CompCytogen 6(2): 107–114 (2012)
doi: 10.3897/CompCytogen.v6i2.2397
www.pensoft.net/journals/compcytogen

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

First cytogenetic characterization of a species of the arboreal ant genus *Azteca* Forel, 1978 (Dolichoderinae, Formicidae)

Danon Clemes Cardoso[1,2], Maykon Passos Cristiano[1,2], Luísa Antônia Campos Barros[1,2], Denilce Meneses Lopes[2], Silvia das Graças Pompolo[2]

1 *Programa de Pós-graduação em Genética e Melhoramento, edifício Arthur Bernardes, subsolo sala 12, Universidade Federal de Viçosa, Viçosa, Minas Gerais, 36570-000, Brazil* **2** *Departamento de Biologia Geral, Av. Peter Henry Rolfs s/n, Universidade Federal de Viçosa, Viçosa, Minas Gerais, 36570-000, Brazil*

Corresponding author: *Danon Clemes Cardoso* (danon.cardoso@ufv.br)

Academic editor: *V. Gokhman* | Received 20 November 2011 | Accepted 30 January 2012 | Published 16 March 2012

Citation: Cardoso DC, Cristiano MP, Barros LAC, Lopes DM, Pompolo SdG (2012) First cytogenetic characterization of a species of the arboreal ant genus *Azteca* Forel, 1978 (Dolichoderinae, Formicidae). Comparative Cytogenetics 6(2): 107–114. doi: 10.3897/CompCytogen.v6i2.2397

Abstract

In this paper we present, for the first time, a detailed karyotype characterization of a species of the genus *Azteca* (Dolichoderinae, Formicidae). Cerebral ganglia from *Azteca trigona* Emery, 1893 were excised and submitted to colchicine hypotonic solution and chromosomal preparations were analyzed through conventional staining with Giemsa, C-banding, silver nitrate staining ($AgNO_3$) and sequential base-specific fluorochromes. The analysis shows that *A. trigona* has a diploid number of 28 chromosomes. The karyotype consists of five metacentric pairs, seven acrocentric pairs and two pseudo-acrocentric pairs, which represents a karyotype formula 2K= 10M + 14A + $4A^M$ and a diploid number of the arms 2AN = 38. The analysis of heterochromatin distribution revealed a positive block on distal region of the short arm of fourth metacentric pair, which was coincident with Ag-NOR band and CMA_3 fluorochrome staining, meaning that rDNA sequences are interspaced by GC-rich base pairs sequences. The C-banding also marked short arms of other chromosomes, indicating centric fissions followed by heterochromatin growth. The karyotype analysis of *A. trigona* allowed the identification of cytogenetic markers that will be helpful in a difficult taxonomic group as *Azteca* and discussion about evolutionary aspects of the genome organization.

Keywords

karyotype, chromosome number, chromosome banding, ants, *Azteca trigona*

Introduction

The subfamily Dolichoderinae presents a great diversity of species throughout the world. Species are distributed in different biogeographic regions, from the Palearctic, Nearctic, Afrotropical region and Malaysia, to the Middle East, Australian and Neotropical regions (Bolton 1994). This subfamily comprises 22 genera, of which ten are found in Brazil, and *Azteca* Forel, 1878 and *Dolichoderus* Lund, 1831 form the two most diverse genera (Cuezzo, 2003). The species of this subfamily represent a common group, but rather inconspicuous of Neotropical ant fauna compared to other more evident groups (Wild 2009, Cardoso and Cristiano 2010). One of the best known species of Dolichoderinae is the ant *Linepithema humile* (Mayr, 1868). This is one of the principal invasive ant species and now occurs in more than 15 countries (Diehl-Fleig and Diehl 2007, Wild 2009).

The genus *Azteca* is strictly Neotropical and very diverse, including around 130 species. They are essentially arboreal and many species have mutualistic associations with particular plant species, where the genus *Cecropia* presents the most conspicuous association (Longino 1991). Taxonomic reviews of the genus are scarce, regional or restricted to specific groups (Longino 2007) and the absence of a more comprehensive taxonomic review of the genus is the main obstacle for understanding the evolutionary basis of the *Azteca-Cecropia* mutualistic relationship.

Cytogenetic characterization offers some of the most reliable taxonomic criteria for some groups of organisms and recently, the application of cytogenetic studies focused on understanding the distribution pattern and evolution of species seems very promising (Mariano et al. 2012). In general, the parameters used in these studies are the number of chromosomes, their morphology, amount of heterochromatin, as well as their composition and the base pairs, obtained by chromosome banding techniques. Extensive information on chromosomes of the order Hymenoptera is available, especially the Formicidae of which there are already many known karyotypes. However cytogenetic information is lacking for a great part of known species. At least 750 from more than 12000 species have been cytogenetically studied (Lorite and Palomeque 2010), and the variation in chromosome number is enormous, ranging from 2n=2 to 2n=120 (Mariano et al. 2008). This variation can provide trustworthy cytotaxonomic markers for evolutionary studies in association with chromosome banding. At least 16 genera of the subfamily Dolichoderinae have their chromosome numbers available (Lorite and Palomeque 2010), where the genus *Iridomyrmex* Mayr, 1862 is the most studied (including the species *Iridomyrmex humile*, now relocated to the genus *Linepithema* Mayr, 1866 (Wild 2009)). The chromosome number in Dolichoderinae is less variable than in other related subfamilies, ranging from 2n= 10 to 2n= 48 (Lorite and Palomeque 2010). Within genera, the chromosome number varies from 2n=14 to 48 in *Iridomyrmex* and represents the largest variation found in the subfamily, while, *Dolichoderus* showed karyotypes ranging from 2n=18 to 30 (Imai et al. 1984a, b).

Even with the immense diversity of species of the genus *Azteca*, including approximately 130 described species, no cytogenetic study is encountered for this genus. Thus,

to contribute to the increased cytogenetic knowledge of Formicidae and further understanding of karyotype evolution, the present study aimed to characterize the karyotype of *Azteca trigona* Emery, 1893, whereas karyotypes of the genera *Anillidris* Santschi, 1936 and *Liometopum* Mayr, 1861 remain totally unknown.

Material and methods

Thirty specimens from two colonies of *Azteca trigona* collected in Ponte Nova (20°25'S, 42°54'W) and Viçosa (20°45'S, 42°52'W), MG, Brazil were analyzed. The colonies were collected in the field and transferred to a plastic container and maintained in a BOD (Biochemical Oxygen Demand) incubator at 25°C following the protocol described by Cardoso et al. (2011) and fed with honey in order to obtain larvae in the pre-pupa stage (post-defecating larvae). The specimens were identified by specialists and Vouchers of the samples collected in this work were deposited in CEPLAC and MZUSP.

Cytogenetic analysis was performed using cerebral ganglia of the larvae selected. Metaphase chromosomes were obtained according to the methodology proposed by Imai et al. (1988). Preparations obtained from fifteen individuals per colony were analyzed. The preparations were stained with Giemsa diluted in Sörensen buffer at (4%) for 20 minutes. On average, ten metaphases were analyzed per slide and ten slides were submitted to banding techniques. C-banding was performed by BSG method (Barium hydroxide/Saline/Giemsa) according to Sumner (1972). The protocol of Schweizer (1980) was used for preparation of sequential fluorochrome staining (CMA_3/DA/DAPI). Identification of nucleolus organizer regions (NOR) was performed according to Howell and Black (1980). The best metaphases were photographed using an Olympus BX60 microscope equipped with a camera Q color 3 Olympus®. Brightness and contrast of the karyotypes were optimized using Photoshop CS4. The karyotypes were mounted in Corel Draw® 13 image editing software. Karyotype structure was described according to the nomenclature proposed by Imai (1991) and Levan et al. (1964). For mounting of the karyotypes, the chromosomes were sorted into three groups: metacentric chromosomes (M), acrocentric chromosomes (A) with heterochromatin located across the length of the short arm of the chromosomes, and pseudo-acrocentric chromosomes (A^M) which possess a long heterochromatic arm (Imai 1991).

Results and discussion

The chromosome number observed for *Azteca trigona* was 2n = 28 (Figure 1). The karyotype of this species consists of five metacentric pairs (M), seven acrocentric pairs and two pseudo-acrocentric pairs (A^M) according to the terminology proposed by Imai (1991). Considering this chromosome classification, the karyotype formula found for the diploid set would be $2K= 10M + 14A + 4A^M$. Thus, the diploid number of the arms according to Imai et al. (1994) was 2AN = 38. However, considering Levan et al. (1964),

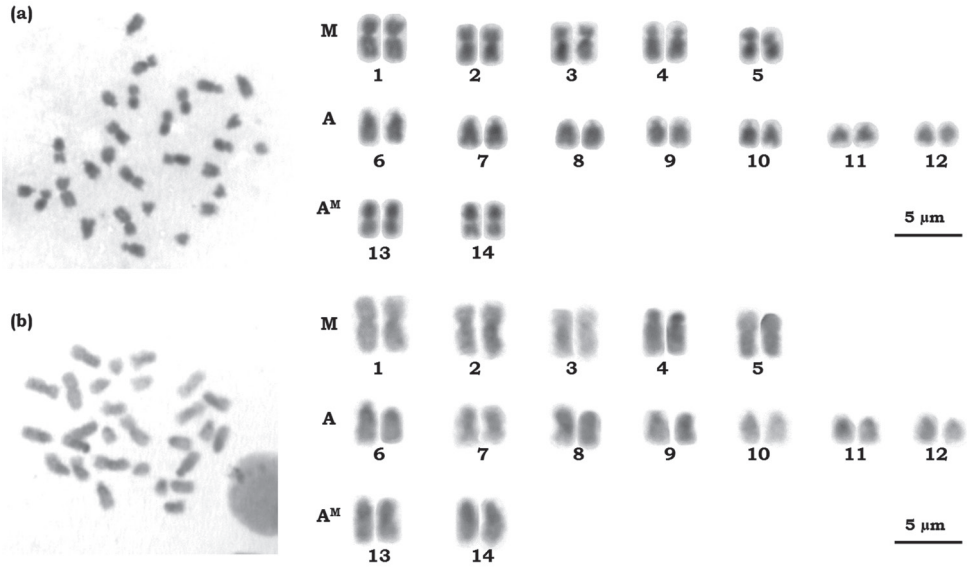

Figure 1. Karyotype of female workers of *Azteca trigona* 2n = 28 sorted according to the classification proposed by Imai (1991) **a** Conventional staining using Giemsa **b** C-banding showing the distribution of heterochromatin.

the arm ratio analysis results in five pairs of metacentric (pairs 1, 2, 3, 13 and 14), two pairs of submetacentric (pairs 4 and 5) and 7 pairs of subtelocentric (pairs 6 to 12). It is important to emphasize that the chromosome classification by Levan et al. (1964) is the most used in cytogenetic studies of Formicidae (Lorite and Palomeque 2010), although classification proposed by Imai (1991) is more informative from an evolutionary view. It was not possible to observe the haploid karyotype (n) since there was no production of alates (reproductive individuals) during the maintenance period of the colony.

Of the subfamilies of Formicidae, Dolichoderinae is the fourth subfamily with the major number of taxa studied. According to Lorite and Palomeque (2010), about 50 taxa in 16 genera have been studied. The diploid karyotype of Dolichoderinae varied from 10 chromosomes in *Tapinoma indicum* Forel, 1895 and *T. melanocephalum* (Fabricius, 1793) (Imai et al. 1984a), up to 48 chromosomes in *Iridomyrmex anceps* (Roger, 1863) (Imai et al. 1984b). However, the latter chromosome number has been questioned due to the great discrepancy from other karyotypes described to subfamily (Lorite and Palomeque 2010). The chromosome number of *Azteca*, 2n = 28, seems consistent with the karyotypic variation of the subfamily, which according to Crozier (1970) and Imai et al. (1977) is characterized by a low to average chromosome number.

Results of the banding techniques indicate positive C-, Ag-NOR and CMA_3-bands, and negative DAPI-bands on the short arm of the fourth pair of metacentric chromosomes (Figs 1–3), indicating that this region is rich in heterochromatin and should correspond to the nucleolus organizer region (NOR) (Fig. 3). This chromosome pair was CMA_3-positive and DAPI-negative, indicating that the marked regions are rich in GC

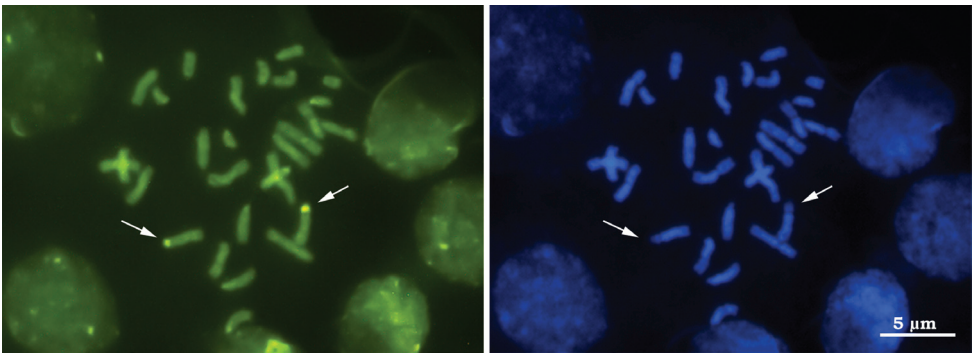

Figure 2. Diploid metaphase of *Azteca trigona* submitted to sequential staining with fluorochromes CMA_3/DA/DAPI **a** Staining with CMA_3, arrows indicate the fourth chromosome pair and the GC^+ regions **b** Staining with DAPI, arrows indicate the same chromosome pair with the negative AT-rich regions.

bases and devoid of AT bases (Fig. 2). Several authors have reported that CG-positive and AT-negative (i.e. auto-complementary) regions are related to nucleolus organizer regions (NORs). This relationship has been reported in grasshoppers (Camacho et al. 1991), bees of the genus *Melipona* (Illiger, 1806) (Rocha et al. 2002) as well as in other neotropical bees (Rocha et al. 2003; Brito et al. 2003) and in ants *Dinoponera lucida* Emery, 1901 (Mariano et al. 2008) and *Tapinoma nigerrimum* (Nylander, 1856) (Lorite et al. 1997), where the latter belongs to the same subfamily of ants as *A. trigona*. In addition, in many studies homology between the Ag-NOR positive regions and probes specific to NORs was revealed by means of the *in situ* hybridization technique (Lorite et al. 1997, Rocha et al. 2002, Mariano et al. 2008). Since this technique uses specific probes, it is much more sensitive to detection of NORs and provides consistent data on the relationships between banding patterns – C-bands, Ag-NOR and fluorochrome staining.

Furthermore, the silver nitrate staining revealed that the fourth metacentric pair also had an Ag-positive block. This finding corroborated that this pair carried NORs, which were heteromorphic between the homologues (Fig. 3). Heteromorphism in NOR size is frequent in a large number of organisms and can be explained by tandem duplications of the ribosomal genes (Sumner 2003). The NOR heteromorphism found in *Azteca trigona* probably resulted from the duplication/amplification or unequal crossover during meiosis of some ribosomal sequences of the homologues.

The results presented here are, to our knowledge, the first cytogenetic data of a species of the genus *Azteca*, and the second known for a species of the subfamily Dolichoderinae in the Neotropics. Previously, cytogenetic data on the species *Dorymyrmex pyramicus* (Roger, 1863) (2n = 18) were presented on the base of only five workers collected in Uruguay (Gőni et al. 1983). According to some authors, cytogenetic data on Neotropical ant species are scarce given the immense biodiversity of this region (Goñi et al. 1983, Lorite et al. 1997). Cytogenetic data are important tools that can be used for phylogenetic inferences (Rocha et al. 2002, Lorite and Palomeque 2010) and solving species identification problems (Borges et al. 2004, Delabie et al. 2008).

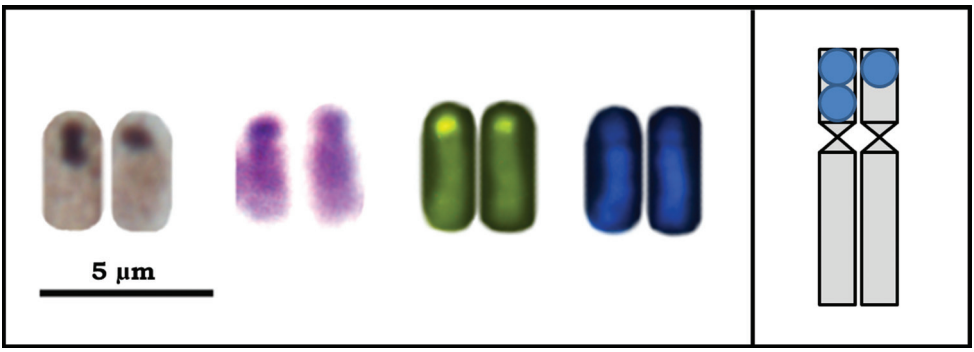

Figure 3. Fourth pair of chromosomes of *Azteca trigona* submitted to different banding techniques: Ag-NOR, C-banding and sequential CMA_3/DA/DAPI fluorochrome staining. The inserted scheme indicates that for all techniques the homologues are heteromorphic for the banding pattern.

In particular, the genus *Azteca* presents a great challenge to taxonomists since identification is practically impossible at the species level in absence of the queen (Longino 2007). Since it is hardly possible to identify the species only by workers, it becomes a limiting factor for studies on biology, ecology and biodiversity of the genus, because the workers they are much more abundant in the colony and the queen is not always collected. Cytogenetic data presented herein are the first records for the genus and can be used for the development of cytogenetic markers. These may be used in phylogenetic inference and applied to taxonomy in order to facilitate the identification of species. Increase in the number of samples and application of other banding techniques to acquire cytogenetic patterns typical of the species of the genus are to be conducted for the better understanding of evolution and taxonomy of this group.

Acknowledgements

The authors are grateful for the critical reading of two anonymous reviewers and Evan Vissar for language reviewing. We also thank Ricardo R. C. Solar for helping us with the ant sampling. This research was supported by Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). We also thank Rodrigo Feitosa and Jacques H.C. Delabie for taxonomic identification.

References

Bolton B (1994) Indentification guide to the ant genera of the world. Harvard University Press, Cambridge, 232 pp.

Borges DS, Mariano CSF, Delabie JHC, Pompolo SG (2004) Estudos citogenéticos em formigas neotropicais do gênero *Gnamptogenys* Roger (Hymenoptera, Formicidae, Ec-

tatomminae). Revista Brasileira de Entomologia 48: 481–484. doi: 10.1590/S0085-56262004000400009

Brito RM, Caixeiro APA, Pompolo SdG, Azevedo GG (2003) Cytogenetic data of *Partamona peckolti* (Hymenoptera, Apidae, Meliponini) by C banding and fluorochrome staining with DA/CMA_3 and DA/DAPI. Genetics and Molecular Biology 26: 53–57. doi: 10.1590/S1415-47572003000100009

Camacho JPM, Cabrero J, Viseras E, Lopez-Leon MD, Navas-Castillo J, Alche JD (1991) G-banding in two species of grasshopper and its relationship to C, N, and fluorescence banding techiques. Genome 34: 638–643. doi: 10.1139/g91-097

Cardoso DC, Cristiano MP (2010) Myrmecofauna of the southern Catarinense Restinga sandy coastal plain: new records of species occurrence for the State of Santa Catarina and Brazil. Sociobiology 55: 229–239.

Cardoso DC, Cristiano MP, Tavares MG (2011) Methodological remarks on rearing basal Attini ants in the laboratory for biological and evolutionary studies: overview of the genus *Mycetophylax*. Insectes Sociaux 58: 427–430. doi: 10.1007/s00040-011-0160-3

Crozier RH (1970) Karyotypes of twenty-one ant species (Hymenoptera; Formicidae), with reviews of the known ant karyotypes. Canadian Journal of Genetics and Cytology 12: 109–128.

Cuezzo F (2003) Subfamilia Dolichoderinae. In: Fernández F (Ed) Introducción a las hormigas de la región Neotropical. Instituto de Investigación de Recursos Biológicos Alexandre von Humbolt, Bogotá, Colombia, 424.

Delabie JHC, Mariano CSF, Mendes LF, Pompolo, SG, Fresneau, D (2008) Problema apontados por estudos morfológicos, ecológicos e citogeneticos no gênero *Pachycondyla* na região neotropical: o caso do complexo *apicalis*. In: Vilela E, Santos IA, Schoereder JH, Serrão JE, Campos LAO, Lino-Neto J (Eds) Insetos Sociais – da biologia a aplicação. Editora UFV, Viçosa, Brazil, 442.

Diehl-Fleig E, Diehl E (2007) Organização social e status de praga da formiga argentina *Linepithema humile* (Mayr 1868) no Rio Grande do Sul. O Biológico 69: 31–34.

Goñi G, Zolessi LC, Imai HT (1983) Karyotype of thirteen ant species from Uruguay (Hymenoptera - Formicidae). Caryologia 36: 363–371.

Howell WM, Black DA (1980) Controlled silver staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 36: 1014–1015. doi: 10.1007/BF01953855

Imai HT (1991) Mutability of constitutive heterochromatin (C-bands) during eukaryotic chromosomal evolution and their cytological meaning. Japanese Journal of Genetics 66: 635–661. doi: 10.1266/jjg.66.635

Imai HT, Crozier RH, Taylor RW (1977) Karyotype evolution in Australian ants. Chromosoma 59: 341–393. doi: 10.1007/BF00327974

Imai HT, Taylor RW, Crosland MWJ, Crozier RH (1988) Modes of spontaneous evolution in ants with reference to the minimum interaction hypothesis. Japanese Journal of Genetics 63: 159–185. doi: 10.1266/jjg.63.159

Imai HT, Taylor RW, Crozier RH (1994) Experimental bases for the minimum interaction theory. I. Chromosome evolution in ants of the *Myrmecia pilosula* species complex (Hymenoptera: Formicidae: Myrmeciinae). Japanese Journal of Genetics 69: 137–182. doi: 10.1266/jjg.69.137

Imai HT, Urbani CB, Kubota M, Sharma GP, Narasimhanna MN, Das BC, Sharma AK, Sharma A, Deodikar GB, Vaidya VG, Rajasekarasetty MR (1984a) Karyological survey of Indian ants. Japanese Journal of Genetics 59: 1–32. doi: 10.1266/jjg.59.1

Imai HT, Kubota M, Brown-Jr. WL, Ihara M, Tohari M, Pranata RI (1984b) Chromosome observations on tropical ants from Indonésia. Annual Report of National Institute of Genetics 35: 46–48.

Levan A, Fredga K, Sandberg AA (1964) Nomenclature for centromeric position on chromosomes. Hereditas 52: 201–220. doi: 10.1111/j.1601-5223.1964.tb01953.x

Longino JT (1991) *Azteca* ants in *Cecropia* trees: Taxonomy, colony structure, and behaviour. In: Huxley CR, Cutler DF (Eds) Ant-Plant Interactions. Oxford University Press, Oxford, 271–288.

Longino JT (2007) A taxonomic review of the genus *Azteca* (Hymenoptera : Formicidae) in Costa Rica and a global revision of the *aurita* group. Zootaxa 1491: 1-63.

Lorite P, Aranega AE, Luque F, Palomeque T (1997) Analysis of the nucleolar organizing regions in the ant *Tapinoma nigerrimum* (Hymenoptera, Formicidae). Heredity 78: 578–582. doi: 10.1038/hdy.1997.96

Lorite P, Palomeque T (2010) Karyotype evolution in ants (Hymenoptera: Formicidae), with a review of the known ant chromosome numbers. Myrmecological News 13: 89–102.

Mariano CDSF, Pompolo SDG, Campos Barros LA, Mariano-Neto E, Campiolo S, Charles Delabie JH (2008) A biogeographical study of the threatened ant *Dinoponera lucida* Emery (Hymenoptera: Formicidae: Ponerinae) using a cytogenetic approach. Insect Conservation and Diversity 1: 161–168. doi: 10.1111/j.1752-4598.2008.00022.x

Mariano CDSF, Pompolo SDG, Silva JG, Delabie JHC (2012) Contribution of cytogenetics to the debate on the paraphyly of *Pachycondyla* spp. (Hymenoptera, Formicidae, Ponerinae). Psyche 2012: 1–9.

Rocha MP, Pompolo SD, Dergam JA, Fernandes A, Campos LAD (2002) DNA characterization and karyotypic evolution in the bee genus *Melipona* (Hymenoptera, Meliponini). Hereditas 136: 19–27. doi: 10.1034/j.1601-5223.2002.1360104.x

Rocha MP, Pompolo SG, Campos LAO (2003) Citogenética da tribo Meliponini (Hymenoptera, Apidae). In: Melo GAR, Alves-dos-Santos I (Eds) Apoidea Neotropica: Homenagem aos 90 Anos de Jesus Santiago Moure. UNESC, Criciúma, Brazil, 311–320.

Schweizer D (1980) Simultaneous fluorescent staining of R bands in a specific heterochromatin regions (DA/DAPI – bands) in human chromosomes. Cytogenetics and Cell Genetics 27: 190–193. doi: 10.1159/000131482

Sumner AT (1972) A simple technique for demonstrating centromeric heterochromatin. Experimental Cell Research 75: 304–306.

Sumner AT (2003) Chromosomes: Organization and Function. Blackwell Publishing, North Berwick, United Kingdom, 287 pp. doi: 10.1016/0014-4827(72)90558-7

Wild AL (2009) Evolution of the Neotropical ant genus *Linepithema*. Systematic Entomology 34: 49–62. doi: 10.1111/j.1365-3113.2008.00435.x

CompCytogen 6(2): 115–125 (2012)
doi: 10.3897/CompCytogen.v6i2.2595
www.pensoft.net/journals/compcytogen

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

First karyotype description of *Hypostomus iheringii* (Regan, 1908): a case of heterochromatic polymorphism

Josiane Baccarin Traldi[1], Marcelo Ricardo Vicari[2], Daniel Rodrigues Blanco[1], Juliana de Fátima Martinez[1], Roberto Ferreira Artoni[2], Orlando Moreira-Filho[1]

1 *Universidade Federal de São Carlos, Departamento de Genética e Evolução, Rodovia Washington Luís Km 235, São Carlos-SP, 13565-905, Brazil* **2** *Universidade Estadual de Ponta Grossa, Departamento de Biologia Estrutural, Molecular e Genética, Av. Carlos Cavalcanti, 4748, Ponta Grossa-PR, 84030-900, Brazil*

Corresponding author: *Josiane Baccarin Traldi* (jositraldi@hotmail.com)

Academic editor: *E. Gornung* | Received 27 December 2011 | Accepted 15 February 2012 | Published 16 March 2012

Citation: Traldi JB, Vicari MR, Blanco DR, Martinez JF, Artoni RF, Moreira-Filho O (2012) First karyotype description of *Hypostomus iheringii* (Regan, 1908): a case of heterochromatic polymorphism. Comparative Cytogenetics 6(2): 115–125. doi: 10.3897/CompCytogen.v6i2.2595

Abstract

In this study, which is the first karyotype analysis of *Hypostomus iheringii,* nine specimens collected in Córrego da Lapa (tributary of the Passa-Cinco River) showed a diploid number of 80 chromosomes. Silver nitrate staining and fluorescence *in situ* hybridization (FISH) with an 18S rDNA probe revealed the presence of multiple nucleolus organizer regions (NORs) (chromosome pairs 13, 20, and 34). FISH with a 5S rDNA probe showed that this cistron was only present in chromosome pair 2. When the karyotypes of individual animals were compared, unique heterochromatic polymorphisms were detected on chromosome pairs 1 and 5. Specifically, specimens had heterochromatic blocks (h+h+) on both chromosomes, one chromosome with heterochromatic blocks (h+h-) or chromosomes that lacked heterochromatic blocks (h-h-). Considering that heteromorphic pattern is not correlated with variation in size, the process of heterochromatinization might act on the long arms of these chromosomes. In summary, all chromosomal markers indicate that the karyotype of *H. iheringii* is highly differentiated and that the heterochromatinization of chromosomal segments may have contributed to its karyotypic differentiation.

Keywords

chromosome variation, fish, heterochromatinization, Hypostomini

Introduction

Loricariidae is a speciose group of Neotropical fishes that is composed of six subfamilies: Hypoptopomatinae, Hypostominae, Lithogeneinae, Loricariinae, Neoplecos-

tominae, and Delturinae (Armbruster 2004, Reis et al. 2006, Cramer et al. 2011). Armbruster (2004) considered the old subfamily Ancistrinae to be a synonym of Hypostominae, a group that currently consists of the tribes Corymbophanini, Rhinelepini, Hypostomini, Pterygoplichthini and Ancistrini. *Hypostomus* is the type genus of Hypostominae and has great morphological (Weber 2003) and cytogenetic (Bueno et al. 2011) diversity. According to Weber (2003), the genus consists of a large number of species that exhibit a high level of morphological and color pattern variation, thus making systematic identification difficult. Of the more than 120 species that have been described within this group (Zawadzki et al. 2010), 21 were reported to reside in the Alto Paraná basin (Weber 2003, Jerep et al. 2007). Ziemniczak et al. (in press) concluded that the karyotypic differentiation of Hypostomini is correlated with the great diversification of form in this tribe and may have been important for genetic and reproductive isolation between species.

Cytogenetic studies in *Hypostomus* indicate that there is great variability in various karyotypic aspects, which contributes to enormous complexity of the group (Artoni and Bertollo 1996). The same chromosomal variations, such as karyotypic formula (Michele et al. 1977, Artoni and Bertollo 1996, Alves et al. 2006, Bueno et al. 2011), heterochromatin distribution (Artoni and Bertollo 1999, Rubert et al. 2008) and nucleolus organizer regions (Artoni and Bertollo 2001, Rubert et al. 2008), occur frequently within populations. However, population polymorphisms are rare and manifest as variations in the karyotypic formula (Artoni and Bertollo 1999).

The amplification and mobility of heterochromatic blocks of chromosomes are well documented in some organisms (Hamilton et al. 1990, Modi 1993). Sequences of satellite DNA appear to play an important role in the evolution of the genome of different organisms by promoting chromosome rearrangements and exhibiting rapid differentiation due to intragenomic mobility taking important role in karyotype evolution and speciation due to gene flow restriction (Wichman et al. 1991, Kantek et al. 2009, Machado et al. 2011).

In this study, the first karyotype analysis of *Hypostomus iheringii* (Regan, 1908) was performed using classic (Giemsa staining, C-banding, and Ag-NOR) and molecular (fluorescence *in situ* hybridization - FISH) cytogenetic methods, emphasizing the distribution of heterochromatic blocks, interrelating and discussing the possible role of heterochromatin in the diversification of the genomes of Loricariidae.

Materials and methods

Animals and mitotic chromosome preparations

Nine specimens (five males and four females) of *H. iheringii* from the Córrego da Lapa, a tributary of the Passa-Cinco River in Ipeúna, São Paulo, Brazil, were analyzed. These specimens were deposited in the Museum of Zoology at the University of São

Paulo, under voucher number MZUSP 106769. The animals were anesthetized with clove oil, according to the method described by Griffiths (2000), and then sacrificed. The procedures were performed following the guidelines of the Committee of Ethics in Animal Experimentation (Process CEUA 07/2011) at Universidade Estadual de Ponta Grossa. Cell suspensions containing mitotic chromosomes were obtained from the cells of the anterior portion of the kidney of these specimens according to the procedures described by Bertollo et al. (1978) and Foresti et al. (1993).

Chromosome staining

The chromosomes were stained with a solution of 5% Giemsa. C-banding was performed following the protocol described by Sumner (1972) with modifications in staining method (Lui et al. 2009). The nucleolus organizer regions (Ag-NORs) were determined according to the method described by Howell and Black (1980). These methods were performed sequentially (conventional staining of the chromosomes with Giemsa, C-banding and Ag-NORs) for accuracy in identifying chromosome pairs.

Chromosome hybridization, probes and karyotype analysis

The physical mapping of 18S and 5S rDNA on the chromosomes was obtained by FISH, as described by Pinkel et al. (1986), with probes obtained from *Prochilodus argenteus* Spix and Agassiz, 1829 (Hatanaka and Galetti Jr. 2004) and *Leporinus elongates* Valenciennes, 1850 (Martins and Galetti Jr. 1999). The probes of 5S and 18S rDNA were labeled with digoxigenin-11-dUTP and biotin-14-dATP, respectively, by *nick translation*, according to the manufacturer's instructions (Roche Applied Science). The hybridization procedure was performed under high stringency conditions (2.5 ng/μL of each probe, 50% deionized formamide, 10% dextran sulphate, 2XSSC, pH 7.0–7.2, at 37°C overnight). After hybridization, the slides were washed in 15% formamide/0.2XSSC at 42°C for 20 min, 0.1XSSC at 60°C for 15 min and 4XSSC/0.05% Tween at room temperature for 10 min. The last step was performed in two 5 min washes. The signal detection was performed using streptavidin-Alexa Fluor 488 (Molecular Probes) against the 18S rDNA and anti-digoxigenin-rhodamine (Roche Applied Science) for the 5S rDNA probes. The chromosomes were counter-stained with a solution of antifading/DAPI (40 μL of antifading + 1 μL of DAPI – 0.2 mg/mL) and analyzed under an Olympus BX50 epifluorescence microscope.

The chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), and acrocentric (a), according to the ratio of arms proposed by Levan et al. (1964), and arranged to form the karyotype in descending order by size. The software Image-Pro Plus 6.3 (Media Cybernetics) was used for image capture.

Results

All of the *H. iheringii* specimens showed a diploid number of 80 chromosomes (8m + 16sm + 28st + 28a, NF=132) without a heteromorphic sexual system (Fig. 1a). C-banding analysis revealed a small section of heterochromatin, preferentially located in

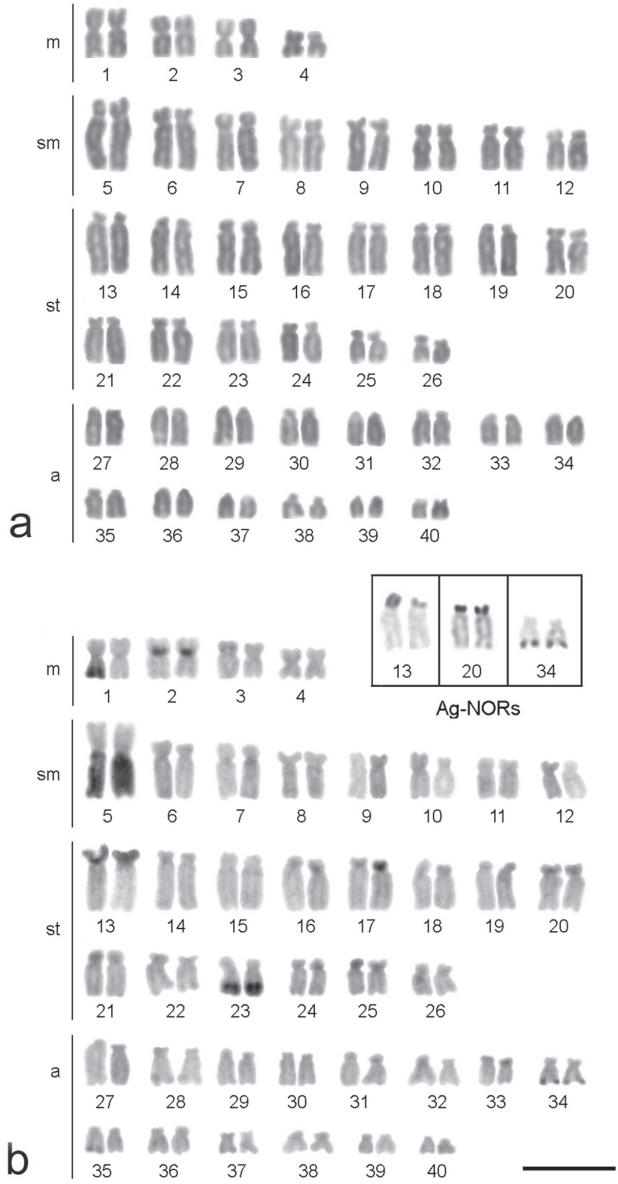

Figure 1. Karyotypes of female *Hypostomus iheringii* arranged from Giemsa-stained (**a**) and C-banded chromosomes (**b**). The chromosome pairs carrying Ag-NORs are boxed. Bar = 5 μm.

the terminal portions of some of the chromosomes (Fig. 1b). Two conspicuous heterochromatic blocks were found in the terminal position of the long arm of chromosome pairs 1 (m) and 5 (sm) with inter-individual variations. Specifically, specimens had heterochromatic blocks (h+h+) on both chromosomes, one chromosome with heterochromatic blocks (h+h-) or chromosomes that lacked heterochromatic blocks (h-h-) (Fig. 2). Silver nitrate staining revealed the presence of multiple nucleolus organizer regions (NORs) located in the terminal portion of the short arm of two pairs of subtelocentric chromosomes (pairs 13 and 20) and in the terminal position of the long arm of an acrocentric chromosome pair (chromosome 34) (Fig. 1, in Box). In addition, a size heteromorphism was found in the Ag-NORs sites of chromosome pair 13 in all individuals analyzed. FISH with the 18S rDNA probe confirmed the silver nitrate staining result for pair 13, but only one of the homologues of the pairs 20 and 34 was marked (Fig. 3a). FISH revealed that the 5S rDNA sites were located in the interstitial portion of the short arm of a metacentric chromosome (pair 2) (Fig. 3b).

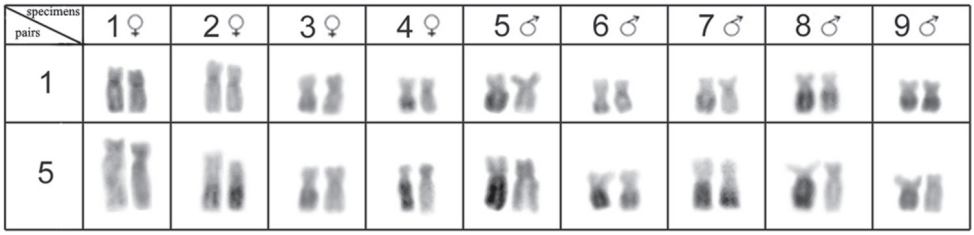

Figure 2. Accentuated heterochromatic polymorphisms on chromosome pairs 1 and 5 of the *H. iheringii*.

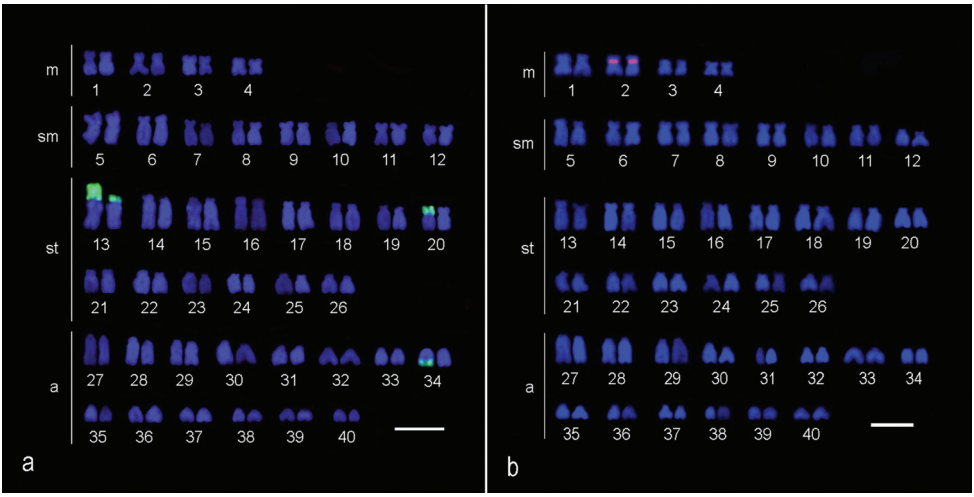

Figure 3. Karyotype of female *H. iheringii* submitted to FISH using 18S rDNA probe (**a**) and submitted to FISH using 5S rDNA probe (**b**). Bars = 5 μm.

Discussion

Cytogenetic studies in Loricariidae reveal a remarkable diversity of chromosomal numbers, formulae and markers. Despite this extensive variation, karyotype analyses of the species in this family have allowed well-defined evolutionary trends and putative group relationships to be inferred (Artoni and Bertollo 2001, Alves et al. 2006, Ziemniczak et al. in press). In *Hypostomus,* the diploid number ranges from 54 chromosomes in *Hypostomus plecostomus* Linnaeus, 1758 (Muramoto et al. 1968) to 84 chromosomes in *Hypostomus* sp. 2 (Cereali et al. 2008). Chromosome sets that are numerically similar to *Hypostomus* sp. 2 were found in *Hypostomus* sp. 3 from the Salobrinha Stream in Mato Grosso do Sul, Brazil (Cereali et al. 2008) (82 chromosomes), *Hypostomus* sp. E from the Mogi-Guaçu River in São Paulo, Brazil (Artoni and Bertollo 1996) (80 chromosomes) and *Hypostomus topavae* (Godoy, 1969) from the Piquiri River in Paraná, Brazil (Bueno et al. 2011) (80 chromosomes). Considering these data, *H. iheringii* (2n = 80) (Fig. 1a) is among the species with the highest chromosome number in the *Hypostomus*. In Loricariidae, the diploid number ranges from 2n=34 chromosomes in *Ancistrus cuiabae* Knaack, 1999 (Mariotto et al. 2011) to 2n=96 chromosomes in *Upsilodus* sp. (Kavalco et al. 2004). The plesiomorphic state is considered to be 2n=54 chromosomes (Artoni and Bertollo 2001). According to Artoni and Bertollo (2001), species of *Hypostomus* with a higher chromosome number are likely to be more derived than species with 2n=54 chromosomes. This hypothesis is supported by the observation that 2n=54 chromosomes is a condition that is shared with the outgroup Trichomycteridae (Ziemniczak et al. in press) and is visualized in all Loricariidae subfamilies as well. Thus, because *H. iheringii* has a high chromosome number, we conclude that it may represent a derived species in this genus.

The increase in the number of st/a chromosomes was postulated to be directly proportional to 2n while the number of m/sm chromosomes is inversely proportional to 2n. This hypothesis would suggest that centric fissions have played a key role in karyotype evolution of this group (Artoni and Bertollo 2001). Recently, the work of Bueno et al. (2011) supported this hypothesis only for species with chromosome numbers higher than or equal to 80 chromosomes. For species with lower chromosome numbers, however, it was not possible to correlate the diploid number of chromosomes with the proportion of st/a chromosomes. Then, other chromosomal rearrangements, such as inversions, deletions, duplications and heterochromatiniztion, could contribute to the chromosomal differentiation of tribe Hypostomini.

Polymorphisms of heterochromatic blocks with maintenance of heteromorphic states are relatively common among Teleost fishes and are correlated to population differentiation and speciation (Hartley and Horne 1984, Mantovani et al. 2000, Vicari et al. 2003, Souza et al. 2007, Kantek et al. 2009, Bellafronte et al. 2011). In Loricariidae, population variation in the number and size of heterochromatic sites was described for *Hisonotus leucofrenatus* Miranda Ribeiro, 1908 (Andreata et al. 2010), *Kronichthys lacerta* Nichols, 1919 and *Isbrueckerichthys duseni* Miranda Ribeiro, 1907 (Ziemniczak et al. in press). This form of variation in *Hypostomus* has been found for

H. iheringii in this study (Fig. 2) and in a previous report for *Hypostomus* sp. B (Artoni and Bertollo 1999), where an extra chromosome with a completely heterochromatic arm was observed in two specimens.

In *H. iheringii*, the polymorphism of the heterochromatic regions in chromosome pairs is not correlated to size variations in these euchromatic chromosomes (Fig. 2). Therefore, the process of heterochromatinization (inactivation by conversion of euchromatin into heterochromatin) might act on the long arms of chromosomes 1 and 5. However, the occurrence of additional amplification in these heterochromatic chromosomal regions cannot be ruled out completely.

Physical mapping of the 45S rDNA multigene family revealed a lack of staining of one homologue of chromosome pairs 20 and 34 (Fig. 3), probably because of unequal crossing over between homologues of these pairs resulting in sites of different sizes (Markovic et al. 1978). Accordingly, FISH failed to detect such sites as a result of its limited ability to detect very small sizes (Schwarzacher and Heslop-Harrison 2000). According to Kavalco et al. (2005), a population of *Hypostomus affinis* (Steindachner, 1877) from Paraíba do Sul river Basin has multiple sites of 18S rDNA, but marking by FISH does not occur in all Ag-NOR positive chromosomes. Thus, the occurrence of small 18S rDNA sites in *Hypostomus* is possible. However, in *H. affinis*, the unequal crossing over was more conspicuous, leading to the emergence of an obvious size heteromorphism among homologous chromosomes. Size differences among NOR sites in this genus are more frequent, and several cases describe variations in the size of sites detectable by silver nitrate staining, similar to what was observed for pair 13 (Fig. 1, Box) (Artoni and Bertollo 1996, Kavalco et al. 2004, 2005, Cereali et al. 2008, Rubert et al. 2008).

Although the literature on the physical mapping of 5S rDNA in *Hypostomus* is not abundant, variations within the group for this marker have been reported. This study identified only one chromosome pair carrying these sites, pair 2 (Fig. 4); however, eight chromosomes bearing such sequences were identified for *H. affinis* (Kavalco et al. 2005), and nine chromosomes were identified for *Hypostomus regani* Ihering, 1905 (Mendes-Neto et al. 2011). The data available for this marker in other genera of Loricariidae indicate that the group has become quite diverse, with some species having a 5S rDNA simple mark, such as *Neoplecostomus micropis* (Steindachner, 1877) and *Harttia loricariformis* Steindachner, 1877 (Kavalco et al. 2004), and others having multiple, such as *Harttia carvalhoi* Miranda Ribeiro, 1939 (Centofante et al. 2006) and *Upsilodus* sp. (Kavalco et al. 2004). In species from the basal groups of Loricariidae, including *K. lacerta*, *I. duseni, Parotocinclus maculicauda* Steindachner, 1877, and the outgroup *Trichomycterus*, the synteny of the major and minor rDNAs has been detected and correlated to the primitive state (Ziemniczak et al. in press). The wide range of diploid number and relocation of the rDNAs in the karyotype of *H. affinis* represent a derived state.

In summary, *H. iheringii* displays evolutionary trends that are characteristic of the genus *Hypostomus*, such as the high number of subtelocentric and acrocentric chromosomes assigned to the species of this genus with high chromosome numbers.

However, the distribution and diversification of heterochromatin suggests new evolutionary trends. All chromosomal markers indicate that the karyotype of *H. iheringii* is highly differentiated and that heterochromatinization of chromosomal segments may contribute to karyotypic differentiation found in this *H. iheringii* population.

Acknowledgements

The authors are grateful to Prof. Dr. Heraldo A. Britski for the identification of the specimens, to the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA/MMA) for the authorization to collect the material; to Pedro L. Gallo e Luiz H. da Silva for collecting effort and to Prof. Dr. Luiz A. C. Bertollo for considerations. This study was financed by FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), Fundação Araucária (Fundação Araucária de Apoio ao Desenvolvimento Científico e Tecnológico do Estado do Paraná) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico).

References

Alves AL, Oliveira C, Nirchio M, Granado A, Foresti F (2006) Karyotypic relationships among the tribes of Hypostominae (Siluriformes: Loricariidae) with description of XO sex chromosome system in a Neotropical fish species. Genetica 128: 1–9. doi: 10.1007/s10709-005-0715-1

Andreata AA, Ferreira DC, Foresti F, Oliveira C (2010) Molecular cytogenetic study of heterochromatin in *Hisonotus leucofrenatus* (Teleostei, Loricariidae, Hypoptopomatinae). Hereditas 147: 10–17. doi: 10.1111/j.1601-5223.2009.2149.x

Armbruster JW (2004) Phylogenetic relationships of the suckermouth armoured catfishes (Loricariidae) with emphasis on the Hypostominae and the Ancistrinae. Zoological Journal of the Linnean Society 141: 1–80. doi: 10.1111/j.1096-3642.2004.00109.x

Artoni RF, Bertollo LAC (1996) Cytogenetic studies on Hypostominae (Pisces, Siluriformes, Loricariidae). Considerantions on kariotype evolution in the genus *Hypostomus*. Caryologia 49: 81–90.

Artoni RF, Bertollo LAC (1999) Nature and distribution of constitutive heterochromatin in fishes, genus *Hypostomus* (Loricariidae). Genetica 106: 209–214. doi: 10.1023/A:1003957719178

Artoni RF, Bertollo LAC (2001) Trends in the karyotype evolution of Loricariidae fish (Siluriformes). Hereditas 134: 201–210. doi: 10.1111/j.1601-5223.2001.00201.x

Bellafronte E, Schemberger MO, Moreira-Filho O, Almeida MC, ArtoniRF, MargaridoVP, Vicari MR (2011) Chromossomal markers and phylogenetic inferences among species of Parodontidae. Review in Fish Biology and Fisheries 21: 559–570. doi: 10.1007/s11160-010-9177-3

Bertollo LAC, Takahashi CS, Moreira-Filho O (1978) Cytotaxonomic considerations on *Hoplias lacerdae* (Pisces, Erythrinidae). Brazilian Journal of Genetics 2: 103–120.

Bueno V, Zawadzki CH, Margarido VP (2011) Trends in chromosome evolution in the genus *Hypostomus* Lacépède, 1803 (Osteichthyes, Loricariidae): a new perspective about the correlation between diploid number and chromosomes types. Reviews in Fish Biology and Fisheries. doi: 10.1007/s11160-011-9215-9

Centofante L, Bertollo LAC, Moreira-Filho O (2006) Cytogenetic characterization and description of na XX/XY_1Y_2 sex chromosome system in catfish *Harttia carvalhoi* (Siluriformes, Loricariidae). Cytogenetic and Genome Research 12: 320–324. doi: 10.1159/000089887

Cereali SS, Pomini E, Rosa RS, Zawadzki CH, Froenlich O, Giluiano-Caetano L (2008) Karyotype of two species of *Hypostomus* (Siluriformes, Loricariidae) of the Planalto da Bodoquena, Brazil. Genetic and Molecular Research 7: 583–591. doi: 10.4238/vol7-3gmr404

Cramer CA, Bonatto SL, Reis RE (2011) Molecular phylogeny of the Neoplecostominae and Hypoptopomatinae (Siluriformes: Loricariidae) using multiple genes. Molecular Phylogenetics and Evolution 59: 43–52. doi: 10.1016/j.ympev.2011.01.002

Foresti F, Oliveira C, Almeida-Toledo LF (1993) A method for chromosome preparations from large specimens of fishes using 'in vitro" short treatment with colchicine. Experientia 49: 810–813.

Griffiths SP (2000) The use of clove oil as an anaesthetic and method for sampling intertidal rockpool fishes. Journal of Fish Biology 57: 1453–1464. doi: 10.1111/j.1095-8649.2000.tb02224.x

Hartley SE, Horne MT (1984) Chromosome polymorphism and constitutive heterochromatin in the Atlantic salmon, *Salmo salar*. Chromosoma 89: 377–380. doi: 10.1007/BF00331255

Hatanaka T, Galetti Jr. PM (2004) Mapping of the 18S and 5S ribosomal RNA genes in the fish *Prochilodus argenteus* Agassiz, 1829 (Characiformes, Prochilodontidae). Genetica 122: 239–244. doi: 10.1007/s10709-004-2039-y

Howell WM, Black DA (1980) Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 36: 1014–1015. doi: 10.1007/BF01953855

Jerep FC, Shibatta OA, Zawadzki CH (2007) A new species of *Hypostomus* Lacépède, 1803 (Siluriformes, Loricariidae) from the upper Rio Paraná basin, Southern Brazil. Neotropical Ichthyology 5: 435–442. doi: 10.1590/S1679-62252007000400002

Kantek DLZ, Vicari MR, Peres WAM, Cestari MM, Artoni RF, Bertollo LAC, Moreira-Filho O (2009) Chromosomal location and distribution of As51 satellite DNA in five species of the genus *Astyanax* (Teleostei, Characidae, Incertaesedis). Journal of Fish Biology 75: 408–421. doi: 10.1111/j.1095-8649.2009.02333.x

Kavalco KF, Pazza R, Bertollo LAC, Moreira-Filho O (2004) Gene mapping of 5S rDNA sites in eight fish species from the Paraiba do Sul river basin, Brazil. Cytogenetic and Genome Research 106: 107–110. doi: 10.1159/000078567

Kavalco KF, Pazza R, Bertollo LAC, Moreira-Filho O (2005) Karyotypic diversity and evolution of Loricariidae (Pisces, Siluriformes). Heredity 94: 180–186. doi: 10.1038/sj.hdy.6800595

Levan A, Fredga K, Sandberg AA (1964) Nomenclature for centromeric position on chromosomes. Hereditas 52: 201–220. doi: 10.1111/j.1601-5223.1964.tb01953.x

Lui RL, Blanco DR, Margarido VP, Moreira-Filho O (2009) First description of B chromosomes in the family Auchenipteridae, *Parauchenipterus galeatus* (Siluriformes) of the São Francisco River basin (MG, Brazil). Mícron 40: 552–559. doi: 10.1016/j.micron.2009.03.004

Machado TC, Pansonato-Alves JC, Pucci MB, Nogaroto V, Almeida MC, Oliveira C, Foresti F, Bertollo LAC, Moreira-Filho O, Artoni RF, Vicari MR (2011) Chromosomal painting and ZW sex chromosomes differentiation in *Characidium* (Characiformes, Crenuchidae). BMC Genetics 12: 65. doi: 10.1186/1471-2156-12-65

Mantovani M, Abel LDS, Mestriner CA, Moreira-Filho O (2000) Accentuated polymorphism of heterochromatin and nucleolar organizer regions in *Astyanax scabripinnis* (Pisces, Characidae): tools for understanding karyotipic evolution. Genetica 109: 161–168. doi: 10.1023/A:1017546601065

Mariotto S, Centofante L, Vicari MR, Artoni RF, Moreira-Filho O (2011) Chromosomal diversification in ribosomal DNA sites in *Ancistrus* Kner, 1854 (Loricariidae, Ancistrini) from three hydrographic basins of Mato Grosso, Brazil. Comparative Cytogenetic 5: 289–300. doi: 10.3897/CompCytogen.v5i4.1757

Markovic VD, Worton RG, Berg JM (1978) Evidence for the inheritance of silver-stained nucleolus organizer regions. Human genetics 41: 181–187. doi: 10.1007/BF00273100

Martins C, Galetti Jr. PM (1999) Chromosomal localization of 5S DNA genes in *Leporinus* fish (Anostomidae, Characiformes). Chromosome Research 7: 363–367. doi: 10.1023/A:1009216030316

Mendes-Neto EO, Vicari MR, Artoni RF, Moreira-Filho O (2011) Description of karyotype in *Hypostomus regani* (Iheringi, 1905) (Teleostei, Loricariidae) from the Piumhi river in Brazil with comments on karyotype variation found in *Hypostomus*. Comparative Cytogenetics 5(2): 133–142. doi: 10.3897/CompCytogen.v5i2.964

Michelle JL, Takahashi CS, Ferrari I (1977) Kariotypic studies of some species of the family Loricariidae (Pisces). Cytologia 42: 536–549. doi: 10.1508/cytologia.42.539

Muramoto I, Ohno S, Atkin, NB (1968) On the diploid state of the fish order Ostariophysi. Chromosoma 24: 59–66. doi: 10.1007/BF00329607

Pinkel D, Straume T, Gray JW (1986) Cytogenetic analysis using quantitative, high-sensitivity, fluorescence hybridization. Proceedings of the National Academy of Sciences of the United States of America 83: 2934–2938. doi: 10.1073/pnas.83.9.2934

Reis RE, Pereira EHL, Armbruster JAW (2006) Delturinae, a new loricariid catfish subfamily (Teleostei, Siluriformes), with revisions of *Delturus* and *Hemipsilichthys*. Zoological Journal of the Linnean Society 147: 277–299. doi: 10.1111/j.1096-3642.2006.00229.x

Rubert M, Zawadski CH, Giuliano-Caetano L (2008) Cytogenetic characterization of *Hypostomus nigromaculatus* (Siluriformes: Loricariidae). Neotropical Ichthyology 6: 93–100. doi: 10.1590/S1679-62252008000100011

Schwarzacher T, Heslop-Harrison P (2000) Practical In Situ Hybridization. Oxford, 203 pp.

Souza IL, Venere PC, Moreira-Filho O (2007) Constitutive heterochromatin and Ag-NOR polymorphisms in the small characid fish *Astyanax scabripinnis* (Jenyns, 1842). Cytologia 72: 63–69. doi: 10.1508/cytologia.72.63

Sumner AT (1972) A simple technique for demonstrating centromeric heterochromatin. Experimental Cell Research 75: 304–306. doi: 10.1016/0014-4827(72)90558-7

Vicari MR, Artoni RF, Bertollo LAC (2003) Heterochromatin polymorphism associated with 18S rDNA: a differential pathway among *Hoplias malabaricus* fish populations. Cytogenetic and Genome Research 101: 24–28. doi: 10.1159/000073413

Weber C (2003) Subfamily Hypostominae. In: Reis RE, Kullander SO, Ferraris CJ (Eds) Checklist of the freshwater fishes of South and Central America. Porto Alegre, Edipucrs, 351–372.

Wichman HA, Payne CT, Ryder OA, Hamilton MJ, Maltbie M, Baker RJ (1991) Genomic distribution of heterochromatic sequences in equids: implications to rapid chromosomal evolution. Journal of Heredity 82: 369–377.

Zawadzki CH, Weber C, Pavanelli CS (2010) A new darksaddled species of *Hypostomus* (Siluriformes: Loricariidae) from the upper rio Paraguay basin. Neotropical Ichthyololgy 8: 719–725. doi: 10.1590/S1679-62252010000400003

Ziemniczak K, Barros AV, Rosa KO, Nogaroto V, Almeida MC, Cestari MM, Moreira-Filho O. Artoni RF, Vicari MR (in press) Comparative cytogenetics of Loricariidae (Actinopterygii: Siluriformes): emphasis in Neoplecostominae and Hypoptopomatinae. Italian Journal of Zoology.

CompCytogen 6(2): 127–139 (2012)
doi: 10.3897/CompCytogen.v6i1.2575
www.pensoft.net/journals/compcytogen

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

A chromosomal investigation of some European Leiodidae (Coleoptera), with particular focus on Spanish subterranean Leptodirini

Robert B. Angus[1,2], David B. Edwards[2], Carlos G. Luque[3], Lucia Labrada[3]

1 *Department of Entomology, The Natural History Museum, Cromwell Road, London SW7 5BD, UK* **2** *School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey TW20 0EX, UK* **3** *Gestión del Patrimonio Bioespeleológico. IMPRESS Group ™ Consulting S.L.; Grupo de Espeleología e Investigaciones Subterráneas ‹‹Carballo-Raba››; P.O. Box 879; 39080 Santander; Spain*

Corresponding author: *R.B. Angus* (r.angus@rhul.ac.uk)

Academic editor: *C. Nokkala* | Received 21 December 2011 | Accepted 29 February 2012 | Published 26 March 2012

Citation: Angus RB, Edwards DB, Luque CG, Labrada L (2012) A chromosomal investigation of some European Leiodidae (Coleoptera), with particular focus on Spanish subterranean Leptodirini. Comparative Cytogenetics 6(2): 127–139. doi: 10.3897/CompCytogen.v6i2.2575

Abstract

Karyotypes are shown for *Leiodes calcarata* (Erichson, 1845), *Catops coracinus* Kellner, 1846, *Cantabrogeus luquei* (Salgado 1993), *Espanoliella luquei* Salgado & Fresneda, 2005, *Fresnedaella lucius* Salgado, Labrada & Luque 2011, *Notidocharis uhagoni* (Sharp, 1872), *Quaestus* (*Quaesticulus*) *pasensis* Salgado, Labrada & Luque, 2010, all of which are shown to have a diploid number of 20 autosomes plus Xy (♂) or XX (♀) sex chromosomes, as well as an as yet undescribed triploid species of the genus *Cantabrogeus* Salgado, 2000. These results are contrasted with published information, all on Leptodirini, which lists 10 species as having diploid numbers of 22 + Xy or XX. It is shown that the higher chromosome number (n = 11 + X or y) previously reported refers exclusively to the more derived Leptodirini ("infraflagellates") whereas the lower number (n = 10 + X or y) refers to the less derived surface-dwelling forms and the less derived Leptodirini ("supraflagellates").

Keywords

Chromosomes, Leiodidae, Leptodirini, *Leiodes* Latreille, 1796, *Catops* Paykull, 1798, *Cantabrogeus* Salgado, 2000, *Espanoliella* Georgiev, 1976, *Fresnedaella* Salgado, Labrada & Luque, 2011, *Notidocharis* Jeannel, 1956, *Quaestus* Schaufuss, 1861, Cantabria, triploid

Introduction

Published information on the chromosomes of Leiodidae is very limited and refers exclusively to subterranean species of the tribe Leptodirini (Durand and Juberthie-Jupeau 1980; Alegre and Escolá 1983; Buzila and Marec 2000). In all data on 10 species have been reported, all with the diploid number 24 (22 + Xy (♂), 22 + XX (♀)). Buzila and Marec (2000) considered this to be the basic chromosome number for the family Leiodidae.

However, investigation of two surface-dwelling forms in 2006 by D.B. Edwards as part of a student project supervised by R.B. Angus, revealed the diploid number 20 + Xy in the males studied. This was a surprise and remained a puzzle until, in December 2009 R.B. Angus received a request from I. Ribera (Barcelona) for help in determining the chromosome number of a cave-dwelling leptodirine from the Cantabria region of northern Spain. Despite intensive searching by C.G. Luque and L. Labrada, only females of this species had been found in the cave so that they suspected the species might be parthenogenetic and wondered if it might be triploid. Investigation of this material showed that the species was indeed triploid, with consistent finding of 33 chromosomes per nucleus. However, while the confirmation that this was a triploid parthenogenetic species was very satisfying, it also added to the puzzle over the diploid number for Leiodidae as this species is was a subterranean member of the tribe Leptodirini, from which the diploid number 24 had been consistently reported. It was therefore decided to extend the investigation to some of the bisexual species to see what the diploid number was in species from Cantabria.

The tribe Leptodirini Lacordaire, 1854 (= Bathysciini Reitter 1906) of the family Leiodidae (Newton 1998, Perreau 2000) is the second largest group (after Carabidae) of subterranean Coleoptera. Nearly all Leptodirini, with a few notable exceptions, inhabit caves or deep soil layers in the Mediterranean basin. This area includes the north and east of the Iberian Peninsula, some Mediterranean islands such as Corsica, Sardinia and Sicily, the southern Alps, Italian and Balkan peninsulas, Carpathian Mountains, southern Russia, the Caucasus, Middle East and Iran (Perreau 2000, 2004; Salgado et al. 2008). The monophyletic origin of the tribe's western Palaearctic core (Leptodirini excl. Platycholeina) is well supported by both morphological (Fresneda et al. 2007) and molecular (Ribera et al. 2010) evidence.

Material and methods

The material investigated is listed in Table 1, and the localities from which the material was obtained are marked on the maps shown in Fig. 1. In all cases the number of specimens from which successful preparations have been obtained is given. As these beetles are frequently very small (2 mm or less) the success rate was generally low and preparations were attempted on considerably more specimens than are listed as suc-

Table 1. Material used, localities, map numbers, numbers of specimens giving successful preparations.

Species	Locality with No. on map	Number giving successful preparations
Leiodes calcarata (Erichson, 1845)	ENGLAND. Surrey: Virginia Water (No. 1)	1♂
Catops coracinus Kellner, 1846	ENGLAND. Surrey: Virginia Water (No. 2)	1♂
Cantabrogeus sp. (triploid)	SPAIN. Cantabria: Municipal District of San Roque de Riomiera, Covallarco cave (No. 3)	7♀♀
C. luquei (Salgado, 1993)	SPAIN. Cantabria: Municipal District of Penagos, Los Gentiles cave (No. 4)	2♂♂, 1♀
Espanoliella luquei Salgado & Fresneda, 2005	SPAIN. Cantabria: Municipal District of Santoña, Merino cave (No. 5)	1♂,1♀
Fresnedaella lucius Salgado, Labrada & Luque, 2011	SPAIN. Cantabria: Municipal District of Selaya, La Canal de la Cubía cave (No. 6)	1♂,1♀
Notidocharis uhagoni (Sharp, 1872)	SPAIN. Cantabria: Municipal District of Ramales, Cullalvera cave-entrance (No. 7)	2♂♂
Quaestus (*Quaesticulus*) *pasensis* Salgado, Labrada & Luque, 2010	SPAIN. Cantabria: Municipal District of Luena, El Rellano del Mazo cave (No. 8)	2♂♂

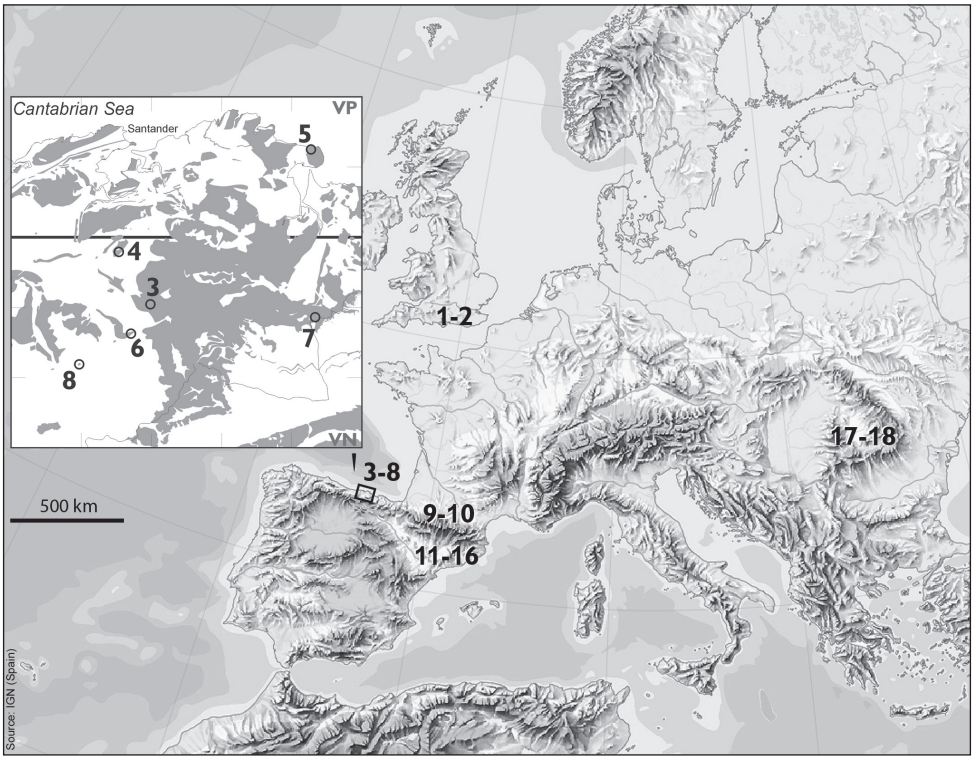

Figure 1. Maps showing the collection sites of the material used in this paper (Nos 1–8), previously published material (Nos 9–18, see Table 2) and areas with carbonate rock outcrops in Cantabria, N Spain (squares 10 × 10 km). See Table 1 for explanation of numbers 1–8, and note that neighbouring sites may share the same number.

cessful. It should also be noted that there was often considerable mortality in transit, even when material was sent express in cooled containers.

The methods of chromosome preparation are as described by Dutton and Angus (2007). The remains of the beetles are either mounted on cards or kept in tubes of 70% ethanol, in the Natural History Museum in London.

For assessment of the chromosomal data in terms of DNA-derived phylogeny we used the dataset from Ribera et al. (2010), plus newly obtained sequences of various Leptodirini species. DNA was extracted from whole specimens with DNeasy Tissue Kits (Quiagen GmbH, Hilden, Germany) in a non-destructive manner to preserve voucher specimens for subsequent morphological study. DNA voucher specimens are deposited in the Institute of Evolutionary Biology (Barcelona, Spain). Seven gene fragments were sequenced: five mitochondrial (3' end of cytochrome c oxidase subunit 1, *cox1*; an internal fragment of cytochrome b, *cyt b*; and 5'end of large ribosomal unit 16S rDNA plus the Leucine transfer RNA gene plus the 3' end of NADH dehydrogenase subunit 1, *rrnL+trnL+nad1*, and two nuclear (5' end of the small ribosomal unit 18S rDNA, *SSU*, and an internal fragment of the large ribosomal unit 28S rDNA, *LSU*). For each fragment both forward and reverse sequences were obtained. Sequences were assembled and edited using Sequencher TM 4.1.4 (Gene Codes, Inc., Ann Arbor, MI). Phylogenetic analysis was conducted using maximum likelihood as implemented in the on-line version of RAxML (which includes an estimation of bootstrap node support, Stamatakis et al. 2008), using GTR+G as the evolutionary model.

Results

Surface forms

Catops coracinus. 2n = 20 + Xy (♂). Giemsa-stained mitotic chromosomes (unbanded), arranged as karyotypes are shown in Fig. 2 a (from mid-gut) and b (from testis). The autosomes are all submetacentric and show an even decrease in length so that pair 10 is about half the length of pair 1. The X chromosome is also submetacentric and is the longest chromosome in the nucleus, about twice the length of autosome 1. The y chromosome is small, almost dot-like. First metaphase of meiosis is shown in Fig. 5 f, which shows the 10 autosomal bivalents and the large X chromosome associated with the y in the typical "parachute" association (Xy_p) of Polyphaga (Smith 1950; Smith and Virkki 1978). Second metaphase of meiosis is shown in Fig. 5, g (♂-determining, with a y chromosome) and h (♀-determining, with an X chromosome). The small y and large X (both labeled) are very distinct in these preparations.

Leiodes calcarata. 2n = 20 + Xy (♂). Unbanded Giemsa-stained mitotic chromosomes from testis are shown in Fig. 2 c. The autosomes and X chromosome are all metacentric or submetacentric. The X chromosome is about the same size as autosome 1, which appears similar in size to that of *Catops coracinus* (compare Fig 1 b and c). The autosomes show an even decrease in length from pairs 1 – 8, which pair 8 about half

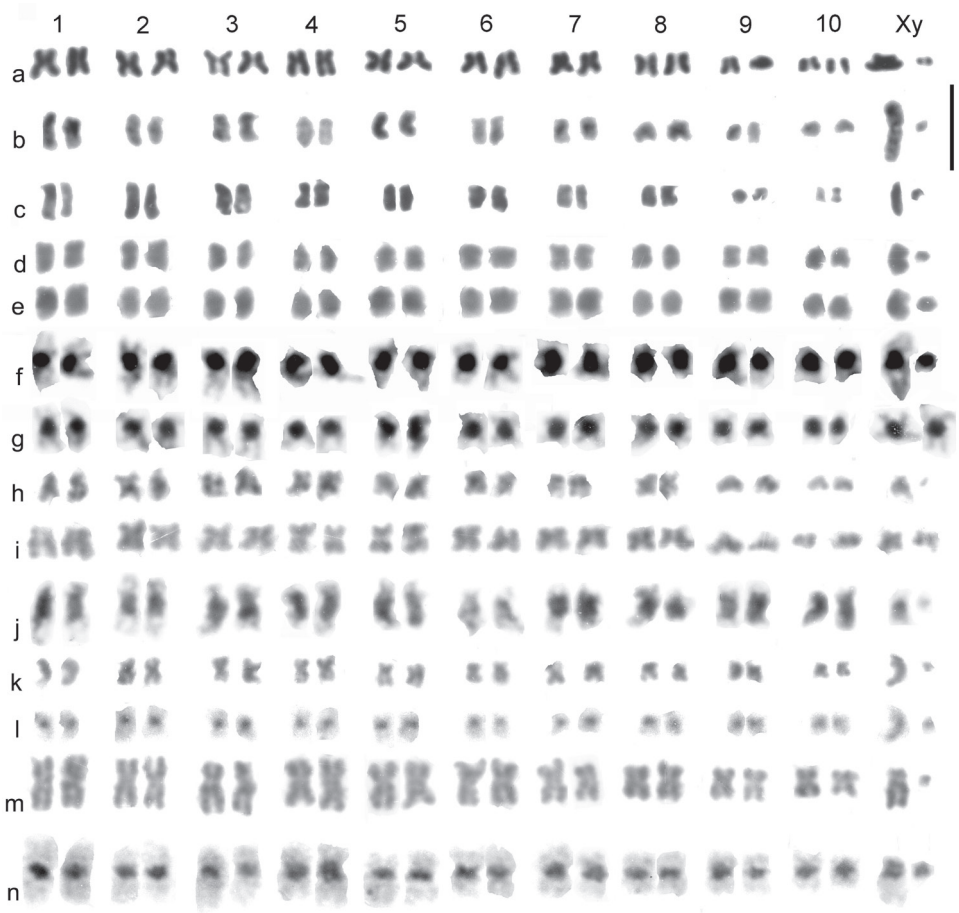

Figure 2. Mitotic chromosomes of Leiodidae, arranged as karyotypes: **a**, **b** *Catops coracinus*, unbanded **a** mid-gut **b** testis **c** *Leiodes calcarata*, testis **d** – **g** *Cantabrogeus luquei*, mid-gut **d**, **e** ♂ unbanded **f** ♂ C-banded **g** ♀ C-banded **h**, **i** *Espanoliella luquei*, mid-gut, unbanded **h** ♂ **i** ♀ **j** *Fresnedaella lucius*, testis, C-banded **k**, **l** *Notidocharis uhagoni*, ♂, mid-gut **k** unbanded **l** the same nucleus C-banded **m**, **n** *Quaestus pasensis*, mid gut **m** unbanded **n** the same nucleus C-banded. Scale bar = 5 µm.

the length of pair 1. There is then an abrupt decrease to pairs 9 and 10, which are about half the length of pair 8. The y chromosome is dot-like. Zygotene of first division of meiosis is shown in Fig. 5 a, where the heavily condensed Xy bivalent is distinct. Fig. 5 b – d shows first metaphase, with the Xy_p bivalent very clear. Fig 5 e shows a second metaphase nucleus, with the y chromosome clearly present.

Diploid cave-dwelling Leptodirini

Cantabrogeus luquei. 2n = 20 + Xy (♂), 20 + XX (♀). Unbanded Giemsa-stained mitotic chromosomes from mid-gut cells are shown as karyotypes in Fig 2, d, e (♂), and

chromosomes from a C-banded ♂ mid-gut nucleus is shown in Fig 2 f. Fig. 2 g shows chromosomes from a C-banded ♀ mid-gut nucleus. The chromosomes shown in Fig 2 e and f are shown as found in Fig. 3 a, b. All the autosomes, and the X chromosome are metacentric with heavy centromeric C-bands. The autosomes show an even decrease in size along the karyotype, with pair 10 about half the length of pair 1. The X chromosome, about 1.5 × the length of autosome 1, is the largest in the nucleus. The y chromosome is a relatively large dot. These preparations are completely consistent with one another and leave no doubt that 20 + Xy or XX is the true diploid number for this species.

Espanoliella luquei. 2n = 20 + Xy (♂), 20 + XX (♀). Unbanded Giemsa-stained mitotic chromosomes from mid-gut cells are shown as karyotypes in Fig. 2 h (♂) and i (♀), and as found in Fig. 3 c, d. No C-banded preparation is available. The chromosomes in these preparations are all rather condensed, but appear either metacentric or submetacentric, with autosome pair 1 distinctly longer than the others, and a gradual decrease in length from pairs 2 – 10. The X chromosome appears similar in length to the medium-sized autosomes and the y chromosome is dot-like. As with *C. luquei*, the preparations are consistent with one another and leave no doubt that the chromosome number reported here is correct.

Fresnedaella lucius. 2n = 20 + Xy (♂), 20 + XX (♀). Fig. 2 j shows spontaneously C-banded Giemsa-stained mitotic chromosomes from testis. Fig 3 e shows these chromosomes as found. The female preparations (not shown) were from mid-gut, completely unbanded and with the chromatids beginning to separate following maximum contraction at metaphase. They are, however, adequate to confirm the chromosome number. Spontaneous C-banding was frequent among testis preparations from these Leptodirini, but in most cases the preparations were not adequate for preparation of karyotypes. The X chromosome is the smallest in the nucleus, apart from the dot-like y. The C-bands are particularly weak in autosome 6 and slightly weaker than most in autosome 2 and the X chromosome, but in the remaining autosomes they are very strong. All the autosomes and the X chromosome are metacentric to submetacentric.

Notidocharis uhagoni. 2n = 20 + Xy (♂). Fig. 2 k, l shows karyotypes from a ♂ mid-gut cell, unbanded and C-banded. These chromosomes are shown as found in Fig. 3 f, g. The autosomes and X chromosome are all metacentric or submetacentric, with small centromeric C-bands. The X chromosome, about twice as long as autosome 1, is the longest in the nucleus, and the autosome lengths decrease fairly evenly along the karyotype, with autosome par 10 about half the length of pair 1. The y chromosome is dot-like. As with the other species, the preparations are consistent and there is no reason to doubt the number obtained.

Quaestus pasensis. 2n = 20 + Xy (♂). Fig. 2 m, n shows karyotypes from a mid-gut cell, unbanded and C-banded, and Fig. 3 h, i shows these chromosomes as found, before and after C-banding. Autosome pair 3 and the X chromosome are more or less subacrocentric while the other autosome pairs are metacentric to submetacentric. The autosome pairs decrease in length evenly along the karyotype, with pair 10 slightly less than half the length of pair 1. The X chromosome is about as long as autosome pair 5

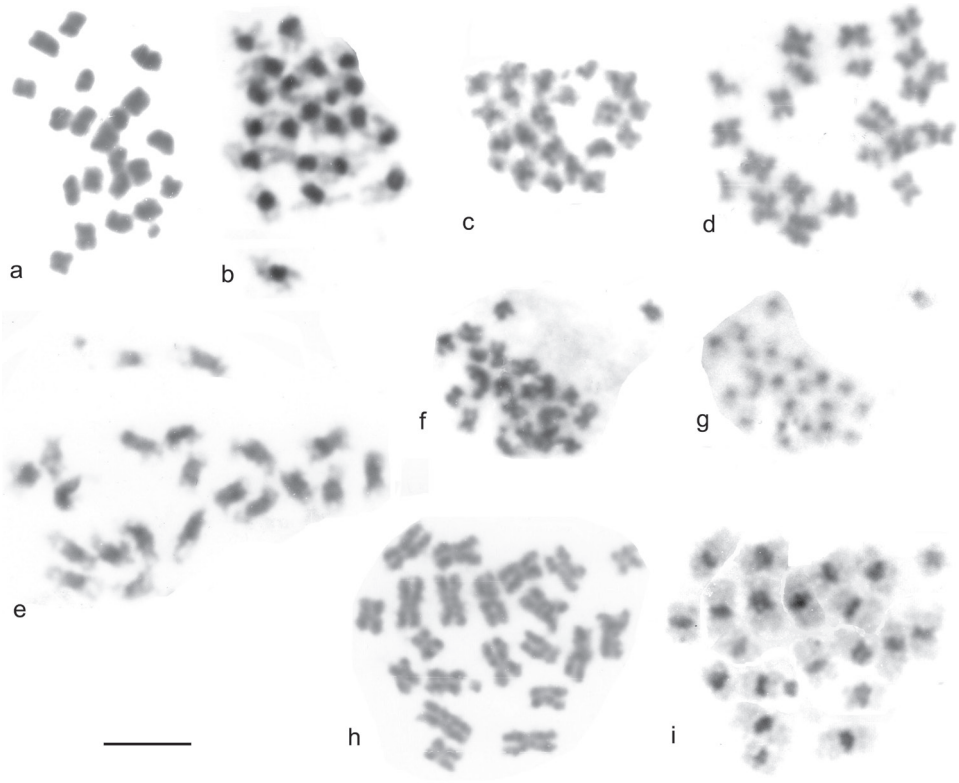

Figure 3. Giemsa stained mitotic chromosomes of Leptodorini as found. **a**, **b** *Cantabrogeus luquei,* mid-gut cell **a** plain **b** C-banded **c**, **d** *Espanoliella luquei*, mid-gut cells, plain **c** ♂ **d** ♀ **e** *Fresnedaella lucius*, testis, C-banded **f**, **g** *Notidocharis uhagoni*, ♂, mid-gut cell **f** plain **g** the same nucleus C-banded **h**, **i** *Quaestus pasensis*, ♂, mid-gut cell **h** plain **i** the same nucleus C-banded. Scale bar = 5 μm.

and the y chromosome is dot-like. The centromeric C-bands are distinct, with those of autosome pairs 1 and 4 about twice the size of the others. As with the other species, the preparations obtained are completely consistent with one another and leave no reason to doubt their accuracy.

Triploid species

Cantabrogeus sp. (Salgado et al. in press) 3n = 33 (♀). Fig. 4 shows mid-gut chromosomes of this species. Two nuclei are shown, both unbanded (Giemsa-stained) and C-banded. Unbanded and C-banded karyotypes from the nucleus shown in Fig. 4 a and b are shown as Fig. 4 e and f. As only females are present it is not possible to identify the X chromosome, but the results are totally consistent with a triploid number and a haploid complement of 10 + X. Pairs 7 and 11 are subacrocentric, pairs 3, 5, 8 and 10 are clearly submetacentric, and the remainder are more or less metacentric. The

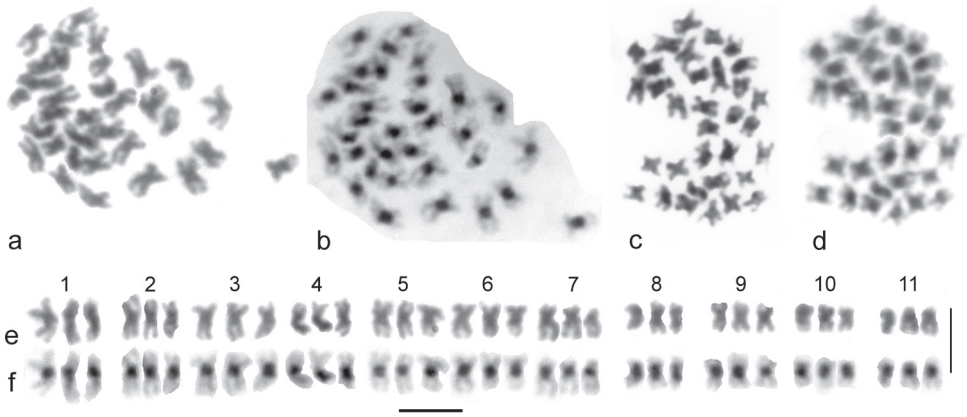

Figure 4. *Cantabrogeus* triploid species, mitotic chromosomes from mid-gut nuclei. **a–d** the chromosomes as found **a**, **c** unbanded **b**, **d** the same nuclei C-banded **e**, **f** karyotypes assembled from the nucleus figured in **a** & **b**. Scale bar = 5 μm.

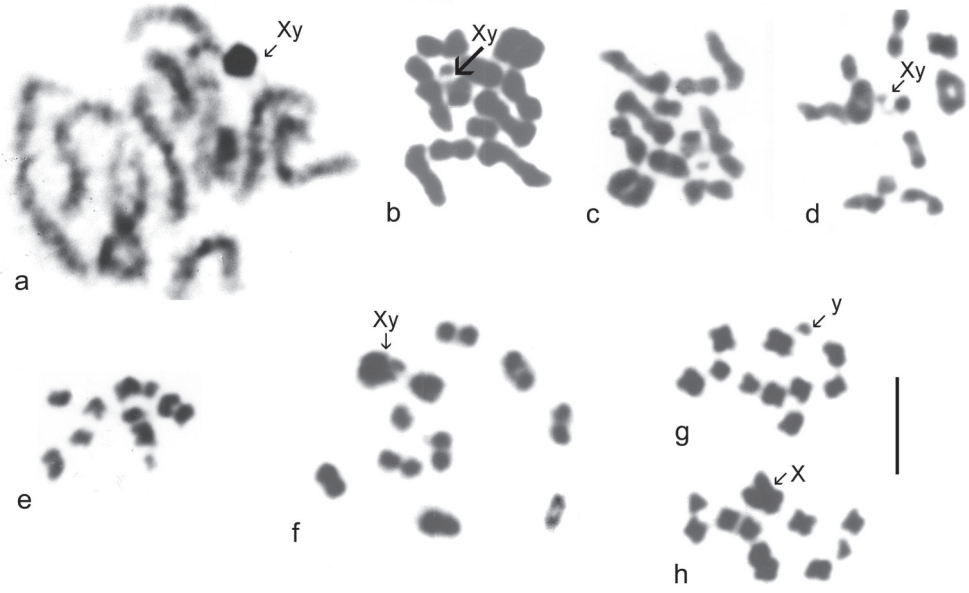

Figure 5. Meiosis of Leiodidae (surface forms). **a–e** *Leiodes calcarata* **f–h** *Catops coracinus* **a** prophase I, zygotene **b–d** metaphase I **e**, metaphase II, ♂-determining, with y chromosome **f** metaphase I **g**, **h** metaphase II **g** ♂-determining, with y chromosome **h** ♀-determining, with X chromosome. Scale bar = 5 μm.

chromosome lengths decrease rather evenly along the karyotype, with pair 11 rather more than half the length of pair 1. The centromeric C-bands are bold and distinct, but slightly smaller than those of *C. luquei*. There is no chromosome of this species which invites obvious comparison with the X chromosome of *C. luquei*, so there is no hint in this material about which is the X chromosome.

Phylogenetic placement of the triploid species.

DNA from a specimen captured on June 6, 2009 (ref. IBE-RA34) was analyzed by I. Ribera (Salgado et al. in press), using the genetic methods described in Ribera et al. (2010) and used to establish the phylogenetic relationships of the new triploid species. Cladistic analysis of the sequences identified the sister relationship between the triploid *Cantabrogeus* sp. and other supraflagellates of the '*Quaestus*' series (Fig. 6). The analysis of the obtained sequences shows that *Cantabrogeus* is the sister group of *Fresnedaella lucius* and *Quaestus pasensis* and this clade, in turn, is the sister group of *Q. minos* and *Q. autumnalis* (Salgado et al. 2011). Moreover, the whole clade has a sister relationship with the genus *Espanoliella*. All clades receive high support values. The sister group relationship between *Cantabrogeus*, *Fresnedaella* and *Quaestus pasensis* is recovered in 99-100% of the bootstrap replicates of RAxML (Fig. 6).

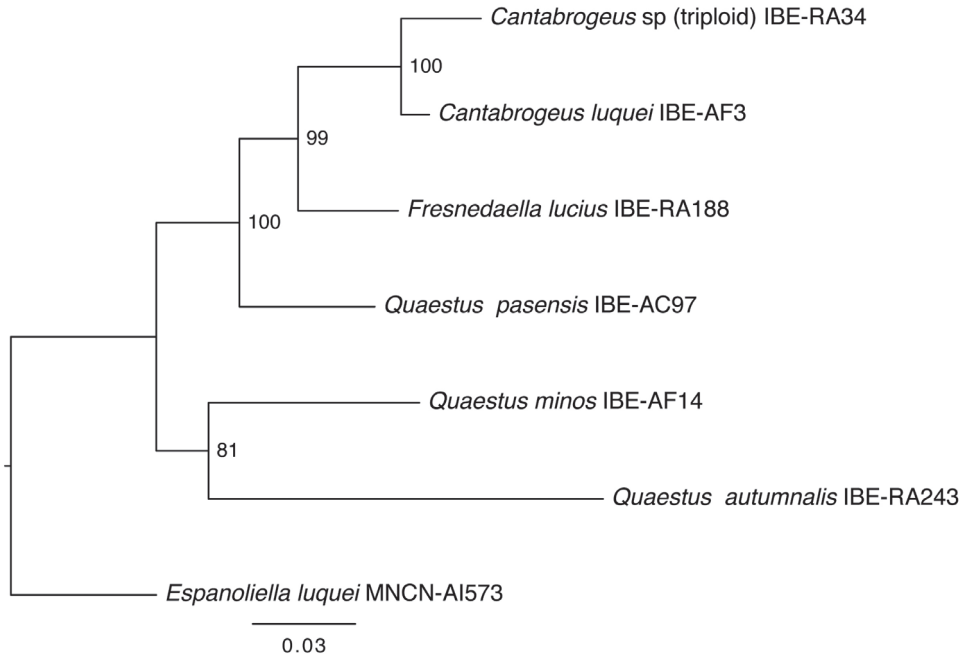

Figure 6. Phylogenetic relationships of triploid *Cantabrogeus* sp., obtained with the same genes and methodology used by Ribera et al. (2010). Courtesy of I. Ribera, Institute of Evolutionary Biology, CSIC–UPF (Barcelona, Spain).

Discussion

As mentioned in the Introduction, the results obtained here appear both surprising and puzzling as all published data indicate a diploid chromosome number of 22 + Xy (♂), which Buzila and Marec (2000) considered to be conservative for the family. The 10 species involved, with their localities of origin and map numbers,

Table 2. Species of Leptodorini for which chromosome details have been published: species with map No. As shown in Fig. 1, area from which the material was collected, and publication reference.

Species (Map No.)	Area	Reference
Speonomus hydrophilus (Jeannel, 1908) (No. 9)	Pyrenees, France-Spain	Durand and Juberthie-Jupeau 1980
S. pyrenaeus (Lespès, 1857) (No.10)		
Parvospeonomus delarouzeei (Fairmaire, 1860) (No.11)		Alegre and Escolà 1983
Troglocharinus elongatus Zariquiey, 1950 (=*variabilis* Bellés, 1978) (No. 12)		
T. jacasi (Lagar, 1966) (No. 13)		
T. schibii (Español, 1972) (No. 14)		
T. ferreri (Reitter, 1908) subspecies *pallaresi* Bellés, 1973 (No. 15)		
T. kiesenwetteri (Dieck, 1869) (No. 16)		
Pholeuon knirschi Breit, 1911 (No. 17)	Carpathians, Romania	Buzila and Marec 2000
Drimeotus kovacsi Miller, 1856 subspecies *viehmanni* Ieniştea, 1955 (No. 18)		

are listed in Table 2. This shows that all these species belong in most derived section of the Leptodirini, the infraflagellates of Jeannel (1955) characterised by the basal region of the internal sac of the aedeagus having a Y-shaped ventral sclerite, the Y-piece. Fresneda et al. (2007), in their cladistic analysis of leiodid morphology, concluded that the infraflagellates were a monophyletic group but that the other of Jeannel's groups, the supraflagellates, characterised by having a dorsal flagellum in the basal region of the internal sac of the aedeagus, was a paraphyletic assemblage of less highly derived species. This arrangement is supported and amplified by the DNA work of Ribera et al. (2010). Moreover, Salgado et al. (2011) published an article in which the phylogenetic relationships of *Fresnedaella* and *Cantabrogeus*, and its allied taxa were mentioned. These authors show that, while all the species previously studied chromosomally, including those from the Carpathians, belong to the derived infraflagellates, those reported here all belong in the more basal supraflagellate assemblage (Figs 6, 7). It therefore seems that the more primitive Leiodidae have a chromosome complement comprising 10 pairs of autosomes and Xy/XX sex chromosomes – the only exception being one triploid species of *Cantabrogeus*. So, far from being the conservative chromosome number for Leiodidae, 11 pairs plus Xy/XX sex chromosomes is a derived feature of the advanced infraflagellates. It is also worth noting that if Smith's (1950) suggestion that the ancestral Polyphagan chromosome complement was 9 pairs + Xy/XX is correct, then the basic number for Leiodidae involves an increase of one pair of autosomes, while the advanced infraflagellates show an additional increase of one pair.

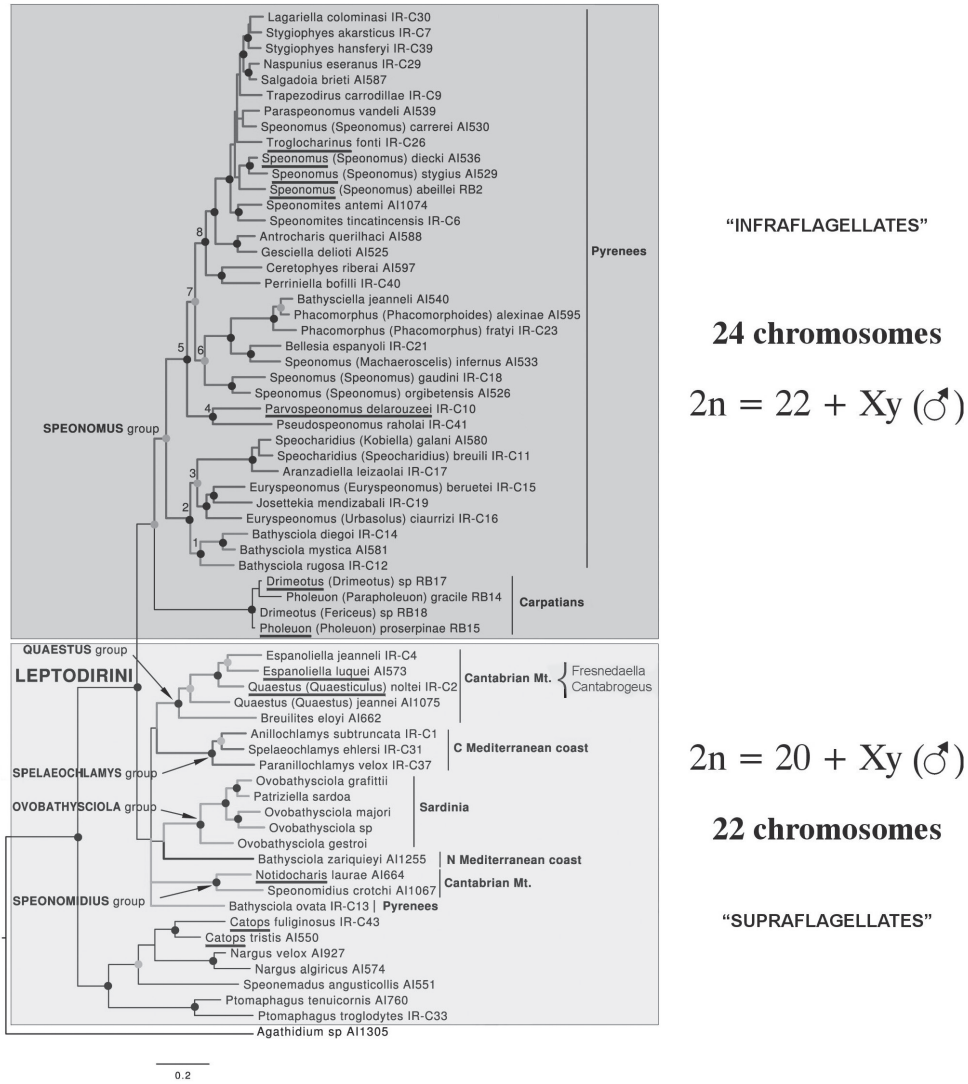

Figure 7. Phylogram obtained from Ribera et al. (2010), modified to show the reconstructed evolution of the chromosome number. Genera whose chromosome complements are known are underlined.

Acknowledgements

The cave-dwelling specimens referred to in this paper were collected during several bio-speleological investigations authorized and funded by the Government of Cantabria (Spain). The studies and explorations in Cantabria were supported in part by grants from the General Direction for Biodiversity of the Regional Government of Cantabria. We thank J.M. Salgado (University of León, Spain), for assistance in collecting speci-

mens, and I. Ribera (Institute of Evolutionary Biology, CSIC–UPF, Barcelona, Spain) who kindly provided information about molecular studies.

The chromosomal work was carried out in the Natural History Museum, London and the School of Biological Sciences, Royal Holloway, University of London, whom we thank for the facilities to carry out these investigations.

References

Alegre C, Escolà O (1983) Étude cytogénetique de cinq espèces de *Troglocharinus* et de *Speonomus delarouzeei* (Col. Catopidae). Mémoires de Biospéologie 10: 363 – 367.

Buzila RA, Marec F (2000) Conservative chromosome number in the Cholevidae family of cave beetles. Mémoires de Biospéologie 27: 21–23.

Durand J, Juberthie-Jupeau L (1980) Étude cytogénétique de deux espèces de *Speonomus* (Coléoptères Bathysciinae). Mémoires de Biospéologie 7: 267–271.

Dutton LA, Angus RB (2007) A karyosystematic investigation of a group of sibling species related to *Stictotarsus griseostriatus* (De Geer) (Coleoptera: Dytiscidae). Comp. Cytogenet. 1: 3 – 16.

Fresneda J, Salgado JM, Ribera I (2007) Phylogeny of the western Mediterranean Leptodirini, with emphasis on genital characters (Coleoptera: Leiodidae: Cholevinae). Systematic Entomology 32 (2): 332 – 358. doi: 10.1111/j.1365-3113.2006.00366.x

Jeannel R (1955) L'édéage, initiation aux recherches sur la systématique des Coléoptères. Publications du Museum National d'Histoire Naturelle, Paris 16: 1–155.

Newton AF (1998) Phylogenetic problems, current classification and generic catalogue of world Leiodidae (including Cholevidae). In: Giachino PM, Peck SB (Eds) Phylogeny and Evolution of Subterranean and Endogean Cholevidae (= Leiodidae Cholevinae). Proceedings of XX International Congress of Entomology, Firenze, 1996. Atti Museo Regionale di Scienze Naturali, Torino, 41–178.

Perreau M (2000) Catalogue des Coléoptères Leiodidae Cholevinae et Platypsyllinae. Mémoires de la Société Entomologique de France 4: 1–460.

Perreau M (2004) Leiodidae. In: Löbl I, Smetana A (Eds) Catalogue of Palaearctic Coleoptera. Volume 2. Apollo Books, Stenstrup, 133–203.

Ribera I, Fresneda J, Bucur R, Izquierdo A, Vogler AP, Salgado JM, Cieslak A (2010) Ancient origin of a Western Mediterranean radiation of subterranean beetles. BMC Evolutionary Biology 10: 29. doi: 10.1186/1471-2148-10-29

Salgado JM, Blas M, Fresneda J (2008) Coleoptera, Cholevidae. Ramos MA et al. (Eds) In: Fauna Ibérica. Volume 31. Museo Nacional de Ciencias Naturales, CSIC, Madrid, 799pp.

Salgado JM, Labrada L, Luque CG (2011) Un nuevo género y nueva especie de Leptodirini troglobio de la Cordillera Cantábrica (Cantabria, España): *Fresnedaella lucius* n. gen., n. sp. (Coleoptera: Leiodidae: Cholevinae). Heteropterus Revista de Entomología 11 (1): 1–12.

Salgado JM, Luque, CG, Labrada L, Fresneda J, Ribera I (in press) Revisión del género *Cantabrogeus* Salgado, 2000 con la descripción de tres nuevas especies hipogeas endémicas de la

Cordillera Cantábrica (Coleoptera, Leiodidae, Cholevinae, Leptodirini). Animal Biodiversity and Conservation 35.

Smith SG (1950) The cyto-taxonomy of Coleoptera. Canadian Entomologist 82 (3): 58 – 68. doi: 10.4039/Ent8258-3

Smith SG, Virkki N (1978) Animal Cytogenetics 3: Insecta 5: Coleoptera. x + 366 pp. Gebrüder Borntraeger, Berlin – Stuttgart.

Stamatakis A, Hoover P, Rougemont J (2008) A rapid bootstrap algorithm for the RAxML web servers. Systematic Biology 57 (5): 758–771. doi: 10.1080/10635150802429642

CompCytogen 6(2): 141–151 (2012)
doi: 10.3897/CompCytogen.v6i2.1769
www.pensoft.net/journals/compcytogen

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Cytogenetic analysis of B chromosomes in one population of the fish *Moenkhausia sanctaefilomenae* (Steindachner, 1907) (Teleostei, Characiformes)

Diogo Teruo Hashimoto[1], Tatiana Aparecida Voltolin[1], Ana Danyelle Noitel Valim de Arruda Paes[1], Fausto Foresti[2], Jehud Bortolozzi[1], Fábio Porto-Foresti[1]

1 *Departamento de Ciências Biológicas, Faculdade de Ciências, Universidade Estadual Paulista (UNESP), Av. Eng. Luiz E. C. Coube, 17033-360, Bauru, SP, Brazil* **2** *Departamento de Morfologia, Instituto de Biociências, Universidade Estadual Paulista (UNESP), Dist. Rubião Júnior, 18618-000, Botucatu, SP, Brazil*

Corresponding author: *Fábio Porto-Foresti* (fpforesti@fc.unesp.br)

Academic editor: *G. Furgala-Selezniow* | Received 2 July 2011 | Accepted 7 March 2012 | Published 9 April 2012

Citation: Hashimoto DT, Voltolin TA, de Arruda Paes ADNV, Foresti F, Bortolozzi J, Porto-Foresti F (2012) Cytogenetic analysis of B chromosomes in one population of the fish *Moenkhausia sanctaefilomenae* (Steindachner, 1907) (Teleostei, Characiformes). Comparative Cytogenetics 6(2): 141–151. doi: 10.3897/CompCytogen.v6i2.1769

Abstract

The aim of this study was to characterize cytogenetically one population of the fish *Moenkhausia sanctaefilomenae* (Steindachner, 1907), with emphasis on the analysis of B chromosomes. The nucleolar activity in the B microchromosomes was characterized, and an analysis of mitotic instability of these microchromosomes was accomplished. The results showed a diploid chromosome number of 50 chromosomes. In all individuals, we observed the presence of B microchromosomes with intra- and inter-individual variability. The analysis of the nucleolus organizing regions (NORs) by silver nitrate staining demonstrated multiple NORs. We observed active sites of ribosomal DNA in the B microchromosomes, with a frequency of 20% in the analyzed cells, which shows gene activity in these chromosomal elements. The analysis of constitutive heterochromatin patterns showed that the B microchromosomes are heterochromatic or euchromatic, which demonstrates differentiation of DNA composition between these genomic elements. The calculation of the mitotic instability index implied that B chromosomes in this species might be in a final stage of instability.

Keywords

fish cytogenetic, NOR expression, supernumerary chromosomes, mitotic instability

Introduction

Moenkhausia Eigenmann, 1903 is considered as *incertae sedis* in Characidae and contains 65 valid species widely distributed in the Neotropical river basins (Lima et al. 2003). Although the genus *Moenkhausia* cannot be characterized as monophyletic, a group consisted of *M. oligolepis* (Günther, 1864), *M. sanctaefilomenae* (Steindachner, 1907), *M. cotinho* Eigenmann, 1908, and *M. pyrophthalma* Costa, 1994 shares a very similar color pattern (Costa 1994). *Moenkhausia* systematic is very complex and nowadays several studies have shown that it needs to be more thoroughly addressed (Benine et al. 2009).

Chromosome studies in the genus *Moenkhausia* are still restricted and cytogenetic data are available only for six species (Portela-Castro et al. 2001). In *M. sanctaefilomenae*, a stable diploid number of 50 chromosomes and few karyotype variations among the different populations analyzed have been reported. Furthermore, some populations of *M. sanctaefilomenae* can show a high inter- and intra-individual variability of the NOR (nucleolus organizer region) phenotypes, as well as conspicuous blocks of constitutive heterochromatin in the pericentromeric region of the chromosomes (Foresti et al. 1989, Portela-Castro et al. 2001, Portela-Castro and Júlio Jr. 2002). However, the occurrence of several B microchromosomes in the genome of this species is the most peculiar feature to be studied in this fish group (Foresti et al. 1989).

B chromosome includes a variety of extra chromosomes that display conspicuous heterogeneity in their nature, behavior, and evolutionary dynamics. This definition highlights some of the most universal properties of B chromosomes: their dispensability (that is, they are not necessary for the host to complete a normal life cycle); their origin from chromosomes (either from within the same species or from other species); and their remarkable differentiation relative to A chromosomes, with which they do not recombine (Camacho 2005).

B chromosomes are widely distributed among eukaryotes and their occurrence has been reported in 10 species of the fungi, nearly 1.300 plants (more than 1.400 when different ploidy levels of the same species are considered separately), and over 500 animals (Camacho 2005). In addition, B chromosomes have been described in 61 species of Neotropical fish to date (Carvalho et al. 2008).

In species of *Moenkhausia*, B chromosomes were documented for *M. sanctaefilomenae* and *M. intermedia* Eigenmann, 1908 (Portela et al. 1988, Foresti et al. 1989). Differently from other microchromosome-bearing fish species, which exhibit a low frequency and a sporadic occurrence (Hashimoto et al. 2008, Oliveira et al. 2009, Hashimoto et al. 2011), several microchromosomes can be found in the genome of *M. sanctaefilomenae* and, in certain situations, the frequency can be related to sex (Portela-Castro et al. 2001). In fact, in Neotropical fish, it is possible to find both B macrochromosomes and B microchromosomes (Oliveira et al. 2009), but in both cases the presence of a large number of B chromosomes in the cells is rare, as was observed in *Prochilodus lineatus* (Valenciennes, 1836) and *M. sanctaefilomenae*,

which presented up to eight microchromosomes in the cells (Foresti et al. 1989, Voltolin et al. 2011).

Another interesting characteristic observed in the B microchromosomes of *M. sanctaefilomenae* is the polymorphism revealed by C-banding. Through this method, these microchromosomes can be characterized in different classes according to the pattern of constitutive heterochromatin; they can be partially and totally heterochromatic, and euchromatic (Foresti et al. 1989). Thus, such polymorphism indicates a distinct DNA composition between these microchromosomes, especially of repetitive DNA.

In the present study, we carried out cytogenetic analyses in one particular population of the fish *M. sanctaefilomenae* focusing on two special features concerning the B microchromosomes: the occurrence of nucleolar activity in the B chromosome of this species and a study about the maintenance of microchromosomes in this population through the calculation of the mitotic instability index (MI).

Material and methods

The cytogenetic analyses were carried out in chromosomal preparations obtained from 15 specimens (8 males and 7 females) of *Moenkhausia sanctaefilomenae*. The individuals were collected from a population of the Batalha River (22°7.02'S, 49°16.01'W), belonging to Tietê River basin, São Paulo State, southeastern Brazil. The voucher specimens were identified and stored in the fish collection of the Laboratório de Genética de Peixes, UNESP, Bauru, SP, Brazil.

Before sacrifice, the animals were inoculated with yeast cell suspension to increase the number of metaphase cells (Oliveira et al. 1988). Chromosomal preparations were obtained from gill and kidney tissues using the technique described by Foresti et al. (1993). Silver staining (Ag-staining) of the nucleolus organizer regions followed the technique of Howell and Black (1980), and C-banding was performed according to Sumner (1972). The chromosomal morphology was determined on the basis of arm ratio, as proposed by Levan et al. (1964) and the chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st), and acrocentric (a).

The index to quantify the mitotic instability of B chromosome, MI, which was calculated as the sum of the absolute values of every deviation in B number with respect to the median (M), and normalized by dividing the median and the number of cells analyzed (N) so that the index is independent of the number of B and the sample size were performed by means of one-way ANOVA.

$$\mathbf{MI = (M\text{-}ni/fi)/M.N}$$

where ni is the numer of B chromosome in the different types of cells that do not coincide with M, and fi is the number of cells of each particular type.

Results and discussion

In the individuals of *Moenkhausia sanctaefilomenae,* our results showed a diploid chromosome number of 50 chromosomes, with karyotypes composed of 6 m, 16 sm and 28 st (fundamental number FN = 100) (Fig. 1). No sex-related karyotype difference was observed. The diploid chromosome number and the karyotypes composed mainly of metacentric and submetacentric chromosomes seem to be a conserved characteristic observed for different *M. sanctaefilomenae* populations (Foresti et al. 1989, Portela-Castro et al. 2001, Portela-Castro and Júlio Jr. 2002).

Extra chromosomes were observed in the genomes of all individuals of *M. sanctaefilomenae*, which were characterized as B microchromosomes (Fig. 1). We detected inter- and intra-individual variation in relation to the number of B chromosomes in the cells, with specimens bearing up to eight microchromosomes. Metaphase counts for 13 individuals showing the variation in supernumerary chromosome numbers are presented in Table 1. The modal numbers were of 2 and 3 microchromosomes. Such variation is in accordance with the pioneer study of Foresti et al. (1989), who also analyzed a population from the Tietê River basin. On the other hand, the specimens from the Paraná River analyzed by Portela-Castro et al. (2001), showed differences because the presence of 0–2 microchromosomes were reported only in males. These polymorphisms concerning the distribution of B chromosomes indicate a process of genetic divergence in distinct populations that likely occurs in some species restricted to small tributaries and streams, as reported for species of *Astyanax* (Moreira-Filho and Bertollo 1991, Vicari et al. 2008, Hashimoto et al. 2011).

Analysis of the constitutive heterochromatin patterns by C-banding showed heterochromatic blocks in the centromeric and pericentromeric regions in the majority of the chromosomes (Fig. 2a, b). Such general heterochromatin pattern was also observed in previous analyses for other *M. sanctaefilomenae* populations (Foresti et al. 1989, Portela-Castro et al. 2001, Portela-Castro and Júlio Jr. 2002), demonstrating that these chromosomal regions present a highly conservative distribution in this species. The supernumerary chromosomes showed different C-banding patterns. We observed euchromatic (Fig. 2a) as well as partially or totally heterochromatic microchromosomes (Fig. 2a, b), evidencing that these B chromosomes can have a different DNA composition, mainly of repetitive sequences. This is a common feature also reported for B chromosomes in other characid species (Néo et al. 2000, Jesus et al. 2003, Moreira-Filho et al. 2004).

The Ag-impregnation revealed intra- (Fig. 3a, b) and inter-individual (Fig. 3c, d) variability for the NOR phenotypes in metaphases of *M. sanctaefilomenae*, ranging from two to five Ag-positive sites, distributed in the interstitial and terminal regions of distinct chromosomes (Fig. 3e). However, the chromosomes 6 always presented active Ag-NORs, and consequently, were considered the major NOR-bearing chromosomes. The minor NORs showed a very variable pattern of activity. Such NOR features were previously reported by Foresti et al. (1989).

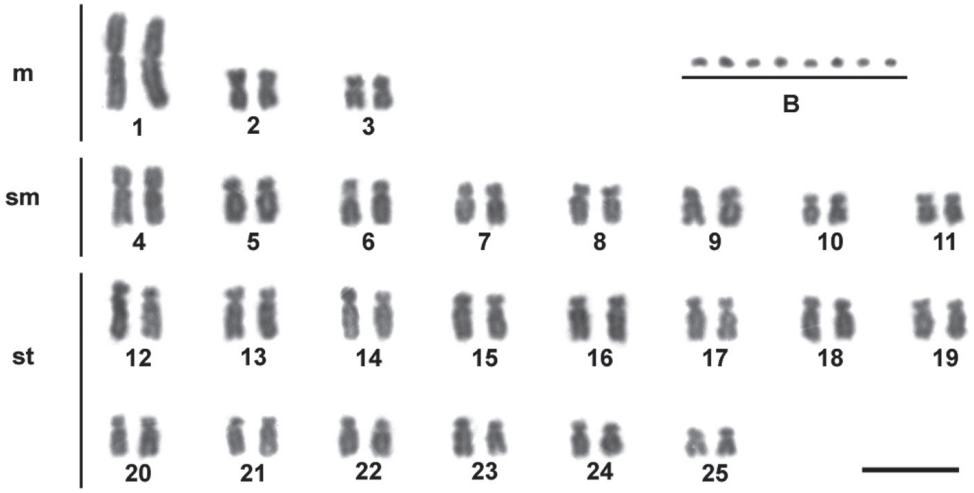

Figure 1. Giemsa-stained karyotype showing 2n = 50 chromosomes of one individual of *Moenkhausia sanctaefilomenae*. In evidence, eight B microchromosomes. Bars = 10 µm.

Table 1. Metaphase counts for 13 specimens of *Moenkhausia sanctaefilomenae* demonstrating the variation in B microchromosome numbers.

Specimen identification	Number of B microchromosomes per cell									Number of cells counted
	0	1	2	3	4	5	6	7	8	
849	6	12	22	-	-	-	-	-	-	40
852	2	32	36	10	-	-	-	-	-	80
853	9	70	-	-	-	-	-	-	-	79
857	3	3	6	10	9	9	2	-	-	42
887	-	3	12	22	4	2	7	13	2	65
888	-	6	6	41	5	10	-	-	-	68
889	-	3	12	63	11	3	-	-	-	92
1233	-	4	10	24	26	29	13	4	-	110
1235	1	6	31	79	15	8	-	-	-	140
1240	8	31	33	26	3	-	-	-	-	101
1241	9	137	175	4	-	-	-	-	-	325
1242	24	85	27	-	-	-	-	-	-	136
1246	5	25	4	5	4	1	-	-	-	44

Indeed, NOR expression was detected in a B chromosome of one individual, which carried only this microchromosome (Fig. 3a, b). We analyzed 60 cells by Ag-staining and observed that about 20% had active ribosomal DNA sites in the B chromosome of this individual. Moreover, this supernumerary chromosome showed to be euchromatic by C-banding. The nucleolar region is a dynamic cell compartment involved in the control of numerous cellular functions that can be visualized after Ag coloration,

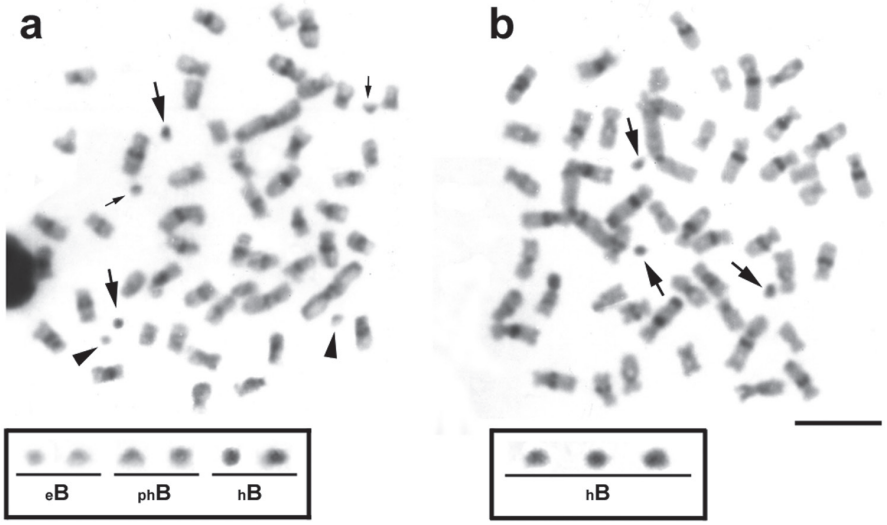

Figure 2. Metaphases from specimens of *Moenkhausia sanctaefilomenae* after C-banding technique. In (**a**), metaphase shows euchromatic ($_{e}$B), partially heterochromatic ($_{ph}$B) and totally heterochromatic ($_{h}$B) microchromosomes. In (**b**), metaphase demonstrates only heterochromatic B chromosomes. The boxes show enlarged B chromosomes. Major and minor arrows indicate totally and partially heterochromatic B microchromosomes, respectively. Arrowheads exhibit euchromatic B microchromosomes. Bars = 10 μm.

when the genes present activities in the interphase that anticipates the mitosis (Roussel et al. 1996, Caperta et al. 2007, Hiscox 2007). Therefore, Ag-staining provides a simple and reliable method to detect ribosomal RNA (rRNA) gene transcription (Bakkali et al. 2001, Teruel et al. 2009). B chromosomes in several species carry rRNA genes (Camacho 2005), including fish species (Baroni et al. 2009, Poletto et al. 2010), and in most of the cases, rRNA has been detected by Ag-staining evidencing the presence of active genes, as demonstrated in the present study. However, further analysis using FISH technique will be necessary to detect positions of additional rDNA genes not only during their activity.

The fact that the NORs located in the chromosomes 6 were always active can suggest that a process of nucleolar dominance can influence the rRNA gene transcription in order to provide the proper amount of rRNA for ribosome assembly. Nucleolar dominance is an epigenetic phenomenon common in interspecific hybrids, in which ribosomal RNA genes set inherited from one parental are rather transcribed in relation to the other (Hashimoto et al. 2009). Nucleolar dominance can also be a consequence of the regulatory process that controls the effective dosage of rRNA genes in pure species (non-hybrid) (Pikaard 2000). Nowadays, the mechanisms by which whole NORs or rRNA genes subsets are selected for inactivation still remains unclear (Preuss and Pikaard 2007).

The chromosome context appears to be important for NOR activity, as deduced from changes in the on/off activity status following chromosome rearrangements moving NORs to new locations (Pikaard 2000). The present findings show that the B chromosome plays an important role in the genome organization of *M. sanctaefilom-*

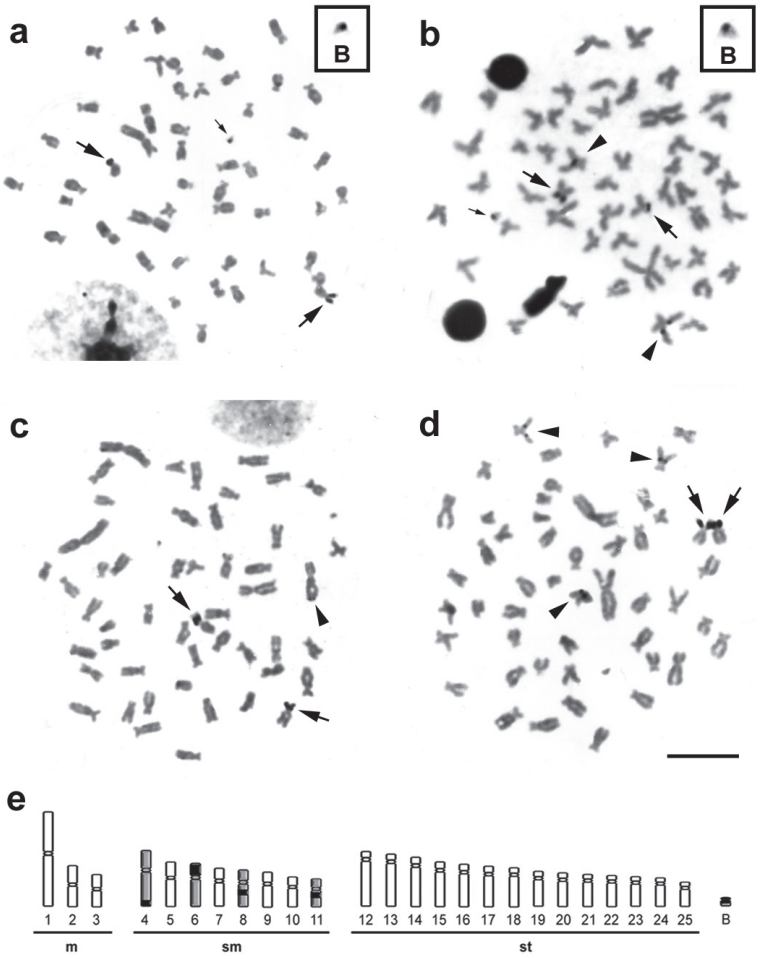

Figure 3. Metaphases from specimens of *Moenkhausia sanctaefilomenae* submitted to the silver coloration. In (**a**) and (**b**), metaphases of one individual show intra-individual variability of active NORs. The boxes exhibit enlarged B chromosomes with nucleolar activity. In (**c**) and (**d**), metaphases of different samples demonstrate inter-individual variability for the NORs. In (**e**), schematic representation shows the NOR-bearing chromosomes (4, 6, 8, 11 and B). Major arrows indicate major NOR-bearing chromosomes (chromosomes 6). Minor arrows show nucleolar activity in the B microchromosomes (**a**) and (**b**). Arrowheads exhibit minor NORs demonstrating a variable pattern of activity in different chromosomes. Bars = 10 μm.

enae, and will be useful for further analyses to determine whether the frequency of B chromosomes expressing their NOR is changing over time and how the B chromosome context can influence A chromosome NOR activity.

In relation to the mitotic instability and maintenance of B chromosomes in *M. sanctaefilomenae*, we compared the results reported by Foresti et al. (1989) with the data described in this study, because both populations were collected from the Tietê River basin (Brazil). In both populations, a pattern of mitotic instability for all ana-

lyzed individuals was observed. The analysis of the standard maintenance of these B chromosomes by calculating the mitotic instability index (MI) revealed that the *M. sanctaefilomenae* population analyzed by Foresti et al. (1989) showed a MI = 0.6; however, the *M. sanctaefilomenae* population analyzed in the present study showed a MI = 0.2. Taking account the high variability of B chromosomes in the genomes of these *M. sanctaefilomenae* specimens, further studies are still necessary to verify if these B chromosomes might be underway towards the neutralization stage, in accordance with the life cycle of B chromosomes described by Camacho et al. (1997).

In fish, the possibility of neutralization through mitotic stabilization of B-chromosomes was also observed in *Prochilodus lineatus*, in the population from the Mogi-Guaçu River (Brazil) (Oliveira et al. 1997). Afterwards, in this same population, Cavallaro et al. (2000) found a drastic temporal decline in the degree of B mitotic instability; Voltolin et al. (2010) showed that the stabilization process was continuous for over 15 years; and currently, the population of *P. lineatus* from the Mogi-Guaçu River presents a total mitotic stability index (MI = 0) and the B chromosomes were considered completely neutralized.

In Neotropical fish, most of the studies about B chromosomes are still descriptive, because many species have not yet been cytogenetically analyzed. Thus, studies focusing B chromosomes in Neotropical fish are extremely necessary to better understand this intriguing class of chromosomes, as has been done for some species, such as *Prochilodus lineatus* and *Astyanax* species (Moreira-Filho et al. 2004, Voltolin et al. 2010, 2011, Hashimoto et al. 2011), towards which efforts are more thoroughly addressed. Thus, our results show that B chromosomes of *M. sanctaefilomenae* are excellent models and also that extensive studies in this species are essential to improve the knowledge of the diversification of B chromosomes.

Acknowledgements

This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

Bakkali M, Cabrero J, López-León MD, Perfectti F, Camacho JPM (2001) Population differences in the expression of nucleolus organizer regions in the grasshopper *Eyprepocnemis plorans*. Protoplasma 217: 185–190. doi: 10.1007/BF01283399

Baroni S, Lopes CE, Almeida-Toledo LF (2009) Cytogenetic characterization of *Metynnis maculatus* (Teleostei; Characiformes): the description in Serrasalminae of a small B chromosome bearing inactive NOR-like sequences. Caryologia 2: 95–101.

Benine RC, Mariguela TC, Oliveira C (2009) New species of *Moenkhausia* Eigenmann, 1903 (Characiformes: Characidae) with comments on the *Moenkhausia oligolepis* species complex. Neotropical Ichthyology 7: 161–168. doi: 10.1590/S1679-62252009000200005

Camacho JPM, Shaw MW, López-Léon MD, Pardo MC, Cabrero J (1997) Population dynamics of a selfish B chromosome neutralized by the standard genome in the grasshopper *Eyprepocnemis plorans*. The American Naturalist 149: 1030–1050. doi: 10.1086/286037

Camacho JPM (2005) B Chromosomes. In: Gregory TR (Ed.) The evolution of the genome. Elsevier, San Diego, 223–286. doi: 10.1016/B978-012301463-4/50006-1

Caperta AD, Neves N, Viegas W, Pikaard CS, Preuss S (2007) Relationships between transcription, silver staining, and chromatin organization of nucleolar organizers in *Secale cereale*. Protoplasma 232: 55–59. doi: 10.1007/s00709-007-0277-4

Carvalho AR, Martins-Santos IC, Dias AL (2008) B chromosomes: an update about their occurrence in freshwater Neotropical fishes (Teleostei). Journal of Fish Biology 72: 1907–1932. doi: 10.1111/j.1095-8649.2008.01835.x

Cavallaro ZI, Bertollo LAC, Perfectti F, Camacho JPM (2000) Frequency increase and mitotic stabilization of a B chromosome in fish *Prochilodus lineatus*. Chromosome Research 8: 627–634. doi: 10.1023/A:1009242209375

Costa WJEM (1994) Description of two new species of the genus *Moenkhausia* (Characiformes: Characidae) from the central Brazil. Zoologischer Anzeiger 232: 21–29.

Foresti F, Almeida-Toledo LF, Toledo SA (1989) Supranumerary chromosome system C-banding pattern characterization and multiple Nucleolus Organizing Region in *Moenkhausia sanctaefilomenae* (Pisces, Characidae). Genetica 79: 107–114. doi: 10.1007/BF00057927

Foresti F, Oliveira C, Almeida-Toledo LF (1993) A method for chromosome preparations from large specimens of fishes using in vitro short treatment with colchicine. Experientia 49: 810–813. doi: 10.1007/BF01923555

Hashimoto DT, Gonçalves VR, Bortolozzi J, Foresti F, Porto-Foresti F (2008) First report of a B chromosome in a natural population of *Astyanax altiparanae* (Characiformes, Characidae). Genetics and Molecular Biology 31: 275–278. doi: 10.1590/S1415-47572008000200021

Hashimoto DT, Laudicina A, Bortolozzi J, Foresti F, Porto-Foresti F (2009) Chromosomal features of nucleolar dominance in hybrids between the Neotropical fish *Leporinus macrocephalus* and *Leporinus elongatus* (Characiformes, Anostomidae). Genetica 137: 135–140. doi: 10.1007/s10709-009-9366-y

Hashimoto DT, Ferguson-Smith MA, Rens W, Foresti F, Porto-Foresti F (2011) Chromosome mapping of H1 histone and 5S rRNA gene clusters in three species of *Astyanax* (Teleostei, Characiformes). Cytogenetic and Genome Research 134: 64–71. doi: 10.1159/000323512

Hiscox JA (2007) RNA viruses: hijacking the dynamic nucleolus. Nature Reviews Microbiology 5: 119–127. doi: 10.1038/nrmicro1597

Howell WM, Black DA (1980) Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experientia 36: 1014–1015. doi: 10.1007/BF01953855

Jesus CM, Galetti Jr PM, Valentini SR, Moreira-Filho O (2003) Molecular characterization and chromosomal location of two families of satelite DNA in *Prochilodus lineatus*

(Pisces, Prochilodontidae), a species with B chromosomes. Genetica 118: 25–32. doi: 10.1023/A:1022986816648

Levan A, Fregda K, Sandbreg AA (1964) Nomeclature for centromeric position on chromosomes. Hereditas 52: 201–220. doi: 10.1111/j.1601-5223.1964.tb01953.x

Lima FCT, Malabarba LR, Buckup PA, Silva JFP, Vari RP, Harold A, Benine R, Oyakawa OT, Pavanelli CS, Menezes NA, et al. (2003) Characidae – genera *incertae sedis* in Characidae. In: Reis RE, Kullander SO, Ferraris Jr CJ (Eds) Check List of the freshwater fishes of the South and Central America. EDIPUCRS. Porto Alegre, 106–169.

Moreira-Filho O, Bertollo LAC (1991) *Astyanax scabripinnis* (Pisces, Characidae): A species complex. Brazilian Journal of Genetics 14: 331–357.

Moreira-Filho O, Galletti Jr. PM, Bertollo LAC (2004) B chromosomes in the fish *Astyanax scabripinnis* (Characidae, Tetragonopterinae): An overview in natural population. Cytogenetic and Genome Research 106: 230–234. doi: 10.1159/000079292

Néo DM, Bertollo LAC, Moreira-Filho O (2000) Morphological differentiation and possible origin of B chromosome in natural Brazilian population of *Astyanax scabripinnis* (Pisces, Characidae). Genetica 108: 211–215. doi: 10.1023/A:1004157901097

Oliveira C, Almeida-Toledo LF, Foresti F, Britski HA, Filho SAT (1988) Chromosome formulae of neotropical freswater fishes. Brazilian Journal of Genetics 11: 577–624.

Oliveira C, Saboya SMR, Foresti F, Senhorini JA, Bernardino G (1997) Increased B chromosome frequency and absence of drive in the fish *Prochilodus lineatus.* Heredity 79: 473–476. doi: 10.1038/hdy.1997.186

Oliveira C, Foresti F, Hilsdorf AWS (2009) Genetics of neotropical fish: from chromosomes to populations. Fish Physiology and Biochemistry 35: 81–100. doi: 10.1007/s10695-008-9250-1

Pikaard CS (2000) The epigenetics of nucleolar dominance. Trends in Genetics 16: 495–500. doi: 10.1016/S0168-9525(00)02113-2

Poletto AB, Ferreira IA, Martins C (2010) The B chromosomes of the African cichlid fish *Haplochromis obliquidens harbor* 18S rRNA gene copies. BMC Genetics 11: 1. doi: 10.1186/1471-2156-11-1

Portela ALBS, Galetti Jr. PM, Bertollo LAC (1988) Considerations on the chromosome evolution of Tetragonopterinae. Brazilian Journal of Genetics 11: 307–313.

Portela-Castro ALBS, Julio HF, Nishiyama PB (2001) New occurrence of microchromosomes B in *Moenkhausia sanctaefilomenae* (Pisces, Characidae) from the Paraná River of Brazil: analysis of the synaptonemal complex. Genetica 110: 277–283. doi: 10.1023/A:1012742717240

Portela-Castro ALB, Júlio–Júnior HF (2002) Karyotype relationships among species of the subfamily Tetragonopterinae (Pisces, Characidae): Cytotaxonomic and evolution aspects. Cytologia 67: 329–336. doi: 10.1508/cytologia.67.329

Preuss S, Pikkard CS (2007) rRNA gene silencing and nucleolar dominance: Insights into a chromosome-scale epigenetic on/off swich. Biochimica et Biophysica Acta 1769: 383–392. doi: 10.1016/j.bbaexp.2007.02.005

Roussel P, Andre C, Comai L, Hernandez-Verdun D (1996) The rDNA transcription machinery is assembled during mitosis in active NORs and absent in inactive NORs. The Journal of Cell Biology 133: 235–246. doi: 10.1083/jcb.133.2.235

Sumner AT (1972) A simple technique for demonstrating centromeric heterochromatin. Experimental Cell Research 75: 304–306. doi: 10.1016/0014-4827(72)90558-7

Teruel M, Cabrero J, Perfectti F, Acosta MJ, Sanchez A, Camacho JPM (2009). Quantitative analysis of NOR expression in a B chromosome of the grasshopper *Eyprepocnemis plorans.* Chromosoma 119: 291–301. doi: 10.1007/s00412-008-0197-x

Vicari MR, Noleto RB, Artoni RF, Moreira-Filho O, Bertollo LAC (2008) Comparative cytogenetics among species of the *Astyanax scabripinnis* complex. evolutionary and biogeographical inferences. Genetics and Molecular Biology 31: 173–179. doi: 10.15902/S1415-47572008000200002

Voltolin TA, Senhorini JA, Oliveira C, Foresti F, Bortolozzi J, Porto-Foresti F (2010) B-chromosome frequency stability in *Prochilodus lineatus* (Characiformes, Prochilodontidae). Genetica 138: 281–284. doi: 10.1007/s10709-009-9420-9

Voltolin TA, Senhorini JA, Foresti F, Bortolozzi J, Porto-Foresti F (2011) Intraspecific crosses resulting in the first occurrence of eight and nine B chromosomes in *Prochilodus lineatus* (Characiformes, Prochilodontidae). Genetics and Molecular Biology 34: 220–224. doi: 10.1590/S1415-47572011005000009

CompCytogen 6(2): 153–161 (2012)
doi: 10.3897/CompCytogen.v6i2.1980
www.pensoft.net/journals/compcytogen

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

A new karyotype for the spiny rat *Clyomys laticeps* (Thomas, 1909) (Rodentia, Echimyidae) from Central Brazil

Alexandra M. R. Bezerra[1], Juliana M. Pagnozzi[2], Ana Paula Carmignotto[3], Yatiyo Yonenaga-Yassuda[4], Flávio H. G. Rodrigues[5]

1 *Departamento de Zoologia, Universidade de Brasília, Campus Darcy Ribeiro, Asa Norte, 70910-900, Brasília, DF, Brazil* **2** *Faculdade Salesiana de Vitória, Av. Vitória 950, Forte São João, 29017-950, Vitória, ES, Brazil* **3** *Universidade Federal de São Carlos, Campus Sorocaba, Rodovia João Leme dos Santos (SP-264), km 110, Bairro Itinga, 18052-780, Sorocaba, SP, Brazil* **4** *Instituto de Biociências, Universidade de São Paulo, Rua do Matão 277, Cidade Universitária, 05508-090, São Paulo, SP, Brazil* **5** *Departamento de Biologia Geral, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, Pampulha, CP 486, 31270-901, Belo Horizonte, MG, Brazil; and Instituto Pró-Carnívoros.*

Corresponding author: *Alexandra M. R. Bezerra* (abezerra@fst.com.br)

Academic editor: *I. Bakloushinskaya* | Received 29 August 2011 | Accepted 23 February 2012 | Published 9 April 2012

Citation: Bezerra AMR, Pagnozzi JM, Carmignotto AP, Yonenaga-Yassuda Y, Rodrigues FHG (2012) A new karyotype for the spiny rat *Clyomys laticeps* (Thomas, 1909) (Rodentia, Echimyidae) from Central Brazil. Comparative Cytogenetics 6(2): 153–161. doi: 10.3897/CompCytogen.v6i2.1980

Abstract

Clyomys Thomas, 1916 is a semifossorial rodent genus of spiny rats represented by only one species, *C. laticeps*, which inhabits the tropical savannas and grasslands of central Brazil and eastern Paraguay. Here we describe a new karyotype of *C. laticeps* found in populations of Emas National Park, Goiás state, Brazil. The four analyzed specimens had a diploid number (2n) of 32 and a fundamental autosome number (FN) of 54. Cytogenetic data include conventional staining, CBG and GTG-banding. The karyotype presents 12 meta/submetacentric pairs (1 to 12) and 3 pairs of acrocentrics (13 to 15) with gradual decrease in size. The X chromosome is a medium submetacentric and the Y is a medium acrocentric. The semifossorial habits together with habitat specificity could have contributed to the karyological variations found on this genus.

Keywords

Clyomys, Cerrado, cytogenetic, Echimyidae, taxonomy, semifossorial habit

Introduction

The genus *Clyomys* Thomas, 1916 has long been represented by two living species, namely *C. laticeps* and *C. bishopi* Avila-Pires et Wutke, 1981distributed in tropical savannas and grasslands from circa 100 to 1,100 m elevation in central Brazil and eastern Paraguay (Woods and Kilpatrick 2005). *C. laticeps* would range from the Paraguayan Chaco to the Brazilian States of Minas Gerais and Bahia throughout the Cerrado domain, whereas *C. bishopi* was restricted to the Cerrado enclaves in São Paulo State, Brazil (Avila-Pires and Wutke 1981). Bezerra and Oliveira (2010) have recently reviewed the genus. These authors considered *Clyomys bishopi* a synonym of *C. laticeps* based on quantitative and qualitative characters of skull, phallic morphology, and pelage patterns.

Spiny rats of the genus *Clyomys* present semifossorial habit and can be identified, together with the other semifossorial echimyids genera *Carterodon* Waterhouse, 1848 and *Euryzygomatomys* Goeldi, 1901 by a set of morphological characters such as a body covered by spinous pelage, short tail and limbs, and long, powerful claws (Bishop 1974). *Clyomys* differ from those genera by its very conspicuous and hypertrophied auditory bullae (Thomas 1916).

Cytogenetic studies of the genus *Clyomys* reported a diploid number (2n) of 34 chromosomes and fundamental autosome number (FN) 60 or 62. The population from State of São Paulo, Brazil, described by Yonenaga (1975) and by Souza and Yonenaga (1984), showed 2n = 34 and FN = 60, while Svartman (1989) analyzed specimens from Distrito Federal, Brazil, and found the same diploid number, but FN = 62.

The present paper describes a different diploid number for *C. laticeps* from a Central Brazilian sample. We also discuss habitat use and biology of this species and their bearing on the observed intraspecific karyotypic variation.

Material and methods

Four wild-caught specimens (2 females and 2 males) of *C. laticeps* from Emas National Park (ENP), state of Goiás, Brazil (18°15'50"S, 52°53'33"W) were karyotypically studied (Fig. 1). The vouchers specimens are deposited at the Museu Nacional (MN), Universidade Federal do Rio de Janeiro, and at the Mammal Collection of the Universidade de Brasília (UNB), Brazil: MN 68165 (female), MN 68164 (male), MN 68167 (male), and UNB 2155 (female). The map was generated using the software GMT (2009).

Mitotic metaphase cells were obtained from bone marrow and spleen after *in vivo* colchicine treatment. Mitotic cells were spread onto clean glass slides, air-dried and stored at -20° C until use. Analysis were performed after routine Giemsa staining, CBG-banding (Sumner 1972) and GTG-banding techniques (Seabright 1971).

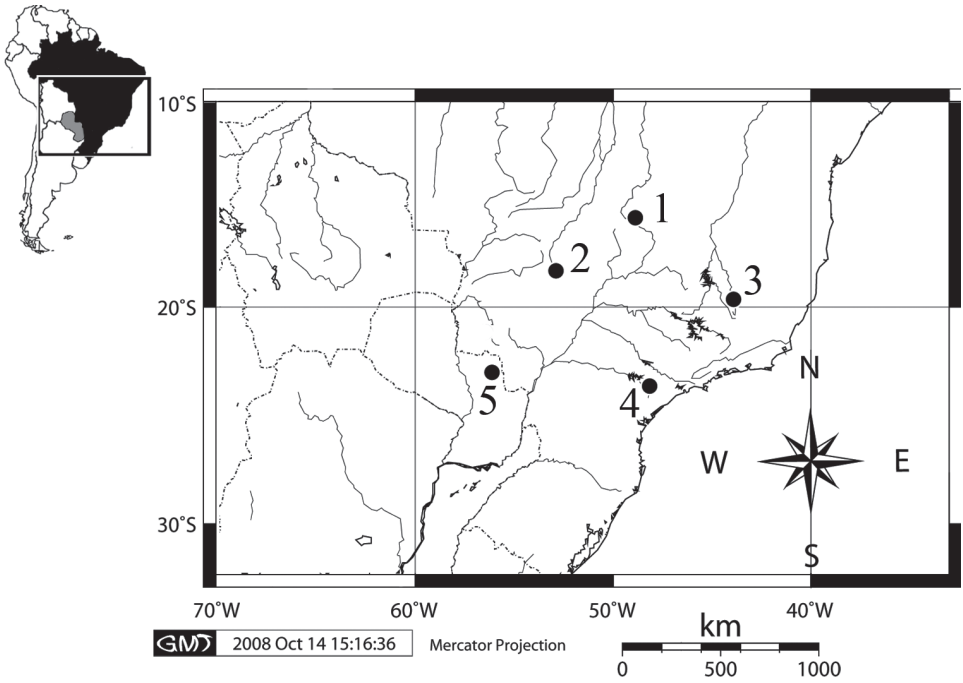

Figure 1. Map of karyotyped populations showing the type localities of *Clyomys laticeps* and its synonyms: Brazil, Distrito Federal, Brasília [**1**] (Svartman 1989); Goiás state, Mineiros, Emas National Park [**2**] (this study); Minas Gerais State, Lagoa Santa [**3**] (type locality of *C. laticeps* – Thomas 1909); São Paulo state, Itapetininga [**4**] (Yonenaga 1975, Souza and Yonenaga 1984; also type locality of *C. bishopi* – Avila-Pires and Wutke 1981); Paraguay, Departamento de San Pedro, Partido de Tacuatí, Acai-Poi [**5**] (type locality of *C. laticeps whartoni* – Moojen 1952). Small map of the South America show the Brazil in black colour and the Paraguay in gray.

Results

Clyomys laticeps from ENP shows a karyotype with 2n = 32 and FN = 54. The autosome complement comprises 12 biarmed pairs (pair 1 is submetacentric with a distal secondary constriction in the long arm, pairs 2 to 12 are metacentric or submetacentric chromosomes) and three acrocentric pairs (pair 13 a heteromorphic medium acrocentric and pairs 14 and 15 are small acrocentrics). The X chromosome is submetacentric and Y is acrocentric, both morphologically distinguishable after G and/or C banding pattern (see below and Fig. 2).

CBG-banding revealed constitutive heterochromatin at the telomeric regions of some autosomes (pairs 2 and 4–10). Additionally, interstitial bands occurred in the pericentromeric region of pairs 2 and 8–10. The distal secondary constriction in the long arm of pair 1 is C-band negative and the proximal region of both arms shows a small amount of faintly stained constitutive heterochromatin. Pair 3 is completely C-band negative.

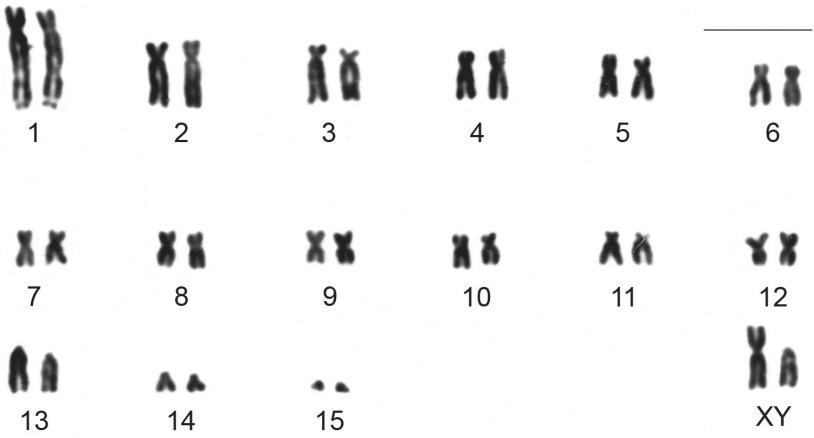

Figure 2. Karyotype of a male of *Clyomys laticeps* (MN 68164) from Emas National Park after conventional staining (2n = 32, FN = 54). Bar = 10 μm.

Pericentromeric positive C-band was present in the pairs 11 and 12, as well as in pairs 13 and 14, which additionally show a large block of heterochromatin in the proximal region of the long arm. The 15th pair is completely heterochromatic. The 13th autosome pair is heteromorphic due to the size of constitutive heterochromatin in all studied specimens (Fig. 2). The X chromosome is identifiable by a large submetacentric with distinctive centromeric heterochromatin and by an unique G-banding pattern characterized by a wide negative G-band at pericentromeric region (Figs 3 and 5). The Y chromosome is an acrocentric similar in size to the smaller acrocentric of the pair 13. It has a conspicuous C-positive band segment in the pericentromeric region and a block at the proximal region of the long arm (Figs 3 and 4). It is readily identifiable only after G-banding since it is G-positive along all its length comparing to the autosome pair 13 (Fig. 5).

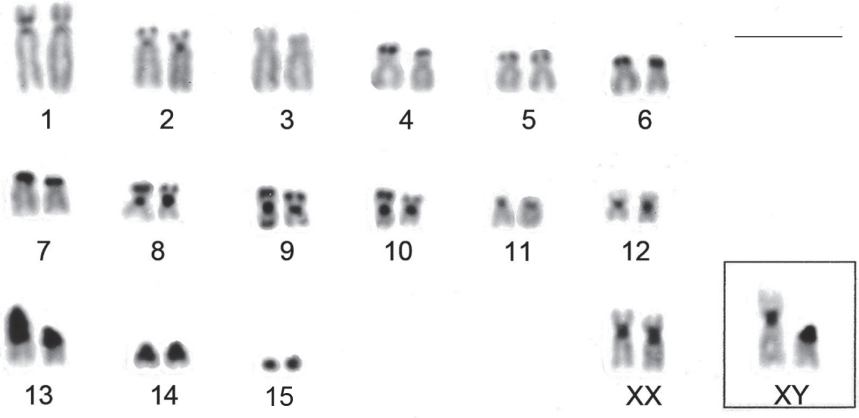

Figure 3. CBG-banded karyotypes of female of *Clyomys laticeps* (UNB 2155) from Emas National Park (2n = 32, FN = 54). Inset: sex chromosomes of a male (MN 68165). Bar = 10 μm.

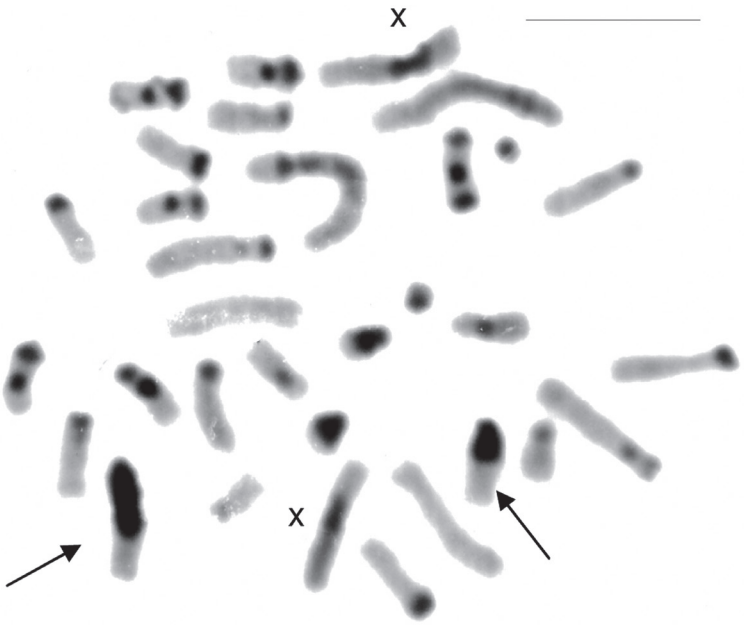

Figure 4. CBG-banded metaphase of a female of *Clyomys laticeps* (UNB 2155) from Emas National Park (2n = 32, FN = 54). The arrows indicate the heteromorphic 13th pair. Bar = 10 μm.

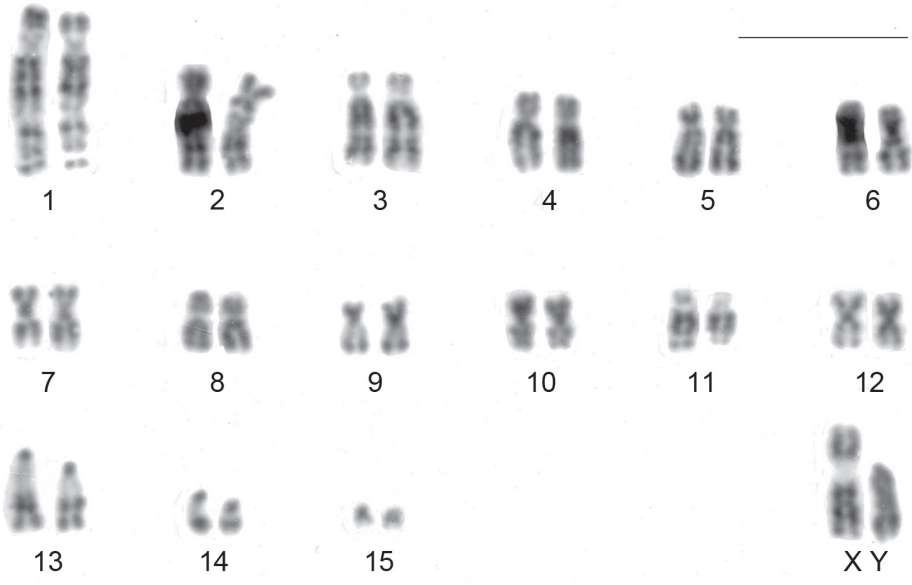

Figure 5. GTG-banded karyotypes of a male of *Clyomys laticeps* (MN 68164) from Emas National Park (2n = 32, FN = 54). Bar = 10 μm.

Discussion

The cytogenetic analysis carried out in *C. laticeps* from Emas National Park, Goiás state, Brazil, revealed a new karyotype, with 2n = 32, FN = 54. The specimens of *C. laticeps* described in the literature from São Paulo state and from Distrito Federal, respectively, shared very similar 2n = 34 karyotypes with a minor difference only in fundamental autosome number (60/62). Specimens from Itapetininga, São Paulo state, the type locality of *C. bishopi*, also showed 2n = 34 and FN = 60 (Yonenaga 1975, Souza and Yonenaga 1984). The autosomes from São Paulo state populations were composed by one large acrocentric pair (pair 1), 13 pairs of metacentrics or submetacentrics and 2 small pairs of acrocentric chromosomes (15 and 16). The specimens from Distrito Federal showed a karyotype with 2n = 34 and FN = 62 (Svartman 1989), with 14 pairs of metacentrics or submetacentrics, one pair of subtelocentric and 1 pair of acrocentric chromosomes. All spiny rats of the family Echimyidae present only one chromosome pair with a large secondary constriction and the karyotypes described for the genus *Clyomys* show this characteristic (Leal-Mesquita et al. 1992, Souza and Yonenaga 1984, Svartman 1989, present study).

The cytogenetic distinction between 2n = 34 karyotypes from São Paulo (with FN = 60) and Distrito Federal (FN = 62) could be the result of a rearrangement such as a pericentric inversion on one pair of chromosomes. The difference in diploid number between the karyotypes of *C. laticeps* with 2n = 32 and 2n = 34, in the other hand, might mostly be related to Robertsonian rearrangements (fusion/fission events).

The origin of the diploid number differences is probably the result of a series of complex rearrangements. The karyotypes with 2n = 34 (from São Paulo specimens) are composed by two small pairs of acrocentric chromosomes (15 and 16) while in specimens with 2n = 32 from ENP there are three pairs of small acrocentrics (13, 14 and 15). There is correspondence between the C-band pattern between the pairs 15 and 16 of *C. laticeps* from São Paulo state and the pairs 14 and 15 from the ones of ENP. Therefore, the karyotypes with the smallest diploid number present an additional small acrocentric pair (13). Moreover, the first pair of chromosomes in the karyotype of São Paulo specimens (2n = 34) is a large acrocentric with a small quantity of heterochromatin in the pericentromeric region, while the first pair in ENP specimens (2n = 32) is a submetacentric with a small amount of faintly stained constitutive heterochromatin in the proximal region of both arms. The X chromosome is also morphologically distinct between the karyotypes analyzed, being an average sized acrocentric in São Paulo specimens and a submetacentric in specimens from ENP. The constitutive heterochromatin in the X chromosome of 2n = 34 karyotypes is located in the pericentromeric region and in the proximal region of the long arm, while in the X chromosome of karyotypes with 2n = 32 a pericentromeric heterochromatic band is present. Thus, events such as addition/deletion of heterochromatin and pericentric inversions associated with centric fission/fusion (Robertsonian rearrangements) might have happened in the evolutionary differentiation of the karyotypes of these two populations.

Cytogenetic variability in fossorial and semifossorial rodents has been widely reported in the literature (e.g., Hafner et al. 1987, Nevo et al. 1990, Sulentich et al. 1991, Garcia et

al. 2000), often inferred as a consequence of population structuring imposed by the specialized fossorious habit (Reig et al. 1990). Spiny rats of the species C. *laticeps* have semifossorial habits (Amante 1975, Carvalho and Bueno 1975, Lacher and Alho 1989) and are the most phylopatric individuals in non-volant small mammal communities (Vieira 1997, Bezerra and Oliveira 2010), suggesting that this rodent is a habitat-specialist that needs soils with a soft structure that permits easily burrowing (Bezerra and Oliveira 2010).

The distinct diploid number shown by *Clyomys* populations (Yonenaga 1975, Souza and Yonenaga 1984, Svartman 1989, this study) could constitute evidence of speciation if one uses a biological species concept. However, additional cytogenetic data, like *in situ* hybridization (FISH) of telomeric sequences, Zoo-FISH, and the chromosomal characterization of other *Clyomys* populations are still necessary to provide us a better comprehension of the mechanisms involved in the chromosome differentiation and, consequently, in the speciation of this genus.

Acknowledgements

We thank João A. Oliveira (MN), Stella Franco (MN), and Jader Marinho-Filho (UnB) for hospitality in the collections and for cataloguing the voucher specimens, respectively; to Albert D. Ditchfield, Riccardo Castiglia, and Marta Svartman for critically reviewing early versions of the manuscript; Reginaldo Constantino provided the database for the map confection. This study was part of the project "Vertebrates of Emas National Park" supported by Fundação O Boticário de Proteção à Natureza/ MacArthur Foundation, Wildlife Conservation Society, BP Conservation/ BirdLife International/ Fauna and Flora International and Conservation International do Brasil/ Anhauser-Bush, and also part of the MSc dissertation of AMRB developed at MN, who received a fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). We are grateful to the IBAMA for the research permission and the support at ENP (proc. 02001.001282/97–01, license 056/1999) AMRB received a postdoctoral fellowship an FHGR a research grant from CNPq (CNPq Procs. 150599/2008–0 and 300664/2008–7, respectively).

References

Amante E (1975) Prejuízos causados pelo roedor *Clyomys laticeps* (Echimydae) em *Pinus* spp., em áreas reflorestadas de Sacramento, Minas Gerais. 27ª Reunião Anual da Sociedade Brasileira para o Progresso da Ciência, São Paulo: 373–374.

Avila-Pires FD, Wutke MRC (1981) Taxonomia e evolução de *Clyomys* Thomas, 1916 (Rodentia, Echimyidae). Revista Brasileira de Biologia 4: 529–534.

Bezerra AMR, Oliveira JA (2010) Taxonomic implications of cranial morphometric variation in the genus *Clyomys* Thomas, 1916 (Rodentia: Echimyidae). Journal of Mammalogy 91(1): 260–272. doi: 10.1644/08-MAMM-A-320R1.1

Bishop IR (1974) An annotated list of caviomorph rodents collected in north-eastern Mato Grosso, Brazil. Mammalia 38: 489–502.

Carvalho CT, Bueno RA (1975) Animais causando danos em plantios (Mammalia, Rodentia). Silvicultura 9: 39–46.

Garcia L, Ponsà M, Egozcue J, Garcia M (2000) Cytogenetic variation in *Ctenomys perrensi* (Rodentia, Octodontidae). Biological Journal of the Linnean Society 71: 615–624. doi: 10.1111/j.1095-8312.2000.tb01281.x

GMT (2009) Mercator Projection. Avaiable in http://gmt.soest.hawaii.edu/.

Hafner MS, Hafner JC, Patton JL, Smith MF (1987) Macrogeographic patterns of genetic differentiation in the pocket gopher *Thomomys umbrinus*. Systematic Zoology 36: 18–34. doi: 10.2307/2413305

Lacher Jr TE, Alho CJR (1989) Microhabitat use among small mammals in the Brazilian Pantanal. Journal of Mammalogy 70: 396–401. doi: 10.2307/1381526

Leal-Mesquita ERRBP, Yonenaga-Yassuda Y, Chu TH, Rocha PLB (1992) Microhabitat use among small mammals in the Brazilian Pantanal. Caryologia 45: 197–212.

Moojen JO (1952) A new *Clyomys* from Paraguay (Rodentia: Echimyidae). Journal of the Washington Academy Science 42: 102.

Nevo E, Filippucci MG, Beiles A (1990) Genetic diversity and its ecological correlates in nature: comparisons between subterranean, fossorial, and aboveground small mammals. In: Nevo E, Reig O (Eds) Evolution of subterranean mammals at the organismal and molecular levels, proceedings of the 5 th International Theriological Congress. Progress in Clinical and Biological Research, Vol. 355. Wiley-Liss, New York, 347–366.

Reig OA, Busch C, Ortells MO, Contreras JR (1990) An overview of evolution, systematics, population biology, cytogenetics, molecular biology and speciation in *Ctenomys*. In: Nevo E, Reig O (Eds) Evolution of subterranean mammals at the organismal and molecular levels, proceedings of the 5 th International Theriological Congress. Progress in Clinical and Biological Research, Vol. 355. Wiley-Liss, New York, 71–96.

Seabright M (1971) A rapid banding technique for human chromosomes. Lancet 2: 971–972. doi: 10.1016/S0140-6736(71)90287-X

Souza MJ, Yonenaga Y (1984) G- and C- band patterns and nucleolus organizer regions in somatic chromosomes of *Clyomys laticeps laticeps* (Rodentia, Echimyidae). Experientia 40: 96–97. doi: 10.1007/BF01959122

Sulentich JM, Williams LR, Cameron GM (1991) *Geomys breviceps.* Mammalian Species 383: 1–4. doi: 10.2307/3504317

Sumner AT (1972) A simple technique for demonstrating centromeric heterochromatin. Experimental Cell Research 75: 304–306. doi: 10.1016/0014-4827(72)90558-7

Svartman M (1989) Levantamento cariotípico de roedores da região do Distrito Federal. Unpublished Master thesis, Departamento de Biologia do Instituto de Biociências, Universidade de São Paulo, 160 pp.

Thomas O (1909) Notes on some South American Mammals, with descriptions of new species. Annals and Magazine of Natural History 4: 230–242. doi: 10.1080/00222930908692666

Thomas O (1916) Some notes on Echimyinae. Annals and Magazine of Natural History 18: 194–303.

Vieira MV (1997) Dynamics of a rodent assemblage in a Cerrado of Southeast Brazil. Revista Brasileira de Biologia 57: 99–107.

Woods CA, Kilpatrick CW (2005) Infraorder Hystricognathi Brandt, 1855. In: Wilson DE, Reeder DM (Eds) Mammals species of the world: a taxonomic and geographic reference. 3rd edition, Vol. 2. John Hopkins University Press, Baltimore, Maryland, 1538–1600.

Yonenaga Y (1975) Karyotypes and chromosome polymorphism in Brazilian rodents. Caryologia 28: 269–286.

CompCytogen 6(2): 163–181 (2012)
doi: 10.3897/CompCytogen.v6i2.1739
www.pensoft.net/journals/compcytogen

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Comparative cytogenetics of ten species of cichlid fishes (Teleostei, Cichlidae) from the Araguaia River system, Brazil, by conventional cytogenetic methods

G. Targino Valente[1], C. de Andrade Vitorino[2], D.C. Cabral-de-Mello[3], C. Oliveira[4], I. Lima Souza[2], C. Martins[1], P.C. Venere[2]

1 *Laboratory of Integrative Genomics, Department of Morphology, Institute of Biosciences, UNESP – Sao Paulo State University, 18618-970, Botucatu, Sao Paulo, Brazil* **2** *Laboratory of Ichthyology, Institute of Biological Sciences and Health, Federal University of Mato Grosso, Campus of Araguaia, 78698-000, Pontal do Araguaia, Mato Grosso, Brazil* **3** *Department of Biology, Institute of Biosciences, UNESP – Sao Paulo State University, 13506-900, Rio Claro, Sao Paulo, Brazil* **4** *Laboratory of Biology and Fish Genetics, Department of Morphology, Institute of Biosciences, UNESP – Sao Paulo State University, 18618-970, Botucatu, Sao Paulo, Brazil*

Corresponding author: *P.C. Venere* (pvenere@uol.com.br)

Academic editor: *A. Maryańska-Nadachowska* | Received 27 June 2011 | Accepted 30 January 2012 | Published 27 April 2012

Citation: Valente GT, Vitorino CA, Cabral-de-Mello DC, Oliveira C, Souza IL, Martins C, Venere PC (2012) Comparative cytogenetics of ten species of cichlid fishes (Teleostei, Cichlidae) from the Araguaia River system, Brazil, by conventional cytogenetic methods. Comparative Cytogenetics 6(2): 163–181. doi: 10.3897/CompCytogen.v6i2.1739

Abstract

Cichlids represent one of the most species-rich families of fishes and have attracted the attention of evolutionary biologists due to the rapid radiation occurring in some groups and the importance of some species in the world aquaculture. Cytogenetic analysis was conducted in 10 cichlid species from the Araguaia River, Amazon Basin, Brazil. The chromosome number was 2n=48 for all analyzed species except for *Laetacara araguaiae* Ottoni et Costa, 2009 (2n=44). Chromosomal polymorphism was detected only in *Geophagus proximus* (Castelnau, 1855), which exhibits an extra large submetacentric and and a dot-like chromosomes. Moreover, the C-banding revealed a general pericentromeric heterochromatic pattern and some additional blocks for some species. The heterochromatic blocks corresponding to AgNOR bearing regions were observed in all species and also corresponded to CMA_3 positive blocks, which were observed in terminal regions. Besides the general conserved chromosomal and heterochromatin patterns for South American cichlids, the presence of GC-rich heterochromatin was quite different in the species *Biotodoma cupido* (Heckel, 1840), *Geophagus proximus*, *Retroculus lapidifer* (Castelnau, 1855), *Crenicichla strigata* Günther, 1862 and *Heros efasciatus* Heckel, 1840. The results suggest that independent events of heterochromatin modification occurred during chromosome evolution in the group, regardless of the conservation of macro-chromosomal structure.

Keywords
chromosome evolution, fish chromosomes, genome, Cichlidae

Introduction

The family Cichlidae includes more than 3000 species comprising one of the most species-rich families of vertebrates (Nelson 2006). Cichlids are distributed mainly in Latin America, Africa and Madagascar, with only few species in South India and the Middle East (Genner et al. 2007). Cichlids found in the great eastern lakes of Africa have served as a model system for the study of evolution (Kornfield and Smitth 2000, Kocher 2004, Genner et al. 2007), and several species have received increasing scientific attention because of their great importance to tropical and subtropical aquaculture (Pullin 1991). This family represents a monophyletic group, and the limits and interrelationships of all four subfamilies (Etroplinae, Ptychochrominae, Cichlinae and Pseudocrenilabrinae) are well supported by molecular and morphological data (Smith et al. 2008).

The African and Neotropical cichlids, Pseudocrenilabrinae and Cichlinae, respectively, are both monophyletic and represent sister groups (Smith et al. 2008). There are 51 genera and 406 species recognized in Neotropical cichlids (Kullander 1998, 2003). The most recent proposed phylogeny of the group denotes the tribes Cichlini, Retroculini, Astronotini, Chaetobranchini, Geophagini, Cichlasomatini and Heroini as members of the Cichlinae clade (Smith et al. 2008).

The chromosome numbers of approximately 135 species of cichlids have been determined. Although more than 60% of the species present karyotypes with 2n=48, the diploid number ranges from 2n=32 to 2n=60 (Poletto et al. 2010, for review). African cichlids have a modal diploid number of 2n=44, whereas the modal number for Neotropical cichlids is 2n=48. Even though chromosomal data are known for several cichlid species, the amount of available data is not representative of the high diversity of species in the group. The chromosomal data already published for the Cichlinae clade focus mostly on the description of chromosome morphology and mapping of 45S rDNA (Poletto et al. 2010), and the heterochromatin patterns of only few species are described (Table 1). The aim of this work was to contribute in the study of the heterochromatin patterns of South American cichlids and their possible involvement in karyotypic diversification in the group.

Material and methods

Specimens and chromosome preparation

It was analyzed 10 South American cichlid species of the subfamily Cichlinae: *Cichla piquiti* Kullander et Ferreira, 2006 (4 individuals: sex not identified), *Retroculus lapidifer* (Castelnau, 1855) (6 individuals: 3 ♀ and 1 ♂, and 2 sex not identified), *Biotodoma cu-*

Table 1. Synthesis of the cichlid species analyzed with respect to the karyotypic formulae, heterochromatin distribution and CMA_3 patterns. m/sm, metacentric and submetacentric chromosomes; st/a, subtelocentric and acrocentric chromosomes; mi, microchromosomes; q, the long arm of a chromosome; p, the short arm of a chromosome; PeriC or C, pericentromeric regions; Prox, proximal portion of a chromosome; Term, Terminal portion of a chromosome; Int, interstitial portion of a chromosome; Adj, adjacent region; NOR, nucleolus organizing region; The numbers in the column "Additional blocks" indicate the number of chromosomes with the described pattern; in some cases, the ranking of these chromosomes are indicated in parentheses.

Tribes and species	Origin of animals	2n	Karyotypic formulae	Heterochromatin distribution		CMA_3^+ blocks	References
				General pattern	Additional blocks		
Cichlini							
Cichla piquiti Kullander et Ferreira, 2006	Das Mortes river, Araguaia basin, MT State, Brazil	48	48st/a	PeriC	NOR; term 2 q	NOR (term)	This work
C. kelberi Kullander et Ferreira, 2006	Araguaia river, MT State, Brazil	48	48st/a	C	NOR; int 1 q	absent	Teixeira et al. 2009
C. monoculus Spix et Agassiz, 1831	Uatumã and Solimões rivers, AM State, Brazil	48	48a	PeriC	NOR; int 1 q	absent	Brinn et al. 2004
C. temensis Humboldt, 1821	Uatumã and Jaú rivers, AM State, Brazil	48	48a	PeriC	NOR; int 1 q	absent	Brinn et al. 2004
Retroculini							
Retroculus lapidifer lapidifer (Castelnau, 1855)	Das Mortes river, Araguaia basin, MT State, Brazil	48	48st/a	PeriC	NOR; term 1 q	NOR (term) and PeriC	This work
Astronotini							
Astronotus ocellatus (Agassiz, 1831)	Tietê river, SP State, Brazil	48	16m/sm + 32st/a	C	NOR	absent	Mazzuchelli and Martins 2009
Geophagini							
Apistogramma trifasciata (Eigenmann et Kennedy, 1903)	Paraná river, Missiones, Argentina	46	16m/sm + 30st/a	PeriC	absent	absent	Roncati et al. 2007
Biotodoma cupido (Heckel, 1840)	Das Mortes river, Araguaia basin, MT State, Brazil	48	4m/sm + 44st/a	PeriC	NOR; some prox blocks	NOR (int)	This work

Tribes and species	Origin of animals	2n	Karyotypic formulae	Heterochromatin distribution		CMA_3^+ blocks	References
				General pattern	Additional blocks		
Crenicichla britskii Kullander, 1982	Jupiá river, PR State, Brazil	48	8m/sm + 40st/a	PeriC	NOR; 1 p almost completely heterochromatic (1st pair)	absent	Benzaquem et al. 2008
C. strigata Günther, 1862	Das Mortes river, Araguaia basin, MT State, Brazil	48	6m/sm + 42st/a	PeriC	NOR; some prox blocks	NOR (term) and PeriC	This work
C. prope *johanna* Heckel, 1840	Negro and Solimões rivers, AM State, Brazil	48	8m/sm + 40st/a	PeriC	NOR; term 1 q (19th pair)	absent	Benzaquem et al. 2008
C. cincta Regan, 1905	Negro and Solimões rivers, AM State, Brazil	48	8m/sm + 40st/a	PeriC	adj NOR	absent	Benzaquem et al. 2008
C. iguassuensis Haseman, 1911	Iguaçu river, PR State, Brazil	48	4m + 4sm + 14st + 26a	PeriC	Some term blocks	NOR	Mizoguchi et al. 2007
C. inpa Ploeg, 1991	Negro and Solimões rives, AM State, Brazil	48	6m/sm + 42st/a	PeriC	adj NOR	absent	Benzaquem et al. 2008
C. lepidota Heckel, 1840	São Gonçalo stream and Polegar lake, RS State, Brazil	48	4m + 4sm + 40st/a	PeriC	term 1 p and 1 q (1st pair); int 1 q (1st pair)	NOR	Perazzo et al. 2010
C. lepidota Heckel, 1840	Porto Rico region, Paraná river basin, PR State, Brazil	48	2m + 4sm + 42st/a	PeriC	int 2 (1st and 5th pairs)	absent	Martins et al. 1995
C. lugubris Heckel, 1840	Negro and Solimões rivers, AM State, Brazil	48	8m/sm + 40st/a	PeriC	NOR; int 1 q (2nd pair)	absent	Benzaquem et al. 2008
C. niederleinii (Holmberg, 1891)	Paraná river, Missiones, Argentina	48	6m/sm + 42st/a	PeriC	absent	absent	Roncati et al. 2007
C. reticulata (Heckel, 1840)	Negro and Solimões river, AM State, Brazil	48	6m/sm + 42st/a	PeriC	adj NOR; int 1 q (10th pair)	absent	Benzaquem et al. 2008
Crenicichla sp.1	Iguaçu river, PR State, Brazil	48	4m + 4sm + 14st + 26a	PeriC	Some term blocks	NOR	Mizoguchi et al. 2007
Crenicichla sp. 2	Iguaçu river, PR State, Brazil	48	4m + 4sm + 14st + 26a	PeriC	Some term blocks	NOR	Mizoguchi et al. 2007

Tribes and species	Origin of animals	2n	Karyotypic formulae	Heterochromatin distribution		CMA_3^+ blocks	References
				General pattern	Additional blocks		
Geophagus brasiliensis (Quoy et Gaimard, 1824)	Socavão and Verde rivers, PR State, Brazil	48	6sm + 42st/a	PeriC/C	absent	NOR	Vicari et al. 2006
G. brasiliensis (Quoy et Gaimard, 1824)	Jaguarriaíva river, PR State, Brazil	48	6sm + 42st/a	PeriC/C	Some int blocks	NOR	Vicari et al. 2006
G. brasiliensis (Quoy et Gaimard, 1824)	Saco da Alemoa, Gasômero, RS State, Brazil	48	4sm + 44st/a	PeriC	NOR	NOR	Pires et al. 2010
G. brasiliensis (Quoy et Gaimard, 1824)	Cambezinho and Três Bocas stream, Tibagi river basin, PR State, Brazil	48	4sm + 44st/a	C	NOR	NOR	Pires et al. 2008
G. brasiliensis (Quoy et Gaimard, 1824)	Pirapo river, Paranapanema basin, PR State, Brazil	48	8sm + 40st/a	PeriC	prox 1 p (10^{th} pair)	absent	Martins et al. 1995
G. proximus (Castelnau, 1855)	Das Mortes river, Araguaia basin, MT State, Brazil	48	4m/sm + 44st/a	PeriC	NOR; 1 p almost completely heterochromatic	NOR (int)	This work
Gymnogeophagus balzanii (Perugia, 1891)	Paraná river, Missiones State, Argentina	48	2m/sm + 46st/a	PeriC	absent	absent	Roncati et al. 2007
G. gymnogenys (Hensel, 1870)	Saco da Alemoa, Barra do Ribeiro, Gasômetro, RS State, Brazil	48	4m + 44st/a; 6m + 42st/a	PeriC	NOR	NOR	Pires et al. 2010
G. labiatus (Hensel, 1870)	Saco da Alemoa, Forqueta river, RS State, Brazil	48	4m + 4sm + 40st/a	PeriC	absent	NOR	Pires et al. 2010
Gymnogeophagus sp.	Paraná river, Missiones, Argentina	48	2m/sm + 46st/a	PeriC	absent	absent	Roncati et al. 2007
Satanoperca jurupari (Heckel, 1840)	Das Mortes river, Araguaia basin, MT State, Brazil	48	4m/sm + 44st/a	PeriC	absent	NOR	This work
S. pappaterra (Heckel, 1840)	Porto rico region, Parana river basin, PR State, Brazil	48	6sm + 42st/a	PeriC	absent	absent	Martins et al. 1995

Tribes and species	Origin of animals	2n	Karyotypic formulae	Heterochromatin distribution		CMA_3^+ blocks	References
				General pattern	Additional blocks		
Cichlasomatini							
Aequidens tetramerus Heckel, 1840	Araguaia river, MT State, Brazil	48	12m/sm + 36st/a	PeriC	absent	NOR	This work
Australoheros facetus (Jenyns, 1842)	São Gonçalo stream and Polegar lake, RS State, Brazil	48	22sm + 26st/a	PeriC/C	absent	NOR	Perazzo et al. 2010
Bujurquina vittata (Heckel, 1840)	Paraná river, Missiones, Argentina	44	22m/sm + 8st/a + 14 mi	PeriC	NOR; p arm of 5th pair completely heterochromatic	absent	Roncati et al. 2007
Cichlasoma dimerus (Heckel, 1840)	Paraná river, Missiones, Argentina	48	8m/sm + 40st/a	PeriC	absent	absent	Roncati et al. 2007
C. facetum (Jenyns, 1842)	Tarumã lake, PR State, Brazil	48	10sm + 38 st/a	PeriC/C	absent	NOR	Vicari et al. 2006
C. paranaense Kullander, 1983	Porto rico region, Parana river basin, PR State, Brazil	48	20sm + 28 st/a	PeriC	prox 2 p (2nd and 9th pairs)	absent	Martins et al. 1995
Laetacara araguaiae Ottoni et Costa, 2009	Araguaia river, MT State, Brazil	44	4m/sm + 40st/a	PeriC	absent	NOR	This work
Laetacara prope *dorsigera* (Heckel, 1840)	Paraná river, PR State, Brazil	43	5m + 38a	C	NOR	absent	Martins-Santos et al. 2005
		44	4m + 40a				
		45	3m + 42a				
		46	2m + 44a				
Heroini							
Heros efasciatus Heckel, 1840	Araguaia river, MT State, Brazil	48	8m/sm + 40st/a	PeriC	absent	NOR (term) and int 1 p	This work
Mesonauta festivus (Heckel, 1840)	Das Mortes river, Araguaia basin, MT State, Brazil	48	14m/sm + 34st/a	PeriC	NOR; term 2 q	NOR (term)	This work
Pterophyllum scalare (Schultze, 1823)	Jari river, PA State, Brazil	48	12m/sm + 36st/a	PeriC/C	1 p almost completely heterochromatic (1st pair)	NOR, some centromeres	Nascimento et al. 2006

Tribes and species	Origin of animals	2n	Karyotypic formulae	Heterochromatin distribution		CMA_3^+ blocks	References
				General pattern	Additional blocks		
Symphysodon aequifasciatus Pellegrin, 1904	Bauana lake, Tefé river, AM State, Brazil	60	8m/sm + 8st/a +4mi; 50m/sm + 6st/a +4mi	PeriC	Some prox blocks; int 1 q (1st pair)	absent	Mesquita et al. 2008
S. discus Heckel, 1840	Boi-boi stream, Negro river, AM State, Brazil	60	50m/sm + 10st/a; 54m/sm + 6st/a	PeriC	Some prox blocks	absent	Mesquita et al. 2008
S. haraldi Schultz, 1960	Manacapuru river, AM State, Brazil	60	52m/sm + 4st/a +4mi	PeriC	Some prox blocks	absent	Mesquita et al. 2008

pido (Heckel, 1840) (5 individuals: 2 ♀, and 3 ♂), *Crenicichla strigata* Günther, 1862 (12 individuals: 5 ♀, 5 ♂, and 2 sex not identified), *Geophagus proximus* (Castelnau, 1855) (9 individuals: 4 ♀, 2 ♂, and 3 sex not identified), *Satanoperca jurupari* (Heckel, 1840) (15 individuals: 7 ♀, 5 ♂, and 3 sex not identified), *Aequidens tetramerus* Heckel, 1840 (44 individuals: 21 ♀, 14 ♂, and 9 sex not identified), *Laetacara araguaiae* Ottoni et Costa, 2009 (5 individuals: 1 ♀, 1 ♂, and 3 sex not identified), *Heros efasciatus* Heckel, 1840 (5 individuals: 5 females) and *Mesonauta festivus* (Heckel, 1840) (5 individuals: 2 ♀, 1 ♂, and 2 sex not identified), which belong to the tribes Cichlini, Retroculini, Geophagini, Cichlasomatini and Heroini (Table 1). All individuals analyzed were not juveniles. Wild specimens were collected in several rivers that are part of the Araguaia River system, which is situated in the quadrant bounded by the coordinates 52°24'00"W, 15°30'S (DMS) and 52°05'00"W, 15°58'S (DMS) in the region of Barra do Garças, Mato Grosso State, Brazil. The sampling of wild animals was performed in accordance with Brazilian laws for environmental protection (wild collection permit, SISBIO/15729–1). The animals were maintained for 24 hours in an aired aquarium at a temperature ranging from 25°C to 28°C before collecting tissue samples. The fish were euthanized with a lethal dose of benzocaine followed by spinal section (Protocol 01204 – Committee of Ethical in Animal Experimentation – UNESP – São Paulo State University, Brazil) before removal of the kidneys for chromosome preparation.

Mitotic chromosome preparations were obtained from kidney cells according to Bertollo et al. (1978). The animals were treated with a 0.0125% solution of colchicine, which was injected at a volume of 1mL/100g of body weight at approximately 45–60 min before euthanasia and chromosome preparation. The kidney tissues were dissected, and the cells were dissociated in a hypotonic solution of KCl 0.075 M with a syringe and remained in the solution for 25 min. The cells were fixed in 3:1 methanol-acetic acid solution and used to prepare slides that were stained with 5% Giemsa solution in phosphate buffer at pH 7 for 10 min.

Differential chromosome staining and banding

The chromosome structure was analyzed through silver nitrate staining, Chromomycin A_3 (CMA_3) staining and C-banding.

To detect nucleolus organizer regions (NORs), the silver staining of the chromosomes was performed according to Howell and Black (1980). The slides were stained with 2% Giemsa for 10 to 15 sec, washed in water and air-dried for later microscopic analysis.

The constitutive heterochromatin was detected using saline solution according to Sumner (1972) with the following adjustments. The slides were initially treated with 0.2 N HCl at 42°C for 5 min, washed in water and rapidly air-dried. The slides were then immersed in 5% barium hydroxide solution that was freshly prepared and filtered at 42°C for 30 sec to 1 min. The treatment was stopped by submerging the slides in 0.2 N HCl and washing them extensively in running water. The slides were immersed in saline solution (2xSSC) at 60°C for 45 min. After completing this step, the slides were

air-dried and stained with 5% Giemsa in phosphate buffer at pH 6.8–7.0. Alternatively, the slides were stained with propidium iodide, which also provides excellent results.

The CMA_3 staining was conducted according to the method by Schweizer (1976) with minor adjustments. This was done by immersing the slides in 0.2% $MgCL_2$ in McIlvaine buffer, pH 7.0, at 25°C for 10 min. The slides were withdrawn, agitated briefly to remove excess solution, mounted with 150 µL of 0.05% CMA_3 in McIlvane buffer under coverslips and then stored in dark boxes for 15 min at 25°C. After this step, the coverslips were removed by washing the slides in McIlvaine buffer. The slides were incubated in a solution of freshly prepared of 0.012% Methyl-green/Hepes for 15 min, rinsed in a solution of Hepes 0.13%/NaCl 0.87% and air-dried. Finally, the slides were mounted with 45–90 µl of glycerol 97.4%/propyl gallate 2.5%. Prior to analysis, the slides were stored in the dark at 4°C for at least one week before analysis by fluorescence microscopy.

Chromosome analysis

The chromosome spreads were analyzed using an Olympus BX 61 microscope, and the images were captured with the Olympus DP71 digital camera with the software Image-Pro MC 6.0. There were analyzed 30 metaphase spreads for all cytogenetic procedures performed for each animal sample. Karyotypes were arranged in the order of decreasing chromosome size, and the chromosomes were classified as either meta/submetacentrics (m/sm) or subtelo/acrocentrics (st/a).

Results

All of the species analyzed have 2n=48 except *L. araguaiae*, which showed a diploid number of 2n=44 and the karyotype formula of 4m/sm + 40st/a. Moreover, chromosomal polymorphism was found in *G. proximus*, which presented two karyotype formulae, 4m/sm + 44st/a or 5m/sm + 42st/a + 1 dot-like chromosome (Fig. 1, Table 1).

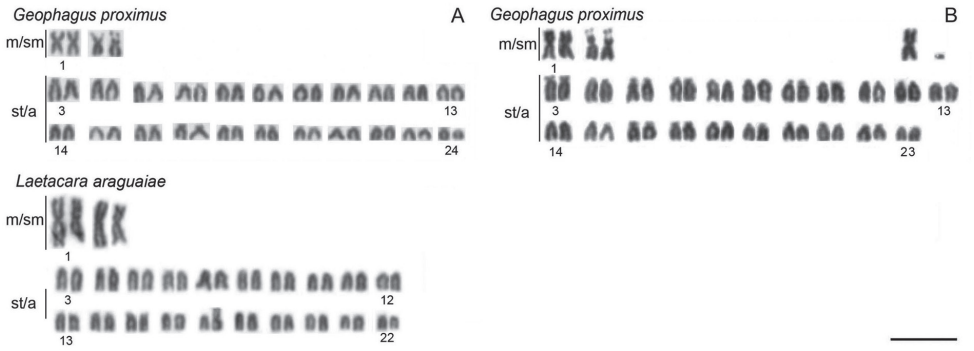

Figure 1. Representative karyotypes of *Geophagus proximus* and *Laetacara araguaiae* species. For *G. proximus*, two karyotypes are presented, a normal (**A**) and a polymorphic karyotype, showing in the upper right corner one extra large metacentric and one dot-like chromosome (**B**). Bar = 10 µm.

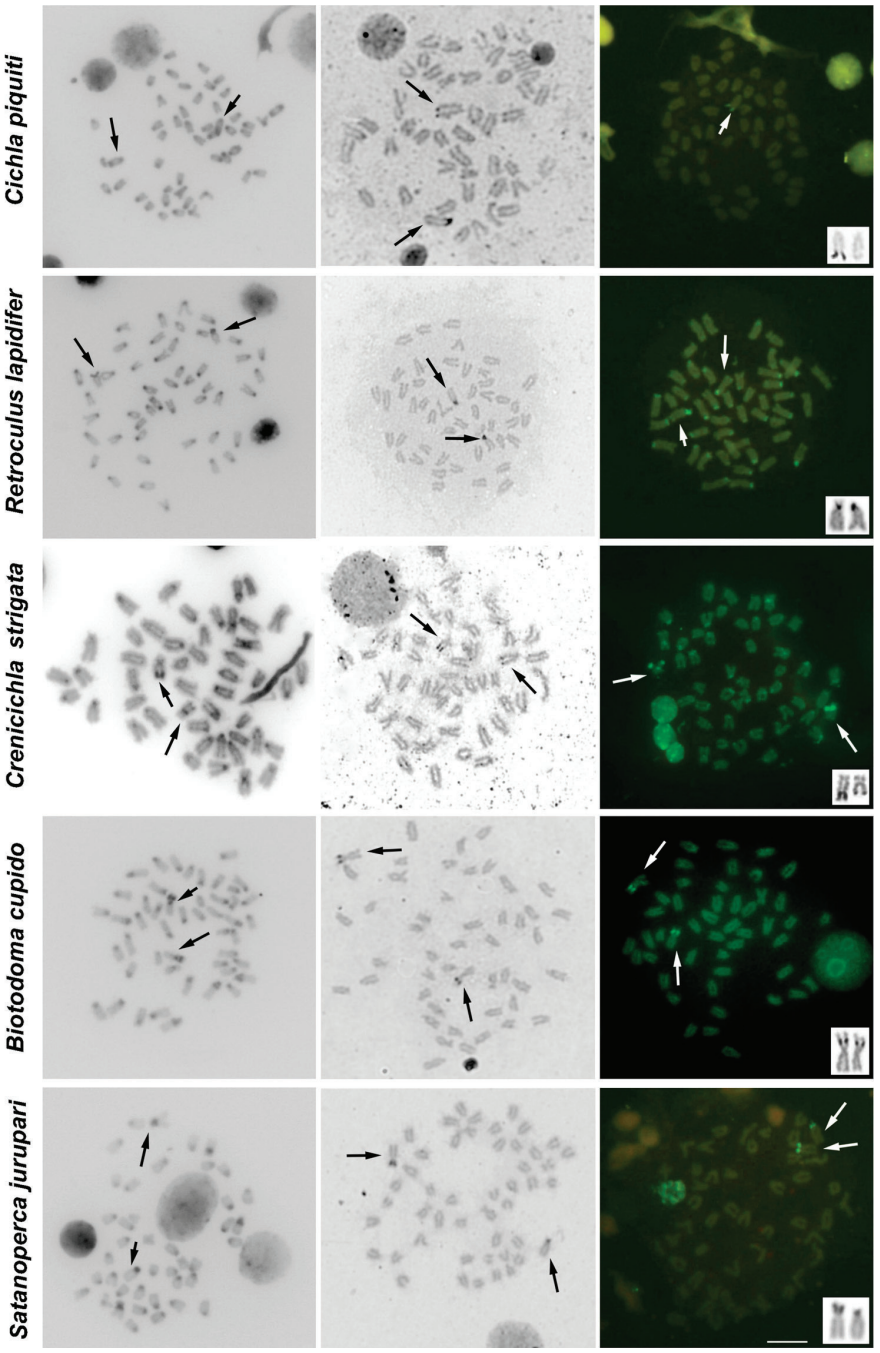

Figure 2. Metaphases of several cichlid species under different chromosome treatments. The species are indicated on the left. The first, second and third columns show C-banded, AgNOR- and CMA_3- stained metaphases, respectively. The third column shows chromosomes bearing AgNORs in the box. The arrows indicate the NOR-bearing chromosomes. Bar = 10 μm.

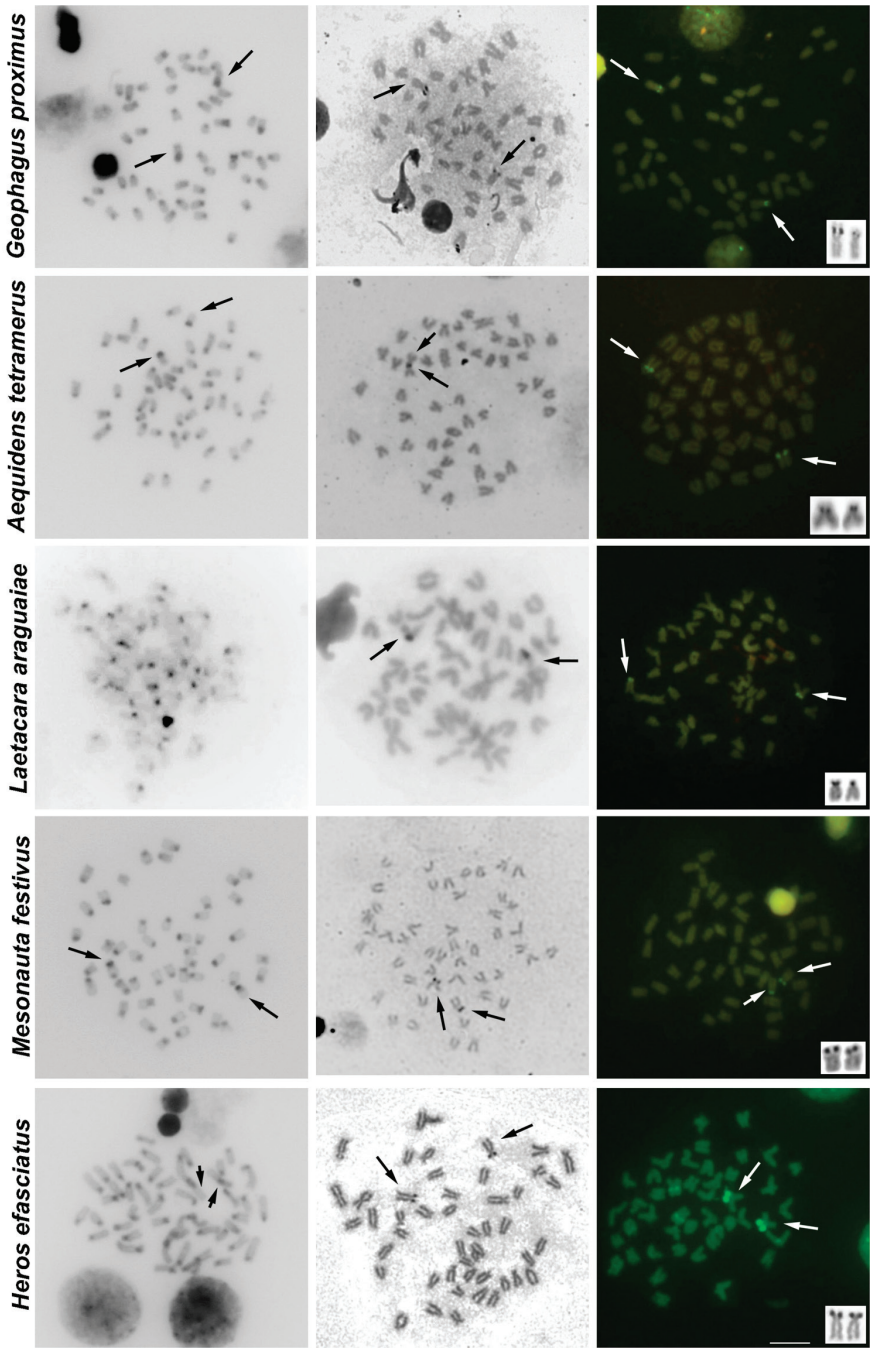

Figure 3. Metaphases of several cichlid species under different chromosome treatments. The species are indicated on the left. The first, second and third columns show C-banded, AgNOR- and CMA_3- stained metaphases, respectively. The third column shows chromosomes bearing AgNORs in the box. The arrows indicate the NOR-bearing chromosomes. For some metaphases (without arrows) it was not possible to identify the NOR-carrying chromosomes. Bar = 10 μm.

The results of C-banding revealed the heterochromatin generally restricted to pericentromeric regions. Additional blocks of heterochromatin were noticed in *C. piquiti*, *R. lapidifer*, *B. cupido*, *C. strigata*, *G. proximus* and *M. festivus* (Figs 2, 3, Table 1).

Characteristic heterochromatic blocks corresponding to AgNOR bearing regions (two blocks, one in each homologue) were observed in all species, and these blocks were consistent with CMA_3 positive (CMA_3^+) blocks (Figs 2, 3, Table 1). These AgNOR/CMA_3^+ blocks were present in terminal regions; however, positional variation was observed in *B. cupido* (Fig. 2) and *G. proximus* (Fig. 3), which the blocks are present in interstitial regions. Moreover, *R. lapidifer* (Fig. 2) and *C. strigata* (Fig. 2) displayed CMA_3^+ blocks in pericentromeric regions of almost all chromosomes, and *H. efasciatus* (Fig. 3) displayed a positive interstitial signal in one chromosome pair. Size variation was also observed in AgNOR/CMA_3^+ blocks between homologous chromosomes in *C. piquiti* (Fig. 2), *C. strigata* (Fig. 2) and *S. jurupari* (Fig. 2). Other chromosomal areas were CMA_3 neutral in all of the species analyzed (Figs 2, 3).

Discussion

The diploid number reported for the species in this study, in general are in agreement with the conserved 2n=48 chromosomes commonly found in South American cichlids and in contrast with the presence of 2n=44 chromosomes in African cichlids. All species, except *Laetacara araguaiae*, had their diploid number already described (Poletto et al. 2010). Moreover, some cichlid species display the occurrence of specific chromosomal rearrangements, such as pericentric inversions, translocations and fission or fusion rearrangements, that occurred during their evolutionary history and deviate their karyotypic formulae from common pattern observed for cichlids (revised by Feldberg et al. 2003, Mesquita et al. 2008, Poletto et al. 2010).

Chromosomal variability was observed in derived lineages, such as the Geophagini and the Cichlasomatini tribes (Feldberg et al. 2003, Poletto et al. 2010). Thus, the diploid number variation observed here in *L. araguaiae* and the polymorphism observed in *Geophagus proximus*, which belong to Cichlasomatini and Geophagini tribes, respectively, could reflect the higher chromosomal variation found in these tribes. In fact, another species of *Laetacara* Kullander, 1986, *Laetacara prope dorsigera* (Heckel, 1840), generally displayed 2n=44 chromosomes with an intraspecific variation in the diploid number that ranges from 2n=43 to 2n=46, which are thought to have originated from centric chromosomal fusions (Martins-Santos et al. 2005). In *G. proximus*, the polymorphism is a consequence of a Robertsonian translocation between two st/a chromosomes that results in a large metacentric chromosome and a dot-like element. However, it is inconclusive if this rearrangement occurred between homologous or non-homologous chromosomes due to the great similarities among the st/a chromosomes in *G. proximus*.

Chromosomal rearrangements such the ones reported here could lead to the karyotypic diversification of the species. In fact, chromosomal rearrangements have contrib-

uted to karyotypic evolution in a range of fishes, including the cichlids *Symphysodon* (Heckel, 1840) (Mesquita et al. 2008, Gross et al. 2009a), salmonids (Allendorf and Thorgaard 1984) and *Gobius fallax* Sarato, 1889 (Thode et al. 1988), among others. Moreover chromosomal rearrangements may result in intraspecific variation as broadly reported in some fish species: in the origin of neo-Y sex chromosomes (Uyeno and Miller 1971, 1972, Bertollo et al. 1983, 1997, Almeida-Toledo et al. 1984, 1988, 2000, Silva and Margarido 2005), in karyotypic diversification of species complex of *Gymnotus carapo* Linnaeus, 1758 (Milhomem et al. 2008), in *Hoplias malabaricus* (Bloch, 1794) (Bertollo et al. 1997) and in *Erythrinus erythrinus* (Bloch et Schneider, 1801) (Bertollo et al. 2004).

Although the cichlid cytogenetics suggests that the ancestral karyotype (2n=48 st/a) could have undergone major changes (pericentric inversions, fusions, fissions and chromosomal translocations) in the macro-structure of the South American species (Feldberg et al. 2003, Poletto et al. 2010), these studies show that this family of fish has a relatively conserved diploid number. Despite of the absence of conclusive data about chromosomal rearrangements rate that occurs in cichlids, it could be suggested that this group has an intermediate level of chromosomal stability compared to birds and mammals, which are more stable and variable, respectively. It is predicted that chromosomal rearrangements can be one of the evolutionary forces that affect the reproductive isolation and speciation processes (Noor et al. 2001, Rieseberg 2001), which create higher levels of species diversity. However, birds and cichlids display greater species richness than what is observed in mammals; this is contrary to the more stable karyotypes of birds and cichlids. Therefore chromosomal rearrangements may be not the most decisive evolutionary process in the cichlids speciation.

C-banding analyses in this study revealed that the conserved pattern of heterochromatin distribution was mostly restricted to the pericentromeric regions of cichlid chromosomes, which has been commonly reported in American and African representatives but with variations in both groups (Kornfield et al. 1979, Majumdar and McAndrew 1986, Feldberg et al. 2003, and others reported in Table 1). Additional heterochromatic blocks were present in almost all species analyzed, and exceptions were observed in *Satanoperca jurupari* (Geophagini), *Aequidens tetramerus* (Cichlasomatini), *Laetacara araguaiae* (Cichlasomatini) and *Heros efasciatus* (Heroini). For all species, one of these blocks was related to AgNOR regions, which seems to be a common feature in cichlids and other fish (Pendás et al. 1993, Artoni et al. 2008, Souza et al. 2008, Venere et al. 2008, among others cited in Table 1).

Concerning the singular heterochromatic blocks reported here, *Cichla piquiti*, *Crenicichla strigata* and *Geophagus proximus* show variability in the positions, extensions and number of these blocks compared to the other species in each genus. Moreover, the divergent patterns are observed in *Crenicichla* Heckel, 1840 and *Geophagus* Heckel, 1840. This variability can be also observed in the *Laetacara* genus; in this case, *L. araguaiae* does not have any additional heterochromatic blocks, whereas *L. prope dorsigera* has heterochromatic NORs as additional blocks (Martins-Santos et al. 2005). Moreover, both of the *Satanoperca* Günther, 1862 species analyzed do not have any additional heterochromatic blocks. Comparisons within every genera *Retroculus* Eigen-

mann et Bray, 1894, *Biotodoma* Eigenmann et Kennedy, 1903, *Aequidens* Eigenmann et Bray, 1894, *Heros* Heckel, 1840 and *Mesonauta* Günther, 1862 are not possible because this is the first C-banding analysis for these genera. Heterochromatic variations can be observed when comparing the additional heterochromatic blocks patterns within the tribes Geophagini, Cichlasomatini and Heroini tribes. This analysis could support the current idea that these groups display some of the highest chromosomal variability for the Cichlidae family (Feldberg et al. 2003, Poletto et al. 2010). However, they are the most studied group concerning heterochromatin analysis, and it is not clear if this variability reflects higher chromosomal variability or a sampling effort (for all comparisons see Table 1).

The fluorochrome CMA_3 showed the presence of GC-rich blocks coinciding with AgNOR sites in all species, which is a common trait in cichlids. The variation in the extension of these blocks also matches the size variation in the AgNOR sites in some species. Additional CMA_3^+ blocks are uncommon patterns in cichlids species, but they have been reported here for some species. In addition, this trait has only been previously reported in the Heroini species *Pterophyllum scalare* (Schultze, 1823) (Nascimento et al. 2006). The general pattern of base-pair richness of the heterochromatin indicates some level of compartmentalization of this genomic content at both intragenomic and intraspecific levels. Finally, based on the present and previously reported data, it seems possible that there is a relationship between CMA_3^+ blocks and AgNOR regions in cichlid species. Furthermore, the variation may be an exception in this group of fish and could suggest that the sequences presented in these regions may possess some dynamism in cichlids genomes.

With respect to AgNOR, length variation between homologous chromosomes could be explained by the duplication or deletion of 45S rDNA repeat units. All AgNOR sites in the species analyzed here are heterochromatic as aforementioned. The length variation detected and extensively observed in other organisms may be caused by the presence of repetitive sequences, errors during the replication process, unequal crossing-over (Ashley and Ward 1993, Pendás et al. 1993, Boron et al. 2006, Gross et al. 2010) and likely non-reciprocal translocation between these regions (revised in Wasko and Galetti 2000).

Conclusion

The heterochromatin, CMA_3^+ blocks and AgNOR regions are classic cases of enriched repetitive elements regions, such as satellite DNA, transposable elements, and rDNA. Among cichlids, it has been reported that the pericentromeric regions, which are commonly evidenced by C-banding, are repositories for a great amount of repetitive elements, such as transposable elements (Gross et al. 2009b, Mazzuchelli and Martins 2009, Teixeira et al. 2009, Valente et al. 2011). Repetitive sequences are highly dynamic in genome evolution; for example, pericentromeric DNA are rapidly evolving

regions in eukaryotic genomes (Haaf and Willard 1997, Csink and Henikoff 1998, Murphy and Karpen 1998) due to the accumulation of repetitive sequences by recombination suppression (Topp and Dawe 2006, Grewal and Jia 2007). In fact, the results reported here and in previous work do not show any phylogenetic relationships in terms of constitutive heterochromatin, NOR and CMA_3^+ blocks; therefore, the actual number, position and length variation of sites are not related to any homology. All of the variation observed in these regions may be related to the intrinsic dynamism of repeated sequences and independent heterochromatin modifications that do not follow the diversification of taxa.

Acknowledgements

The study was supported by Fundação de Amparo à Pesquisa do Estado de Mato Grosso (FAPEMAT), Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq).

References

Allendorf FW, Thorgaard GH (1984) Tetraploidy and the evolution of Salmonid fishes. In: Turner BJ (Eds) Evolution of Fishes. Plenum, New York, 1–53.

Almeida-Toledo LF, Foresti F, Daniel MFVZ, Toledo-Filho SA (2000) Sex chromosome evolution in fish: the formation of the neo-Y chromosome in *Eigenmannia* (Gymnotiformes). Chromosoma 109(3): 197-200. doi: 10.1007/s004120050428

Almeida-Toledo LF, Foresti F, Toledo-Filho SA (1984) Complex sex chromosome system in *Eigenmannia* sp. (Pisces, Gymnotiformes). Genetica 64(3): 165–169. doi: 10.1007/BF00115340

Almeida-Toledo LF, Viegas-Pequignot E, Foresti F, Toledo-Filho SA, Dutrillaux B (1988) BrdU replication patterns demonstrating chromosome homeologies in two fish species, genus *Eigenmannia*. Cytogenetic and Cell Genetic 48(2): 117–120. doi: 10.1159/000132603

Artoni RF, Gross MC, Schneider CH, Vicari MR, Almeida MC, Matoso DA (2008) Epifluorescence and light microscopy evidencing structural and functional polymorphism of ribosomal DNA in fish (Teleostei: *Astyanax fasciatus*). Micron 39(8): 1156–1159. doi: 10.1016/j.micron.2008.05.011

Ashley T, Ward DC (1993) A "hot spot" of recombination coincides with interstitial telomeric sequence in the Armenian hamster. Cytogenetic and Cell Genetic 62(2–3): 169–171. doi: 10.1159/000133464

Bertollo LAC, Fontes MS, Fenocchio AS, Cano J (1997) The X1X2Y sex chromosome system in the fish *Hoplias malabaricus*. I. G-, C- and chromosome replication banding. Chromosome Research 5(7): 493–499. doi: 10.1023/A:1018477232354

Bertollo LAC, Oliveira C, Molina WF, Margarido VP, Fontes MS, Pastori MC, Falcão JN, Fenocchio AS (2004) Chromosome evolution in the erythrinid fish, *Erythrinus erythrinus* (Teleostei: Characiformes). Heredity 93: 228–233. doi: 10.1038/sj.hdy.6800511

Bertollo LAC, Takahashi CS, Moreira-Filho O (1978) Cytotaxonomic consideration on *Hoplias lacerdae* (Pisces, Erythrinidae). Brazilian Journal of Genetics 1(2): 103–120. [http://web2.sbg.org.br/GMB/edicoesAnteriores/Indice/indiceVol01N2.html]

Bertollo LAC, Takahashi CS, Moreira-Filho O (1983) Multiple sex chromosomes in the genus *Hoplias* (Pisces, Erythrinidae). Cytologia 48(1): 1–12. [http://www.journalarchive.jst.go-.jp/english/jnltoc_en.php?cdjournal=cytologia1929&cdvol=48&noissue=1]

Benzaquem DC, Feldberg E, Porto JIR, Gross MC, Zuanon JAS (2008) Cytotaxonomy and karyoevolution of the genus *Crenicichla* (perciformes, cichlidae). Genetics and Molecular Biology 31(1): 250–255. doi: 10.1590/S1415-47572008000200016

Boron A, Ozouf-Costaz C, Coutanceau JP, Woroniecka K (2006) Gene mapping of 28S and 5S rDNA sites in the spined loach *Cobitis taenia* (Pisces, Cobitidae) from a diploid population and a diploid-tetraploid population. Genetica 128(1–3): 71–79. doi: 10.1007/s10709-005-5536-8

Brinn MNA, Porto JIR, Feldberg E (2004) Karyological evidence for interspecific hybridization between *Cichla monoculus* and *C. temensis* (perciformes, cichlidae) in the amazon. Hereditas 141(3): 252–257. doi: 10.1111/j.1601-5223.2004.01830.x

Csink AK, Henikoff S (1998) Something from nothing: the evolution and utility of satellite repeats. Trends in Genetics 14(5): 200–204. doi: 10.1016/S0168-9525(98)01444-9

Feldberg E, Porto JIR, Bertollo LAC (2003) Chromosomal changes and adaptation of cichlid fishes during evolution. In: Val AL, Kapoor BG (Eds). Fish adaptations. Science Publishers INC, New Delhi, New York, 285–308.

Genner MJ, Seehausen O, Lunt DH, Joyce DA, Shaw PW, Carvalho GR, Turner GF (2007) Age of cichlids: new dates for ancient lake fish radiations. Molecular Biology and Evolution 24(5): 1269–1282. doi: 10.1093/molbev/msm050

Grewal SIS, Jia S (2007) Heterochromatin revisited. Nature Reviews Genetics 8: 35–46. doi: 10.1038/nrg2008

Gross MC, Feldberg F, Cella DM, Schneider MC, Schneider CH, Porto JIR, Martins C (2009a) Intriguing evidence of translocations in Discus fish (*Symphysodon*, Cichlidae) and report of the largest meiotic chromosomal chain observed in vertebrates. Heredity 102: 435–441. doi: 10.1038/hdy.2009.3

Gross MC, Schneider CH, Valente GT, Porto JIR, Martins C, Feldberg E (2009b) Comparative Cytogenetic Analysis of the Genus *Symphysodon* (Discus Fishes, Cichlidae): Chromosomal Characteristics of Retrotransposons and Minor Ribosomal DNA. Cytogenetic and Genome Research 127(1): 43–53. doi: 10.1159/000279443

Gross MC, Schneider CH, Valente GT, Martins C, Feldberg E (2010) Variability inter and intraspecific of 18S rDNA locus among *Symphysodon* fishes: chromosomal rearrangements. Journal of Fish Biology 76(5): 1117–1127. 2

Haaf T, Willard HF (1997) Chromosome-specific alpha-satellite DNA from the centromere of chimpanzee chromosome 4. Chromosoma 106(4): 226–232. doi: 10.1007/s004120050243

Howell WM, Black DA (1980) Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Cellular and Molecular Life Sciences 36(8): 1014–1015. doi: 10.1007/BF01953855

Kocher TD (2004) Adaptive evolution and explosive speciation: the cichlid fish model. Nature Reviews Genetics 5(4): 288–298. doi: 10.1038/nrg1316

Kornfield IL, Ritte U, Richler C, Wahrman J (1979) Biochemical and cytological differentiation among cichlid fishes of the Sea of Galilee. Evolution 33(1): 1–14. [http://www.jstor.org/pss/2407360]

Kornfield I, Smith PF (2000) African cichlid fishes: Model system for evolutionary biology. Annual Review of Ecology and Systematics 31: 163–196. doi: 10.1146/annurev.ecolsys.31.1.163

Kullander SO (1998) A phylogeny and classification of the South American Cichlidae (Teleostei: Perciformes). In: Malabarba LR, Reis RE, Vari RP, Lucena ZM, Lucena CAS (Eds) Phylogeny and classification of Neotropical fishes. EDIPUCRS, Porto Alegre, 461–498.

Kullander SO (2003) Family Cichlidae. In: Reis RE, Kullander SO, Ferraris CJ (Eds) Check list of the freshwater fishes of South and Central America. EDIPUCRS, Porto Alegre, 605–654.

Majumdar KC, McAndrew BJ (1986) Relative DNA content of somatic nuclei and chromosomal studies in three genera, *Tilapia*, *Sarotherodon*, and *Oreochromis* of the tribe Tilapiini (Pisces, Cichlidae). Genetica 68(3): 175–188. doi: 10.1007/BF02424441

Martins IC, Portella-Castro ALB, Julio Jr. HF (1995) Chromosome analysis of 5 species of the Cichlidae family (Pisces, Perciformes) from the Paraná River. Cytologia 60(3): 223–231. [http://www.journalarchive.jst.go.jp/english/jnltoc_en.php?cdjournal=cytologia1929&cdvol=60&noissue=3]

Martins-Santos IC, Portela-Castro ALB, Julio Jr. HF (2005) Chromosomal polymorphism and speciation in *Laetacara* cf. *dorsigera* (Teleostei, Perciformes, Cichlidae) from the river Paraná PR Brazil. Caryologia 58(2): 95–101. [http://www1.unifi.it/caryologia/past_volumes/58_2/58_2abst.html]

Mazzuchelli J, Martins C (2009) Genomic organization of repetitive DNAs in the cichlid fish *Astronotus ocellatus*. Genetica 136(3): 461–469. doi: 10.1007/s10709-008-9346-7

Mesquita DR, Porto JIR, Feldberg E (2008) Chromosomal variability in the wild ornamental fish *Symphysodon* spp. (Perciformes, Cichlidae) from Amazon. Neotropical Ichthyology 6(2): 181–190. doi: 10.1590/S1679-62252008000200005

Milhomem SSR, Pieczarka JC, Crampton WGR, Silva DS, Souza ACP, Carvalho Jr. JR, Nagamachi CY (2008) Chromosomal evidence for a putative cryptic species in the *Gymnotus carapo* species-complex (Gymnotiformes, Gymnotidae). BMC Genetics 9: 1–75. doi: 10.1186/1471-2156-9-75

Mizoguchi SMHK, Portela-Castro ALB, Martins-Santos IC (2007) Cytogenetic characterization of *Crenicichla* (Pisces, Perciformes) of the Iguaçu River. Genetics and Molecular Research 6(3): 650–656. [http://www.geneticsmr.com/issue/6/all]

Murphy TD, Karpen GH (1998) Centromeres take flight: alpha satellite and the quest for the human centromere. Cell 93(3): 317–320. doi: 10.1016/S0092-8674(00)81158-7

Nascimento A, Souza ACP, Feldberg E, Carvalho Jr. JR, Barros RMS, Pieczarka JC, Nagamachi CY (2006) Cytogenetic analysis on *Pterophyllum scalare* (perciformes, cichlidae)

from Jari river, Pará state. Caryologia 59(3): 138–143. [http://www1.unifi.it/caryologia/past_volumes/59_2/59_2abst.html]

Nelson JS (2006) Fishes of the world. John Wiley and Sons, New York, 601 pp.

Noor MA, Grams KL, Bertucci LA, Reiland J (2001) Chromosomal inversions and the reproductive isolation of species. Proceedings of the National Academy of Sciences of the United States of America 98(21): 12084–12088. doi: 10.1073/pnas.221274498

Pendás AM, Morán P, Garcia-Vasquez E (1993) Ribosomal RNA genes are interspersed throughout a heterochromatic chromosome arm in Atlantic salmon. Cytogenetic and Cell Genetics 63(2): 128–130. doi: 10.1159/000133517

Perazzo G, Noleto RB, Vicari MR, Machado PC, Gava A, Cestari MM (2010) Chromosomal studies in *Crenicichla lepidota* and *Australoheros facetus* (Cichlidae, Perciformes) from extreme Southern Brazil. Reviews in Fish Biology and Fisheries 21(3): 509–515. doi: 10.1007/s11160-010-9170-x

Pires LB, Giuliano CL, Dias AL (2008) Karyotype similarities among two populations of *Geophagus brasiliensis* (Perciformes, Cichlidae) from the Tibagi River basin/PR/Brazil. Caryologia 61(2): 135–138. [http://www1.unifi.it/caryologia/past_volumes/61_2/61_2abst.html]

Pires LB, Giuliano CL, Dias AL (2010) Cytogenetic characterization of *Geophagus brasiliensis* and two species of *Gymnogeophagus* (Cichlidae:Geophaginae) from Guaíba Lake, RS, Brazil. Folia biologica 58(1–2): 29–34. doi: 10.3409/fb58_1-2.29-34

Poletto AB, Ferreira IA, Cabral-de-Mello DC, Nakajima RT, Mazzuchelli J, Ribeiro HB, Venere PC, Nirchio M, Kocher TD, Martins C (2010) Chromosome differentiation patterns during cichlid fish evolution. BMC Genetics 11: 1–50. doi: 10.1186/1471-2156-11-50

Pullin RSV (1991) Cichlids in aquaculture. In: Keenleyside MHA (Eds) Cichlid fishes: behaviour, ecology and evolution. Chapman & Hall, London, 280–309.

Rieseberg LH (2001) Chromosomal rearrangements and speciation. Trends in Ecology & Evolution 16(7): 351–358. doi: 10.1016/S0169-5347(01)02187-5

Roncati HA, Pastori MC, Fenocchio AS (2007) Cytogenetic Studies and Evolutive Considerations on Fishes of the Family Cichlidae (Perciformes) from Parana River (Argentina). Cytologia 72(4): 379–384. doi: 10.1508/cytologia.72.379

Schweizer D (1976) Reverse fluorescent chromosome banding with chromomycin and DAPI. Chromosoma 58(4): 307–324. doi: 10.1007/BF00292840

Silva EB, Margarido VP (2005) An $X_1X_1X_2X_2/X_1X_2Y$ multiple sex chromosome system in a new species of the genus *Gymnotus* (Pisces, Gymnotiformes). Environmental Biology of Fishes 73(3): 293–297. doi: 10.1007/s10641-005-2144-5

Smith WL, Chakrabarty P, Sparks JS (2008) Phylogeny, taxonomy, and evolution of Neotropical cichlids (Teleostei: Cichlidae: Cichlinae). Cladistics 24(5): 625–641. doi: 10.1111/j.1096-0031.2008.00210.x

Souza IL, Santos-Silva LK, Venere PC, Moreira-Filho O (2008) Molecular cytogenetics of *Salminus* fish (Characiformes) based on 5S and 18S rRNA genes hybridization, fluorochrome staining and C-banding. Micron 39(7): 1036–1041. doi: 10.1016/j.micron.2007.09.007

Sumner AT (1972) A simple technique for demonstrating centromeric heterochromatin. Experimental Cell Research 75(1): 304–306. doi: 10.1016/0014-4827(72)90558-7

Teixeira WG, Ferreira IA, Cabral-de-Mello DC, Mazzuchelli J, Valente GT, Pinhal D, Poletto AB, Venere PC, Martins C (2009) Organization of repeated DNA elements in the genome of the cichlid fish *Cichla kelberi* and its contributions to the knowledge of fish genomes. Cytogenetic and Genome Research 125(3): 224–234. doi: 10.1159/000230006

Thode G, Martinez G, Ruiz JL, Lopéz JR (1988) A complex chromosomal polymorphism in *Gobius fallax* (Gobiidae, Perciformes). Genetica 76(1): 65–71. doi: 10.1007/BF00126011

Topp CN, Dawe RK (2006) Reinterpreting pericentromeric heterochromatin. Current Opinion in Plant Biology 9(6): 647–653. doi: 10.1016/j.pbi.2006.09.008

Uyeno T, Miller RR (1971) Multiple sex chromosomes in a Mexican Cyprinodontidae fish. Nature 231: 452–453. doi: 10.1038/231452a0

Uyeno T, Miller RR (1972) Second discovery of multiple sex chromosome among fishes. Experientia 28(2): 223–225. doi: 10.1007/BF01935773

Valente GT, Mazzuchelli J, Ferreira IA, Poletto AB, Fantinatti BEA, Martins C (2011) Cytogenetic Mapping of the Retroelements Rex1, Rex3 and Rex6 among Cichlid Fish: New Insights on the Chromosomal Distribution of Transposable Elements. Cytogenetic and Genome Research 133(1): 34–42. doi: 10.1159/000322888

Venere PC, Souza IL, Silva LKS, Dos Anjos MB, De Oliveira RR, Galetti Jr. PM (2008) Recent chromosome diversification in the evolutionary radiation of the freshwater fish family Curimatidae (Characiformes). Journal of Fish Biology 72(8): 1976–1989. doi: 10.1111/j.1095-8649.2008.01814.x

Vicari MR, Artoni RF, Moreira-Filho O, Bertollo LAC (2006) Basic and molecular cytogenetics in freshwater Cichlidae (Osteichthyes, Perciformes). Karyotypic conservadorism and divergence. Caryologia 59(3): 260–266. [http://www1.unifi.it/caryologia/past_volumes/59_3/59_3abst.html]

Wasko AP, Galetti Jr. PM (2000) Mapping 18S ribosomal genes in fish of the genus *Brycon* (Characidae) by fluorescence in situ hybridization (FISH). Genetics and Molecular Biology 23(1): 135–138. doi: 10.1590/S1415-47572000000100025

CompCytogen 6(2): 183–197 (2012)
doi: 10.3897/CompCytogen.v6i2.2950
www.pensoft.net/journals/compcytogen

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Karyotype analysis of four jewel-beetle species (Coleoptera, Buprestidae) detected by standard staining, C-banding, AgNOR-banding and CMA3/DAPI staining

Gayane Karagyan[1], Dorota Lachowska[2], Mark Kalashian[1]

1 *Institute of Zoology of Scientific Center of Zoology and Hydroecology, National Academy of Sciences of Armenia, P. Sevak 7, Yerevan 0014, Armenia* **2** *Department of Entomology, Institute of Zoology Jagiellonian University, Ingardena 6, 30-060 Krakow, Poland*

Corresponding author: *Gayane Karagyan* (gaykaragyan@yahoo.com)

Academic editor: *Robert Angus* | Received 15 February 2012 | Accepted 17 April 2012 | Published 27 April 2012

Citation: Karagyan G, Lachowska D, Kalashian M (2012) Karyotype analysis of four jewel-beetle species (Coleoptera, Buprestidae) detected by standard staining, C-banding, AgNOR-banding and CMA3/DAPI staining. Comparative Cytogenetics 6(2): 183–197. doi: 10.3897/CompCytogen.v6i2.2950

Abstract

The male karyotypes of *Acmaeodera pilosellae persica* Mannerheim, 1837 with 2n=20 (18+neoXY), *Sphenoptera scovitzii* Faldermann, 1835 (2n=38–46), *Dicerca aenea validiuscula* Semenov, 1895 – 2n=20 (18+Xy_p) and *Sphaerobothris aghababiani* Volkovitsh et Kalashian, 1998 – 2n=16 (14+Xy_p) were studied using conventional staining and different chromosome banding techniques: C-banding, AgNOR-banding, as well as fluorochrome Chromomycin A_3 (CMA_3) and DAPI. It is shown that C-positive segments are weakly visible in all four species which indicates a small amount of constitutive heterochromatin (CH). There were no signals after DAPI staining and some positive signals were discovered using CMA_3 staining demonstrating absence of AT-rich DNA and presence of GC-rich clusters of CH. Nucleolus organizing regions (NORs) were revealed using Ag-NOR technique; argentophilic material mostly coincides with positive signals obtained using CMA_3 staining.

Keywords

Coleoptera, Buprestidae, karyotypes, Ag-banding, C-banding, CMA_3-staining, DAPI-staining

Introduction

The family Buprestidae (Coleoptera) is one of the largest groups of Polyphagan beetles containing over than 14500 nominal species worldwide (Bellamy 2008a-d, 2009). Until now, karyotypes have been published for 88 species (34 from Armenia) of jewel-

beetles belonging to 22 genera and 14 tribes of the subfamilies Julodinae, Polycestinae, Chrysochroinae, Buprestinae and Agrilinae (Smith and Virkki 1978; Karagyan and Kuznetsova 2000; Karagyan 2001; Karagyan et al. 2004; Karagyan and Lachowska 2007; Moura et al. 2008). In the works listed the data were obtained by study of gonads of imagos. Only one work was based on the study of hemocytes of the larva of *Chalcophora mariana* Linnaeus, 1758 (Chrysochroinae, Chrysochroini) with diploid chromosome number 2n=22 (Baragaño 1978; this work was omitted in all previous reviews). The diploid chromosome numbers (2n) in the family Buprestidae range between 12 and 46. The modal number is 2n=20 found in 16 species, 8 genera, 6 tribes and 4 subfamilies. The most frequent sex chromosome system is XX/XY. The XY system of males is diverse (Xy_p, Xy_r, "XY", neo-XY and multiple X- and Y- sex chromosomes). The Xy_p "parachute" type is the most common and occurs in 64 species, 15 genera, 10 tribes and 4 subfamilies.

Up till now, cytogenetic studies on jewel-beetles have been carried out using conventional staining techniques mainly, with few exceptions. Karagyan (2001) reported the first data on AgNOR- staining in the karyotypes of two species of the genus *Sphenoptera* Dejean, 1833 and four species of *Acmaeoderella* Cobos, 1955, and Moura et al. (2008) studied karyotype of *Euchroma gigantea* Linnaeus, 1758 using Ag-banding, C-banding and fluorescent *in situ* hybridization (FISH) with a rDNA probe.

The present paper is dedicated to study of karyotypes of *Acmaeodera pilosellae persica* Mannerheim, 1837, *Sphenoptera scovitzii* Faldermann, 1835, *Dicerca aenea validiuscula* Semenov, 1895 and *Sphaerobothris aghababiani* Volkovitsh & Kalashian, 1998 using conventional and differential staining techniques. $AgNO_3$-banding was used to reveal the nucleolus organizing chromosomes and to locate the NORs in them. C-banding method was used to study the distribution of constitutive heterochromatin (CH). To characterize the molecular composition of the CH chromosomes were stained with DNA-specific fluorochromes DAPI and CMA_3 which selectively stain AT-rich and GC-rich DNA fragments, respectively. In the present study the fluorochromes CMA_3 and DAPI were applied for the first time to jewel-beetles chromosomes.

Material and methods

The buprestid beetles were collected in 2006 in Southern Armenia (Vayotsdzor and Syunik provinces). Males' gonads were dissected in several drops of hypotonic 0.9% sodium citrate solution containing 0.005% colchicine and incubated for 30-45 min at room temperature. Then the gonads were fixed in 3:1 ethanol-acetic acid mixture. Karyological slides were made according to the method proposed by Rożek (1994) with minor modifications (Rożek and Lachowska 2001). Slides were examined with phase contrast optics and the best of them with well spread chromosomes were chosen for further treatments. Depending on the specific task, various staining techniques were used. First, to determine the number and morphology of chromosomes, karyo-

types were examined using 4% Giemsa solution in phosphate buffer (pH 6.8). Then a series of chromosome banding techniques was applied as follows:

The Ag-banding method (Howell and Black 1980)

The method is based on selective silver staining of NORs containing clusters of functional rRNA genes. The slides were subjected to hydrolysis in 2N formic acid for 10 min, rinsed in running water and dried. Then the slides were treated by putting of 4-5 drops of 50% aqueous silver nitrate ($AgNO_3$) solution and 2 drops of colloidal developer solution (0.2 g gelatin, 10 ml distilled water and 0.1 ml concentrated formic acid – HCOOH). The slides were covered with a coverslip and incubated on a hotplate for 3-4 min at 60^0C in a moist chamber (warmed beforehand). After rinsing in distilled water, the slides were dried and stained by 4% Giemsa solution in phosphate buffer (pH 6.8) for 10-20 sec.

The C-banding method (Rożek 2000)

The slides were treated for 1-3 min. in 0.2N HCL at room temperature then rinsed in distilled water. Thereafter the slides were placed in 5% $Ba(OH)_2$ solution at 20^0C for approximately 3 min, then carefully rinsed in streaming and distilled water, and for 1 min in 2xSSC solution (0.3 M sodium chloride containing 0.03 M tri-sodium citrate). Then the slides were incubated in 2xSSC solution at 50^0C for 1 hr and rinsed in distilled water. Some of these slides were stained with 4% Giemsa solution in phosphate buffer (pH 6.8) and the others were dyed with DNA-specific fluorochromes DAPI and CMA_3.

To characterize the molecular composition of the constitutive heterochromatin, we stained the chromosomes with DNA-specific fluorochromes **CMA_3** (the antibiotic chromomycin A_3) and **DAPI** (4'-6-diamidino-2-phenylindol) that selectively stain GC-rich and AT-rich DNA fragments, respectively (Schweizer 1976; Donlon and Magenis 1983). Staining with fluorochromes was performed after C-banding procedure as described above. After 2xSSC treatment slides were subsequently rinsed in distilled water and dried. Then slides were immersed in McIlvaine buffer (pH 7.0) for 5 min. After this, slides were stained with CMA_3 at a final concentration of 5 μg/ml in 10 mM McIlvaine buffer at pH 7.0 (25 μl of 96% methanol was added to 500 μl of final staining solution) for 30 min, rinsed in buffer and stained with DAPI at a final concentration of 0.4 μg/ml in 10 mM McIlvaine buffer (pH 7.0) for 5 min. After staining, slides were rinsed in distilled water and mounted in anti-fade medium consisting of 1% n-propylgallate in a 10 M McIlvaine buffer (pH 7.0) solution with 70% glycerol. To improve the fluorochrome staining, 0.5% methanol was added to the fluorescent dye (Kuznetsova et al. 2001).

The slides were analyzed and photographed with a Nikon Eclipse 400 light microscope and CCD DS-U1 camera using the software Lucia Image 5.0.

Data resources

The data underpinning the analyses reported in this paper are deposited in the Dryad Data Repository at doi: 10.5061/dryad.hc52qb12.

Results

Subfamily Polycestinae
Tribe Acmaeoderini

Acmaeodera pilosellae persica, 2n=20, n ♂ = 9 + neo-XY.

In prometaphase I/metaphase I nine autosomal bivalents formed a series of gradually decreasing sizes and large heteromorphic neo-XY sex-bivalent were observed (Fig. 1A).

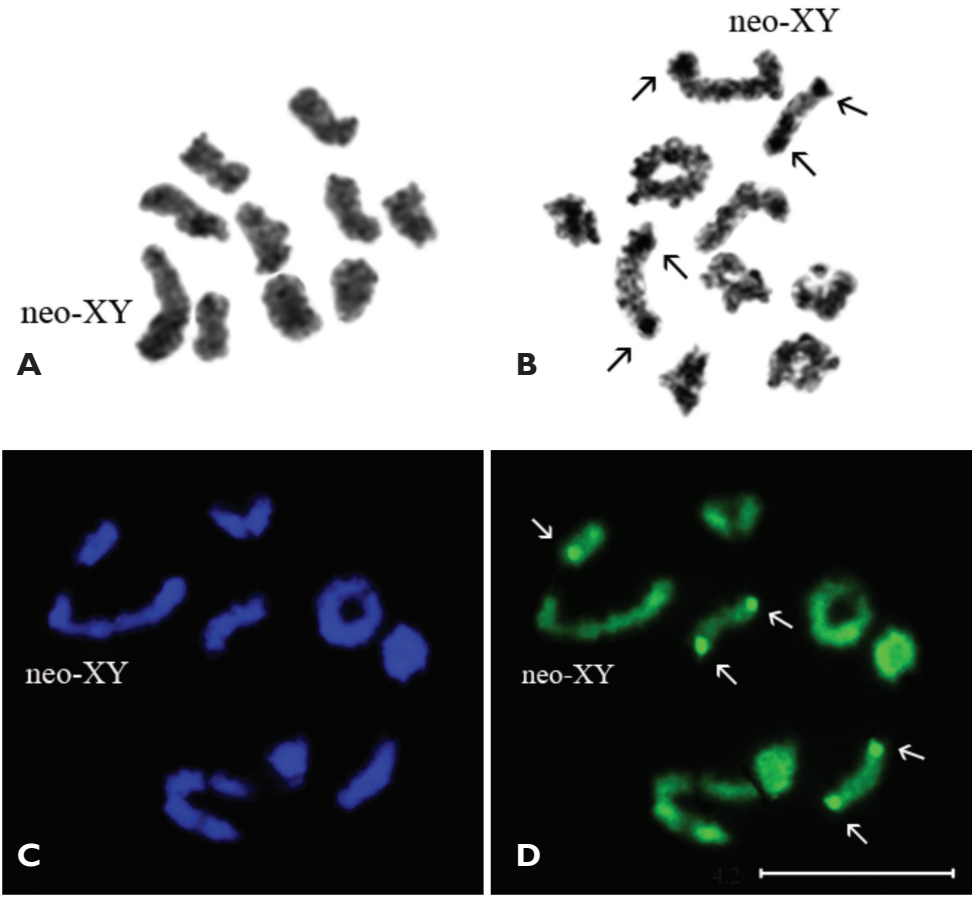

Figure 1A–D. *Acmaeodera pilosellae persica*, n=9AA+ neo-XY. **A** prometaphase I/metaphase I **B** late diakinesis (Ag-banding) **C** prometaphase I (DAPI staining) **D** (CMA_3 staining). Bar = 10 μm.

In late diakinesis / prometaphase I the 5–6 rod-shaped bivalents have most likely a terminal chiasma and 3–4 ring-shaped bivalents – two chiasmata (Fig. 1B). The X-chromosome seems to be submetacentric, Y-chromosome is most probably acrocentric and similar in size to the shorter arm of X-chromosome.

The C-banding technique revealed a very small amount of constitutive heterochromatin in the majority of the chromosomes (most probably, in pericentromeric regions) which do not form any distinct blocks. The DAPI staining did not reveal any positive signal and therefore absence of AT-rich clusters of DNA was demonstrated (Fig. 1C). Application of CMA_3 staining revealed small positive signals on telomeric regions of both homologues of two or sometimes three rod-shaped autosomal bivalents. Besides, there is small ring-shaped bivalent which is brightly visible in the majority of photographs (Fig. 1D). These signals correspond to argentophilic material revealed by the AgNOR-banding technique (Fig. 1B) and probably associated with NORs. Besides, the Y-chromosome of neo-XY bivalent is dyed argentophilic.

Subfamily Chrysochroinae
Tribe Sphenopterini

Sphenoptera scovitzii, 2n=38–46.

The karyotype of *Sph. scovitzii* studied using Ag-banding was published earlier (Karagyan 2001) and the data on karyotype of this species obtained in that study were confirmed. Unfortunately, until now, the male diploid karyotype of the species can not be determined with certainty. Thus, it seems that karyotype consists of 38–46 chromosomes, most probably of 46; the sex chromosomes could not be identified.

Some of chromosomes have a very small amount of constitutive heterochromatin weakly visible in pericentromeric regions and do not form distinct blocks. The DAPI staining of chromosomes did not reveal any positive signal (Fig. 2A), yet fluorescence after CMA_3 staining was discovered (Fig. 2B). In metaphase I, three or sometimes four rod-shaped bivalents showed distinct CMA_3 positive signals on telomeric regions of both homologues. These signals are quite stable in the largest and in two of the middle-sized bivalents and correspond to Ag-positive material revealed by the AgNOR-banding technique (Fig. 2C). Weak CMA_3 positive signals were also at times visible in some other small bivalents.

Tribe Dicercini

Dicerca aenea validiuscula, 2n=20 (18+Xy_p).

The male mitotic metaphase displayed 20 chromosomes including 9 autosomal pairs and X- and Y- sex chromosomes (Fig. 2D). All autosomes were biarmed: one pair large

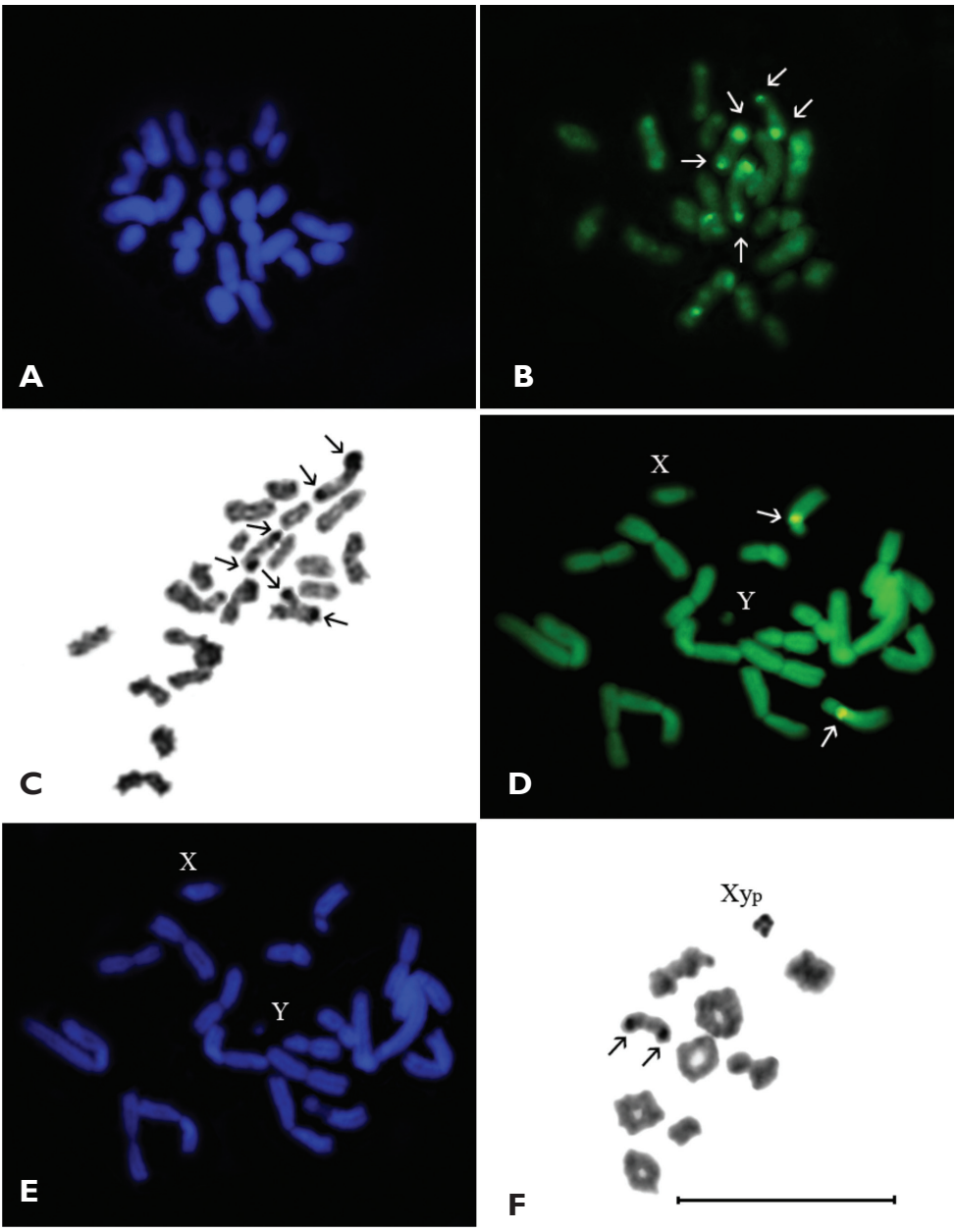

Figure 2A–F. **A–C** *Sphenoptera scovitzii*, n=19-23, metaphase I. **A** DAPI staining; **B** CMA_3 staining; **C** Ag-banding **D–F** *Dicerca aenea validiuscula*, n=9AA+Xy_p **D** mitotic metaphase (CMA_3 staining) **E** mitotic metaphase (DAPI staining) **F** diakinesis/prometaphase I (Ag-banding). Bar = 10 μm.

and 5 pairs of middle-sized metacentrics, 2 pairs of middle-sized submetacentrics and 1 pair of middle-sized subtelocentric. The X-chromosome was middle-sized and acrocentric, Y-chromosome was dot-like with unclear morphology. In mitotic metaphase

CMA_3 positive signals were found in the pericentromeric region of long arm of middle-sized homologous pair of subtelocentric autosomes (Fig. 2D). The DAPI staining did not reveal any positive signal (Fig. 2E).

In diakinesis/prometaphase I nine autosomal bivalents and heteromorphic sex-bivalent most probably of "parachute" Xy_p type were observed (Figs 2F, 3A). The autosomal bivalents formed a series of gradually decreasing sizes. There were 5–6 ring-shaped autosomal bivalents with two chiasmata, 1–2 rod-shaped bivalents possessed most likely one terminal chiasma and 1–2 were cross-shaped with an interstitial chiasma. The heterovalent Xy_p was rather small. In the middle-sized rod-shaped bivalent (which, most likely, formed by subtelocentric autosomes) the CMA_3 positive signals were visible in terminal regions of both homologues (Fig. 3A). At this stage staining by AgNOR-banding revealed Ag-positive signals on the same bivalent, besides the sex bivalent was nearly homogenously argentophilic (Fig. 2F).

Metaphase II showed 10 chromosomes of which 7 chromosomes were meta- and submetacentric, one of chromosome was subtelocentric (which showed CMA_3 positive signal on pericentromeric region), whereas the morphology of other chromosomes was vague (Figs 3B, 3C). The C-banding revealed weakly visible constitutive heterochromatin localized in the pericentromeric region of the same subtelocentric chromosome (Fig. 3C).

Subfamily Buprestinae
Tribe Chrysobothrini

Sphaerobothris aghababiani, 2n=16 (14+Xy_p).

In diakinesis/prometaphase I seven autosomal bivalents and a sex chromosome heterovalent of the Xy_p type were observed (Fig. 3D). The bivalents gradually decreased in size. The Xy_p sex heterovalent was smallest element in the set. The majority of autosomal bivalents were rod-shaped, but in some cells one or two ring-shaped autosomal bivalents were observed.

The C-banding revealed a small amount of constitutive heterochromatin most probably in the pericentromeric regions and do not form distinct blocks. The DAPI staining did not reveal any positive signals (Fig. 3E). In metaphase I bright and distinct CMA_3 positive signal was visible on "parachute" sex heterovalent, probably connected with the Y-chromosome (Fig. 3F). In addition, weak CMA_3 positive signals were observed, as a rule they were situated in telomeric or near telomeric regions of some of rod-shaped autosomal bivalents. Ag-banding revealed the bright argentophilic material connected with whole "parachute" type sex heterovalent (Fig. 3D). Small and weak argentophilic blocks were visible in telomeric or near telomeric regions of some autosomal bivalents.

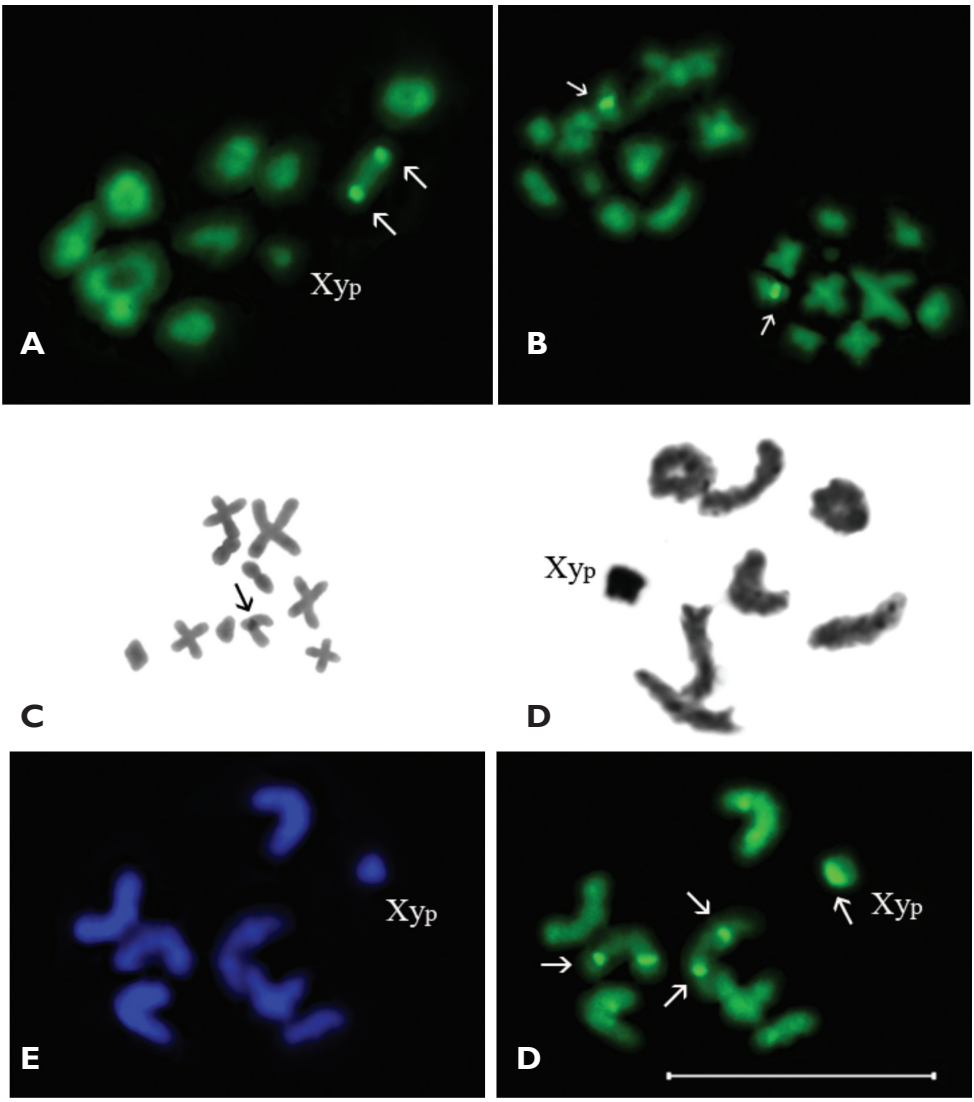

Figure 3A–F. A–C *Dicerca aenea validiuscula*, **A** diakinesis/prometaphase I (CMA_3 staining) **B** metaphase II (CMA_3 staining) **C** metaphase II (C-banding) **D–F** *Sphaerobothris aghababiani*, n=7AA+Xy_p **D** diakinesis/prometaphase I (Ag-banding) **E** metaphase I (DAPI staining) **F** metaphase I (CMA_3 staining). Bar = 10 μm.

Discussion

In total, the karyotypes of 92 species of jewel-beetles belonging to 23 genera, 14 tribes of 5 subfamilies are presently described. Generalization of data including new ones shows that the modal diploid chromosome number in Buprestidae is 2n=20 (9AA + X- and Y- sex chromosome heterovalent in males) so far found in 18 species belonging

to 8 genera, 6 tribes, 4 subfamilies. The Xy_p type of sex chromosome heterovalent is modal and occurs in 66 species, 16 genera, 10 tribes, 4 subfamilies. The most common karyotype 2n=20 (19+Xy_p) can be considered as modal. The new data confirm modality of this karyotype within the family. This karyotype occurs in a large number of beetles from different families (Smith and Virkki 1978; James and Angus 2007; Schneider et al. 2007; Cabral-de-Mello et al. 2008; et al.).

Application of C-banding technique showed that the studied species of jewel-beetles are characterized by a small amount of heterochromatin localized, most probably, in pericentromeric regions of chromosomes, as in most studied species of the order Coleoptera (Smith and Virkki 1978; Rożek et al. 2004; Lachowska et al. 2009; Arcanjo et al. 2009; et al.). On the other hand, in *Euchroma gigantea* large C-blocks were observed in multiple sex chromosomes ($X_1X_2X_3$-$Y_1Y_2Y_3$) (Moura et al. 2008). The presence of large C-blocks in chromosomes is uncommon in Coleoptera as a whole and has been observed in a few species only (Juan et al. 1991; Rożek 1992; Rożek and Rudek 1992; Plohl et al. 1993; Rożek and Lachowska 2001, 2003; Rożek et al. 2004; Bione et al. 2005a; Cabral-de-Mello et al. 2010).

Until now, the karyotypes of only seven species of jewel-beetles studied by using Ag-NOR-staining technique have been published (Karagyan 2001; Moura et al. 2008). In the present paper Ag-banding was applied for study of karyotypes of another three species.

Even this restricted material showed noticeable variability of distribution of argentophilic material (probably NOR) in the karyotypes of jewel-beetles. The argentophilic material is located on:

1) the autosomes: *Sphenoptera scovitzii* (2n=38–46), *Sph. mesopotamica* Marseul, 1865 (2n=24, Xy_p).
2) both on the sex chromosomes and on the autosomes: *Acmaeoderella villosula* Steven, 1830 (described as *Acmaeoderella boryi* Brullé, 1832 in Karagyan 2001) (2n=18, Xy_r), *Acmaeodera pilosellae persica* (2n=20, neo-XY), *Sphaerobothris aghababiani* (2n=16, Xy_p), *Dicerca aenea validiuscula* (2n=20, Xy_p).
3) sex chromosomes only, situating either on one of the sex chromosomes or localized between sex chromosomes of bivalent: *Acmaeoderella flavofasciata* (Piller & Mitterpacher, 1783) (2n=18, Xy_r), *A. gibbulosa* Ménétriés, 1832 (2n=18, Xy_r), *A. vetusta* (Ménétriés, 1832) (2n=18, Xy_r). In *Euchroma gigantea* (2n=32, 36, $X_1X_2X_3Y_1Y_2Y_3$ – Moura et al. 2008) argentophilic material labeled the multiple sex chromosomes chain.

As commonly understood, in Coleoptera NORs may be located in some autosomal pair and/or sex chromosomes (Almeida et al. 2000; Moura et al. 2003; Bione et al. 2005b; Holecová et al. 2008). The most common pattern in Coleoptera is the location of the nucleolus organizer region in one autosomal pair (Virkki 1983; Virkki et al. 1984; Vitturi et al. 1999; Colomba et al. 2000; Moura et al. 2003; Almeida et al. 2006; Schneider 2007; et al.). On the other hand, the argentophilous body has been repeatedly observed between the sex-chromosomes of Xy_p type (Postiglioni and Brum-Zorrilla 1988; Virkki et al. 1990, 1991; Maffei et al.

2001; Moura et al. 2003; et al.). Among Buprestidae, the whole Xy_p sex bivalent is brightly argentophilic and probably bears NOR only in *Sphaerobothris aghababiani* and *Dicerca aenea validiuscula*. However, the lack of relationship between nucleolus and sex chromosome system of Xy_p has also been demonstrated for some Coleoptera including one species of jewel-beetles, *Sphenoptera mesopotamica* (Karagyan 2001). According to some authors, the "parachute" can be strongly marked by the silver nitrate during different phases of meiosis independent of whether or not the NORs are located in Xy_p bivalent (Virkki et al. 1991; Moura et al. 2003; Bione et al. 2005b; Mendes-Neto et al. 2010). This phenomenon is probably related to the presence of argentophilic substance (proteins) that theoretically facilitates the configuration and segregation of the sex chromosomes of the Xy_p system (Virkki et al. 1990, 1991; Juan et al. 1993; Petitpierre 1996; Moura et al. 2003; Bione et al. 2005a, b; Schneider et al. 2007).

Chromosome staining by DNA base specific fluorochromes has been used in cytogenetic studies of Coleoptera (Juan and Petitpierre 1989; Vitturi et al. 1999; Colomba et al. 2000; Moura et al. 2003; Schneider et al. 2007; Lachowska 2008; Mendes-Neto et al. 2010; et al) but has never before been applied to Buprestidae.

The correlation between NORs and CMA_3 bands is quite common in some insects, including beetles (Camacho et al. 1991; Vitturi et al. 1999; Colomba et al. 2000; Maffei et al. 2001; Kuznetsova et al. 2001; Araújo et al. 2002; Brito et al. 2003; Nechayeva et al. 2004). Silver staining mainly reveals transcriptionally active NORs (Sumner 1990), as opposed to fluorochrome CMA_3 staining which labels NORs independently of their activity (Schmid and Guttenbach 1988). In this study, the fluorescent signals after CMA_3 staining were positive in all four species of jewel-beetles. While in *Sphenoptera scovitzii* and *Sphaerobothris aghababiani* they were nearly fully correlated with argentophilic material observed on silver dyed chromosomes, in *Acmaeodera pilosellae persica* CMA_3 signals correlated with argentophilic blocks on 2–3 autosomal bivalents but not with Y-chromosome which was dyed argentophilic as well. Meanwhile in *Dicerca aenea validiuscula* CMA_3 signal was correlated with one of autosomal pairs when argentophilic material was revealed in the same autosomal pair as well as on sex bivalent.

Fluorochrome DAPI staining in all studied species of jewel-beetles did not reveal any particular bright regions on chromosomes which allows for preliminary suggestion that CH have no distinct AT-rich DNA clusters. In general, there is variable distribution of AT- or GC-rich clusters of CH among beetles studied by fluorochromes staining. For instance, positive CMA_3 and negative DAPI signals were found in some Elateridae (Schneider et al. 2006, 2007), and most of studied Scarabaeoidea (Vitturi et al. 1999; Moura et al. 2003; Bione et al. 2005b; Cabral-de-Mello et al. 2010). Positive DAPI signals were found only in some other Scarabaeoidea (Moura et al. *l. c.*) and in majority of the Curculionidae studied (Lachowska 2008; Lachowska et al. 2008). Rarely both DAPI and CMA_3 positive signals were revealed in Scarabaeidae (Bione et al. 2005b), in a few Curculionidae (Lachowska 2008) and Chrysomelidae (Almeida et al. 2006).

In conclusion, data of the present study offer important insights into the karyotypes characteristics of jewel-beetles which may be useful in elucidation of relationships both among the species of the family itself as well as between jewel-beetles and the representatives of other coleopteran families.

Acknowledgements

This study was supported by Józef Mianowski Fund (Poland). We would like to express our greatest gratitude to our colleagues from the Institute of Systematics and Evolution of Animals, Polish Academy of Sciences Prof. dr hab. Elżbieta Warchałowska-Śliwa, Prof. dr hab. Anna Maryańska-Nadachowska, Dr. Maria Rożek and Prof. dr hab. Adam Nadachowski for their invaluable support of this study. We gratefully acknowledge to Dr. Alexander Danchenko (Moscow State University, Moscow, Russia) for his kind help in collecting some of the specimens studied.

References

Almeida MC, Zacaro AA, Cella DM (2000) Cytogenetic analysis of *Epicauta atomaria* (Meloidae) and *Palembus dermestoides* (Tenebrionidae) with Xy_p sex determination system using standard staining, C-bands, NOR and synaptonemal complex microspreading techniques. Hereditas 133: 147–157. doi: 10.1111/j.1601-5223.2000.00147.x

Almeida MC, Campaner C, Cella DM (2006) Karyotype characterization, constitutive heterochromatin and nucleolus organizer regions of *Paranaita opima* (Coleoptera, Chrysomelidae, Alticinae). Genetics and Molecular Biology 29(3): 475–481. doi: 10.1590/S1415-47572006000300013

Arcanjo AP, Cabral-de-Mello DC, Silva AEB, Moura RC (2009) Cytogenetic characterization of *Eurysternus caribaeus* (Coleoptera: Scarabaeidae): evidence of sex-autosome fusion and diploid number reduction prior to species dispersion. Journal of Genetics 88(2): 177–182. doi: 10.1007/s12041-009-0025-y

Araújo SMR, Pompolo SG, Perfectti F, Camacho JPM (2002) Genetic load caused by variation in the amount of rDNA in a wasp. Chromosome Research 10: 607–613. doi: 10.1023/A:1020970820513

Baragaño R (1978) Nuevo método para el estudio de cromosomas en Coleoptera a partir de hemocitos de estados larvarios. Boletín del Servicio de Plagas 4: 23–33.

Bellamy CL (2008a) A world catalogue and bibliography of the jewel beetles (Coleoptera: Buprestoidea). Volume 1: Introduction; Fossil Taxa; Schizopodidae; Buprestidae: Julodinae - Chrysochroinae: Poecilonotini. Pensoft Publishers, Moscow-Sofia, 606 pp.

Bellamy CL (2008b) A world catalogue and bibliography of the jewel beetles (Coleoptera: Buprestoidea). Volume 2: Chrysochroinae: Sphenopterini through Buprestinae: Stigmoderini. Pensoft Publishers, Moscow-Sofia, 614 pp.

Bellamy CL (2008c) A world catalogue and bibliography of the jewel beetles (Coleoptera: Buprestoidea). Volume 3: Buprestinae: Pterobothrini through Agrilinae: Rhaeboscelina. Pensoft Publishers, Moscow-Sofia, 632 pp.

Bellamy CL (2008d) A world catalogue and bibliography of the jewel beetles (Coleoptera: Buprestoidea). Volume 4: Agrilinae: Agrilina through Trachyini. Pensoft Publishers, Moscow-Sofia, 722 pp.

Bellamy CL (2009) A world catalogue and bibliography of the jewel beetles (Coleoptera: Buprestoidea). Volume 5: Appendices, Bibliography, Indices. Pensoft Publishers, Moscow-Sofia, 582 pp.

Bione E, Camparoto ML, Simões ZLP (2005a) A study of the constitutive heterochromatin and nucleolus organizer regions of *Isocopris inhiata* and *Diabroctis mimas* (Coleoptera: Scarabaeidae, Scarabaeinae) using C-banding, $AgNO_3$ staining and FISH techniques. Genetics and Molecular Biology 28(1): 111–116. doi: 10.1590/S1415-47572005000100019

Bione E, Moura RC, Carvalho R, Souza MJ (2005b) Karyotype, C-and fluorescence banding pattern, NOR location and FISH study of five Scarabaeidae (Coleoptera) species. Genetics and Molecular Biology 28(3): 376–381. doi: 10.1590/S1415-47572005000300006

Brito RM, Caixerio APA, Pompolo SG, Azevedo GG (2003) Cytogenetic data of *Partamona peckolti* (Hymenoptera, Apidae, Meliponini) by C-banding and fluorochrome staining with DA/CMA_3 and DA/DAPI. Genetics and Molecular Biology 26(1): 53–57. doi: 10.1590/S1415-47572003000100009

Cabral-de-Mello DC, Oliveira SG, Ramos IC, Moura RC (2008) Karyotype differentiation patterns in species of the subfamily Scarabaeinae (Scarabaeidae, Coleoptera). Micron 39: 1243–1250. doi: 10.1016/j.micron.2008.04.002

Cabral-de-Mello DC, Moura RC, Souza MJ (2010) Amplification of repetitive DNA and origin of a rare chromosomal sex bivalent in *Deltochilum* (*Calhyboma*) *verruciferum* (Coleoptera, Scarabaeidae). Genetica 138: 191–195. doi: 10.1007/s10709-009-9409-4

Camacho JPM, Cabrero J, Viseras E (1991) G Banding in two species of grasshoppers and its relationships to C, N, and fluorescence banding techniques. Genome 34: 638–643. doi: 10.1139/g91-097

Colomba MS, Vitturi R, Zunino M (2000) Karyotype analyzes, banding, and fluorescent *in situ* hybridization in the Scarab beetle *Gymnopleurus sturmi* McLeady (Coleoptera, Scarabaeoidea, Scarabaeidae). The Journal of Heredity 91: 260–264. doi: 10.1093/jhered/91.3.260

Donlon TA, Magenis RE (1983) Methyl green is a substitute for distamycin A in the formation of distamycin A/DAPI C-bands. Human Genetics 65: 144–146. doi: 10.1007/BF00286651

Holecová M, Rożek M, Lachowska D (2008) The first cytogenetic report on *Laena reitteri* Weise, 1877 (Coleoptera, Tenebrionidae, Lagriinae) with notes on karyotypes of darkling beetles. Folia biologica (Kraków) 56(3–4): 213–217.

Howell WM, Black DA (1980) Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-srep method. Experientia 36: 1014–1015. doi: 10.1007/BF01953855

James LV, Angus RB (2007) A chromosomal investigation of some British Cantharidae (Coleoptera). Genetica 130: 293–300. doi: 10.1007/s10709-006-9106-5

Juan C, Gonsálves J, Mezzanotte R, Petitpierre E (1991) Cytological and biochemical characterization of the in situ endonuclease digestion of fixed *Tenebrio molitor* chromosomes. Chromosoma 100: 432–438. doi: 10.1007/BF00364553

Juan C, Petitpierre E (1989) C-banding and DNA content in seven species of Tenebrionidae (Coleoptera). Genome 32: 834–839. doi: 10.1139/g89-519

Juan C, Pons J, Petitpierre E (1993) Localization of tandemly repeated DNA sequences in beetle chromosomes by fluorescent *in situ* hybridization. Chromosome Research 1: 167–174.

Karagyan G (2001) Ag-banded karyotypes in six species of jewel-beetles (Buprestidae, Coleoptera). Folia biologica (Kraków) 49(1–2): 42–48. doi: 10.1007/BF00710770

Karagyan GH, Kuznetsova VG (2000) Chromosome numbers and sex chromosome systems in buprestid beetles (Coleoptera, Buprestidae). Entomological Review 80(1): 38–49. Translated from Entomologicheskoe Obozrenie 79(1): 96–106.

Karagyan G, Kuznetsova VG, Lachowska D (2004) New cytogenetic data on Armenian buprestids (Coleoptera, Buprestidae) with a discussion of karyotype variation within the family. Folia biologica (Kraków) 52(3–4): 151–158. doi: 10.3409/1734916044527601

Karagyan GH, Lachowska D (2007) New karyotypes of five species of jewel-beetles and presumptive ancestral state of karyotypes of the subfamilies Polycestinae, Chrysochroinae and Buprestinae (Coleoptera: Buprestidae). Comparative Cytogenetics 1(2): 121–127. (www.zin.ru/journals/compcyt)

Kuznetsova VG, Westendorff M, Nokkala S (2001) Patterns of chromosome banding in the sawfly family Tenthredinidae (Hymenoptera, Symphyta). Caryologia 54(3): 227–233.

Lachowska D (2008) Karyotypes and chromosome rearrangements in two tribes of weevils (Coleoptera, Curculionidae: Sciaphilini and Brachydrini). Folia biologica (Kraków) 56(3–4): 219–225. doi: 10.3409/fb.56_3-4.219-225

Lachowska D, Rożek M, Holecová M (2008) Cytotaxonomy and karyology of the tribe Otiorhynchini (Coleoptera: Curculionidae). European Journal of Entomology 105: 175–184.

Lachowska D, Rożek M, Holecová M (2009) Chromosomal similarities and differences among three sibling species of the *Acalles echinatus* group (Coleoptera, Curculionidae, Cryptorhynchinae). Zootaxa 1985: 63–68.

Maffei EMD, Pompolo SG, Campos LAO, Petitpierre E (2001) Sequential FISH analysis with rDNA genes and Ag-NOR banding in the lady beetle *Olla* v-*nigrum* (Coleoptera, Coccinellidae). Hereditas 135: 13–18. doi: 10.1111/j.1601-5223.2001.00013.x

Mendes-Neto EO, Vicari MR, Campaner C, Nogaroto V, Artoni RF, Almeida MC (2010) Cytogenetic analysis of *Astylus antis* (Perty, 1830) (Coleoptera, Melyridae): karyotype, heterochromatin and location of ribosomal genes. Genetics and Molecular Biology 33(2): 237–243. doi: 10.1590/S1415-47572010005000050

Moura RC, Souza MJ, Melo NF, Lira-Neto AC (2003) Karyotypic characterization of representatives from Melolonthinae (Coleoptera, Scarabaeidae): Karyotypic analysis, banding and fluorescent *in situ* hybridization (FISH). Hereditas 138: 200–206. doi: 10.1034/j.1601-5223.2003.01611.x

Moura RC, Melo NF, Souza MJ (2008) High levels of chromosomal differentiation in *Euchroma gigantea* L., 1735 (Coleoptera, Buprestidae). Genetics and Molecular Biology 31(2): 431–437. doi: 10.1590/S1415-47572008000300007

Nechayeva GA, Kuznetsova VG, Nokkala S (2004) New data on the karyotype of *Pseudococcus viburni* (Sign.) (Homoptera, Coccinea). Entomological Review 84(4): 393–400.

Petitpierre E (1996) Molecular cytogenetics and taxonomy of insects, with particular reference to the Coleoptera. International Journal of Insect Morphology and Embryology 25: 115–134. doi: 10.1016/0020-7322(95)00024-0

Plohl M, Lucijanic-Justic V, Ugarkovic D, Petitpierre E, Juan C (1993) Satellite DNA and heterochromatin of the flour beetle *Tribolium confusum*. Genome 36: 467–475. doi: 10.1139/g93-064

Postiglioni A, Brum-Zorrilla N (1988) Non-relationship between nucleolus and sex chromosome system Xy_p in *Chelymorpha variabilis* Boheman (Coleoptera, Chrysomelidae). Genetica 77: 137–141. doi: 10.1007/BF00057764

Rożek M (1992) Karyological analysis of *Acupalpus* (*Stenolophus*) *teutonus* (Schrank) and *Pogonus presicus* (Chaud.) (Coleoptera, Carabidae). Folia biologica (Kraków) 40(3–4): 113–118.

Rożek M, Rudek Z (1992) Karyotype analysis and C-banding pattern in two species of Carabid (Coleoptera, Carabidae). Folia biologica (Kraków) 40: 47–52.

Rożek M (1994) A new chromosome preparation technique for Coleoptera (Insecta). Chromosome Researche 2: 76–78. doi: 10.1007/BF01539458

Rożek M (2000) The C-banding technique for Coleoptera (Insecta). Folia biologica (Kraków) 48(1–2): 29–31.

Rożek M, Lachowska D (2001) C-bands on chromosomes of four beetle species (Coleoptera: Carabidae, Silphidae, Elateridae, Scarabaeidae). Folia biologica (Kraków) 49: 179–182.

Rożek M, Lachowska D (2003) C-banded chromosomes of genus *Bembidion* Latr. (Coleoptera, Carabidae). Folia biologica (Kraków) 51: 143–146.

Rożek M, Lachowska D, Petitpierre E, Holecová M (2004) C-bands on chromosomes of 32 beetle species (Coleoptera: Elateridae, Cantharidae, Oedemeridae, Cerambycidae, Chrysomelidae and Curculionidae). Hereditas 140: 1–10. doi: 10.1111/j.1601-5223.2004.01810.x

Schmid M, Guttenbach M (1988) Evolutionary diversity of reverse (R) fluorescent chromosome bands in vertebrates. Chromosoma 77: 101–114. doi: 10.1007/BF00327367

Schneider MC, Almeida MC, Rosa SP, Costa C, Cella DM (2006) Evolutionary chromosomal differentiation among four species of *Conoderus* Eschscholtz, 1829 (Coleoptera, Elateridae, Agrypninae, Conoderini) detected by standard staining, C-banding, silver nitrate impregnation, and CMA_3/DA/DAPI staining. Genetica 128: 333–346. doi: 10.1007/s10709-006-7101-5

Schneider MC, Rosa SP, Almeida MC, Costa C, Cella DM (2007) Chromosomal similarities and differences among four Neotropical Elateridae (Conoderini and Pyrophorini) and other related species, with comments on the NOR patterns in Coleoptera. Journal of Zoological Systematics and Evolutionary Research 45(4): 308–316. doi: 10.1111/j.1439-0469.2006.00398.x

Schweizer D (1976) Reverse fluorescent chromosome banding with chromomycin and DAPI. Chromosoma (Berl.) 58: 307–324. doi: 10.1007/BF00292840

Smith SG, Virkki N (1978) Animal cytogenetics. 3. Insecta 5. Coleoptera. Berlin-Stuttgart, 366 pp.

Sumner AT (1990) Chromosome banding. Unwyn Hyman, London, XIV + 434 pp.

Virkki N (1983) Banding of Oedionychna (Coleoptera, Alticinae) chromosomes: C- and Ag-bands. Journal of Agriculture of the University of Puerto Rico 67: 221–225.

Virkki N, Flores M, Escudero J (1984) Structure, orientation and segregation of the sex trivalent in *Pyrophorus luminosus* (Coleoptera, Elateridae). Canadian Journal of Genetics and Cytology 26: 326–330.

Virkki N, Mazzella C, Denton A (1990) Staining of substances adjacent to the Xy_p sex bivalent of some weevils (Coleoptera: Curculionidae). Journal of Agriculture of the University of Puerto Rico 74: 405–418.

Virkki N, Mazzella C, Denton A (1991) Silver staining of the coleopteran Xy_p sex bivalent. Cytobios 67: 45–63.

Vitturi R, Colomba MS, Barbieri R, Zunino M (1999) Ribosomal DNA location in the scarab beetle *Thorectes intermedius* (Costa) (Coleoptera, Geotrupidae) using banding and fluorescent *in-situ* hybridization. Chromosome Research 7: 255–260. doi: 10.1023/A:1009270613012

CompCytogen 6(2): 192–211 (2012)
doi: 10.3897/CompCytogen.v6i2.3107
www.pensoft.net/journals/compcytogen

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Chromosome number variation of the Italian endemic vascular flora. State-of-the-art, gaps in knowledge and evidence for an exponential relationship among even ploidy levels

Gianni Bedini[1], Fabio Garbari[1], Lorenzo Peruzzi[1]

1 *Dipartimento di Biologia, Unità di Botanica generale e sistematica, Università di Pisa, via Luca Ghini 5, 56126, Pisa, Italy*

Corresponding author: *Lorenzo Peruzzi* (lperuzzi@biologia.unipi.it)

Academic editor: *Joan Vallés* | Received 21 March 2012 | Accepted 17 April 2012 | Published 7 May 2012

Citation: Bedini G, Garbari F, Peruzzi L (2012) Chromosome number variation of the Italian endemic vascular flora. State-of-the-art, gaps in knowledge and evidence for an exponential relationship among even ploidy levels. Comparative Cytogenetics 6(2): 192–211. doi: 10.3897/CompCytogen.v6i2.3107

Abstract

The Italian endemic vascular flora is composed of 1,286 specific and subspecific taxa. From the critical analysis of "Chrobase.it", 711 of them (about 55%) have been studied from a karyological point of view. These taxa belong to 52 out of 56 families and 204 out of 284 genera. These data suggest that endemic species are more studied than the flora as a whole. Mean chromosome number for Italian endemics is 2n = 30.68 ± 20.27 (median: 2n = 26, mode: 2*n* = 18). These values are very close to those known for the whole flora. Similar variation ranges, among endemics and species with wider distribution, are likely to reflect similar evolutionary trends. Known chromosome numbers in Italian endemics range from 2n = 8 to 2n = 182. About 9% of taxa show more than one cytotype and the frequency of Bs in the Italian endemic vascular flora is 3.3%. These values are slightly smaller compared with the whole Italian flora. Finally, for the basic chromosome numbers x = 7, 8, 9, the proportion of diploids (2n = 2*x*) to even polyploids (2*n* = 4x, 6x, 8x and 10x) can be described by the exponential function $f(p) = e^{(5.539 - 0.637p)}$ ($R^2 = 0.984$).

Keywords

B-chromosomes, cytotaxonomy, endemics, evolution, Italy, polyploidy

Introduction

The number of chromosome count databases, either hard-printed or online, matches current research trends, and attests to the usefulness of chromosome data in current taxonomic, genetic and cytogeographic research (Del Prete et al. 1999; Guerra 2008; Stuessy 2009; Bedini et al. 2012a, b). Recent studies (Peruzzi et al. 2011; Bedini et al. 2012a, b) exemplified the potentialities of such databases in inferring evolutionary and cytogeographic relationships. Italian vascular flora coverage, in terms of karyological knowledge, is about 35% (Peruzzi et al. 2011; Bedini et al. 2012a, b).

The Italian endemic vascular flora, according to on-going research carried out in collaboration with CRFA (Centro di Ricerca per la Flora dell'Appennino) of Barisciano (L'Aquila, Italy), comprises 1,286 specific and subspecific taxa (L. Peruzzi, F. Bartolucci and F. Conti, unpublished data), including those species which eventually occur also in Corse (France). The present work aims to summarize the karyological knowledge focusing on the endemic component of Italian vascular flora, extracted from the online database "Chrobase.it" (Bedini et al. 2010 onwards).

Methods

Source of chromosome data

Data about chromosome numbers (*n* and/or 2*n*) and B-chromosomes about Italian vascular flora are stored in the online database "Chrobase.it" (Bedini et al. 2010 onwards). Up to March 20, 2012, the database consisted of 7,560 records, derived from 1,364 literature references, dating back to 1925 (Garbari et al. 2012). They refer to 3,035 accepted taxa, according to the nomenclature of Conti et al. (2005, 2007). Data for endemic taxa were obtained by querying "Chrobase.it" with the list of endemics and relative synonyms (the complete list of endemics, including those lacking karyological knowledge, is available at request from the authors). The number of available counts and taxa were calculated for Italy as a whole, for each of the 20 Italian administrative regions, and for each family and genus. Counts in multiple copy (i.e., the same chromosome number for the same species) were excluded from further analyses, while different chromosome counts for the same taxon (i.e. cytotypes) were retained. Any n count was transformed to 2n and then included in the dataset. 25 counts, referring to 25 different taxa, obtained from Corsican populations were also included, given the high number of endemics shared by Sardinia, Corse and Tuscan Archipelago. From 24 of these units no counts were available for the Italian territory.

Concerning the list of endemics, for the difficult and critical genera *Hieracium* L. (1753) and *Pilosella* Vaill. (1754) (Asteraceae) the subspecies rank was not considered.

Analysis of data

Mean (± standard deviation), median, modal chromosome number, and frequencies (histograms) were calculated for the entire dataset of Italian vascular flora endemics. Frequency and mean number (± standard deviation) of B-chromosomes for the whole dataset and for each genus were also calculated. The frequencies of basic chromosome numbers (x) in those complements (2n) where more than one basic number can occur were obtained by a taxon by taxon screening of relevant karyological literature quoted in Chrobase.it (Bedini et al. 2010 onwards). Frequencies of even ploidy levels from 2*x* to 10*x*, in the three most frequent basic numbers x = 7, x = 8 and x = 9, were arranged in a linear plot, and their relationships between ploidy levels was tested by means of linear and non linear models based on least-squares estimates in R software (www.r-project.org; Rossiter 2009): lm function was used to fit a straight line, nls function was used for power, logarithmic, and exponential curves. Odd ploidy levels were not considered since they were very rare in our data set, with the only exception of 2n = 27 (see over).

Results

Chromosome counts are available for 711 out of the 1,286 (ca. 55%) currently accepted specific and infraspecific endemic taxa, resulting in 839 different cytotypes; they are representative of 204 out of the 284 genera (72%) – and 52 out of the 56 families (91%) – encompassing the endemic taxa. The geographic distribution of counts is shown in Fig. 1. The most intensely investigated regions are Sicily, Tuscany and Sardinia, where more than half of the Italian endemic flora growing in the respective territories was karyologically studied. Table 1 shows the number of taxa studied for each region.

The distribution of the 711 taxa across families is shown in Fig. 2. The most represented families are Asteraceae (19%), Plumbaginaceae (14%), Fabaceae (8%), Brassicaceae and Caryophyllaceae (6%). For all the endemics from Rosaceae (26 taxa), Thymelaeaceae (2 taxa), Aspleniaceae, Berberidaceae, Cannabaceae, Convolvulaceae, Cucurbitaceae, Oleaceae, Papaveraceae and Pinaceae (1 taxon each), no karyological data is available in the literature.

For 92 genera, of which 7 have ≥ 5 endemics, 100% of Italian endemics was covered (Table 2). Other 29 genera, hosting ≥ 5 endemics, showed coverage ranging from 93% in *Genista* L. (1753) to only 10% in *Hieracium*. The highest number of studied taxa occurs in the genera *Limonium* Mill. (1754) (84 *taxa*), *Centaurea* L. (1753) (46 taxa) and *Genista* (26 taxa).

Chromosome numbers range from 2n = 8, reported in 8 endemic taxa [*Bellevalia dubia* (Guss.) Schult. & Schult. f. (1830) s.s., *Crepis vesicaria* subsp. *hyemalis* (Biv.) Babc. (1941), *Crocus etruscus* Parl. (1860), *C. ilvensis* Peruzzi & Carta (2011), *C. siculus*

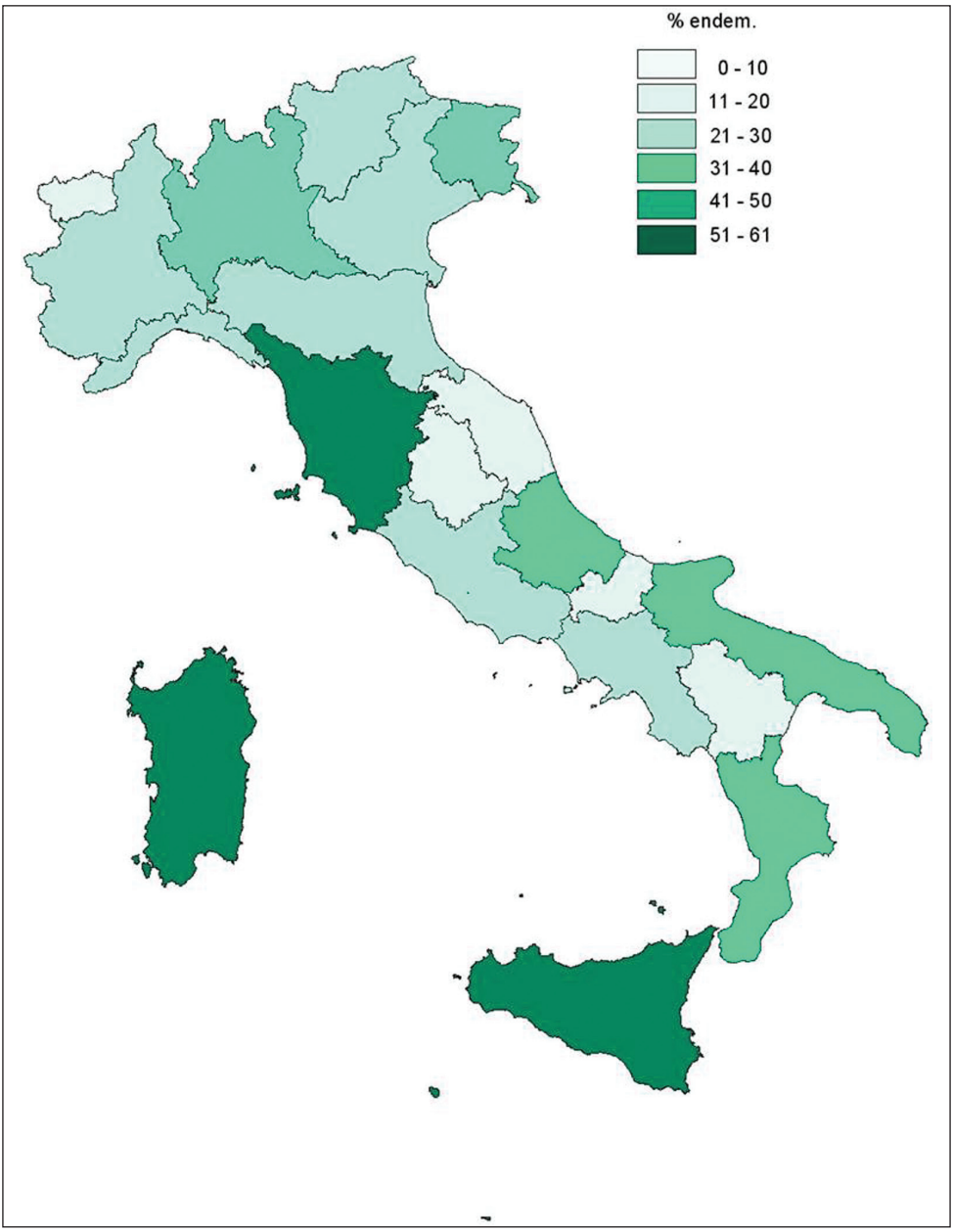

Figure 1. Map of Italian regions, showing the % endem. of Italian vascular flora karyologically studied with respect to the Italian endemics growing in each region (derived from Conti et al. 2005).

Tineo (1832), *Hypochaeris robertia* (Sch. Bip.) Fiori (1910), *Leontodon anomalus* Ball (1850) and *L. intermedius* Porta (1879)], to 2n = 180, 182, reported for *Colchicum gonarei* Camarda (1978).

Table 1. Number of karyologically studied Italian endemic vascular flora for each region. The sequence of regions is the same as the one used in Conti et al. (2005).

Region	**No. taxa studied**
Valle d'Aosta	0
Piedmont	7
Lombardy	13
Trentino-Alto Adige	9
Veneto	10
Friuli-Venezia Giulia	6
Liguria	9
Emilia-Romagna	9
Tuscany	89
Marche	10
Umbria	9
Latium	26
Abruzzo	61
Molise	2
Campania	26
Apulia	33
Basilicata	10
Calabria	75
Sicily	193
Sardinia	156

The mean chromosome number is 2n = 30.68 ± 20.27, with median 2n = 26 and mode 2n = 18. Fig. 3 shows the most frequent chromosome numbers in the whole dataset of the Italian endemics. The six most frequent chromosome numbers are 2n = 14, 2n = 16, 2n = 18, 2n = 27, 2n = 32 and 2n = 36. Taken together, they account for more than one half (51%) of all the counts available.

Concerning chromosome complements where more than one basic number can occur, the most heterogeneous, among those up to 2n = 72 (Table 3), are: 2n = 24, which in most cases represents diploids with x = 12, but also triploids with x = 8 and rarely tetraploids with x = 6; 2n = 36, which in most cases represents tetraploids with x = 9, then diploids with x = 18 and rarely triploids with x = 12; 2n = 40, with tetraploids, pentaploids and diploids; 2n = 48, representing mostly by diploids with x = 24, but also tetraploids with x = 12 and hexaploids with x = 8; 2n = 56, representing mostly tetraploids with x = 14, but also diploids with x = 28 and octoploids with x = 7. The most frequent chromosome numbers, not reported in Table 3, include only diploids (2n = 2x = 14, 2n = 2x = 18) or triploids (2n = 3x = 27). When we apply these observations to the six most frequent chromosome numbers 2n = 14, 2n = 16 and 2n = 18, 2n = 27, 2n = 32 and 2n = 36, pooled together, we obtain 73.1% diploids, 15.9% tetraploids and 11% triploids.

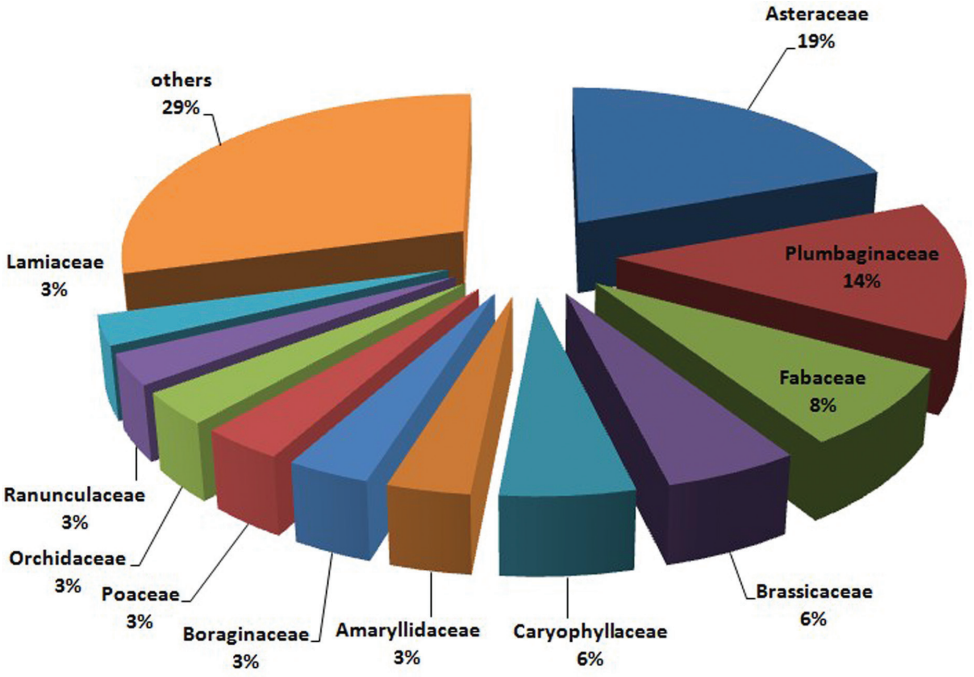

Figure 2. Pie plot showing the distribution of karyologically studied Italian endemic taxa across families. Families with less than 20 studied taxa were merged.

Table 2. Most karyologically studied genera of Italian endemics (≥ 5 taxa). They are arranged firstly according their decreasing % of coverage, secondly their number of taxa studied and, thirdly, alphabetically.

Genus	No. taxa studied	Coverage
Anchusa L. (1753)	7	100%
Ornithogalum L. (1753)	7	100%
Crocus L. (1753)	6	100%
Santolina L. (1753)	6	100%
Sesleria Scop. (1760)	6	100%
Stachys L. (1753)	6	100%
Onosma L. (1762)	5	100%
Genista L. (1753)	26	93%
Allium L. (1753)	18	90%
Viola L. (1753)	17	85%
Limonium Mill. (1754)	83	84%
Cardamine L. (1753)	5	83%
Erysimum L. (1753)	5	83%
Iris L. (1753)	8	80%
Campanula L. (1753)	14	77%

Genus	No. taxa studied	Coverage
Biscutella L. (1753)	10	77%
Anthemis L. (1753)	8	75%
Pinguicula L. (1753)	6	75%
Brassica L. (1753)	8	73%
Cerastium L. (1753)	8	73%
Euphorbia L. (1753)	10	71%
Jacobaea Mill. (1754)	5	71%
Armeria Willd. (1809)	11	69%
Astragalus L. (1753)	9	69%
Senecio L. (1753)	6	67%
Centaurea L. (1753)	45	66%
Helichrysum Mill. (1754)	6	60%
Taraxacum F.W. Wigg. (1780)	9	53%
Festuca L. (1753)	8	53%
Asperula L. (1753)	6	50%
Ranunculus L. (1753)	16	48%
Saxifraga L. (1753)	7	47%
Ophrys L. (1753)	14	38%
Silene L. (1753)	10	38%
Dianthus L. (1753)	8	30%
Hieracium L. (1753)	5	10%

Genera with 100% karyological coverage but with less than 5 Italian endemics: *Cynoglossum* L. (1753) (4), *Erodium* L'Hér. (1789) (3), *Gagea* Salisb. (1806) (3), *Plantago* L. (1753) (3), *Arenaria* L. (1753) (2), *Athamanta* L. (1753) (2), *Borago* L. (1753) (2), *Buphthalmum* L. (1753) (2), *Globularia* L. (1753) (2), *Hypericum* L. (1753) (2), *Isoëtes* L. (1753) (2), *Moltkia* Lehm. (1817) (2), *Muscari* Mill. (1754) (2), *Oncostema* Raf. (1837) (2), *Orchis* L. (1753) (2), *Paeonia* L. (1753) (2), *Ptilostemon* Cass. (1816) (2), *Pulmonaria* L. (1753) (2), *Rhaponticoides* Vaill. (1754) (2), *Ribes* L. (1753) (2), *Soldanella* L. (1753) (2), *Thapsia* L. (1753) (2), *Tragopogon* L. (1753) (2), *Veronica* L. (1753) (2), *Acer* L. (1753) (1), *Adonis* L. (1753) (1), *Adoxa* L. (1753) (1), *Ajuga* L. (1753) (1), *Anemonoides* Mill. (1754) (1), *Arum* L. (1753) (1), *Arundo* L. (1753) (1), *Bellium* L. (1771) (1), *Bituminaria* Fabr. (1759) (1), *Calendula* L. (1753) (1), *Callianthemum* C.A. Mey. (1830) (1), *Chiliadenus* Cass. (1825) (1), *Coristospermum* Bertol, (1838) (1), *Cryptotaenia* DC. (1829) (1), *Digitalis* L. (1753) (1), *Diplotaxis* DC. (1821) (1), *Drymochloa* Holub (1984) (1), *Echinops* L. (1753) (1), *Edraianthus* (A. DC.) A. DC. (1839) (1), *Erucastrum* C. Presl (1826) (1), *Ferula* L. (1753) (1), *Goniolimon* Boiss. (1848) (1), *Jasione* L. (1753) (1), *Jurinea* Cass. (1821) (1), *Klasea* Cass. (1825) (1), *Lactuca* L. (1753) (1), *Lagurus* L. (1753) (1), *Lamium* L. (1753) (1), *Lamyropsis* (Kharadze) Dittrich (1971) (1), *Leucojum* L. (1753) (1), *Limodorum* Boehm. (1760) (1), *Mentha* L. (1753) (1), *Morisia* J. Gay (1832) (1), *Nananthea* DC. (1838) (1), *Nepeta* L. (1753) (1), *Nigritella* Rich. (1817) (1), *Oenanthe* L. (1753) (1), *Oxytropis* DC. (1802) (1), *Petagnaea* Caruel (1894) (1), *Phleum* L. (1753) (1), *Pimpinella* L. (1753) (1), *Plagius* DC. (1838) (1), *Ptilotrichum* C.A. Mey. (1831) (1), *Prospero* Salisb. (1866) (1), *Pseudoscabiosa* Davesa (1984) (1), *Ptychotis* W.D.J. Koch (1924) (1), *Quercus* L. (1753) (1), *Retama* Raf. (1838) (1), *Rhizobotrya* Tausch (1836) (1), *Ruta* L. (1753) (1), *Salicornia* L. (1753) (1), *Sideritis* L. (1753) (1), *Solenanthus* Ledeb. (1829) (1), *Solidago* L. (1753) (1), *Symphytum* L. (1753) (1), *Teucrium* L. (1753) (1), *Trachelium* L. (1753) (1), *Tripolium* Nees (1832) (1), *Urtica* L. (1753) (1), *Vinca* L. (1753) (1), *Zelkova* Spach (1841) (1).

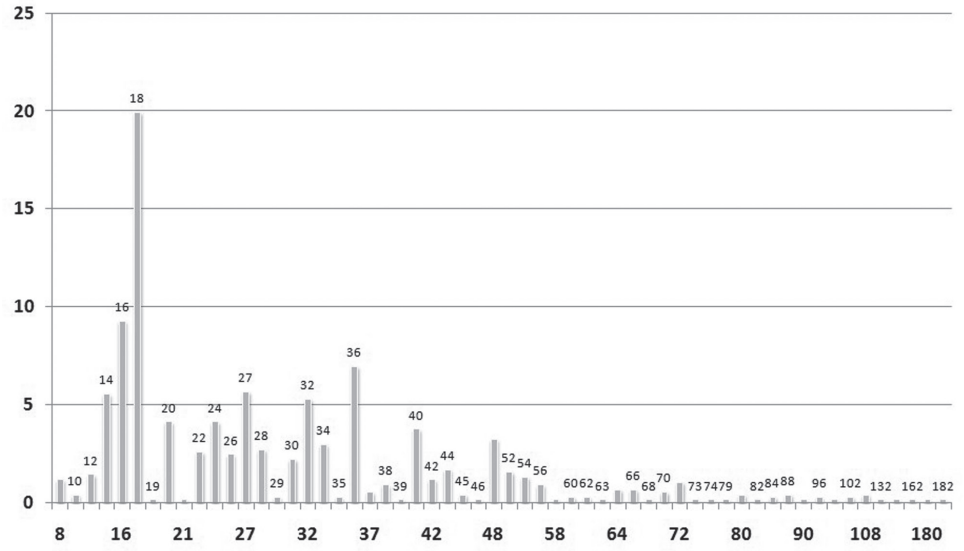

Figure 3. Histograms showing the percentage frequencies (y-axis) of 2n chromosome numbers (x-axis) known for the Italian endemic vascular flora.

Table 3. Chromosome complements up to 2n = 72, where more than one basic number (x) can occur, and respective x frequencies. The frequencies were obtained by a taxon-per-taxon literature screening. Higher chromosome numbers (very rare, in our dataset) were not considered.

2n	**Basic chromosome numbers (x)**												
	4	**5**	**6**	**7**	**8**	**9**	**10**	**12**	**14**	**16**	**18**	**20**	**21**
8	100%												
12			100%										
16	2.8%				97.2%								
20		3.1%					96.9%						
24			4.2%		20.8%			71%					
28				51%					49%				
32					71.9%					28.1%			
36						62.8%		2.3%			34.9%		
40					20.7%		62%					17.3%	
42				22%					11%				67%
	6	**7**	**8**	**9**	**11**	**12**	**13**	**14**	**18**	**22**	**28**	**32**	
44					92.3%					7.7%			
48			12%			88%							
54				90%					10%				
56		25%	12.5%					37.5%			25%		
64			80%									20%	
70		75%						25%					
72	12.5%			87.%									

The relationships among the different even ploidy levels, within each considered basic chromosome number (x = 7, x = 8, x = 9), was best described by an exponential function (Table 4). The power function provided the second-best fit, with a slightly higher RSS, followed by the logarithmic and linear functions, whose RSS is higher by an order of magnitude. Hence, diploids are much more frequent than polyploids, and frequency gradually decreases with increasing levels of polyploidy (Fig. 4).

Table 4. Goodness of fit of different models. The coefficients (a, b) matching the least-squares estimates are given for each model. RSS = Residual Sum of Squares. **= significant at 0.01 level.

formula	coef. a	coef. b	RSS	R-squared
y = a*x + b	7.786	66.714	3353.531	0.6844**
y = a*x^b	297.600	2.060	235.400	0.9780**
y = a + b*log(x)	91.940	-43.580	1422.000	0.8660**
y = e^(a + b*x)	5.539	-0.637	170.400	0.9840**

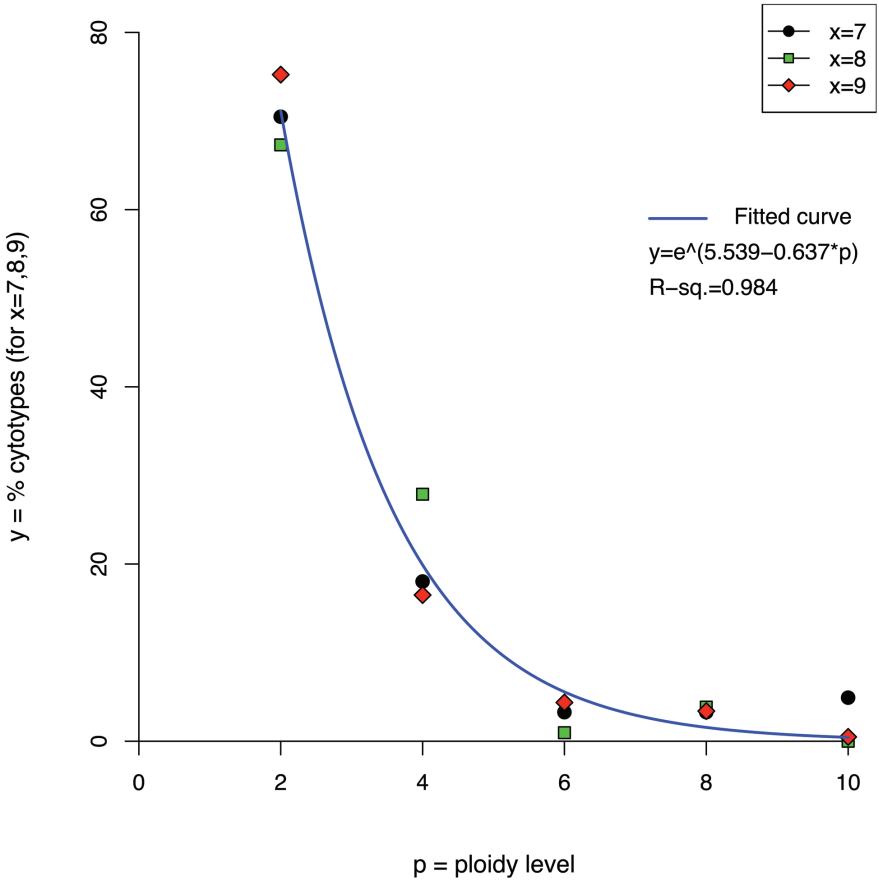

Figure 4. Plot showing the percentage frequencies (y-axis) of even ploidy levels from 2x to 10x (p-axis), for three frequent basic chromosome numbers (x = 7, x = 8 and x = 9) and the curve fitted to the data points by nonlinear least-square estimate.

More than one cytotype was shown by 65 out of 711 taxa, with a maximum number of seven in *Crocus minimus* DC. (1804) (2n = 24, 25, 26, 27, 28, 29, 30); six in *Ornithogalum etruscum* Parl. (1857) subsp. *etruscum* (Asparagaceae): 2n = 72, 73, 74, 79, 90, 108 and in *Genista sulcitana* Vals. (1986) (Fabaceae): 2n = 18 + 0–2B, 27 + 0–2B; in 12 taxa, the variation is due to the possible presence of B-chromosomes.

B-chromosomes occur in 16/711 taxa and in 24 cytotypes (3.3% of the dataset), grouped in 10/284 genera (3.5%), 9/56 families (16%). Among the taxa showing B-chromosomes, their mean number is 1.73 ± 0.91, mode = 1 and median = 1.5. The highest number of B-chromosomes is 4, from a single accession of *Agrostis monteluccii* (Selvi) Banfi (2005) (Poaceae) and a single accession of *Rhinanthus wettsteinii* (Sterneck) Soó (1929) (Orobanchaceae). Table 5 shows the families and genera involved, with respective number of taxa showing B-chromosomes. Many records are concentrated in the genus *Genista*.

Table 5. Families and genera showing B-chromosomes in Italian vascular flora endemics, with the respective number of taxa (cytotypes), and the range of Bs.

Family	Genus	No. cytotypes with B	range of Bs
Fabaceae	*Genista* L. (1753) (5 taxa)	10	1–2
Poaceae	*Agrostis* L. (1753)	4	1–4
Asparagaceae	*Ornithogalum* L. (1753)	2	1, 3
Boraginaceae	*Onosma* L. (1762)	2	1
Boraginaceae	*Pulmonaria* L. (1753)	1	2
Lamiaceae	*Scutellaria* L. (1753)	1	2
Orchidaceae	*Orchis* L. (1753)	1	1
Orobanchaceae	*Rhinanthus* L. (1753)	1	4
Primulaceae	*Primula* L. (1753)	1	1
Ranunculaceae	*Ranunculus* L. (1753)	1	1

Discussion

The taxonomic and geographic distribution of karyological knowledge in Italian endemics closely parallels that dealing with the whole Italian vascular flora (Bedini et al. 2012a). However, according to our data, Italian endemic species are significantly more studied than the whole flora, whose karyological coverage is about 35% (Bedini et al. 2012a). Central tendencies values of endemics (mean, median, mode) are very close to those known for the whole Italian flora (2n = 30.52 ± 22.09, median: 2n = 24, mode: 2n = 18) (Bedini et al. 2012a). This accounts for similar variation ranges among endemics and species with wider distribution and, probably, similar evolutionary trends. The frequency of B-chromosomes in endemics is on the contrary slightly smaller with respect to the whole Italian flora (Bedini et al. 2012a), but still much higher in comparison to other geographical areas with exceptionally high rate of endemism, such as

for instance New Zealand (Peruzzi et al. 2011). Among the taxa not yet studied, we can point out an otherwise well known species such as *Abies nebrodensis* (Lojac.) Mattei (1908), the whole endemic component of several families (i.e. Rosaceae), and many species of critical Asteraceae genera (*Centaurea*, *Hieracium*).

The precise relationship [exponential function $f(p) = e^{(5.539 - 0.637p)}$ ($R^2 = 0.984$)] found among even ploidy levels from 2*x* to 10*x* within the frequent basic chromosome numbers x = 7, x = 8 and x = 9 was never reported before in literature, as far as we are aware (see also Levin 2002 for a recent review on chromosomal changes in plant evolution). At evidence, it seems that higher the (even) ploidy level, much lower is its frequency of occurrence. This could imply a sort of evolutionary constraint avoiding high ploidy levels. This point certainly deserves further investigations. Chromosome number evolution models take into account the fixation rates of polyploids at population level (Ramsey and Schemske 1998), or the reconstruction of ancestral chromosome numbers and the expected number of polyploidization events and single chromosome changes that occurred along a phylogeny (Mayrose et al. 2010; Cusimano et al. 2012). However, the latter works may suffer from a bias toward high ancestral chromosome number estimation (Guerra 2012). In any case, no attempt has ever been made to model the ratio of different even ploidy levels on a whole flora, albeit a similar work was recently done for Polish angiosperms (Gacek et al. 2011). The latter authors, however, mainly grossly estimated ploidy levels across their dataset by means of pre-established threshold numbers.

Odd ploidy levels are generally very rare in our dataset, with the noteworthy exception of triploids with 2n = 27. As already evidenced by Bedini et al. (2012a), this is due to a high number of (endemic) apomictic taxa within the genera *Hieracium* (Asteraceae) and *Limonium* (Plumbaginaceae).

The meaning of the precise relationship found in this work must be clarified by further analyses on large datasets of chromosome counts (e.g. PhytoKaryon; Bareka et al. 2008). If confirmed, it might provide evolutionary cytogenetic research with new insights as regards the frequency of polyploidization in vascular plants and its biological implications.

Conclusions

Despite the efforts of Italian and foreign botanists in studying the endemic flora, the data here highlighted clearly show how much work is still to be done, concerning the karyological knowledge of Italian endemics. However, we were able to summarize the up-to-date knowledge, which accounts for more than one half of the endemic flora, and to suggest that these species likely followed karyological evolutionary processes similar to the whole flora. Moreover, as far as we are aware, it is the first time that a precise quantitative relationship between (even) ploidy levels is shown to occur. We demonstrated indeed that, for the frequent basic chromosome

numbers x = 7, x = 8 and x = 9 the diploids dominate and are related to higher even ploidy levels by an exponential relationship. In our mind, this intriguing phenomenon opens a new line of investigation in cytogenetics, aimed to clarify the evolutionary mechanisms giving rise to these constant relationships among increasing even ploidy levels.

Acknowledgements

Financial funding (EX60%) from University of Pisa is gratefully acknowledged.

References

Bareka P, Mitsainas GP, Constantinidis P, Kamari G (2008) Phytokaryon: a karyological database of European and Mediterranean plants. Flora Mediterranea 18: 109–116.

Bedini G, Garbari F, Peruzzi L (Eds) (2010 onwards) Chrobase.it – chromosome numbers for the Italian flora. Available from http://www.biologia.unipi.it/chrobase [accessed 20 March 2012].

Bedini G, Garbari F, Peruzzi L (2012a) Karyological knowledge of Italian vascular flora as inferred by the analysis of Chrobase.it. Plant Biosystems 145. doi: 10.1080/11263504.2011.611182

Bedini G, Garbari F, Peruzzi L (2012b) Does chromosome number count? Mapping karyological knowledge on Italian flora in a phylogenetic framework. Plant Systematics and Evolution 298: 739–750. doi: 10.1007/s00606-011-0585-1

Conti F, Abbate G, Alessandrini A, Blasi C (Eds) (2005) An annotated checklist of the Italian vascular flora. Roma, Palombi Editori, 420 pp.

Conti F, Alessandrini A, Bacchetta G, Banfi E, Barberis G, Bartolucci F, et al. (2007) Integrazioni alla checklist della flora vascolare italiana. Natura Vicentina 10 (2006): 5–74.

Cusimano N, Sousa A, Ronner SS (2012) Maximum likelihood inference implies a high, not a low, ancestral haploid chromosome number in Araceae, with a critique of the bias introduced by "*x*". Annals of Botany (London) 109(4): 681–692. doi: 10.1093/aob/mcr302

Del Prete C, Sgarbi E, Bedini G, Dallai D (1999) A database for mapping karyological data of European and Mediterranean orchids. Acta Botanica Fennica 162: 155–159.

Gacek P, Góralski G, Joachimiak AJ (2011) Chromosome numbers and polyploidy in Polish angiosperms. Acta Biologica Cracoviensia, series Botanica 53(2): 37–49. doi: 10.2478/v10182-011-0022-3

Garbari F, Bedini G, Peruzzi L (2012) Chromosome numbers of the Italian flora. From the Caryologia foundation to present. Caryologia 65. (In press). doi: 10.1080/00087114.2012.678090

Guerra M (2008) Chromosome numbers in plant cytotaxonomy: concepts and implications. Cytogenetics and Genome Research 120: 339–350. doi: 10.1159/000121083

Guerra M (2012) Cytotaxonomy: the end of childhood. Plant Biosystems 146(3). (In press).

Levin DA (2002) The role of chromosomal change in plant evolution. New York: 230, Oxford University Press, 230 pp.

Mayrose I, Barker MS, Otto SP (2010) Probabilistic models of chromosome number evolution and the inference of polyploidy. Systematic Biology 59(2): 132–144. doi: 10.1093/sysbio/syp083

Peruzzi L, Dawson MI, Bedini G (2011) Chromosome number variation in two antipodean floras. AoB Plants plr020, doi: 10.1093/aobpla/plr020

Ramsey J, Schemske DW (1998) Pathways, mechanisms, and rates of polyploid formation in flowering plants. Annual Review of Ecology and Systematics 29: 467–501. doi: 10.1146/annurev.ecolsys.29.1.467

Rossiter DG (2009) Technical note: Curve fitting with the R Environment for Statistical Computing. Enschede (NL): 17, International Institute for Geo-information Science & Earth Observations.

Stuessy TF (2009) Plant Taxonomy. The systematic evaluation of comparative data. New York, Columbia University Press, 539 pp.

CompCytogen 6(2): 213–225 (2012)
doi: 10.3897/CompCytogen.v6i2.1679
www.pensoft.net/journals/compcytogen

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
A peer-reviewed open-access journal
International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Karyotypic variation in *Rhinophylla pumilio* Peters, 1865 and comparative analysis with representatives of two subfamilies of Phyllostomidae (Chiroptera)

Anderson José Baia Gomes[1], Cleusa Yoshiko Nagamachi[1], Luís Reginaldo Ribeiro Rodrigues[2], Solange Gomes Farias[3], Jorge Dores Rissino[1], Julio Cesar Pieczarka[1]

1 *Laboratory of Cytogenetics, ICB. Guamá Campus, UFPA. Perimetral Avenue, sn. Belém, PA, Brazil, 66075-900* **2** *Laboratory of Genetics and Biodiversity, UFOPA, Tapajós Campus. Vera Paz Street, sn, Salé district, Santarém, PA, Brazil. 68.035-150* **3** *Mammals Collection, Santa Cruz State University, UESC. Ilheus-Itabuna Highway, sn. Itabuna district. Ilhéus, BA, Brazil. 45650-000*

Corresponding author: *Julio Cesar Pieczarka* (julio@ufpa.br)

Academic editor: *A. Polyakov* | Received 8 July 2011 | Accepted 24 February 2012 | Published 23 May 2012

Citation: Gomes ASB, Nagamachi CY, Rodrigues LRR, Farias SG, Rissino JD, Pieczarka JC (2012) Karyotypic variation in *Rhinophylla pumilio* Peters, 1865 and comparative analysis with representatives of two subfamilies of Phyllostomidae (Chiroptera). Comparative Cytogenetics 6(2): 213–225. doi: 10.3897/CompCytogen.v6i2.1679

Abstract

The family Phyllostomidae belongs to the most abundant and diverse group of bats in the Neotropics with more morphological traits variation at the family level than any other group within mammals. In this work, we present data of chromosome banding (G, C and Ag-NOR) and Fluorescence *In Situ* Hybridization (FISH) for representatives of *Rhinophylla pumilio* Peters, 1865 collected in four states of Brazil (Amazonas, Bahia, Mato Grosso and Pará). Two karyomorphs were found in this species: 2n=34, FN=64 in populations from western Pará and Mato Grosso states and 2n=34, FN=62 from Amazonas, Bahia, and northeastern Pará and Marajó Island (northern). Difference in the Fundamental Number is determined by variation in the size of the Nucleolar Organizer Region (NOR) accompanied with heterochromatin on chromosomes of pair 16 or, alternatively, a pericentric inversion. The C-banding technique detected constitutive heterochromatin in the centromeric regions of all chromosomes and on the distal part of the long arm of pair 15 of specimens from all localities. FISH with a DNA telomeric probe did not show any interstitial sequence, and an 18S rDNA probe and silver staining revealed the presence of NOR in the long arm of the pair 15, associated with heterochromatin, and in the short arm of the pair 16 for all specimens. The intra-specific analysis using chromosome banding did not show any significant difference between the samples. The comparative analyses using G-banding have shown that nearly all chromosomes

of *R. pumilio* were conserved in the chromosome complements of *Glossophaga soricina* Pallas, 1766, *Phyllostomus hastatus* Pallas, 1767, *Phyllostomus discolor* Wagner, 1843 and *Mimon crenulatum* Geoffroy, 1801, with a single chromosomal pair unique to *R. pumilio* (pair 15). However, two chromosomes of *M. crenulatum* are polymorphic for two independent pericentric inversions. The karyotype with 2n=34, NF=62 is probably the ancestral one for the other karyotypes described for *R. pumilio*.

Keywords
Biodiversity, Amazon rainforest, Chiroptera, cytogenetics

Introduction

Traditionally, the subfamily Carolliinae (*sensu* Wetterer et al. 2000) encompasses two genera: *Carollia* Gray, 1838 (10 species) and *Rhinophylla* Peters, 1865 (3 species) with wide distribution throughout South America. *Rhinophylla* consists of the smallest animals in the subfamily and has three currently recognized species: *Rhinophylla pumilio* Peters, 1865 and *Rhinophylla fischerae* Carter, 1966, with distribution on the east side of Andes in South America, and *Rhinophylla alethina* Handley, 1966 with distribution on the Pacific slope and lowlands of Colombia and Ecuador (McLellan and Koopman 2007).

Cytogenetic studies in Carolliinae have shown different rates of chromosomal evolution between both genera. The genus *Carollia* has two karyomorphs: 2n=20/21 with a multiple sex chromosome system (XX/XY_1Y_2), observed in most species (Yonenaga et al. 1969, Pathak et al. 1973, Stock 1975, Baker 1979, Varella-Garcia et al. 1989, Pieczarka et al. 2005), and 2n=22 with simple sex chromosome system found only in *Carollia benkeithi* Solari & Baker, 2006. On the other hand, the genus *Rhinophylla* has diversified karyotypes with four karyomorphs for *R. pumilio* (Tables 1 and 2) and two for *R. fischerae* (Baker and Bleier 1971, Baker 1979, Baker et al. 1987, Gomes et al. 2010). No karyotype has been described for *R. alethina*.

The monophyly of the subfamily Carolliinae and the sister-group relationships of *Carollia* and *Rhinophylla* have been supported by a phylogenetic analysis based on morphological data (Baker et al. 1989, Wetterer et al. 2000, Jones et al. 2002), however molecular data are in disagreement with the advanced hypotheses (Wright et al. 1999, Baker et al. 2000, 2003b). Additionally, classical cytogenetic markers failed to provide a support for the phylogenetic relationships between *Carollia* and *Rhinophylla*, since the chromosomal homeologies could not be assigned because of the reshuffled genome of *Carollia*. In contrast, *Rhinophylla* is quite comparable to other lineages and shares a lot of chromosomal characters with representatives of the subfamilies Phyllostominae, Glossophaginae, Stenodermatinae and Desmodontinae (Baker and Bickham 1980, Baker et al. 1987, 1989).

Therefore, we analyzed, through conventional cytogenetic (G-, C- banding and Ag-NOR staining) techniques and Fluorescence *In Situ* Hybridization (FISH) with rDNA and Telomere probes, two karyotypes of *R. pumilio* and discussed the biogeographical chromosome variation by comparing karyotypes of this species with representatives of two subfamilies of Phyllostomidae (Glossophaginae and Phyllostominae).

Material and methods

Specimens analyzed

Cytogenetic preparations of *R. pumilio* were obtained from 40 specimens collected in four states in Brazil: Pará state – 16 males and 13 females, Amazonas state – 1 male and 4 females, Mato Grosso state – 1 male and 4 females, Bahia state – 1 male (Fig. 1, Table 1). The bats were collected in the field using mist nets during the expeditions to faunal inventories. Comparative cytogenetic analyses were performed with *Glossophaga soricina* Pallas, 1766 (from Santa Barbara), *Phyllostomus hastatus* Pallas, 1767 (from Peixe-Boi), *Phyllostomus discolor* Wagner, 1843 (from Belém) and *Mimon crenulatum* Geoffroy, 1801 (from Faro). Chromosomal preparations and tissue biopsies were sent to the Cytogenetics Laboratory at Universidade Federal do Pará. Animals were fixed in 10% formalin preserved in 70% ethanol and deposited in the mammal's collection of the Museum Paraense Emilio Goeldi, mammal's collection of the Santa Cruz State University, Ilhéus-Bahia, Zoology Museum of the Mato Grosso Federal University and Zoology Museum of the West Pará Federal University.

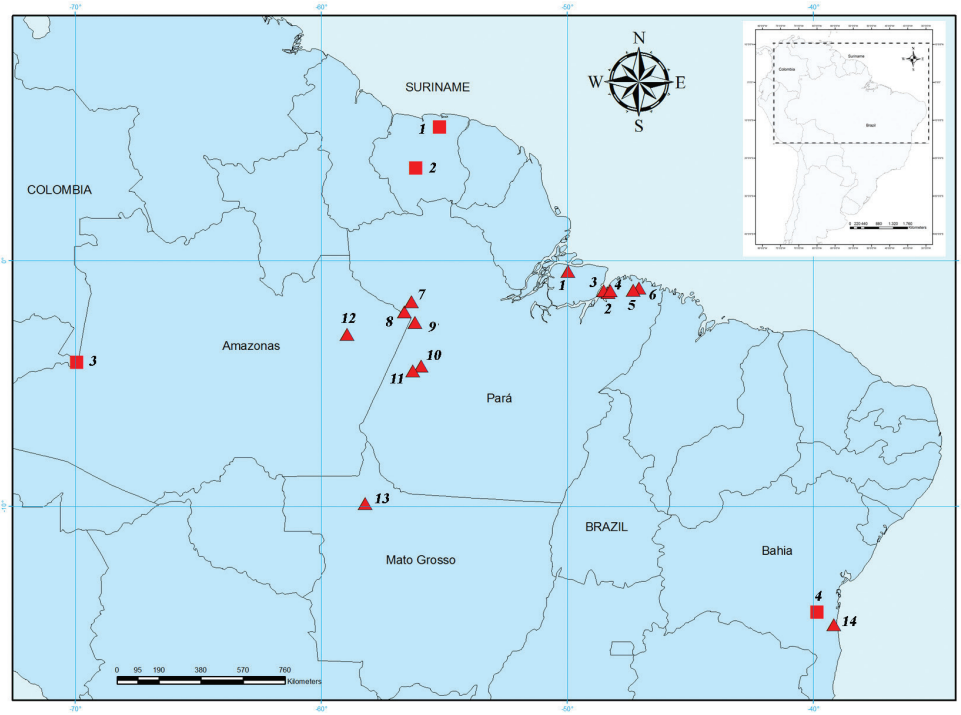

Figure 1. Map of collected samples of *Rhinophylla pumilio*. Squares indicate the sites from where previous cytogenetic descriptions were performed whereas triangles represent the cytogenetic samples studied herein (see Tables 1 and 2 for locality details). Numbers of sites correspond to numbers on Tables 1 and 2.

Table 1. Cytogenetic samples of *Rhinophylla pumilio* from different localities. Numbers of sites correspond to numbers of triangles on the map (Fig. 1).

Site	n	Locality/State	2N/FN	Methods	Geographical coordinates
1	1♂+1♀	Chaves, Pará	34/62	G	00°24'55.3"S; 49°58'44.1"W
1	3♀		34/62		
2	1♂	Marituba, Pará	34/62	G, C	01°16'37.5"S; 48°20'14.9"W
3	1♂	Belém, Pará	34/62	G, C, NOR, Telomere, rDNA, CMA3	01°13'29.3"S; 48°32'59.0"W
3	1♂		34/62	G, C	
4	1♂+1♀	Santa Barbara, Pará	34/62	G	01°13'57.4"S; 48°16'34.4"W
4	4♂+2♀		34/62		
5	1♀	Capanema, Pará	34/62	C	01°24'02.5"S; 48°29'02.4"W
6	1♂	Peixe-Boi, Pará	34/62	G, C	01°11'11.0"S; 47°19'28.5"W
6	1♂		34/62	G, C, rDNA, CMA3	
7	2♂+1♀	Oriximiná, Pará	34/62	G, C	01°39'03.3"S; 56°20'30.6"W
8	1♀	Faro, Pará	34/62	G, C	02°03'53.1"S; 56°37'57.4"W
9	1♂	Juruti, Pará	34/64	G, C, NOR, rDNA	02°29'38.8"S; 56°11'27.1"W
9	1♀		34/64	G, C, rDNA	
10	1♀	Itaituba, Pará	34/64		04°16'26.6"S; 55°56'47.6"W
10	1♂		34/64	G, C, rDNA, CMA3	
11	1♂+1♀	Itaituba, Pará	34/64	G, C	04°28'20.5"S; 56°17'03.7"W
12	1♂+3♀	Itacoatiara, Amazonas	34/62	G, C	02°58'49.6"S; 58°57'51.0"W
12	1♀		34/62		
13	1♂+4♀	Potriguaçú, Mato Grosso	34/64	G, C	09°51'53.7"S; 58°13'06.8"W
14	1♂	Ilhéus, Bahia	34/62	G, C, NOR	14°47'52.0"S; 39°10'15.0"W

Chromosomal preparation and cell culture

The chromosome spreads were obtained from bone marrow following Baker et al. (2003a) and fibroblast primary culture following the protocols by Moratelli et al. (2002), and conventionally stained. The G-banding patterns were obtained with pepsin solution, subsequent incubation in saline solution (0,5 X SSC) at 60ºC and staining with Wright's solution following Verma and Babu (1995). The C-banding was carried out following Sumner (1972), detection of Nucleolar Organizer Regions was performed according to Howell and Black (1980) and double staining with DAPI - CMA_3 was performed according to Schweizer (1980).

Fluorescence *In Situ* Hybridization (FISH)

Fluorescence *In Situ* Hybridization using digoxigenin-labeled telomeric probes (All Human Telomere Probes, Oncor) was performed according to the manufacturer's protocol. To confirm the position of the NORs, 18S rDNA probes were amplified by

BACs (Bacterial Artificial Chromosomes), labeled by nick translation and subsequently detected with avidin-Cy3 or anti-digoxigenin- FITC. Briefly, the slides were incubated in RNAse and pepsin solutions following Martins and Galetti (1998). The slides were dehydrated in ethanol series (70%, 90% and 100%), aged in a 65°C incubator for one hour, and denatured in 70% formamide/2 X SSC for one minute. The labeled probe (2 µl) was diluted in 10 µl of hybridization buffer (50% deionized formamide, 10% dextran sulfate, 0,5 M phosphate buffer 7,3 pH, 1x Denhardt's solution), denatured at 70ºC for 15 minutes, and dropped on the slide with the denatured chromosome preparation, which was then mounted with a 24 × 24 mm coverslip. Slides then were incubated overnight at 37ºC. The hybridization signal was detected with avidin-Cy3 as described previously (Yang et al. 1995, Pieczarka et al. 2005). The images were captured with an Axiocam Mrm CCD camera coupled on a Zeiss Axioplan 2 microscope using the Axiovision 3.0 software. The chromosomes were identified according to their morphology and inverted banding patterns using DAPI (4',6-diamidino-2-phenylindole).

Results

All studied specimens of *R. pumilio* have the same chromosome number – 2n=34. The autosomal complement consists of 15 pairs biarmed (metacentric and submetacentric) and one pair of acrocentric chromosomes (pair 16) in samples collected from Bahia, Amazonas, northeastern Pará and Marajó Island (north of Para) (Fig. 2a). In contrast, the chromosome pair 16 of specimens from west Pará and Mato Grosso is biarmed (Fig. 3a). The X chromosome is a medium-sized metacentric chromosome and the Y is a small acrocentric.

The constitutive heterochromatin was found in the centromeric regions of all chromosomes and at the distal part of the long arm of pair 15 for all specimens (Fig. 2b). Telomere sequences were observed at the tips of chromosomes (Fig. 2d). The rDNA probes and staining with silver nitrate confirmed the presence of NORs in the long arm of the pair 15 and short arm of the pair 16 (Fig. 2c). The FISH with rDNA and subsequent double staining with DAPI and CMA_3 are in agreement with the patterns of G-bands and R-bands, respectively, where the R-bands show the tips of the chromosomes and its association with the NOR (Fig. 3b).

The comparative analysis with *P. hastatus, P. discolor*, *M. crenulatum* (Phyllostominae) and *G. soricina* (Glossophaginae) (Fig. 4a) suggests that the karyotypes of *R. pumilio* here described have nearly all chromosome pairs shared with these species, although one pair was autapomorphic to *R. pumilio* (Fig. 4b). Analyzed species are different in the number of chromosomes (34 in *R. pumilio* and 32 in other species) and the fundamental number (58 in *P. hastatus*, 60 in *M. crenulatum*, *P. discolor*, *G. soricina* and 62/64 in *R. pumilio*). The heterochromatin presents in the centromeric regions of all species with additional blocks in the short and long arms of the 15th pair of *M. crenulatum* and *G. soricina*, respectively. Chromosomes of 5th and 6th pairs of *M. crenulatum* exhibit two polymorphic conditions derived probably from pericentric

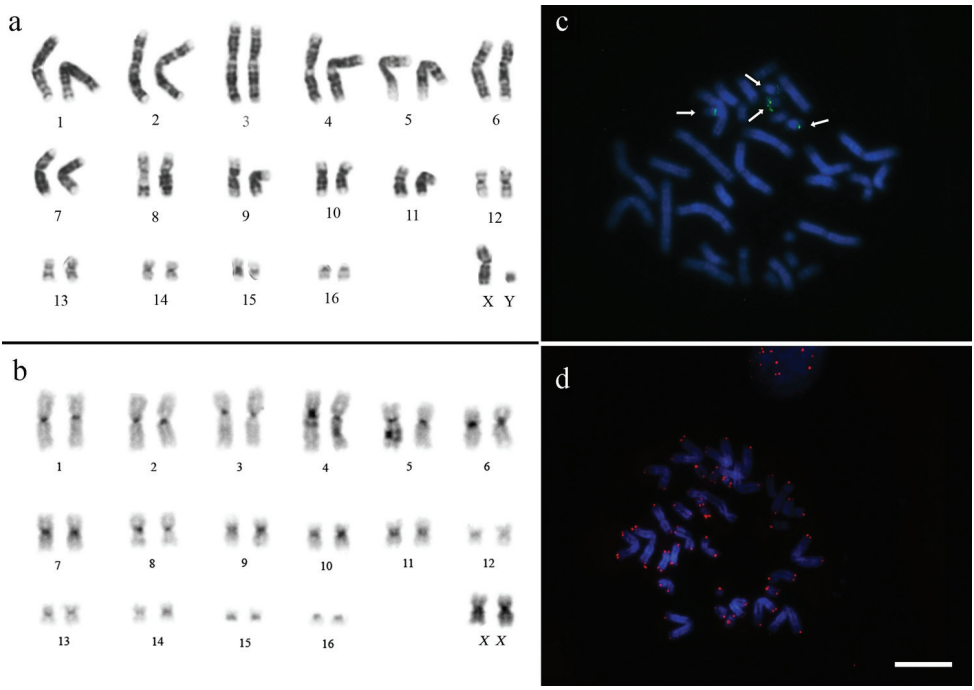

Figure 2. Karyotypes of *Rhinophylla pumilio* from northeastern Pará (except C-banding obtained from specimens from Amazonas state) **a** G-banding **b** C-banding **c** 18S rDNA FISH and **d** telomeric FISH. Arrows show NORs in the chromosome pairs 15 and 16. Bar = 10 μm.

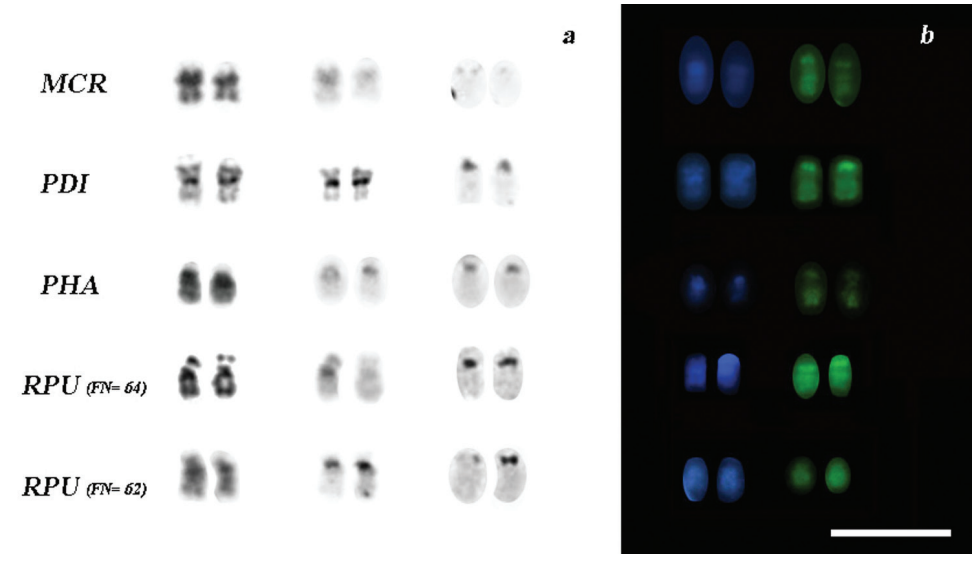

Figure 3. Variation of chromosome pair 15 (16 in *Rhinophylla pumilio*) in the analyzed species **a** chromosomes after G, C and Ag-NOR sequential staining **b** patterns of double staining with DAPI-CMA_3. Bar = 10 μm.

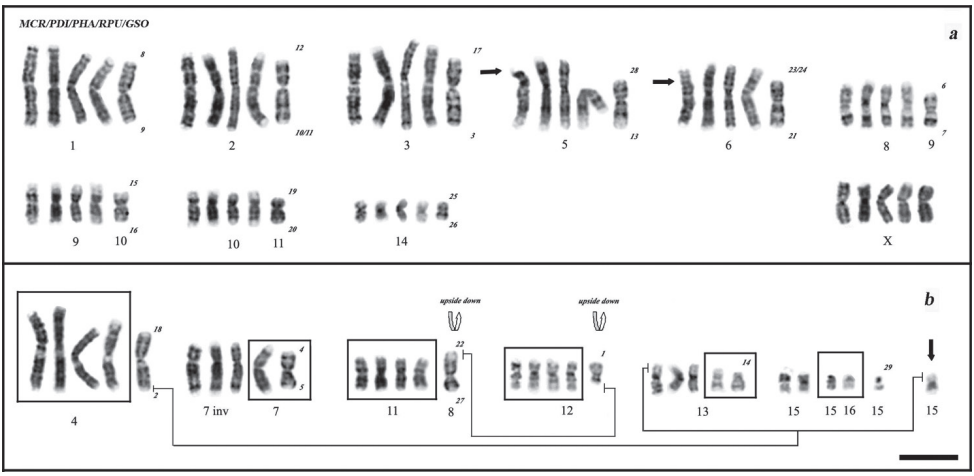

Figure 4. Comparative analysis using G-banded chromosomes of *Mimon crenulatum*, *Phyllostomus discolor*, *Phyllostomus hastatus*, *Rhinophylla pumilio* and *Glossophaga soricina*, from left to right **a** Conserved chromosomes among species, arrows show the centromeric position in *M. crenulatum* **b** Chromosomal differences among species. Black arrow indicates autapomorphic chromosome in *R. pumilio*. Numbers (beside *G. soricina*) correspond to the chromosomal nomenclature applied to arms of *Macrotus waterhousii* in *G. soricina* according to Baker and Bass (1979). Bar = 10 μm.

inversions that could cause the acrocentric and subtelocentric forms, respectively. Both specimens are heterozygous for 6th pair and homozygous for normal and rearranged forms of 5th chromosome pair. The NORs in this species are localized in the short arm of 15th pair and in the Y chromosome.

Discussion

Intraspecific variation in *Rhinophylla pumilio*

Our G-, C-, and Ag-NOR banding analyses have shown two distinct karyotypes for specimens of *R. pumilio* from localities ranging more than 1000 km. The differences between these karyotypes may be caused by a pericentric inversion in the chromosome pair 16 or, alternatively, an amplification of rDNA cistrons accompanied with a faint block of heterochromatin in *R. pumilio* with FN=64 (Fig. 3a). This segment is coincident with CMA_3 positive staining for NOR and DAPI positive to the heterochromatic block (Fig. 3b).

Comparative analysis of karyotypes from different geographic localities (Table 2) allows discussing the morphology and number of chromosomes. Since only data of conventional staining or karyotype formula were described in the literature we had to restrict our comparisons to number and basic morphology of chromosomes. In this way, specimens of *R. pumilio* collected on the Marajó island and northeastern Pará (Fig. 1, triangles 1, 2, 3, 4, 5, and 6) in the left side of the Amazon basin on Pará and

Table 2. Previous cytogenetic studies on *Rhinophylla pumilio*. Numbers of sites correspond to numbers of squares on the map (Fig. 1).

Site	Region	Geographical coordinates	2n/FN	References
1	Suriname	05°27'00"S; 55°12'00"W	34/64	Honeycutt et al. 1980, Baker et al. 1981
2	Suriname	03°46'00"S; 56°10'00"W	34/56	Baker and Bickham 1980
3	Colombia	04°07'43"S; 69°56'37"W	36/62	Baker and Bleier 1971
4	Brazil-Bahia	14°17'29"S; 39°51'18"W	26/48	Toledo 1973

Amazonas (triangles 7, 8 and 12) and Bahia (triangle 14) have 2n=34 and FN=62. Meanwhile, the samples from western Pará (triangles 9, 10 and 11) and Mato Grosso (triangle 13) presented the same fundamental number as specimens collected from Suriname, with 2n=34, FN=64 (Honeycutt et al. 1980, Baker et al. 1981, square 1).

Karyotype with 2n=26 and FN=48 described by Toledo (1973) (Fig. 1, Bahia, square 4) was found only in 100 km from the collection site of our sample with 2n=34 and NF=62. Varella-Garcia et al. (1989) suggested that the chromosome differences between populations of *R. pumilio* described by Toledo (1973) and Baker and Bleier (1971) would be enough to reach the reproductive isolation between them. Nevertheless, analysis of mithocondrial DNA did not reveal sufficient genetic distance (0,3%) between two specimens from Northeastern Brazil (Pernambuco and Bahia) (Ditchfield 2000). Such distance is commonly observed within a breeding population. A re-analysis of the chromosome data from Toledo (1973) showed a disagreement with respect to the small size of the X chromosome and discordant number of chromosomes in mitotic and meiotic cells.

Another cytogenetic study on specimens of *R. pumilio* from Colombia described a karyotype with 2n=36 and FN=62, (Baker and Bleier 1971, Fig. 1, square 3), differing from populations with 2n=34 and FN=62 probably by a chromosome fusion/fission event. Bats with karyotypes 2n=34, FN=56 (Baker and Bickham 1980, square 2) and 2n=34, FN=64 (Honeycutt et al. 1980, Baker et al. 1981, square 1) could be probably found in sympatry on the territory of Suriname.

Intergeneric comparative analysis

Comparative analysis of chromosome banding patterns of *R. pumilio* was undertaken with representatives of two other subfamilies of Phyllostomidae bats: *P. hastatus*, *P. discolor*, *M. crenulatum* (Phyllostominae) and *G. soricina* (Glossophaginae). Karyotypes of these species supposed to be ancestral for their respective subfamilies (Patton and Baker 1978, Baker and Bass 1979, Baker and Bickham 1980, Haiduk and Baker 1982, Baker et al. 1989) and karyotype of *R. pumilio* with 2n=34 and FN=56 described by Baker and Bickham (1980) revealed several characters shared with the above mentioned species.

Comparative analysis revealed that there are an extensive number of conserved chromosomes shared among these species. However, *R. pumilio* shared more charac-

ters with Phyllostominae species than *G. soricina* (Fig. 4b). Based on outgroup comparisons, Baker and Bickham (1980) proposed that the most primitive karyotype for the family Phyllostomidae is identical to that of *Macrotus waterhousii* Gray, 1843. This hypothesis together with the basal position of *M. waterhousii* in recent phylogenies (Baker et al. 2000, 2003b, Datzmann et al. 2010) allows to suppose the most basal nature of chromosome pairs 12 and 8q of *G. soricina* because they are homologous to the acrocentric element 22 and to short arm of the biarmed element 1/2 of *M. waterhousii*, respectively (in Baker and Bass 1979). However, we suggest that in the basal branch that led to peculiarity of chromosome pairs 11 and 12 of *P. hastatus*, *P. discolor*, *M. crenulatum* and *R. pumilio*, the same chromosomes (12 and 8q of *G. soricina*) could be involved in a simple translocation from a segment on the long arm of pair 8 to short arm of the pair 12 of *G. soricina*. Alternatively, the same chromosomes would be synapomorphic in *G. soricina*, as well as in some species of the Glossophaginae subfamily, and symplesiomorphic in other species analyzed here.

Furthermore, other differences among karyotypes (Fig. 4b) are a pericentric inversion on pair 7 of *P. hastatus* (Patton and Baker 1978) and a simple translocation involving the pairs 4 and 13 of this species as was observed by Pieczarka et al. (2005). Such events are symplesiomorphic in *G. soricina*, synapomorphic in Phyllostominae species and probably autoapomorphic in *R. pumilio* (pair 15). Integration of data derived from multidirectional chromosome painting with chromosome probes of *Carollia brevicauda* Schinz, 1821 and *P. hastatus* on metaphase spreads of *G. soricina* and chromosome map using probes of human chromosomes in the last species (Volleth et al. 1999) have shown that the basal position of *G. soricina* is supported by the fact that the pair 6 of human chromosomes was not disrupted. This chromosome has been assumed to be disrupted and subsequently fused with chromosome 13 of the Phyllostominae group, whereas this small segment forms an independent pair 15 in *R. pumilio* (unpublished data).

Another interesting problem in our comparative analysis is the pair 16 in *R. pumilio*, which has two chromosomal traits similar to those observed within representatives of genus *Phyllostomus* Lacépède, 1799. The difference between the karyotypes of *P. hastatus* and *P. discolor* consists of a pericentric inversion of the pair 15 (Patton and Baker 1978, Rodrigues et al. 2000). This chromosome is biarmed in *P. discolor* and acrocentric in *P. hastatus*, *P. elongatus* Geoffroy, 1810, *P. latifolius*_Thomas, 1901 and *Phylloderma stenops* Peters, 1865 (Baker 1979, Baker and Bickham 1980, Honeycutt et al. 1980, Santos et al. 2002). Rodrigues et al. (2000) suggested that the biarmed state of pair 15 of *P. discolor* could be most basal, because it has been shared with *M. crenulatum*, considered the most basal for the genus, and because this chromosome seems to be the result of a fusion of two acrocentric chromosomes of *M. waterhousii* (Patton and Baker 1978). The other species of *Phyllostomus* along with *P. stenops* form a clade supported by the acrocentric form of the pair 15. However, the three species analyzed in this work showed different forms of the biarmed pair 15 (16 in *R. pumilio*). The short arm of *M. crenulatum* represents a block of heterochromatin followed by the NOR, whereas in *R. pumilio* the NOR appears before the heterochromatin. On the other hand, in *G. soricina* the NOR is represented at

the long arm near the centromeric region accompanied by a heterochromatic block. Figure 3 shows the pattern of G- C and NOR sequential staining of pair 15 (16 in *R. pumilio*) as well as the pattern of A/T-G/C evidenced by double staining with fluorescence DAPI and CMA_3. The more plausible explanation is that the biarmness appeared in different branches of Phyllostomidae bats by amplification of rDNA cistrons accompanied or not with addition of heterochromatin, and possibly with other types of rearrangements.

Baker et al. (1972) defined three morphological types (submetacentric, acrocentric and subtelocentric) for the 5th chromosome pair of *M. crenulatum* at localities encompassing a wide geographic distance (Trinidad, Peru and Colombia). In this work, we have collected two specimens geographically apart from sites studied by Baker et al. (1972). We have found similar morphological types but G-banding analysis revealed that the acrocentric chromosome belonged to the 5th pair and the subtelocentric – to the 6th pair. That means that this polymorphism is defined by two pairs of chromosomes instead of one as it was suggested earlier.

Among species of genus *Carollia* karyotypes are highly rearranged and after the reciprocal chromosome painting Pieczarka et al. (2005) found only two chromosomes conserved *in toto* between *C. brevicauda* (pairs 7 and 9) and *P. hastatus* (pairs 11 and 14). This finding suggests that they represent probably a part of the ancestral karyotype of Phyllostomidae, since they are preserved in such phylogenetically remote species. In the genus *Rhinophylla* these shared chromosomes are also presented by pairs 11 and 14 and can be also observed in others species studied herein except for the 8th pair of *G. soricina* that is partially homologous to the 11th pair of *R. pumilio*. Therefore an analysis of the chromosomes homology among other species, especially those closely related to the genus *Carollia*, will be necessary to corroborate the sister group relationships of the genus *Carollia* and *Rhinophylla*.

Finally, we believe that variation of karyotypes along the area of *R. pumilio* is correlated with intraspecific variation where the karyomorphs would be derived from ancestral karyotype with 2n=34, FN=62, since this karyotype is similar to other close related species at the chromosome level. However, additional analyses will be necessary to elucidate the biogeographical patterns related to the chromosome variation in *R. pumilio*.

Acknowledgments

We thank to Manoel Rodrigues (Juris Ambientes Consultores), Flávio Eduardo Pimenta and Ana lima (Aotus Consultoria), Pablo Suarez, Dionísio Pimentel, Heriberto Figueria and the staff of CNEC- Juruti for Help us in the field expedition in the area of Alcoa's Juruti Bauxite mine. We thank to Biodinamica-Rio, Aotus Consultoria and Talita Ribas, Eloiza Soarez, Fabio Sarmento, Fabio Augustos and Ramon Araujo for help us in the bats expedition in the Electric power transmission from Oriximina (Pará state) to Silves (Amazonas state) and Thayse Benathar for the chromosome preparation from Mato Grosso state and in the area TERFRON project on Itaituba. We are grateful

to Cibele Sotero-Caio for a critical analysis of this work. This research was supported by CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), CAPES (Conselho Aperfeiçoamento de Pessoal de Nível Superior), UFOPa (Universidade Federal do Oeste do Pará) and UFPa (Universidade Federal do Pará).

References

Baker RJ, Bleier WJ (1971) Karyotypes of bats of the subfamily Carolliinae (Mammalia; Phyllostomatidae) and their evolutionary implications. Experientia 27: 220–222. doi: 10.1007/BF02145907

Baker RJ, Gardner AL, Patton JL (1972) Chromosomal polymorphism in the phyllostomatid bat, *Mimon crenulatum* (Geoffroy). Experientia 28: 969–970. doi: 10.1007/BF01924978

Baker RJ, Bass RA (1979) Evolutionary relationship of the Brachyphyllinae to Glossophaginae genera *Glossophaga* and *Monophyllus*. Journal of Mammalogy 60: 364–372. doi: 10.2307/1379808

Baker RJ (1979) Karyology. In: Baker RJ, Jones JK Jr., Carter DC (Eds), Biology of the bats of the New World, family Phyllostomatidae, p. III. Special Publications of the Museum of Texas Tech University 16: 1107–155.

Baker RJ, Bickham JW (1980) Karyotipic Evolution in bats: Evidence of extensive and conservative chromosomal evolution in closely related taxa. Systematic Zoology 29: 239–253. doi: 10.2307/2412660

Baker RJ, Genoways HH, Seyfarth PA (1981) Results of the Alcoa Foundation–Suriname Expeditions. VI. Additional chromosomal data for bats (Mammalia: Chiroptera) from Suriname. Annals of Carnegie Museum 50: 333–344.

Baker RJ, Qumsiyeh MB, Hood CS (1987) Role of chromosomal banding patterns in understanding mammalian evolution. In: Genoways HH (Ed) Current Mammalogy. Plenum Press, New York, 67–96.

Baker RJ, Hood CS, Honeycutt RL (1989) Phylogenetic relationships and classification of the higher categories of the New World bat family Phyllostomidae. Systematic Zoology 38: 228–238. doi: 10.2307/2992284

Baker RJ, Porter CA, Patton JC, Van Den Bussche RA (2000) Systematics of bats of the family Phyllostomidae based on RAG2 DNA sequences. Occasional Paper of the Museum of Texas Tech University 202: 1–16.

Baker RJ, Hamilton M, Parish DA (2003a) Preparations of Mammalian karyotypes under field conditions. Occasional Paper of the Museum of Texas Tech University 228: 1–7.

Baker RJ, Hoofer SR, Porter CA, Van Den Bussche RA (2003b) Diversification among New World leaf-nosed bats: an evolutionary hypothesis and classification inferred from digenomic congruence of DNA sequence. Occasional Paper of the Museum of Texas Tech University 230: 1–32.

Datzmann T, Helversen OV, Mayer F (2010) Evolution of nectarivory in phyllostomid bats (Phyllostomidae Gray, 1825, Chiroptera: Mammalia). BMC Evolutionary Biology 10: 165. doi: 10.1186/1471-2148-10-165

Ditchfield AD (2000) The comparative phylogeography of Neotropical mammals: patterns of intraspecific mitochondrial DNA variation among bats contrasted to nonvolant small mammals. Molecular Ecology 9: 1307–1318. doi: 10.1046/j.1365-294x.2000.01013.x

Gomes AJB, Rodrigues LRR, Rissino JD, Nagamachi CY, Pieczarka JC (2010) Biogeographical karyotypic variation of *Rhinophylla fischerae* (Chiroptera, Phyllostomidae) suggests the occurrence of cryptic species. Comparative Cytogenetics 4: 79–85. doi: 10.3897/compcytogen.v4i1.24

Haiduk MW, Baker RJ (1982) Cladistical analysis of G-banded chromosomes of nectar-feeding bats (Glossophaginae, Phyllostomidae). Systematic Zoology 31: 252–265. doi: 10.2307/2413231

Honeycutt RL, Baker RJ, Genoways HH (1980) Results of the Alcoa Foundation-Suriname expeditions. III. Chromosomal data for bats (Mammalia: Chiroptera) from Suriname. Annals of Carnegie Museum 49: 237–250.

Howell WM, Black DA (1980) Controlled silver-staining of nucleolar organizer regions with protective colloidal developer: a 1-step method. Experientia 36: 1014–1015. doi: 10.1007/BF01953855

Jones JK, Purvis A, Maclarnon A, Bininda-Emonds ORP, Simmons NB (2002) A phylogenetic supertree of the bats (Mammalia: Chiroptera). Biological Reviews 77: 223–259. doi: 10.1017/S1464793101005899

Martins C, Galetti PM Jr (1998) Karyotype similarity between two sympatric Schizodon fish species (Anostomidae, Characiformes) from Paraguay River basin. Genetics and Molecular Biology 21: 355–360. doi: 10.1590/S1415-47571998000300011

McLellan LJ, Koopman KF (2007) Subfamily Carolliinae Miller, 1924. In: A. L. Gardner (ed) Mammals of South America. Volume 1. University of Chicago Press, Chicago, 208–218.

Moratelli R, Andrade CM, Armada JLA (2002) A technique to obtain fibroblast cells from skin biopsies of living bats (Chiroptera) for cytogenetic studies. Genetics and Molecular Research 2: 128–130.

Pathak S, Hsu TC, Utakoji T (1973) Relationships between patterns of chromosome banding and DNA synthetic sequences: a study on the chromosomes of the Seba's fruit bat *Carollia perspicilata*. Cytogenetics and Cell Genetics 12: 157–164. doi: 10.1159/000130451

Patton JC, Baker RJ (1978) Chromosomal homology and evolution of phyllostomatoids bats. Systematic Zoology 27: 449–462. doi: 10.2307/2412927

Pieczarka JC, Nagamachi CY, O'Brien PCM, Yang F, Ren W, Barros RMS, Noronha RCR, Rissino J, Oliveira EHC, Ferguson-Smith MA (2005) Reciprocal chromosome painting between two South American bats: *Carollia brevicauda* and *Phyllostomus hastatus* (Phyllostomidae, Chiroptera). Chromosome Research 13: 349–347. doi: 10.1007/s10577-005-2886-0

Rodrigues LRR, Barros RMS, Marques-Aguiar S, Assis MFL, Pieczarka JC, Nagamachi CY (2000) Chromosome comparison between two species of Phyllsotomus (Chiroptera-Phyllostomidae) from Eastern Amazonia, with some phylogenetic insights. Genetics and Molecular Biology 3: 593–599.

Santos N, Fagundes V, Yonenaga-Yassuda Y, Souza MJ (2002) Localization of rDNA genes in Phyllostomidae bats reveals silent NORs in *Artibeus obscurus*. Hereditas 136: 137–143. doi: 10.1034/j.1601-5223.2002.1360208.x

Schweizer D (1980) Simultaneous fluorescent staining of R bands and specific heterochromatic region. l (DA-DAPI bands) in human chromosomes. Cytogenetics and Cell Genetics 27: 190–193. doi: 10.1159/000131482

Solari S, Baker RJ (2006) Mitochondrial DNA sequence, karyotypic, and Morphological variation in the *Carollia castanea* species complex (Chiroptera: Phyllostomidae) with description of the new species. Occasional Paper of the Museum of Texas Tech University 254: 1–16.

Stock AD (1975) Chromosome banding pattern homology and its phylogenetic implications in the bat genera *Carollia* and *Choeroniscus*. Cytogenetics and Cell Genetics 14: 34-41. doi: 10.1159/000130317

Sumner AT (1972) A simple technique for demonstrating centromeric heterochromatin. Experimental Cell Research 75: 304–306. doi: 10.1016/0014-4827(72)90558-7

Toledo LA (1973) Estudos citogenéticos em morcego brasileiros (Mammalia Chiroptera). Doctorship Thesis, Faculdade de Ciências Medicas e Biológicas de Botucatu, USP, Botucatu, Sao Paulo, Brasil, 90pp.

Varella-Garcia M, Morielle-Versute E, Taddei VA (1989) A survey of cytogenetic data on brazilian bats. Revista Brasileira de Genética 12: 761–793.

Verma RS, Babu A (1995) Human chromosomes: Principles and techniques. 2nd ed., Mcgrew-Hill, inc. Health profession division, New York, 419 pp.

Volleth M, Klett C, Kollak A, Dixkens C, Winter Y, Just W, Vogel W, Hameister H (1999) ZOO-FISH analysis in a species of the order Chiroptera: *Glossophaga soricina* (Phyllostomidae). Chromosome Research 7: 57–64. doi: 10.1023/A:1009227428727

Wetterer AL, Rockman MV, Simmons NB (2000) Phylogeny of phyllostomid bats (Mammalia: Chiroptera): data from diverse morphological systems, sex chromosomes and restriction sites. Bulletin of the American Museum of Natural History, New York, 248: 1–200. doi: 10.1206/0003-0090(2000)248<0001:POPBMC>2.0.CO;2

Wright AJ, Van Den Bussche RA, Lim BK, Engstrom MD, Baker RJ (1999) Systematics of the genera C*arollia* and *Rhinophylla* based on the cytochrome-b gene. Journal of Mammalogy 80:1202–1213. doi: 10.2307/1383171

Yang F, Carter NP, Shi L, Ferguson-Smith MA (1995) A comparative study of karyotypes of muntjacs by chromosome painting. Chromosoma 103: 642–652. doi: 10.1007/BF00357691

Yonenaga Y, Frota-Pessoa O, Lewis KR (1969) Karyotypes of seven species of Brazilian bats. Caryologia 22: 63–78.