in terminal regions of both chromosomal arms of complement A and also in chromosome B, and it suggests that possible rearrangements (centric fusions and/or paracentric inversions) able to lead to interstitial locations of ITS are not frequent, or even that interstitial telomeric sequences are quickly eliminated from the genome of these fishes.

Furthermore, an accumulation of repetitive sequences in the chromosome 2 of the three studied populations and also in the chromosome 16 of the (LP) population was evident. Piscor and Parisi-Maltempi (2016) proposed evolutionary pathways for

microsatellites in the genome of *Astyanax*. The authors propose that the association of 5S rDNA-GATA can stabilize the structure of DNA and act as 'hot spots' for chromosomal recombination. Our data contrast with the Piscor and Parisi-Maltempi (2016) and show that GATA sequences are not restricted to co-location with the 5S rDNA and may occur with sex-specific location in *A. scabripinnis*.

The location of $(GATA)_n$ repetitive sequences revealed a slightly dispersed pattern in chromosomes of *A. scabripinnis*, but with preferential accumulation in terminal and interstitial regions of the three studied populations. Dispersed signal of GATA sequences in fishes are verified by FISH in *Solea senegalensis* Kaup, 1858 (Cross et al. 2006), as well as in species of genus *Hypostomus* Lacépède, 1803 (Traldi et al. 2013) and are conspicuous in a unique chromosomal pair in *Halobatrachus didactylus* Bloch & Schneider, 1801 (Merlo et al. 2007). Jones and Singh (1985) assumed that sequences of the family *Bkm* – the major component of the satellite DNA isolated of snakes by Epplen et al. (1982) – tend to accumulate over the sex chromosomes W and Y of the organisms. Afterwards, Subramanian et al. (2003) suggested that the $(GATA)_n$ sequences have gradually accumulated in the genome of the organisms in the evolutionary scale and they could regulate the expression of close genes by means of chromatin reorganization. This last hypothesis favors the explanation for the widely dispersed location of $(GATA)_n$ sequences over fish chromosomes, but it raises a suspicion of link with sex determination in the case of *Astyanax scabripinnis,* wherein the location of these sequences by FISH appears to be sex-specific. It is worth highlighting that $(GATA)_n$ sequences are rarely coincident to heterochromatic regions in the *A. scabripinnis* populations studied herein.

The presence of B chromosome in *A. scabripinnis* confirmed the heterochromatic pattern found in different populations (see Moreira-Filho et al. 2004 for review). However, the $(GATA)_n$ sequence, ITS and moderately repetitive sequences such as histones and ribosomal DNAs were not identified in these supernumerary chromosomes. Nevertheless, the location of microsatellite sequences ($(CA)_n$; $(CAC)_n$; $(GA)_n$) showed different patterns and it suggests the heterogeneous nature of the chromatin for B chromosomes studied herein.

The mapping of microsatellites in the chromosomes may help in investigating the chromatin nature. Similarly, to data found in chromosomes of *A. scabripinnis* (LP), there might be similar patterns among different species, such as the dinucleotide $(CA)_{15}$, which is found at the centromeric region of chromosome W in lizards *Eremias velox* Eremchenko & Panfilov, 1999 (Pokorná et al. 2011) and in telomeric regions with some interstitial sites in *Erythrinus erythrinus* Bloch & Schneider, 1801 (Yano et al. 2014). The *As*51 satellite DNA is presented in large pericentromeric distal blocks when it is hybridized over B chromosome in this specie (Mestriner et al. 2000, Vicari et al. 2011). As it was described by Mestriner et al. (2000), the repetitive DNA of the complex *Astyanax scabripinnis* by 59% AT bases. In contrast, the current study evidenced the preferential accumulation of dinucleotide $(CA)_{15}$ and $(GA)_{15}$ and the trinucleotide $(CAC)_{10}$ for B chromosome. The compartmentalization of microsatellites may also be verified in the heteromorphic W sex chro-

mosomes of *Triportheus* Cope, 1872 and *Leporinus* Spix & Agassiz, 1829 (Cioffi et al. 2012, Poltronieri et al. 2014). These evidences of microsatellite sequences organization in heterochromatinized chromosomes and non-recombinant suggest the involvement of this DNA class in the amplification and differentiation of these chromosomes.

Thus, the study brings up the required association condition and co-location of different group of repetitive DNAs expressed and non-encoding. Although these sequences may be related to the evolution of genome and karyotype, they still suggest a possible relation with sex determination in the species.

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Supplementary material 1

Karyotype data of the three *Astyanax* aff. *scabripinnis* populations analyzed: diploid number, karyotype formulae, Ag-NORs and repetitive DNAs locations

Authors: Patrícia Barbosa, Eliza Viola Leal, Maelin da Silva, Mara Cristina de Almeida, Orlando Moreira-Filho, Roberto Ferreira Artoni

Data type: karyotype data

Supplementary material 2

DNA sequences of the histone PCR product of the three *Astyanax* aff. *scabripinnis* populations employed as probes for FISH in this study

Authors: Patrícia Barbosa, Eliza Viola Leal, Maelin da Silva, Mara Cristina de Almeida, Orlando Moreira-Filho, Roberto Ferreira Artoni

Data type: molecular data

Explanation note: LFS: Lavrinha Farm Stream; LP: Lake of Pedalinho; RP: Ribeirão das Perdizes

Supplementary material 3

Fluorecence in situ Hybridization in metaphases of the three *Astyanax* aff. *scabripinnis* populations with telomeric probe (TTAGGG)n

Authors: Patrícia Barbosa, Eliza Viola Leal, Maelin da Silva, Mara Cristina de Almeida, Orlando Moreira-Filho, Roberto Ferreira Artoni

Data type: karyotype data

Explanation note: a: Lavrinha Farm Stream – LSF, b: Lake of Pedalinho – LP, c: Ribeirão das Perdizes – RP.

Supplementary material 4

Metaphases of the three *Astyanax* aff. *scabripinnis* populations (a, d); (b, e); (c, f) showing C-banding. Staining with propidium iodide converted to grayscale

Authors: Patrícia Barbosa, Eliza Viola Leal, Maelin da Silva, Mara Cristina de Almeida, Orlando Moreira-Filho, Roberto Ferreira Artoni

Data type: karyotype data

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RESEARCH ARTICLE

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First karyotype description and nuclear 2C value for *Myrsine* (Primulaceae): comparing three species

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Abstract

Cytogenetic studies in Primulaceae are mostly available for herbaceous species, and are focused on the chromosome number determination. An accurate karyotype characterization represents a starting point to know the morphometry and class of the chromosomes. Comparison among species within *Myrsine*, associating these data with the nuclear 2C value, can show changes that led the karyotype evolution. Here, we studied three *Myrsine* species [*Myrsine coriacea* (Swartz, 1788) Brown ex Roemer et Schultes, 1819, *Myrsine umbellata* Martius, 1841 and *Myrsine parvifolia* Candolle, 1841] that show different abilities to occupy the varied types of vegetation within the Brazilian Atlantic Forest. Cytogenetic characterization showed some individuals with 2n = 45 chromosomes for *M. parvifolia* and *M. coriacea*, with most individuals of the three species having 2n = 46. The first karyograms for *Myrsine* were assembled and presented morphologically identical and distinct chromosome pairs. In addition, differences in the mean 2C nuclear value and chromosome morphometry were found. Therefore, the first description of the *Myrsine* karyotype has been presented, as well as the nuclear 2C value. The procedures can be applied to other *Myrsine* species for future investigations in order to better understand its effects on the differential spatial occupation abilities shown by the species in Brazilian Atlantic Forest.

Keywords
Atlantic Forest, cytogenetics, flow cytometry, karyogram, Myrsinaceae, *Rapanea*

Introduction

Previous studies regarding the chromosome number in Primulaceae (s. APG 2016) are available for some genera, as: *Cyclamen* Linnaeus, 1753 (Bennett and Grimshaw 1991, Ishizaka 2003), *Anagallis* Linnaeus, 1753 (Aguilera et al. 2011, Bennett and Leitch 2012), *Lysimachia* Linnaeus, 1753 (Baltisberger and Kocyan 2010, Bennett and Leitch 2012, Chalup and Seijo 2013), *Androsace* Linnaeus, 1753 (Chepinoga et al. 2009), *Elingamita* Baylis, 1951 (Dawson 1995), *Trientalis* Linnaeus, 1753 (Vickery and Miller 2008), *Ardisia* Swartz, 1788 (Koyama and Kokubugata 1998), *Primula* Linnaeus, 1753 (Abou-El-Enain 2006, Casazza et al. 2012, Theodoridis et al. 2013), and *Dodecatheon* Linnaeus, 1753 (Oberle et al. 2012), and *Myrsine* Linnaeus, 1753 (Beuzenberg and Hair 1983, Dawson 1995, 2000, Hanson et al. 2003, Rice et al. 2015). Except the genus *Cyclamen* and *Myrsine,* these taxa comprise annual and biennial herbaceous species.

The cosmopolitan *Myrsine* Linnaeus is one of the main genera of Primulaceae, considering species richness, represented by tree and shrub species (Heenan and Lange 1998). Its members are generally dioecious plants, characterized by ramiflorus and congested inflorescences, and flowers with oppositipetalous stamens. Despite *Myrsine* being one of the largest and most important genera of Primulaceae, only eighteen species, among the 300 estimated from this genus, have been studied regarding cytogenetic aspects. Fifteen of these species occur in the African, Asian and Oceania continents (*M. coxii* Cochayne, 1902, *M. divaricata* Cunningham, 1839, *M. kermadecensis* Cheeseman, 1887, *M. nummularia* (Hooker f.) Hooker f., 1867, *M. salicina* (Hooker f.) Hooker f., 1864, *M. argentea* Heenan et de Lange, 1998, *M. oliveri* Allan, 1961, *M. chathamica* Mueller, 1864; *M. africana* Linnaeus, 1753; *M. sandwicensis* Candolle, 1841, *M. seguinii* Léveille, 1914, *M. semiserrata* Wallich, 1824, *M. australis* (A. Richard, 1832) Allan, 1947, *M. capitellata* Wallich, 1824), and just three occurs in America continent (*M. matensis* (Mez, 1902) Otegui, 1998; *M. guianensis* (Aublet, 1775) Kuntze, 1891, *M. coriacea* (Swartz, 1788) Brown ex Roemer et Schultes, 1819. The chromosome number (2n = 46 or 2n = 48) was the only karyotype data reported, without any images of the chromosomes. In addition, the evolutionary aspects that culminated in the karyotype diversification within the genus are poorly understood.

One interesting ecological aspect observed in Neotropical species of *Myrsine* that occur in Brazil is that some of them occur in more than one biome, as Cerrado, Atlantic Forest, and Amazonian Forest, while others are restricted of one of these biomes, as Atlantic Forest (BFG 2015). Among species that occur in Atlantic Forest, for example, some are able to occupy different types of vegetation within this biome, including Restinga Vegetation, High Altitude Campos, Rocky Outcrops, Ombrophyllous and Mixed Ombrophyllous Forests, while others are able to occupy just one type of vegeta-

tion (Freitas and Kinoshita 2015). Considering the distinct ecological aspects, cytogenetic studies are relevant to show other differences between these species.

Studies combining cytogenetics and nuclear DNA content have offered data for understanding evolutionary processes in different species (Clarindo and Carvalho 2008, Kolář et al. 2013). Measurement of the nuclear DNA content is complementary to cytogenetic information and is useful for detecting genome size variations between related species (Marhold et al. 2010, Kolář et al. 2013). Fine adjustments in cytogenetic procedures, combining advances in microscopy and image analysis systems, can provide accurate karyotype characterization for *Myrsine* species. Here, we study three species of *Myrsine* that occur in contrasting types of vegetation of the Brazilian Atlantic Forest, aiming to determine the chromosome number, describe the karyotype and measure the nuclear DNA.

Material and methods

Plant samples

Three species were selected for this study: 1. *Myrsine coriacea* (Voucher – T.T. Carrijo 1458, VIES herbarium), which is a widespread species in Atlantic Forest found in all types of vegetation, including open areas within Ombrophyllous and Mixed Ombrophyllous Forests, Rock Outcrops, High Altitude Campos, and Restinga Vegetation; 2. *Myrsine umbellata* (Voucher – T.T. Carrijo 1467, VIES herbarium), which is found in mostly all types of vegetation of *M. coriacea*, except High Altitude Campos; and 3. *Myrsine parvifolia* (Voucher – T.T. Carrijo 2232, VIES herbarium), a species restricted to Restinga vegetation (BFG 2015).

Fruits and leaves of all species were collected. *Myrsine coriacea* and *M. umbellata* were sampled from October 2012 to July 2015 in a forest remnant located in Iúna municipality, Espírito Santo (ES) State, Brazil (20°21'6"S – 41°31'58"W), characterized as Rocky Outcrops, at 600 (*M. coriacea*) and 1,100 m.s.m (*M. umbellata*). *M. parvifolia* was collected in a forest remnant located in Guarapari municipality, ES, Brazil (20°36'15"S – 40°25'27"W), characterized as coastal sandy plains vegetation (Restinga) at sea level. Leaves and fruits of *Solanum lycopersicum* L. and *Pisum sativum* L. (internal standards for flow cytometry – FCM, 2C = 2.00 pg and 2C = 9.16 pg, respectively, Praça-Fontes et al. 2011) were supplied by Dr. Jaroslav Doležel (Experimental Institute of Botany, Czech Republic).

In vitro plantlet recovering

Fruit pericarp was manually removed and the seeds were desinfested according to Oliveira et al. (2013) and germinated in a medium composed of MS salts (Sigma) and vitamins (Murashige and Skoog 1962), 30 g L^{-1} sucrose (Sigma), 7 g L^{-1} agar and

2.685 μM naphthaleneacetic acid (NAA, Sigma). *Solanum lycopersicum* and *P. sativum* seeds were subjected to the same disinfestation procedure and inoculated in medium without NAA. Germination was done at 25 °C under a 16/8 hours (light/dark) regime.

Nuclear 2C value measurement

In order to adapt the FCM for *Myrsine*, the following procedures were done: (a) initially, from leaves collected in the field of male and female individuals (samples) and of the two standards; (b) afterward, replacing the dithiothreitol antioxidant by polyethylene glycol (PEG) in nuclei isolation buffer; and (c) from leaves of the samples and *P. sativum* plantlets in vitro cultivated.

Nuclei suspensions were obtained by co-chopping (Galbraith et al. 1983) leaf fragments (1 cm^2) cut from each sample (*Myrsine* species) and standard (*S. lycopersicum* or *P. sativum*). The suspensions were processed and stained following Otto (1990) and Praça-Fontes et al. (2011) and analyzed with the flow cytometer Partec PAS II/III (Partec GmbH). *Myrsine* genome size was measured by multiplying the 2C value of the internal standard using the fluorescence intensity corresponding to G_0/G_1 nuclei peak. Mean 2C values were compared by the *F* test at 5% probability.

Cytogenetic analysis

Roots were cut from the in vitro plantlets, treated with 5.0 μM amiprofos-methyl (APM) (Agrochem KK Nihon Bayer) for 12, 15, 18 or 24 h at 4°C, rinsed in distilled water (dH_2O) for 20 min and fixed in methanol:acetic acid (3:1) for 24 h. The fixative solution was changed three times and the material was stored at -20°C (Carvalho et al. 2007). The roots were washed, macerated in 1:5 pectinase solution (enzyme:dH_2O) for 3 h at 34°C, or 1:20 enzymatic pool (4% cellulase – Kinki Yakult MFG, 1% macerozyme – Kinki Yakult MFG, and 0.4% hemicellulase – Sigma) for 1 h 30 min or 1 h 45 min at 34°C, washed in dH_2O, fixed, and stored at -20°C.

Slides were prepared and stained according to Carvalho et al. (2007) and analyzed on a Nikon eclipse Ci-S microscope (Nikon). Prometaphases and metaphases were captured using the 100× objective and a CCD camera (Nikon Evolution™) coupled to a Nikon microscope 80i (Nikon). About 100 slides were analyzed for each *Myrsine* species. Chromosome morphometry was characterized and the class was determined as proposed by Levan et al. (1964) and reviewed by Guerra (1986).

Using chromosome morphometric data (total, short and long arm length), the standardized Euclidean Distance and Unweighted Pair-Group Method Average (UPGMA) was applied to each species. In addition, the value of the relative size (% size in relation to sum of the mean values of total length, Table 1) of each chromosome was compared among species by the Scott-Knott test at 5% probability. Analyses were made using the software R 3.2.4 (R Core Team 2016).

Table 1. Morphometry and chromosome class performed at least 10 prometaphases/metaphases. In all species were found chromosomes morphologically indentical, similar and distinct.

	M. parvifolia						*M. coriacea*						*M. umbellata*					
Chrom.	**Total ± SD**	**Short**	**Long**	**r**	**Class**	**Relative size (%)**	**Total ± SD**	**Short**	**Long**	**r**	**Class**	**Relative size (%)**	**Total ± SD**	**Short**	**Long**	**r**	**Class**	**Relative size (%)**
1	2.64 ± 0.29	1.01	1.63	1.61	SM	5.60	2.79 ± 0.09	1.24	1.55	1.25	M	6.14	2.72 ± 0.06	1.14	1.59	1.39	M	6.60
2	2.47 ± 0.23	1.09	1.37	1.25	M	5.24	2.45 ± 0.11	1.02	1.42	1.38	M	5.38	2.67 ± 0.06	1.14	1.54	1.35	M	6.48
3	2.45 ± 0.22	0.86	1.59	1.85	SM	5.19	2.35 ± 0.10	1.09	1.26	1.15	M	5.17	2.13 ± 0.16	0.94	1.19	1.26	M	5.16
4	2.44 ± 0.27	0.68	1.75	2.55	SM	5.17	2.30 ± 0.05	1.02	1.27	1.24	M	5.06	2.13 ± 0.08	0.84	1.29	1.53	SM	5.16
5	2.24 ± 0.18	0.71	1.53	2.13	SM	4.76	2.29 ± 0.08	0.99	1.30	1.30	M	5.04	2.08 ± 0.14	0.74	1.34	1.80	SM	5.04
6	2.21 ± 0.17	0.73	1.48	2.00	SM	4.69	2.22 ± 0.17	0.86	1.36	1.57	SM	4.88	1.88 ± 0.11	0.64	1.24	1.92	SM	4.56
7	2.18 ± 0.25	0.81	1.37	1.68	SM	4.62	2.17 ± 0.12	0.78	1.39	1.77	SM	4.77	1.83 ± 0.11	0.79	1.04	1.31	M	4.44
8	2.16 ± 0.27	0.61	1.55	2.51	SM	4.59	2.12 ± 0.11	0.78	1.34	1.71	SM	4.67	1.83 ± 0.09	0.59	1.24	2.08	SM	4.44
9	2.15 ± 0.29	0.86	1.29	1.49	M	4.55	2.04 ± 0.15	0.81	1.23	1.50	SM	4.49	1.83 ± 0.09	0.59	1.24	2.08	SM	4.44
10	2.13 ± 0.25	0.61	1.51	2.45	SM	4.51	2.00 ± 0.10	0.78	1.23	1.56	SM	4.41	1.78 ± 0.12	0.59	1.19	2.00	SM	4.32
11	2.09 ± 0.22	0.79	1.31	1.65	SM	4.44	2.00 ± 0.17	0.75	1.26	1.67	SM	4.41	1.68 ± 0.13	0.59	1.09	1.83	SM	4.08
12	1.99 ± 0.16	0.75	1.23	1.63	SM	4.22	1.89 ± 0.10	0.71	1.18	1.64	SM	4.16	1.68 ± 0.08	0.59	1.09	1.83	SM	4.08
13	1.97 ± 0.23	0.66	1.31	1.96	SM	4.19	1.89 ± 0.07	0.57	1.32	2.31	SM	4.16	1.68 ± 0.10	0.49	1.19	2.40	SM	4.08
14	1.95 ± 0.14	0.65	1.30	2.00	SM	4.14	1.84 ± 0.06	0.55	1.29	2.32	SM	4.06	1.68 ± 0.14	0.66	1.02	1.52	SM	4.08
15	1.93 ± 0.16	0.72	1.21	1.67	SM	4.11	1.81 ± 0.11	0.65	1.16	1.78	SM	3.98	1.58 ± 0.06	0.64	0.94	1.46	M	3.84
16	1.85 ± 0.13	0.65	1.20	1.82	SM	3.93	1.81 ± 0.08	0.57	1.24	2.17	SM	3.98	1.58 ± 0.06	0.69	0.89	1.29	M	3.84
17	1.85 ± 0.23	0.72	1.13	1.56	SM	3.93	1.78 ± 0.04	0.66	1.11	1.66	SM	3.91	1.58 ± 0.09	0.69	0.89	1.29	M	3.84
18	1.84 ± 0.19	0.70	1.15	1.63	SM	3.92	1.71 ± 0.13	0.65	1.06	1.63	SM	3.77	1.58 ± 0.11	0.59	0.99	1.67	SM	3.84
19	1.82 ± 0.22	0.63	1.20	1.89	SM	3.87	1.68 ± 0.11	0.55	1.13	2.03	SM	3.70	1.58 ± 0.08	0.59	0.99	1.67	SM	3.84
20	1.75 ± 0.18	0.68	1.06	1.55	SM	3.71	1.67 ± 0.09	0.58	1.09	1.85	SM	3.69	1.58 ± 0.13	0.49	1.09	2.20	SM	3.84
21	1.68 ± 0.14	0.79	0.89	1.13	M	3.56	1.55 ± 0.16	0.49	1.06	2.17	SM	3.42	1.43 ± 0.14	0.59	0.84	1.42	M	3.48
22	1.66 ± 0.16	0.58	1.08	1.85	SM	3.53	1.55 ± 0.04	0.35	1.20	3.33	A	3.42	1.38 ± 0.11	0.49	0.89	1.80	SM	3.36
23	1.66 ± 0.30	0.58	1.08	1.86	SM	3.53	1.52 ± 0.07	0.39	1.13	2.88	SM	3.34	1.28 ± 0.10	0.59	0.69	1.17	M	3.13
Sum	47.22	16.99	30.23			100.00	45.53	16.96	28.57			100.00	41.30	15.79	25.51			100.00

Chrom = chromosomes; Total = total length; SD = standard deviation; Long/Short = arm length; r = arm ratio – long/short; M = metacentric; SM = submetacentric; A = acrocentric; Relative size = % size in relation to sum of the mean values of total length; Sum = sum of the mean values.

Results

In vitro plantlet recovering

Approximately 60 days after in vitro inoculation, plantlets were obtained for the three *Myrsine* species. All plantlets exhibited sufficient and morphologically normal leaves and roots for FCM and cytogenetic analyses, respectively.

Nuclear 2C value measurement

FCM analysis performed on leaves collected in the field did not result in histograms showing profile G_0/G_1 peaks. So, dithiothreitol antioxidant was replaced by PEG in the nuclei isolation buffer OTTO I. This change provided G_0/G_1 peaks, exhibiting a coefficient of variation (CV) less than 5% for *M. umbellata* and the two internal standards. The channel of the *P. sativum* G_0/G_1 peak however was closer to *M. umbellata* than *S. lycopersicum*. Thus, based on linearity international criteria for FCM, *P. sativum* was the standard chosen for the next measurements. The mean 2C value of the male (2C = 6.65 pg ± 0.02) and female (2C = 6.67 pg ± 0.11) *M. umbellata* individuals were statistically identical by the *F* test. Considering these previous results, the 2C value was measured from leaves of in vitro plantlets. The mean values were 2C = 4.81 pg ± 0.05 for *M. parvifolia*, 2C = 6.60 pg ± 0.14 for *M. coriacea* and 2C = 6.63 pg ± 0.13 for *M. umbellata*. The mean value of the *M. umbellata* in vitro plantlets was statistically identical to the males and females in the field. Therefore, the mean value adopted for this species was 2C = 6.65 pg, which was statistically equal to the *M. coriacea*.

Cytogenetic analysis

Roots exposed to a 12 h APM provided prometaphases, exhibiting chromosomes at a distinct chromatin compact level, and metaphases. Enzymatic maceration in 1:5 pectinase solution ensured the chromosomes remained inside the cell, allowing an accurate determination of 2n = 45 or 2n = 46. Chromosome number of 2n = 45 was found for 12.60% individuals of *M. parvifolia* and 8.45% of *M. coriacea*, with 2n = 46 for the three species. Based on these results, the next slides were made from roots of particular seedlings with 2n = 45 or 2n = 46. Root maceration with 1:20 enzymatic pool for 1h 30 min supplied chromosomes no damage to the chromatin structure, without overlapping, with well-defined centromeres and free of cytoplasm debris (Fig. 1).

Karyotype characterization was possible only after carefully testing the time and concentration of the APM antitubulin and cell wall enzymes. *Myrsine parvifolia* presented a greater total sum of the length of the chromosomes despite having less nuclear DNA content. For this species only, we found prometaphase chromosomes showing low level of chromatin compaction (Fig. 2a), resulting in a higher sum of the total

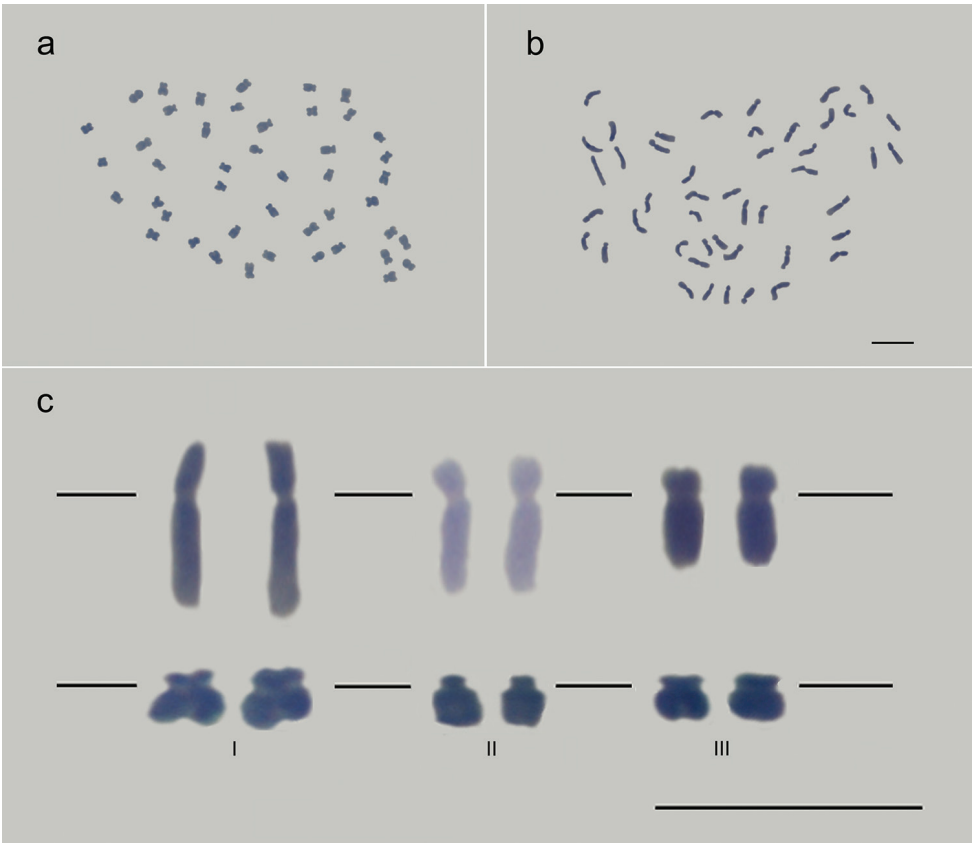

Figure 1. First images of the *Myrsine* chromosomes. Karyotype of a *M. parvifolia* individual with 2n = 45 (**a**) and another with 2n = 46 (**b**) chromosomes. Note the different levels of chromatin compaction between the chromosomes of the two karyotypes. The distinct chromatin compact level was highlighted in (**c**), where the same submetacentric chromosome of *M. parvifolia* (above) and the same acrocentric chromosome of *M. coriacea* (below) were taken from two different prometaphases (I and II) and one metaphase (III). Bar = 5 μm.

length (Table 1). *Myrsine coriacea* and *M. umbellata* did not show pronounced variation in chromatin compaction, but the quality of the chromosomes allowed us to characterize the karyotype and to assemble the karyogram (Fig. 2b–c, Table 1).

Morphometric analysis was used to classify the chromosomes and evidence similarities and differences among species karyotypes. *Myrsine parvifolia* presented three metacentric (2, 9 and 21) and 20 submetacentric (1, 3–8, 10–20, 22 and 23) chromosome pairs, *M. coriacea* showed five metacentric (1–5), 17 submetacentric (6–21 and 23) and one acrocentric (22) chromosome pairs, and *M. umbellata* displayed nine metacentric (1–3, 7, 15–17, 21 and 23) and 14 submetacentric (4–6, 8–14, 17, 18, 20 and 22) chromosome pairs (Fig. 2, Table 1).

Morphologically similar and identical chromosomes groups were found in all species. *Myrsine parvifolia* presented sets of two chromosome pairs (5–6, 13–14, 16–17

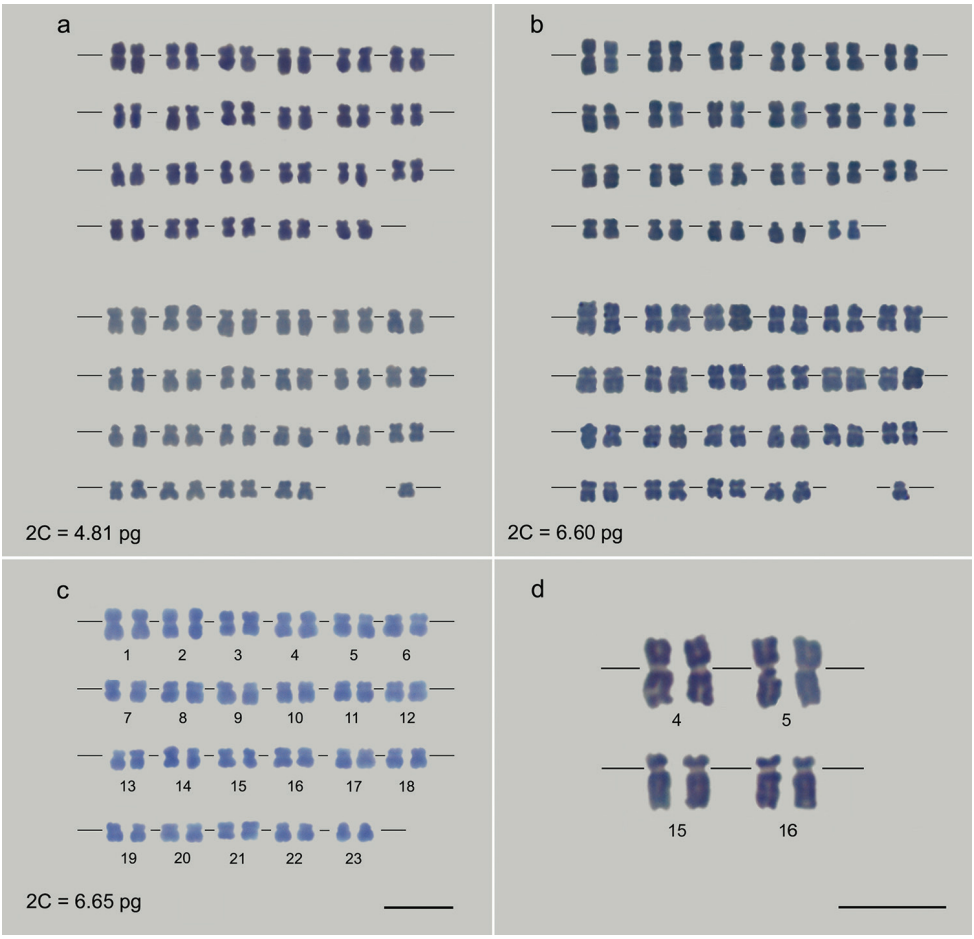

Figure 2. *Myrsine* karyograms displaying 2n = 45 (**a** *M. parvifolia* and **b** *M. coriacea*) or 2n = 46 chromosomes (**a**–**c** the three species). In all *M. parvifolia* (**a**) and *M. coriacea* (**b**) individuals with 2n = 45, the odd chromosome number was well-marked by absence of the homologue pair of the chromosome 23. Metacentric and submetacentric chromosomes prevailing in the karyograms of the three species, with only one acrocentric chromosome was identified in *M. coriacea* (**b** chromosome 22). Although showing approximately 2C = 1.50 pg less DNA, *M. parvifolia* (a) displayed the same chromosome number in relation to the other species (**b** *M. coriacea* **c** *M. umbellata*). For all species, morphometric analyses showed identical, similar and distinct chromosome pairs with regard to morphometry and class. The similarity of some chromosomes was highlighted from the metacentric chromosome pairs 4 and 5 (**d** above) and submetacentric 15 and 16 (**d** below) of *M. coriacea*. In contrast, other chromosomes showed singular morphology, as the chromosome 1 and 2 of all species, the 22 of *M. coriacea*, which is the single acrocentric chromosome, and the chromosome 23. Bar = 5 μm.

and 22–23), as did *M. coriacea* (4–5, 10–11, 13–14, 15–16 and 19–20), and *M. umbellata* presented three sets of two (11–12, 16–17 and 18–19) and one set of three chromosome pairs (8–10). The other chromosome pairs in each species were con-

Table 2. Chromosome groups of the *Myrsine* karyotype suggested from karyogram evaluation (Fig. 2 and Table 1) and confirmed by UPGMA clustering (Fig. 3a–c).

Species	*Karyogram evaluation	**UPGMA clustering	***Confirmed chromosome groups
M. parvifolia	5–6; 13–14; 16–17; and 22–23	1 and 2; 3–11; and 12–23	5–6; 13–14; 16–17; and 22–23
M. coriacea	4–5; 9–10; 13–14; 15–16; and 19–20	1; 2–11; and 12–23	4–5; 9–10; 13–14; 15–16; and 19–20
M. umbellata	8–10; 11–12; 16–17; and 18–19	1 and 2; 3–5, 7; and 6, 8–23	8–10; 11–12; 16–17; and 18–19

* Chromosome groups morphologically identical or similar defined from all morphometric data (total length, short and long arms, r = ratio long/short arm, chromosomal class; relative size) and observation of the karyogram.

** Chromosome groups formed by UPGMA clustering method using data about total, short and long arms length.

*** Common chromosome groups evidenced by two analyses (qualitative *x* quantitative).

sidered morphologically distinct (Fig. 2, Table 1, 2). Using morphometric data and applying the UPGMA statistical analysis, the chromosomes of each *Myrsine* species were grouped in three clusters in all species (Fig. 3a–c, Table 2). Chromosome groups formed by qualitative analysis of all species were clustered by UPGMA, supporting previous findings.

As the mean 2C values of *M. coriacea* (6.60 pg) and *M. umbellata* (6.65 pg) were statistically identical, the Scott-Knott test was used to compare the relative size (Table 1) of each chromosome of these species. Chromosomes 1, 2, 6, 7, 11, 14, 19 and 23 differed between the species, while the others were statistically identical (Fig. 3d, Table 2).

Discussion

The first step in FCM was to define the best antioxidant and internal standard. The presence of secondary metabolites in the *Myrsine* leaves, such as tannins, saponins, flavonoids and steroids (Abbi et al. 2011) made this challenging. These compounds probably prevented us from measuring the 2C value in individuals from the field when the OTTO I buffer (Otto 1990) was supplemented with dithiothreitol. Cytosolic compounds can reduce or inhibit the interaction of the fluorochromes and DNA during the nuclei staining step (Noirot et al. 2003). Antioxidants inhibit this interference, preserving the chromatin structure (Shapiro 2003). Nevertheless, the dithiothreitol was not efficient at providing nuclei suspensions suitable for FCM. Thus, this compound, which is more specific for molecules that possess free sulfhydryl groups, was replaced by PEG because of its wide spectrum for antioxidant activities, an effect called PEGylation (Term Fisher Scientific 2016). Due to this effect, PEG was more efficient at inhibiting the action of cytosolic compounds, resulting in G_0/G_1 peaks for *M. umbellata* and *P. sativum* with CV below 5%. Owing to the linearity parameter, *P. sativum* was a more adequate standard relative to *S. lycopersicum*, which reduced measurement errors.

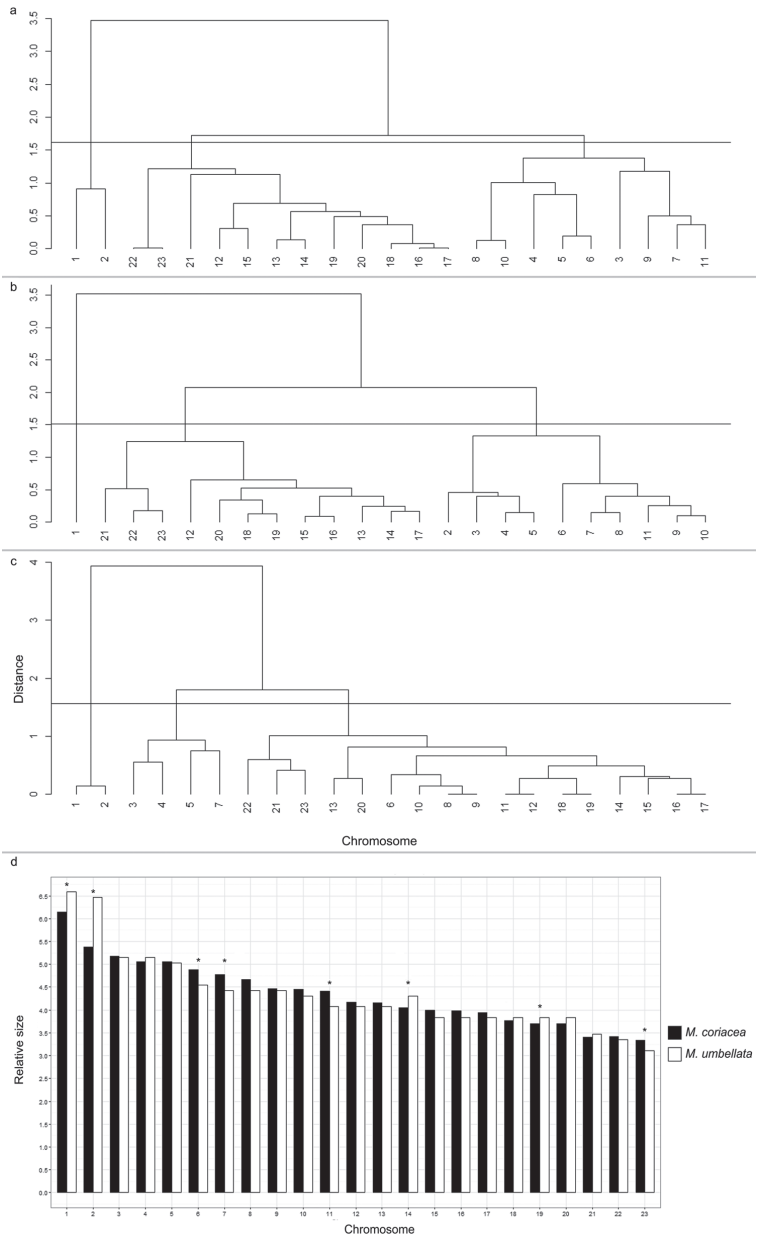

Figure 3. a–c Multivariate clustering generated from chromosome morphometric data (total, long and short arms length). Mojena's criteria showed three clusters for *M. parvifolia* (**a**), *M. coriacea* (**b**) and *M. umbellata* (**c**) with cut point between 1.5 to 1.8. This analysis confirmed the morphological discrepancy of the chromosome 1, and the similarity of other chromosomes (**d**) Graphic provided by comparison between mean relative size (% size in relation to sum of the mean values of total length, Table 1) of each chromosome of *M. coriacea* and *M. umbellata*. The chromosomes 1, 2, 6, 7, 11, 14, 19 and 23 (*) between the species are statistically different in relation to mean relative size according to Scott Knott test at 5% of probability.

Secondary metabolite interference was completely resolved for other *Myrsine* species using in vitro plantlets propagated in a controlled environment. FCM measurements from leaves collected in the field may have been influenced by environmental conditions. Secondary metabolite production is influenced by humidity, temperature, light intensity and the availability of water and nutrients (Akula and Ravishankar 2011). Thus, the conditions at each elevation gradient can be associated with the FCM result, suggesting a differentiated production of secondary metabolic compounds for *Myrsine* at distinct altitudes.

Genome size in *Myrsine* had only been reported for *M. africana* as 2C = 2.46 pg (Hanson et al. 2003), which was measured by Feulgen microdensitometry using *Vigna* sp. as standard. Levels of endoreduplication in cells of *V. radiata*, varying from 2C to 64C, were reported by Pal et al. (2004). Thus, the differences, which were about 200% between the values found for *Myrsine* species in this study and the value observed for *M. africana*, can be related to the C value of *V. radiata* used as reference.

Values close to *M. umbellata* and *M. coriacea* species were reported for *Cyclamen purpurascens* Mill. (2C = 6.60 pg) and *Dodecatheon meadia* L. (2C = 5.58 pg). Higher DNA contents were described for *Cyclamen coum* Mill. (2C = 13.56 pg), *Soldanella pusilla* Baumg. (2C = 12.36 pg), and lower values for *Soldanella hungarica* Simonk (2C = 3.16 pg) and *Primula vulgaris* Huds (2C = 0.47 pg) (Bennett and Leitch 2012). The interspecific variation for the 2C DNA value found in this study, as for other species of Primulaceae (Bennett and Leitch 2012), suggests the occurrence of karyotype changes.

As well as for FCM, karyotype data about *Myrsine* species in the literature are very limited, with only the chromosome number reported (Beuzenberg and Hair 1983, Dawson 1995, Dawson 2000, Molero et al. 2002, Molero et al. 2006, Rice et al. 2015). In vitro *Myrsine* plantlets were fundamental for providing sufficient quantities of roots for the cytogenetic study independent of the reproductive period. Meticulous standardization of the antimitotic agent and enzymatic maceration were also essential for accurate chromosomal characterization.

Chromosome number 2n = 46 (Beuzenberg and Hair 1983, Dawson 1995, Dawson 2000, Molero et al. 2002, Rice et al. 2015, present study) and 2n = 48 (Molero et al. 2006) had been reported, but this was the first record of 2n = 45. The odd chromosome number 2n = 45 was well-marked by absence of the homologue pair of the chromosome 23 (Fig. 2). So, other cytogenetic approaches should be performed from *Myrsine* individuals separately to know the cause of this aneuploidy.

Some chromosome groups determined by statistical analysis are morphologically distinct, such as chromosomes 22 and 23 of *M. coriacea*. Although clustered (Fig. 3b), these chromosomes are cytogenetically distinct, with 22 being acrocentric and 23 submetacentric (Fig. 2b, Table 2). Likewise, distinct chromosomes clustered in *M. parvifolia* (Fig. 3a, Table 2) and *M. umbellata* (Fig. 3c, Table 2). Chromosome 1 of *M. coriacea* and 1 and 2 of *M. parvifolia* and *M. umbellata* presented the highest contrast, considering the morphology and Euclidean distances (Fig. 2, Fig. 3). Similarities and differences regarding relative size (% size in relation to sum

of the mean values of total length, Table 1) were shown between *M. coriacea* and *M. umbellata* through the Scott-Knott test. The similarities, which were shown for some chromosomes, imply that these species could have originated from a common ancestor. The distinct chromosomes are likely to be attributed to karyotype changes that happened throughout their evolution, altering the chromosome relative size and contributing to taxa diversification. Comparative investigations of the karyotypes of related species have usually been applied to infer the evolutionary role of karyotypic modifications in different taxa and to describe the pattern and directions of chromosomal evolution within a group (Stebbins 1971, Soltis and Soltis 2012, Amaral-Silva et al. 2016).

Based on the constant chromosome number displayed by *Myrsine* species, interspecific variation of the nuclear 2C value between *M. parvifolia* compared to *M. coriacea* and *M. umbellata* was also caused by karyotype alterations. The changes to the nuclear DNA content have also been attributed to structural rearrangements and/or heterochromatin polymorphisms (Pellicer et al. 2014, Amaral-Silva et al. 2016).

In conclusion, the first karyotype description and data about nuclear 2C value were shown for three *Myrsine* species. Besides of the comparison between them, these data represent the basis to understand karyotype evolution in *Myrsine*.

Author contribution statement

The authors Carvalho RF, Amaral-Silva PM, Spadeto MS and Clarindo WR conceived, designed and conducted the tissue culture, flow cytometry and cytogenetic approaches. Carvalho CR contributed the flow cytometry analysis. Amaral-Silva PP and Carrijo TT collected and identified the *Myrsine* species. Nunes ACP did the statistical analysis. All authors contributed equally to manuscript editing and revision and approved the final manuscript for submission.

Conflict of interest

The authors declare they have no conflict of interest.

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RESEARCH ARTICLE

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A cytogenetic study of three parasitic wasp species (Hymenoptera, Chalcidoidea, Eulophidae, Trichogrammatidae) from Brazil using chromosome morphometrics and base-specific fluorochrome staining

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Abstract

Chromosomes of three chalcid wasp species from Brazil, *Palmistichus elaeisis* Delvare et LaSalle, 1993, *Trichospilus diatraeae* Cherian et Margabandhu, 1942 (both belonging to the family Eulophidae) and *Trichogramma pretiosum* Riley, 1879 (Trichogrammatidae), were studied using chromosome morphometrics and base-specific fluorochrome staining. The present study confirmed that these species respectively have 2n = 12, 14 and 10. Chromomycin A_3 / 4', 6-diamidino-2-phenylindole (CMA_3/DAPI) staining revealed a single CMA_3-positive and DAPI-negative band within haploid karyotypes of both *P. elaeisis* and *T. pretiosum*. This CG-rich band clearly corresponds to the nucleolus organizing region (NOR). Moreover, analogous multiple telomeric bands found on all chromosomes of *T. diatraeae* may also represent NORs. Certain features of karyotype evolution of the phylogenetic lineage comprising both Eulophidae and Trichogrammatidae are discussed. The results obtained during the present study demonstrate the importance of chromosome research on tropical parasitoids that remain poorly known in this respect.

Keywords

Parasitoids, chromosomes, karyotypes, NORs, CMA_3, DAPI

Introduction

Parasitoid Hymenoptera are one of the most species-rich, taxonomically complicated and economically important insect groups (Godfray 1994), with their species number in the world fauna probably approaching one million (Quicke 1997). Despite rapid accumulation of karyotypic data, they are still available for just about 500 parasitoid species (Gokhman 2009). Moreover, chromosome sets of only a few members of this group coming from tropical regions are studied up to now (see e.g. Silva-Junior et al. 2000a), which substantially hampers our knowledge of worldwide patterns of karyotype structure and evolution in parasitoid Hymenoptera. Furthermore, research of this kind rarely goes beyond the chromosome numbers and some other results of conventional staining (Silva-Junior et al. 2000b, Santos et al. 2015), and when it does, it often reveals previously unknown karyotypic features (Carabajal Paladino et al. 2013). To promote better understanding of the above-mentioned patterns, we have recently studied chromosome sets of three polyphagous parasitoids from Brazil, i.e., *Palmistichus elaeisis* Delvare et LaSalle, 1993, *Trichospilus diatraeae* Cherian et Margabandhu, 1942 (both belong to the family Eulophidae) and *Trichogramma pretiosum* Riley, 1879 (Trichogrammatidae) using both routine (Giemsa) and base-specific fluorochrome staining.

Material and methods

Origin of parasitoids

Parasitoid strains were kept as lab stocks in the Laboratory of Biological Control of Insects of the Faculdade de Ciências Biológicas e Ambientais of the Universidade Federal da Grande Dourados, Dourados, Mato Grosso do Sul, Brazil, in climate-controlled chambers at 25 ± 1°C, 70 ± 10% relative humidity, and 14-hour photoperiod. All examined strains were maintained on lepidopteran hosts. Specifically, both studied members of the family Eulophidae and *T. pretiosum* were respectively bred on the pupae of *Diatraea saccharalis* (Fabricius, 1794) (Crambidae) and eggs of *Ephestia kuehniella* (Zeller, 1879) (Pyralidae). Parasitoids were identified by Marcelo Teixeira Tavares (Eulophidae) and Ranyse Barbosa Querino da Silva (Trichogrammatidae).

Preparation of chromosomes

Chromosomal preparations were obtained from cerebral ganglia of parasitoid prepupae taken from dissected hosts generally following the protocol developed by Imai et al. (1988) with certain modifications (see Gokhman et al. 2016). Five to seven individuals of each species were studied. Ganglia were extracted from insects dissected in 0.5% hypotonic sodium citrate solution containing 0.005% colchicine. The extracted ganglia were then transferred to a fresh portion of hypotonic solution and incubated

for 30 min at room temperature. The material was transferred onto a pre-cleaned microscope slide using a Pasteur pipette and then gently flushed with Fixative I (glacial acetic acid: absolute ethanol: distilled water 3:3:4). The tissues were disrupted using dissecting needles in an additional drop of Fixative I. Another drop of Fixative II (glacial acetic acid: absolute ethanol 1:1) was applied to the center of the area, and the more aqueous phase was blotted off the edges of the slide. The slides were then dried for approximately half an hour and stored at -20°C.

Chromosome staining

For conventional staining, preparations were stained with freshly prepared 3% Giemsa solution in 0.05M Sørensen's phosphate buffer (Na_2HPO_4 + KH_2PO_4, pH 6.8) for about 15 min. Fluorochrome staining with chromomycin A_3 and 4', 6-diamidino-2-phenylindole (CMA_3/DAPI) was performed according to Schweizer (1976) with certain modifications by Guerra and Souza (2002). The slide was flooded with CMA_3 staining solution (0.5 mg/ml in McIlvaine's buffer), covered with a coverslip, and incubated at room temperature in the dark for 1 hour. The coverslip was then removed, and the slide was briefly rinsed with distilled water and air-dried. The slide was then flooded with DAPI solution (2 µg/ml in McIlvaine's buffer), covered with a coverslip, and stained in the dark at room temperature for 30 min. The coverslip was then removed, and the slide was briefly rinsed with distilled water before being air-dried. The preparation was then mounted in VECTASHIELD anti-fade medium (Vector Laboratories). The slide was stored in the dark prior to examination for a minimum of three days.

Image acquisition and analysis

Metaphase plates were analyzed under an Olympus BX51 microscope. Images of chromosomes were taken with an Olympus DP72 camera using ImageProPlus software. To prepare illustrations, the resulting images were arranged and enhanced using Adobe Photoshop 8.0. The same software was also used for taking measurements from selected metaphase plates with good chromosome morphology. Statistical analysis was performed using STATISTICA 5.5. The chromosomes were classified following guidelines provided by Levan et al. (1964).

Results

Palmistichus elaeisis

Five pairs of large metacentric chromosomes of similar size and a much smaller pair of acrocentrics were found in this species with 2n = 12 (Fig. 1a; Table 1). Fluoro-

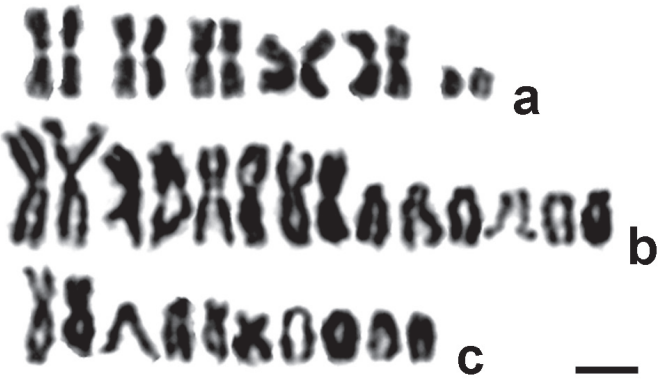

Figure 1. Diploid karyotypes of parasitoids. **a** *Palmistichus elaeisis* **b** *Trichospilus diatraeae* **c** *Trichogramma pretiosum*. Bar = 5 μm.

Table 1. Relative lengths (RL) and centromeric indices (CI) of parasitoid chromosomes (mean ± SD). For each species, numbers of analyzed diploid metaphase plates are given in brackets.

Chr. no.	*P. elaeisis* (6)		*T. diatraeae* (4)		*T. pretiosum* (15)	
	RL	CI	RL	CI	RL	CI
1	20.44 ± 0.78	48.43 ± 2.42	20.28 ± 0.72	44.28 ± 4.08	25.94 ± 1.48	42.32 ± 2.92
2	19.47 ± 0.42	47.57 ± 1.67	18.38 ± 0.72	45.17 ± 3.72	20.55 ± 0.83	0
3	18.68 ± 0.30	47.64 ± 1.86	16.75 ± 0.91	44.44 ± 3.75	18.97 ± 0.78	44.89 ± 2.73
4	17.88 ± 0.32	47.61 ± 1.59	14.88 ± 0.68	44.51 ± 4.45	18.66 ± 0.84	0
5	16.98 ± 0.45	46.61 ± 1.97	11.20 ± 0.72	0	15.88 ± 0.95	0
6	6.55 ± 0.39	0	10.18 ± 0.66	0	-	-
7	-	-	8.33 ± 0.66	0	-	-

chrome staining of the diploid karyotype revealed a single pericentromeric CMA_3-positive and DAPI-negative band on both homologous medium-sized metacentric chromosomes, probably the second longest ones (Fig. 2a–c). In addition, both CMA_3- and DAPI-positive arms were visualized on smaller chromosomes of another metacentric pair.

Trichospilus diatraeae

The karyotype of this species contains four pairs of large metacentrics of approximately the same size (the first pair is slightly longer than the remaining ones) and three considerably smaller pairs of acrocentric chromosomes; the chromosome number in *T. diatraeae* is thus 2n = 14 (Fig. 1b; Table 1). Surprisingly, fluorochrome staining visualized multiple telomeric CMA_3-positive and DAPI-negative bands on virtually all chromosomes (Fig. 2d–f).

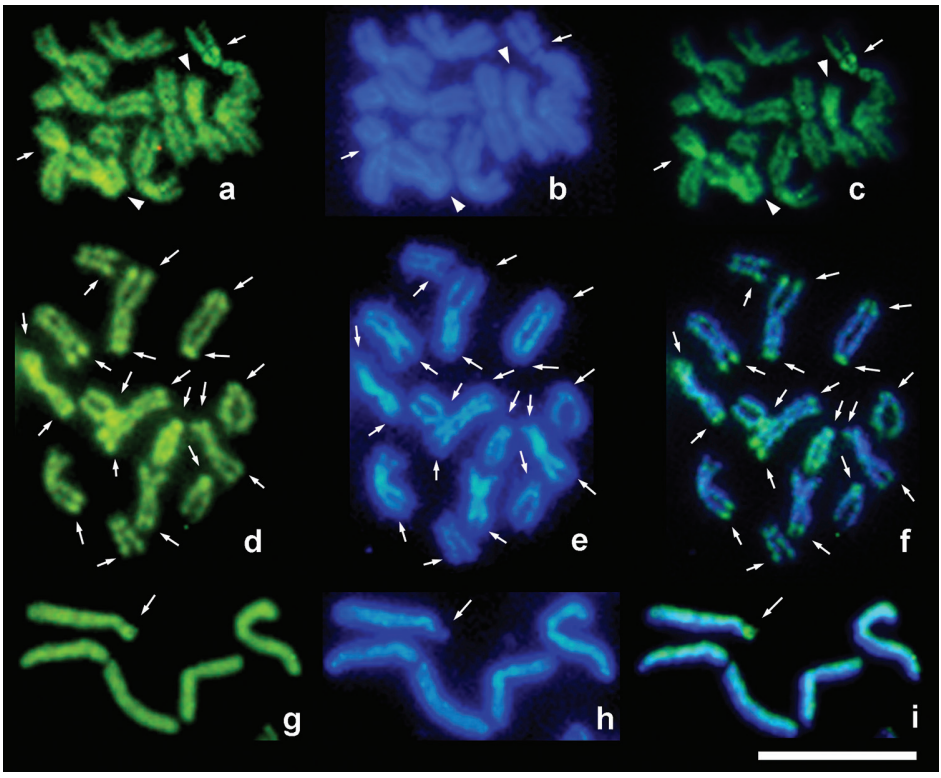

Figure 2. CMA_3/DAPI-stained metaphase plates of parasitoids. **a–c** *P. elaeisis*, female **d–f** *T. diatraeae*, female **g–i** *T. pretiosum*, male **a, d, g** CMA_3 staining **b, e, h** DAPI staining **c, f, i** merged CMA_3/DAPI staining. Arrows and arrowheads respectively indicate CMA_3-positive and DAPI-negative bands (NORs) as well as both CMA_3- and DAPI-positive chromosome arms. Bar = 10 μm.

Trichogramma pretiosum

The largest chromosome pair in the karyotype of this species is metacentric, whereas the remaining four pairs are clearly smaller; three of them are somewhat similar in size, and the fourth one is substantially shorter (2n = 10). Most these chromosomes are acrocentric (pairs no. 2, 4, and 5) except for the third pair of metacentric chromosomes which is more or less equal in length to the fourth pair of acrocentrics (Fig. 1c; Table 1). As in *P. elaeisis*, a single CMA_3-positive and DAPI-negative band is revealed on chromosomes of *T. pretiosum* using fluorochrome staining (Fig. 2g–i). This pericentromeric band is localized on the largest acrocentric.

Discussion

Chromosome sets of *P. elaeisis* and *T. diatraeae* were first studied by Silva-Junior et al. (2000a) who respectively found 2n = 12 and 14 in these species. We therefore

confirm these results. The karyotype structure of *P. elaeisis* is similar to that of many other members of the subfamily Tetrastichinae. Moreover, this structure is apparently the commonest in the family Eulophidae in general (Gebiola and Bernardo 2008; Gokhman 2009, Gokhman and Gumovsky 2009, Bolsheva et al. 2012, Gokhman et al. 2014b) which is, in turn, one of the best karyotypically studied chalcid families (see e.g. Gokhman 2009, Gebiola et al. 2012). The chromosome set of *T. diatraeae* (Eulophinae) is also structurally similar to those of most members of the family Eulophidae, except for the two additional pairs of acrocentrics instead of a larger pair of metacentric chromosomes. Silva-Junior et al. (2000a) therefore hypothesized that this particular feature had probably resulted from a centric fission, and we agree with this assumption. Interestingly, another tropical/subtropical parasitoid, *Chelonus insularis* Cresson, 1865 (Braconidae), also shows n = 7 (Silva-Junior et al. 2000b), as opposed to n = 6 in all other studied species of temperate Cheloninae (Gokhman 2009), and this is also interpreted as a case of centric fission.

The chromosome set of *T. pretiosum* was previously studied by Hung (1982) together with three other members of this genus. All these parasitoids had n = 5, and this author also postulated highly similar karyotype structure of those species in terms of relative chromosome lengths and centromeric indices. Specifically, Hung (1982) reported that haploid chromosome sets of those parasitoids harbored a larger submetacentric, a smaller metacentric and three smallest acrocentric chromosomes of the same size (one of them with an obvious shorter arm), with approximate RLs of 26.5, 21 and 3 × 17.5 percent respectively (see Fig. 7 in Hung 1982). However, images of metaphase plates provided by this author suggest that chromosomal morphology of certain *Trichogramma* Westwood, 1833 species can substantially differ from the above-cited pattern. It is therefore not surprising that we have found a slightly different karyotype structure in *T. pretiosum* as well (Table 1). In particular, the first pair of chromosomes is clearly metacentric. Moreover, another medium-sized pair of metacentric chromosomes is the third/fourth longest one within the karyotype (Fig. 1c), not the second, as it was shown in Hung (1982). In addition, no acrocentric chromosome carries a clearly visible shorter arm, and all acrocentric pairs seemingly differ in their lengths in this species.

Staining of *P. elaeisis* karyotype with certain base-specific fluorochromes was apparently performed for the first time by Silva-Junior et al. (2000a). However, these authors neither published images of stained chromosomes nor gave a detailed description of the results obtained. Within previously studied parasitoid karyotypes, GC-rich, i.e. CMA_3-positive (and DAPI-negative) regions corresponded only to NORs (Bolsheva et al. 2012, Gokhman et al. 2016). Our results therefore demonstrate presence of the single NOR in the chromosome set of *P. elaeisis*. As far as other members of the subfamily Tetrastichinae are concerned, AgNOR-banding also visualized the only NOR on a larger metacentric chromosome (perhaps the second longest one) in the karyotype of *Melittobia australica* Girault, 1912 (Maffei et al. 2001). In addition, CMA_3/DAPI staining revealed a single NOR in the chromosome set of another member of the same genus, *Melittobia hawaiiensis* Perkins, 1907 (Silva-Junior et al. 2000a). However,

NORs are localized on the smallest acrocentrics within karyotypes of some other Eulophidae, i.e. two *Entedon* Dalman, 1820 (Entedoninae) and several *Pnigalio* Schrank, 1802 (Eulophinae) species (Bernardo et al. 2008, Bolsheva et al. 2012, Gebiola et al. 2012). On the other hand, occurrence of multiple telomeric CMA_3-positive bands found in the karyotype of *T. diatraeae* is apparently derived and quite unusual for parasitoids, but is known for aculeate wasps (Menezes et al. 2011) and bees (Duarte et al. 2009). In the latter study, most CMA_3-positive bands are attributed to NORs, and this is probably true for *T. diatraeae* as well. As for both CMA_3- and DAPI-positive chromosome arms found in *P. elaeisis*, segments of that kind detected in other Hymenoptera are usually heterochromatic (see e.g. Menezes et al. 2011). Interestingly, fully heterochromatic chromosome arms in parasitic wasps were also revealed, for example, on a particular pair of metacentric chromosomes of three species of the genus *Nasonia* Ashmead, 1904 (Gokhman and Westendorff 2000).

Karyotypes of *Trichogramma* species have never been examined using base-specific fluorochromes. Nevertheless, NORs in this genus were previously studied by van Vugt et al. (2005, 2009) who examined the chromosome set of *T. kaykai* Pinto et Stoutamer, 1997 using FISH with a 45S rDNA probe. In essence, two NORs were detected within the karyotype of this species (a third one was visualized on a particular B chromosome). However, we observed a single NOR on the chromosomes of *T. pretiosum*, indicating that the genus *Trichogramma* can harbor different species which karyotypes have either one or two NORs. Variation of this kind was earlier detected within the chalcid genus *Eurytoma* Illiger, 1807 (Eurytomidae; Gokhman et al. 2014a).

The chalcid families Eulophidae and Trichogrammatidae belong to the same phylogenetic lineage that also includes Aphelinidae and a few smaller groups of which the chromosomes are as yet unknown (Heraty et al. 2013). The present study can therefore add to our knowledge of the karyotype evolution within this lineage. In particular, both Eulophidae and Aphelinidae appear to have a single NOR per haploid karyotype (Baldanza et al. 1999, Baldanza and Giorgini 2001, Giorgini and Baldanza 2004, Bernardo et al. 2008, Bolsheva et al. 2012, Gebiola et al. 2012, Gokhman et al. 2017). On the contrary, haploid chromosome sets of various *Trichogramma* species studied in this respect can carry either one or two rDNA sites (van Vugt 2005, 2009 and the present paper). However, since higher numbers of rDNA clusters are probably ancestral for the superfamily Chalcidoidea as well as for parasitic wasps in general (Gokhman et al. 2014a), presence of a single NOR in all these groups may represent a derived state.

In conclusion, we would like to stress the importance of studying chromosome sets of tropical parasitic wasps for our understanding of the karyotype structure and evolution in parasitoid Hymenoptera in general. For example, the chromosome set of *T. diatraeae* demonstrates deviating karyotypic features in terms of both Giemsa and CMA_3/DAPI staining. In particular, the presence of multiple CMA_3-positive telomeric bands on all chromosomes of this species was not previously detected in any other parasitoid. Moreover, this study reveals for the first time considerable karyotypic differences within the speciose and practically important genus *Trichogramma*, again in terms of both chromosome morphometrics and the number of NORs, thus potentially

contributing to our knowledge of its taxonomy and phylogeny. Our data therefore confirm that further research of parasitoid Hymenoptera from various geographical regions will undoubtedly demonstrate more variation in the overall karyotype structure and distribution of different chromosome segments in this group.

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