CompCytogen 6(4): 335–340 (2012)
doi: 10.3897/CompCytogen.v6i4.3519
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 analysis of four species of Chilean Chrysomelinae (Coleoptera, Chrysomelidae)

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Academic editor: *C. Nokkala* | Received 12 July 2012 | Accepted 21 September 2012 | Published 5 October 2012

Citation: Petitpierre E, Elgueta M (2012) A chromosomal analysis of four species of Chilean Chrysomelinae (Coleoptera, Chrysomelidae). Comparative Cytogenetics 6(4): 335–340. doi: 10.3897/CompCytogen.v6i4.3519

Abstract

Four species of Chilean leaf beetles in the subfamily Chrysomelinae have been cytogenetically analyzed, *Blaptea elguetai* Petitpierre, 2011, *Henicotherus porteri* Bréthes, 1929 and *Jolivetia obscura* (Philippi, 1864) show 2n = 28 chromosomes and a 13 + Xy_p male meioformula, and *Pataya nitida* (Philippi, 1864) has the highest number of 2n = 38 chromosomes. The karyotype of *H. porteri* is made of mostly small meta/submetacentric chromosomes, and that of *Jolivetia obscura* displays striking procentric blocks of heterochromatin at pachytene autosomic bivalents using conventional staining. These findings are discussed in relation to previous cytogenetic data and current taxonomy of the subfamily.

Keywords

Coleoptera, Chrysomelidae, Chrysomelinae, karyotypes, cytotaxonomy

Introduction

The subfamily Chrysomelinae is a group of mostly quite large or medium sized leaf beetles mainly distributed in cool and temperate regions of the world, which are composed of 133 genera (Daccordi 1994), and nearly 3000 species worldwide (Farrell 1998; Reid et al. 2009)

From the cytogenetic standpoints, this subfamily is relatively well-known since nearly 260 taxa and chromosomal races in 38 genera have been surveyed to date (Petitpierre 2011a). In a previous cytogenetic study, we analyzed three Chilean species of Chrysomelinae (Petitpierre and Elgueta 2006), belonging to three of the ten genera so

far found in the country (Daccordi 1994). We have here enlarged this research with four additional species and genera from Chile, of which three, *Henicotherus* Bréthes, 1929, *Jolivetia* Bechyné, 1946 and *Pataya* Bechyné, 1946, are endemics for this geographic subregion in the Neotropics (Daccordi 1994), and the fourth, *Blaptea* Weise, 1915, has only one other species, in Colombia and Brazil (Daccordi 1994, Petitpierre 2011b).

Material and methods

The checked species and their origins are reported in Table 1.

The surveyed individuals of *Blaptea elguetai, Jolivetia obscura* and *Pataya nitida* were caught by sweeping on their host plants *Tropaeolum brachyceras* Hook. et Arn., 1830 (Tropaeolaceae), *Aristeguietia salvia* (Colla) R.M. King et H. Rob., 1975 (Asteraceae) and *Ageratina glechonophylla* (Less.) R.M. King et H. Rob., 1970 (Asteraceae), in October 2009, October 2006 and November 2007, respectively, and those of *Henicotherus porteri* were caught by hand under stones in October 2009. At least two individuals from each species have been cytogenetically studied.

The chromosome analyses were only performed on male living individuals brought from Chile to our laboratory in Palma de Mallorca (Spain), where they were killed with ethyl acetate. Then, the procedure to get the conventional staining preparations was the same used before in our previous paper (Petitpierre and Elgueta 2006). Finally, we obtained micrographs by a ZEISS AXIOSKOP photomicroscope and subsequently enlarged them for printing at X1500.

Table 1. Chromosomally checked species and their Chilean geographical sources.

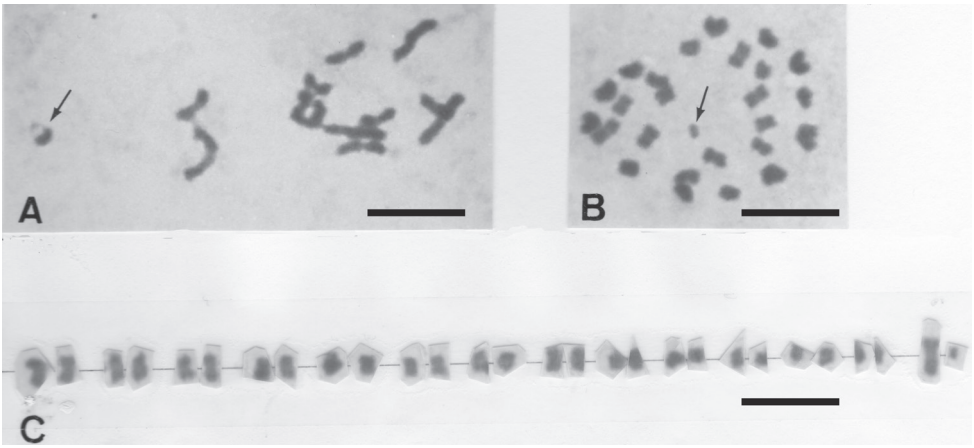
Blaptea elguetai Petitpierre, 2011	Isla Negra, prov. San Antonio, Reg. Valparaíso
Henicotherus porteri Bréthes, 1929	Mincha 2 km W, prov. Choapa, Reg. Coquimbo
Jolivetia obscura (Philippi, 1864)	Isla Negra, prov. San Antonio, Reg. Valparaíso
Pataya nitida (Philippi, 1864)	Isla Negra, prov. San Antonio, Reg. Valparaíso

Results

Tribe Chrysomelini
Subtribe Entomoscelina

Blaptea elguetai has 2n = 28 chromosomes and a 13 + Xy_p male meioformula of medium and small autosomal bivalents plus the Xy_p "parachute" sex-chromosome system where most of these autosomic bivalents are rod-shaped (Fig. 1A).

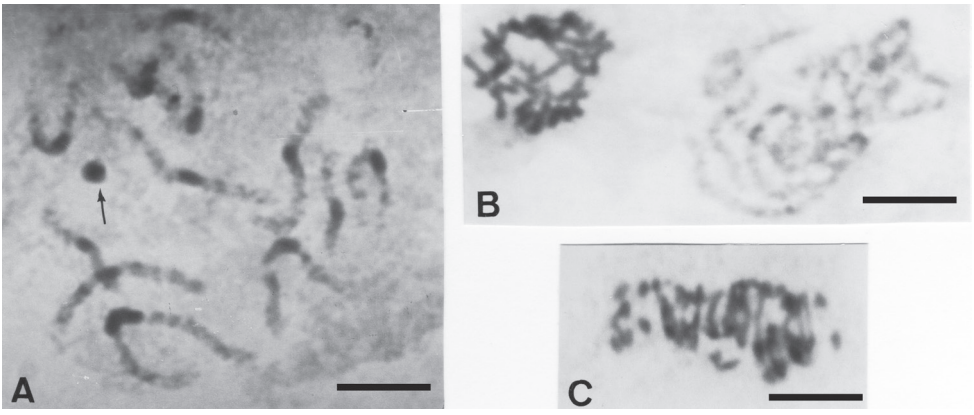
Henicotherus porteri has also 2n = 28 chomosomes at spermatogonial metaphases (Fig. 1B), from which a karyogram has been obtained, made of medium and small metacentrics of gradually decreasing sizes, including the largest X-chromosome and the smallest y-chromosome elements (Fig. 1C). Confirming what was expected, the metaphases I comprise 13 autosomic bivalents and the Xy_p sex-chromosome system (not shown).



Figures 1A–C. A *Blaptea elguetai* metaphase I showing 13 + Xy_p, the Xy_p is arrowed **B** *Henicotherus porteri* spermatogonial metaphase with 2n = 28, the y-chromosome is arrowed.**C** karyogram showing small meta/submetacentric chromosome pairs, the medium-sized X and the smallest y-chromosome are in the extreme right. Bar = 10 μm

Tribe Chrysomelini
Subribe Chrysomelina

Jolivetia obscura displays 2n = 28 chromosomes as in the two previous species, from pachytene meiotic cells where 14 bivalents are distinguishable and among them the Xy_p sex-chromosome system. Each of the 13 pachytene autosomal bivalents show a remarkable band of procentric heterocromatin, and the presumed Xy_p sex-chromosome system appears as a strongly heterochromatic round bulk under the conventional staining technique (Fig. 2A).



Figures 2A–C. A *Jolivetia obscura* pachytene showing 13 + Xy_p with striking procentric heterochromatic bands in the autosome bivalents, and the presumed Xy_p arrowed **B** *Pataya nitida* spermatogonial metaphase (left) and pachytene (right), some autosome bivalents show small procentric bands of heterochromatin **C** anaphase I showing 2n = 38 chromosomes. Bar = 10 μm

Conversely, *Pataya nitida* displays a higher diploid number of 19 pachytene bivalents, a few of which having heterochromatic bands (Fig. 2B), and 2n = 38 small chromosomes at spermatogonial anaphase (Fig. 2C).

Discussion

The diploid number of chromosomes and male sex-chromosome system of *Blaptea elguetai* 2n = 28 (Xy_p) agrees with our findings in *Microtheca ochroloma* Stål, 1860 (Petitpierre 1988), *Blaptea* and *Microtheca* Stål, 1860, both American genera, are closely related taxa within the subtribe Entomoscelina (Daccordi 1994). *Henicotherus porteri*, also belonging to the same subtribe Entomoscelina as the former (Daccordi 1994), shares again a 2n = 28 (Xy_p) diploid number and male sex-chromosome system, and its karyotype is made up of meta/submetacentric chromosomes of small size mostly. These meta/submetacentric chromosome shapes are the prevalent elements in beetle karyotypes (Smith and Virkki 1978; Virkki 1984), and more particularly, in the leaf beetles of the subfamily Chrysomelinae too (Petitpierre 2011a).

Among the different subtribes of Chrysomelinae (Daccordi 1994), the Entomoscelina have been scarcely surveyed from cytogenetic standpoints, with only seven checked species (Barabás and Bezo 1978; Petitpierre 1988; Petitpierre and Grobbelaar 2004), in five genera including the two present ones, among the total of 27 genera described to date (Daccordi 1994). However, it might seem that this subtribe is rather conservative in chromosome number and sex-chromosome system because five species have 2n = 28 (Xy_p) and two 2n = 26 (Xy_p), contrary to most other subtribes of Chrysomelinae, which exhibit a wide range of haploid chromosome numbers, namely from 9 to 22 in Timarchina, 10 to 25 in Chrysolinina, and 6 to 18 in Doryphorina (Petitpierre 2011a).

As reported above, *Jolivetia obscura* and *Pataya nitida* are classified in a different subtribe, Chrysomelina, than the two previous species (Daccordi 1994), and they have 2n = 28 (Xy_p) and 2n = 38 chromosomes, respectively. Among the 35 chromosomally studied species belonging to 12 genera in this subtribe, there is again a rather wide range of haploid numbers from 12 to 19, but with a clear modal value at n = 17 (65.7%) (Petitpierre 2011a). Therefore, *Pataya nitida* displays the highest so far found number and *Jolivetia obscura* one of the lowers within subtribe Chrysomelina. It is also remarkable, that even though both species, *Jolivetia obscura* and *Pataya nitida*, are taxonomically and morphologically related (Daccordi 1994), feeding on Asteraceae host plants as mentioned above, they are characterized with so diverse chromosome numbers.

The procentric bands of heterochromatin found in pachytene autosomal bivalents of *Jolivetia obscura* and in some of those of *Pataya nitida,* using conventional staining, are common feature in beetle chromosomes, as it has been recently demonstrated in several families of Coleoptera including Chrysomelidae by C-banding techniques (Rozek et al. 2004; Karagyan et al. 2012).

The sex-chromosome system found in our sampled species of Chilean chrysomelines was the parachute-type $Xy_{p,}$ except in *Pataya nitida* which has not been identified. Thus,

they agree with those found in the three previously analysed species of Chilean chrysomelines (Petitpierre and Elgueta 2006), and follow the prevalent rule in the subfamily Chrysomelinae, where almost 80% of the nearly 260 examined taxa display this sex-chromosome system (Petitpierre 2011a), as well as it also occurs in most beetles of the suborder Polyphaga (Smith and Virkki 1978; Virkki 1984; Dutrillaux and Dutrillaux 2009).

Acknowledgements

We are very much indebted to Marcelo Guerrero (Santiago, Chile) and Mauro Daccordi (Verona, Italy) who helped to the authors in collecting some of these four species in Isla Negra and Mincha, respectively. Miguel Angel Conesa (UIB, Palma de Mallorca) has given a valuable aid in arranging the micrographs.

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CompCytogen 6(4): 341–346 (2012)
doi: 10.3897/CompCytogen.v6i4.4058
www.pensoft.net/journals/compcytogen

SHORT COMMUNICATIONS

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

The first finding of $(TTAGG)_n$ telomeric repeat in chromosomes of true bugs (Heteroptera, Belostomatidae)

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Academic editor: *I. Gavrilov-Zimin* | Received 27 September 2012 | Accepted 2 October 2012 | Published 5 October 2012

Citation: Kuznetsova VG, Grozeva SM, Anokhin BA (2012) The first finding of $(TTAGG)_n$ telomeric repeat in chromosomes of true bugs (Heteroptera, Belostomatidae). Comparative Cytogenetics 6(4): 341–346. doi: 10.3897/CompCytogen.v6i4.4058

Abstract

Using the fluorescence *in situ* hybridization (FISH), the presence of $(TTAGG)_n$ telomeric sequence was detected in the chromosomes of *Lethocerus patruelis* (Stål, 1854) belonging to the family Belostomatidae (Heteroptera: Nepomorpha). This sequence was exclusively present at the ends of chromosomes in this species. This is the first evidence of the insect-type TTAGG telomeric repeats in Heteroptera.

Keywords

Chromosomes, FISH, $(TTAGG)_n$ telomeric repeat, true bugs, Nepomorpha, Belostomatidae, *Lethocerus patruelis*

Introduction

Telomeres are specific nucleoprotein structures at the ends of chromosomes and are responsible for their stability. Information on the telomere structure and function is presently available for many animals, plants and fungi (Fuchs et al. 1995, McKnight and Shippen 2004, Traut et al. 2007, Zakian 2012). The telomeres of insect species are predominantly composed of a pentanucleotide sequence repeat $(TTAGG)_n$ (reviewed in

Frydrychová et al. 2004). On the other hand, there are some higher taxa known to have lost this telomeric motif during their evolution, and Heteroptera are repeatedly referred to as one of such groups (Sahara et al. 1999, Frydrychová et al. 2004, Vitková et al. 2005, Lukhtanov and Kuznetsova 2010, Grozeva et al. 2011, Kuznetsova et al. 2011).

In this paper we report the molecular structure of telomeres at the physical ends of chromosomes in *Lethocerus patruelis* (Stål, 1854) (Nepomorpha: Belostomatidae).

Material and methods

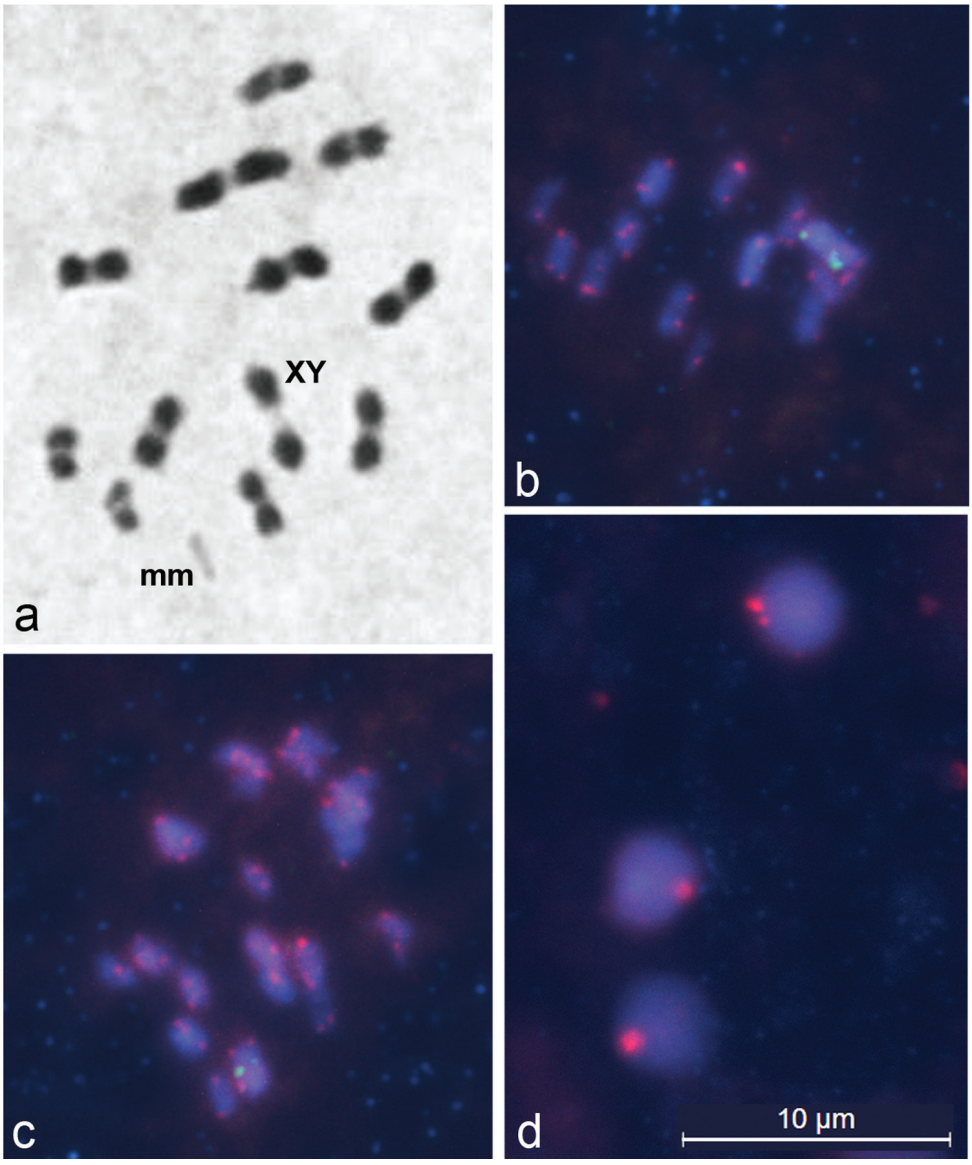
Spread chromosome preparations were made from testes of *L. patruelis* and stained using a Shiff-Giemsa method as described in Grozeva et al. (in press). The molecular structure of telomeres was investigated by fluorescence *in situ* hybridization of chromosomes (FISH) with a $(TTAGG)_n$ probe. In addition, we used an 18S rDNA probe to reveal the location of ribosomal clusters, NORs, on *L. patruelis* chromosomes. In these experiments we followed the protocol described in Grozeva et al. (2011). Fluorescence images were taken with a Leica DFC 345 FX camera using Leica Application Suite 3.7 software with an Image Overlay module.

Results

At first metaphases in *L. patruelis* males, 11 autosomal bivalents, each with one (sometimes two) terminal or subterminal chiasmata, a bivalent of m-chromosomes (microchromosomes) and a XY- pseudo-bivalent could be seen (Fig. 1a). Figures 1b-d show the results of fluorescence *in situ* hybridization with pentanucleotide $(TTAGG)_n$ and 18S rDNA probes to several meiotic spreads. At metaphase nuclei, TTAGG fluorescent signals (red) are clearly seen at all chromosomal ends, whereas rDNA clusters (green) are clearly evident on the X and Y chromosomes (Fig. 1b, c). Prominent telomere clustering at the periphery of spermatid nuclei (Fig. 1d) creates one large while sometimes a small number of TTAGG signals (red).

Discussion

The standard karyotype of *Lethocerus patruelis* males is 2n = 22A + 2m + XY as it was recently shown by Grozeva et al. (in press). We found that *L. patruelis* displayed FISH rDNA sites both on X and Y chromosomes. This is as expected since CMA_3-staining performed by Grozeva et al. (in press) has revealed GC rich clusters (typically pointed to NORs) on the sex chromosomes in this species. In other Belostomatidae species studied in this respect, NORs are known to be located either on sex chromosomes or on a pair of autosomes, the co-generic species sometimes differing in this pattern (reviewed in Grozeva et al. 2011).



Figures 1. Meiotic chromosomes of *Lethocerus patruelis* subjected to standard staining (**a**) and FISH (**b–d**). **a** metaphase I showing n = 11AA + mm + XY; **b–d** representative FISH images of metaphase I chromosomes (**b, c**) and spermatids (**d**) hybridized with probes against 18S rDNA and telomeres, showing ribosomal clusters (green) on X and Y chromosomes (**b, c**), and TTAGG repeats (red) located at the ends of chromosomes (**b, c**) and clustered at the periphery of spermatid nuclei (**d**).

DNA of the telomeres consists of short nucleotide motifs (combinations) repeated thousands and millions of times. Comparative analysis of these motifs in various groups of organisms has shown that they are evolutionarily stable, and, having once appeared during the evolution, mark taxa and phylogenetic lineages of high rank (Traut et al. 2007).

Quite recently, Frydrychová et al. (2004) assembled and analyzed the data available on the telomeric sequences in Insecta, and, together with some original observations, they interpreted these character data in a phylogenetic framework. Conclusions in that work are largely congruent with those previously proposed by Sahara et al. (1999). The great majority of insect species share the telomeres composed of $(TTAGG)_n$ repeat. Since the same telomere composition is characteristic of the rest of arthropods, the $(TTAGG)_n$ telomeric motif is considered an ancestral one in Insecta. Many higher-level insect groups preserved this motif; however several orders, e.g. Dermaptera, Heteroptera, Diptera and some others, are suggested to have lost this telomeric sequence during the evolution (Sahara et al. 1999, Frydrychová et al. 2004, Vitková et al. 2005, Lukhtanov and Kuznetsova 2010).

We emphasize, however, that the problem of telomere composition in different insect orders is still not adequately explored and in most cases, the available data concern one or more species only (see Fig. 6 in Frydrychová et al. 2004). On the other hand, in one of the better studied orders, Coleoptera (data are available for more than 20 species), both $(TTAGG)_n$-positive and $(TTAGG)_n$-negative species have been reported (Frydrychová and Marec 2002, Frydrychová et al. 2004).

In Heteroptera, the absence of the $(TTAGG)_n$ telomeric motif was firstly shown for *Halyomorpha halys* (Stål, 1855) (Pentatomidae) studied using Southern hybridization (Okazaki et al. 1993: as *H. mista* (Uhler, 1860)) and *Pyrrhocoris apterus* (Linnaeus, 1758) (Pyrrhocoridae) subjected to both Southern hybridization and FISH (Sahara et al. 1999). On the other hand, this sequence was revealed in telomeres of non-heteropteran Hemiptera and some other Paraneoptera (Frydrychova et al. 2004).

Originally proposed by Sahara et al. (1999) and accepted at a later time by other authors (Frydrychova et al. 2004, Vitková et al. 2005, Lukhtanov and Kuznetsova 2010), the hypothesis for the loss of $(TTAGG)_n$ sequence in true bugs has received further support owing to the discovery of Grozeva et al. (2011) that five more species studied by FISH and Dot-blot hybridization are also $(TTAGG)_n$- negative. Based on evidence provided by Okazaki et al. (1993), Sahara et al. (1999) and Grozeva et al. (2011), $(TTAGG)_n$ motif is known to be absent in seven species of true bugs. These species represent phylogenetically distant families, such as Pentatomidae (*Halyomorpha halys*, *Eurydema oleracea* (Linnaeus, 1758), *Graphosoma lineatum* (Linnaeus, 1758)) and Pyrrhocoridae (*Pyrrhocoris apterus*) belonging to the infraorder Pentatomomorpha and also Miridae (*Deraeocoris rutilus* (Herrich-Schaffer, 1838), *Megaloceroea recticornis* (Geoffroy, 1785)) and Cimicidae (*Cimex lectularius* (Linnaeus, 1758) belonging to the infraorder Cimicomorpha.

Our results of FISH with a $(TTAGG)_n$ probe strongly demonstrated that $(TTAGG)_n$ sequence was located at the telomeres of all chromosomes in *L. patruelis*. The finding of the insect-type $(TTAGG)_n$ telomeric motif in *L. patruelis* is thus clearly indicative of the heterogeneity of Heteroptera in telomere organization. The family Belostomatidae, to which this species belongs, is classified within the infraorder Nepomorpha (or true water bugs). The data on telomeres imply that true water bugs preserved the plesiomorphic telomere structure, whereas Cimicomorpha and Pentatomomorpha have

the apomorphic state of this character, which can be considered a synapomorphy of these infraorders. This conclusion is consistent with the generally accepted opinion that Cimicomorpha and Pentatomomorpha represent a monophyletic lineage, and Nepomorpha has a basal position within Heteroptera (Wheeler et al. 1993; Mahner 1993, Scherbakov and Popov 2002, Xie et al. 2008, Weirauch and Schuh 2011).

Acknowledgements

The study was supported (for VK and BA) by the RFBR (grant 11-04-00734), programs of the Presidium of the RAS "Gene Pools and Genetic Diversity" and "Origin of the Biosphere and Evolution of Geo-biological Systems" and the Ministry of Education and Science of the Russian Federation, and (for SG) by DO-02-259 NSF Sofia, BAS.

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CompCytogen 6(4): 347–358 (2012)
doi: 10.3897/CompCytogen.v6i4.3183
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RESEARCH ARTICLE

COMPARATIVE Cytogenetics
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The chromosomes of Tsing-Ling pika, *Ochotona huangensis* Matschie, 1908 (Lagomorpha, Ochotonidae)

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Academic editor: *N. Shapoval* | Received 3 April 2012 | Accepted 18 September 2012 | Published 8 October 2012

Citation: Vakurin AA, Korablev VP, Xue-Long J, Grigor'eva TV (2012) The chromosomes of Tsing-Ling pika, *Ochotona huangensis* Matschie, 1908 (Lagomorpha, Ochotonidae). Comparative Cytogenetics 6(4): 347–358. doi: 10.3897/CompCytogen.v6i4.3183

Abstract

The karyotype of the Tsing-Ling (Huanghe) pika, *Ochotona huangensis* Matschie, 1908 from the forest habitats of the Qinling Mountains (Shaanxi Province, China) was described for the first time. The chromosome set contains 42 chromosomes (NFa=80). The autosomes are 15 meta-submetacentric pairs and 5 subtelocentric pairs. The X chromosome is a medium sized submetacentric; the Y chromosome is a small sized acrocentric. C-banding revealed a localization of heterochromatin in the pericentromeric regions of all autosomes.

Keywords

Ochotona huangensis, pika, karyotype, chromosome, C-banding

Introduction

The pikas *Ochotona* Link, 1795 are small (12–28 cm long) mammals of the order Lagomorpha Brandt, 1855. The developed sound signaling is a characteristic feature of most northern *Ochotona* species. They live either alone or in colonies, preferring taluses or open plains. The pikas find refuges in the crevices between rocks or dig burrows (Sokolov et al. 1994, Hoffmann and Smith 2005).

These animals occur in North America from Alaska to New Mexico. In the Old World pikas are distributed from the Arctic coast to the northern regions of Iran, Afghanistan, Pakistan, India and Burma, from the Polar Urals in the West to the Pacific coast in the East, including Chukotka, Kamchatka peninsula, Hokkaido Island and also in territory of North Korea (Sokolov et al. 1994, Hoffmann and Smith 2005).

The pikas are one of the most ancient groups of the placental mammals (Gureev 1964, Ivanitskaya 1989, Lopatin and Aver'yanov 2008, Rose et al. 2008). The morphological criteria of species diagnosis were ascertained for many described *Ochotona* species. The pikas have rather distinct interspecific differentiation of karyotypes (2n=38-68) that helps to solve controversial taxonomic issues. Most species have stable karyotypes without geographic variability and intrapopulation polymorphism (Ivanitskaya 1989). However, two chromosomal forms of uncertain taxonomic rank were revealed for *O. alpina* group (Formozov et al. 2006).

The majority of modern taxonomists recognize 30 species of pikas and they divide them into three subgenera: *Pika* Lacepede, 1799, *Ochotona* Link, 1795 and *Conothoa* Lyon, 1904 (Hoffmann and Smith 2005). The karyotypes at least of 17 pika species were described (Table 1). These species are mainly from northern and temperate

Table 1. Subgenera system of the genus *Ochotona* and variability of the diploid chromosome number (2n). NF – the fundamental number of chromosomal arms.

Subgenus	Species	2n	NF	Banding methods	References
Pika	*O. argentata* Howell, 1928	38	76	C, NOR	Formozov et al. 2004
	O. hoffmanni Formozov et al., 1996	38	76	G, C	Formozov and Baklushinskaya 1999
	O. pallasi (=*pricei*) Gray, 1867	38	–	–	
			76	G, C	Ivanitskaya 1991
	O. hyperborea Pallas, 1811	40	–	–	Hayata and Shimba 1969
			–	–	Vorontsov and Ivanitskaya 1973
			76	C	Ivanitskaya 1991
	O. alpina Pallas, 1773	42	–	–	Vorontsov and Ivanitskaya 1973
			78	G, C	Ivanitskaya 1991
			–	–	Formozov et al. 2006
	O. collaris Nelson, 1893	68	90	–	Rausch and Ritter 1973
	O. princeps Richardson, 1828	68	86	–	Adams 1971
				G, C	Stock 1976
Ochotona	*O. huangensis* Matschie, 1908	42	84	C	**Our data**
	O. curzoniae Hodgson, 1858	46	68	G, C	Tan and Bai 1987
	O. nubrica Thomas, 1922	48	–	–	Formozov et al. (personal communication)
	O. dauurica Pallas, 1776	50	–	–	Vorontsov and Ivanitskaya 1973
			72	G, C	Ivanitskaya 1991
	O. pusilla Pallas, 1769	68	–	–	Vorontsov and Ivanitskaya 1973
			106	G, C	Ivanitskaya 1991

Subgenus	Species	2n	NF	Banding methods	References
Conothoa	*O. forresti* Thomas, 1923	54	–	DAPI	Ye et al. 2011
	O. rufescens Gray, 1842	60	86	–	Nadler et al. 1969
			–	–	Vorontsov and Ivanitskaya 1973
			–	G, C	Kimura et al. 1983
			90	G, C	Ivanitskaya 1991
	O. roylei Ogilby, 1839	62	–	G, NOR	Capanna et al. 1991
	O. macrotis Gunther, 1875	62	86	–	Vorontsov and Ivanitskaya 1973
	O. rutila Severtsov, 1873	62	–	–	Vorontsov and Ivanitskaya 1973
			86	G, C	Ivanitskaya 1991
	O. ladacensis Gunther, 1875	68	–	–	Formozov et al. (personal communication)

latitudes. Information on the differential staining of chromosomes is available for 13 species. Comparative analysis of G-banding pika chromosomes showed a high degree of similarity between the karyotypes of several species: *O. alpina – O. pallasi, O. pusilla – O. princeps, O. rutila – O. rufescens* (Ivanitskaya 1991).

Up to 24 species of pika inhabit China (Wang 2003), but the karyotypes of only five species have been described for this territory: *O. curzoniae* Hodgson, 1858 (Tan and Bai 1987), *O. ladacensis* Gunther, 1875, *O. nubrica* Thomas, 1922 (Formozov et al., personal communication), *O. argentata* Howell, 1928 (Formozov et al. 2004), *O. forresti* Thomas, 1923 (Ye et al. 2011).

During the last four decades, the systematics of the northern Palearctic and North American pikas has been well developed, but the system of subgenera and superspecies groups was periodically reconsidered with increase of number of morphological, morpho-ecological features and descriptions of karyotypes (Ivanitskaya 1991). Later it was corrected by multiple molecular data (Yu et al. 2000, Niu et al. 2004, Formozov et al. 2006, Lissovsky et al. 2007, Lanier and Olson 2009).

In this paper the karyotype of *Ochotona huangensis* Matschie, 1908 is described for the first time. This species has a few synonyms of common names: Tsing-Ling pika, Huanghe pika, Qinling pika. We will use the common name as Tsing-Ling pika, before conducting the full revision of this species. We adhered to intrageneric taxonomy proposed by Hoffmann and Smith (2005), in which *O. huangensis* belongs to the subgenus *Ochotona*. A level of variation of the diploid chromosome numbers in subgeneric groups of the genus *Ochotona* is discussed on the basis of our own and literature data.

Material and methods

One male of *Ochotona huangensis* was used as a material for this study. It was caught on Sept. 12, 2005 during the joint Russian-Chinese expedition to the Qinling Mountains

near the Foping village of Shaanxi Province, China. The pika was caught on a glade of the pine-oak forest, at height less than 1800 m (33°28'36,3"N, 108°30'18,6"E). This was slightly below the typical habitat for the Tsing-Ling (Huanghe) pika: a birch-fir forest located above 2000 m (Qin et al. 2007). This specimen is stored under the № 0509391 in the museum of Kunming Institute of Zoology. The karyotype of one male of *Ochotona dauurica* Pallas, 1776 was studied for comparison. The Daurian pika was caught in 2004 near the Tsagan-Oluy village (50°30'N, 117°3'25"E) of Borzya Distr. Transbaikalia, Russia.

Identification of the pika from the Qinling Mountains was performed by morphological characters. We used a molecular express analysis of the cytochrome *b* gene of mtDNA for confirming of taxonomic status of this specimen to the species *O. huangensis*. Total genomic DNA was extracted from liver tissue by standard protocol (Arrighi et al. 1968). We used a standard polymerase chain reaction (PCR) for full-length sequences cytochrome *b* gene (1140 bp) amplification with specially designed primers:

L14075och 5' – gta tgt cat aat tct tac atg ga – 3'
H15374och 5' – gta agc cga ggg cgt ctt tg – 3'

The primers were designed according to published whole mitochondrial sequence of pika *O. collaris* (GenBank NCBI (www.ncbi.nlm.nih.gov) № NC_003033). The PCR program consisted of 94 °C for 5 min followed by 35 cycles at 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 3 min. A final amplification step completed the PCR at 72 °C for 7 min.

The PCR products were purified by Sin Column PCR Product Purification Kit (Evrogen, Moscow, Russia). The directly sequencing of the purified PCR products was performed using ABI PRISM BigDye™ Terminator v3.1 (Applied Biosystems, Inc., Foster City, California) with an automatic DNA sequencer (Model ABI PRISM 3100-Avant Genetic Analyzer; Applied Biosystems, Inc., Foster City, California). The same primers were used for sequencing PCR from both directions.

The obtained sequence (GenBank NCBI № JN645147) was compared with full-length cytochrome *b* (1140bp) of 23 pikas species published by different authors in GenBank. The alignment of sequences was conducted by the program BIOEDIT v7.0.9 (Tom Hall, Ibis Biosciences). The genetic distances were estimated with neighbor-joining method, using Kimura two-parameter model. The tree was constructed by including all transitions and transversions with TREECON v3.1b (Yves Van De Peer, Germany). A rabbit *Oryctolagus cuniculus* Linnaeus, 1758 was selected as an outgroup (Fig. 1).

Method of cell division stimulation in the red bone marrow with baker's yeast solution was used for preparation of chromosomal slides (Lee and Elder 1980). The slides were made by standard method (Ford and Hamerton 1956). The procedure of differential staining (C-banding) was held for detection of structural heterochromatin (Sumner 1972).

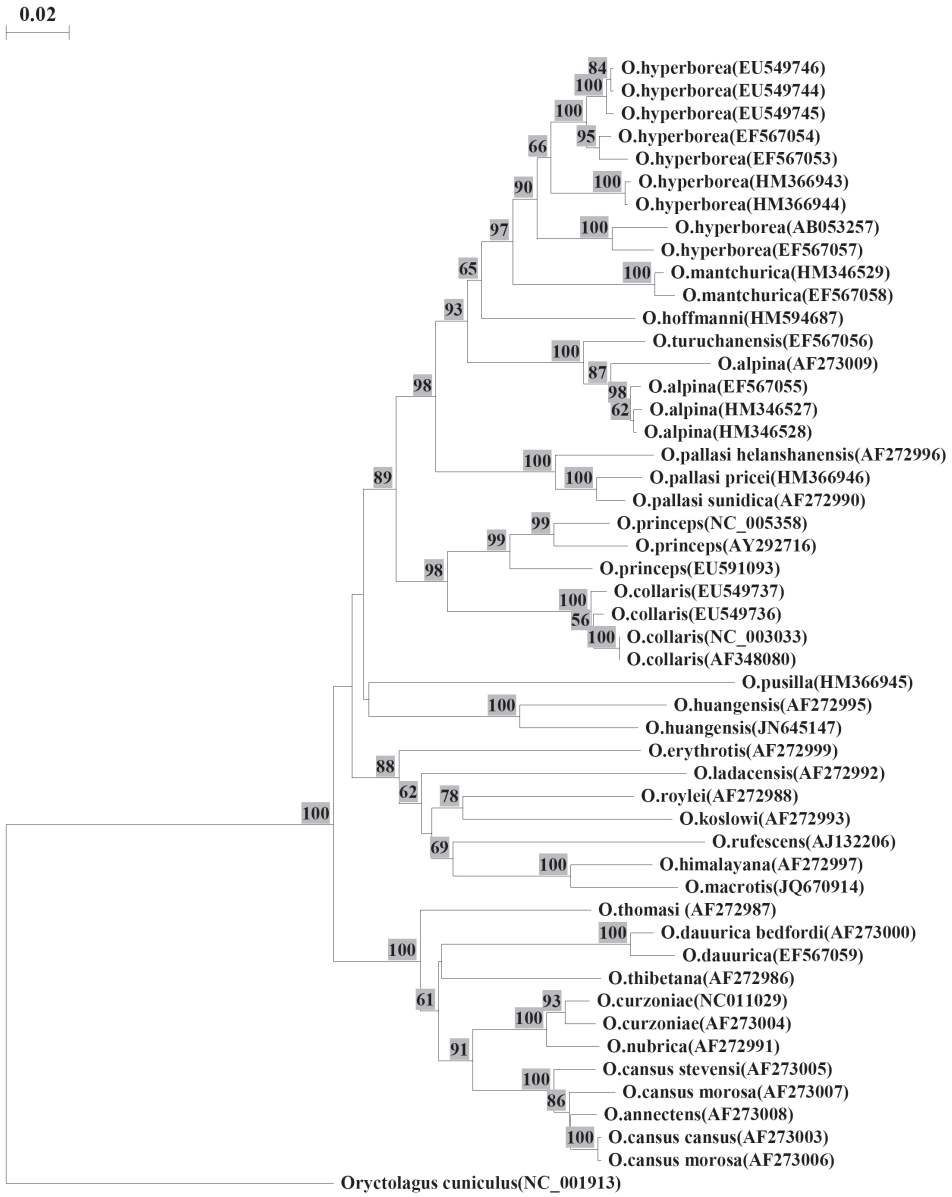

Figure 1. Neighbor-joining distance tree constructed using the Kimura two-parameter model for complete sequence cytochrome b (1140 bp). Numbers on branches indicate bootstrap support; values less than 50 are not shown. Numbers following the species names indicate the GenBank accession numbers.

The chromosomal slides were analyzed on light microscope AxioSkop 40 with lens x100. Photographs were performed with the digital camera AxioCamHR using the program AXIOVISION 4.7 (Carl Zeiss MicroImaging GmbH, Germany). The morphology of the chromosomes was assessed visually without measurements (Orlov 1974).

Results and discussion

Independence of the *O. huangensis* taxon was suggested by molecular studies (Yu et al. 2000, Niu et al. 2004), but the morphological revision of specimens used in these articles was never done, so we can stick to only one fact. Our data of the molecular express analysis showed that the sequence of our specimen had maximum similarity to that sequence of specimen which was identified as *O. huangensis* by Yu (2000) (Fig. 1).

According to the results of counting on 40 metaphase plates, the diploid chromosome number of *O. huangensis* is 42 (NFa=80). Morphologically two groups of autosomes were identified. The first group consists of 15 pairs (3 large, 8 medium and 4 small) meta-submetacentric chromosomes. The second group consists of 5 pairs rather large, gradually decreasing in size, subtelocentric chromosomes. The X chromosome is a medium sized submetacentric, the Y chromosome is a small acrocentric (Fig. 2a).

Nineteen metaphase cells stained for the structural heterochromatin (C-banding) were analyzed. The clearly stained pericentromeric heterochromatic blocks, which sizes were approximately the same, were identified at all chromosomes of *O. huangensis*. The heteromorphism was detected by localization of heterochromatic blocks on the 8-th pair of autosomes. An intercalary heterochromatic block was always detected in the long arm of one homologue of the 8-th pair. Also, that homologue had the pericentromeric block of heterochromatin. In the second homologue of this pair, the intercalary heterochromatic block was detected in nine metaphase cells. In the remaining cells, only the larger pericentromeric heterochromatic block was detected in this homologue. By that, the euchromatic site, which separates the intercalary heterochromatic block, was broader on the first homologue than that on the second homologue (Fig. 4). We can't characterize this phenomenon in details and discuss about its nature, because of the absence of sufficient material. So we leave it only as an observed fact. The X chromosome has a pericentromeric block of heterochromatin. The heterochromatic region occupies 2/3 of the lower arm on the Y chromosome (Fig. 3a).

The Daurian pika, which like *O. huangensis* belongs to the subgenus *Ochotona,* was studied for a comparative karyotype analysis. The karyotype of *O. dauurica* contains 50 chromosomes (NFa=68) which are grouped in 10 meta-submetacentric pairs (3 large, 2 medium and 5 small) and 14 subtelo- and acrocentric pairs of autosomes. The X chromosome is a submetacentric, similar in size to the 3-rd or 4-th pairs of autosomes, the Y chromosome is a very small acrocentric (Fig. 2b). The karyotype of the Daurian pika does not differ from that which was previously described in the literature (Vorontsov and Ivanitskaya 1969, 1973).

Analysis of 15 C-stained metaphase plates showed that all autosomes of *O. dauurica* have the large pericentromeric heterochromatic blocks which were intensively stained. The 10-th and 15-th – 22-th pairs of autosomes have completely heterochromatic short arms. The last two small pairs of autosomes (23-th and 24-th) are composed of heterochromatin entirely. The large pericentromeric block of the X chromosome occupies 1/3 of the long arm. Heterochromatic structure of the Y chromosome was not confirmed (Fig. 3b) compared with published data (Ivanitskaya 1991).

Figure 2. Routine stained karyotypes of *Ochotona huangensis* (**a**) and *O. dauurica* (**b**): **M-Sm** meta-submetacentric chromosomes, **St** subtelocentric chromosomes, **St-Ac** subtelo- and acrocentric chromosomes. Bar = 5 μm.

An obvious resemblance between the karyotypes of *O. huangensis* and *O. dauurica* was seen by the routine staining, despite of some bigger size of the first pair of *O. huangensis*. The first four meta-submetacentric pairs of *O. dauurica* are similar to the 2nd – 5-th pairs of *O. huangensis* autosomes. The remaining five meta-submetacentric pairs of *O. dauurica*, except the 10-th pair, are similar to the last five pairs of the first group of *O. huangensis* autosomes. The 11-th – 15-th autosomes of *O. dauurica* are very similar to the second subtelocentric group of *O. huangensis* by morphology and sizes, with a loss of the little part of the upper arm on the 20-th pair. The absence of G-stained chromosomes not allows us to do unambiguous conclusion about the relationship between the karyotypes of *O. dauurica* and *O. huangensis*. Such species as *O. alpina* (2n=42), *O. hyperborea* (2n=40), *O. pallasi* (2n=38) and *O. argentata* (2n=38) of the subgenus *Pika* (Vorontsov and Ivanitskaya 1973, Ivanitskaya 1991) are close to *O. huangensis* by the diploid chromosome number. However, they have more significant differences in relation of morphological groups and sizes of chromosomes.

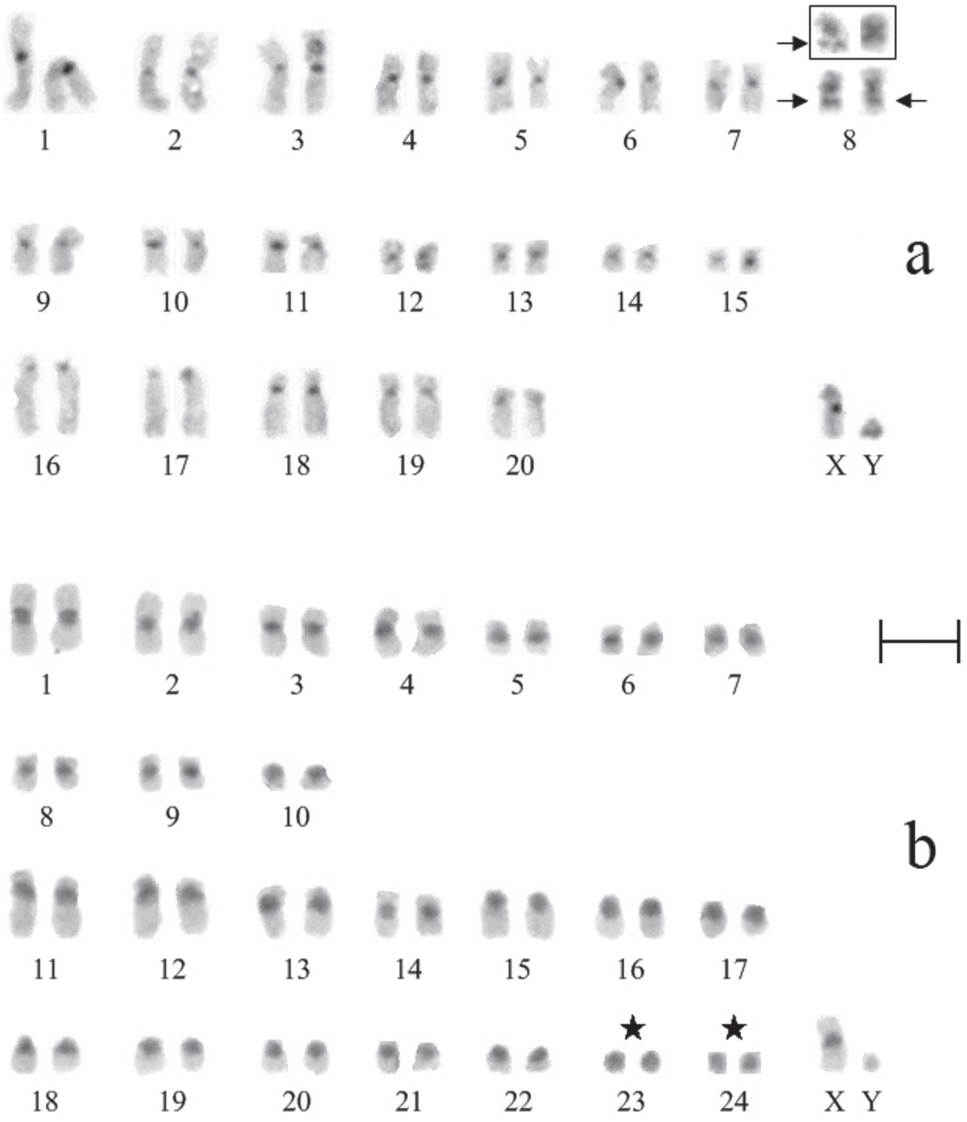

Figure 3. C-banded karyotypes of *Ochotona huangensis* (**a**) and *O. dauurica* (**b**): ➙ – intercalary heterochromatic blocks, ★ – autosomes entirely consisted of heterochromatin. Bar = 5 μm.

The C-banding patterns of *O. dauurica* specimens from Transbaikalia (near the station Armagotuy) and Mongolia (Selenge aimag, near Shamar) (Ivanitskaya 1991) differs slightly from the specimen studied by us. Four pairs of subtelo-acrocentric autosomes have euchromatic material on the short arms in our pika. According to the data obtained by Ivanitskaya (1991), euchromatic material was on the short arms only on one pair. This pair is the largest and it corresponds to our 11-th pair. In addition, Ivanitskaya (1991) described three completely heterochromatic pairs, but according to our data, only last two pairs of autosomes have such features. These differences may be due

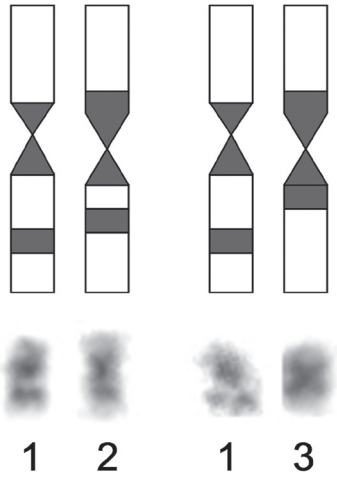

Figure 4. Scheme of localization of heterochromatic blocks on 8-th pair of *Ochotona huangensis*: 1 – the first homologue, 2 and 3 – the second homologue in two variants.

to interpopulation variability as well as influence of different C-staining procedures of chromosomal slides. However, the reason of these differences remains unclear, because of the absence of sufficient material at present.

A tendency of heterochromatin decreasing is confirmed in row of pikas: from species with a large number of chromosomes to species with a smaller number, while comparing the overall C-banding pattern of *O. dauurica* and *O. huangensis* (Formozov et al. 2004). Perhaps, this indicates a loss of the heterochromatic material as a result of the chromosomal rearrangements.

The species *O. alpina* (subgenus *Pika*) is similar to *O. huangensis* by the diploid chromosome number, but it has another arrangement of heterochromatin. Pericentromeric heterochromatin is detected only on 6 submetacentric and 5 subtelocentric pairs of *O. alpina* autosomes (Ivanitskaya 1991). Four submetacentric pairs of *O. alpina* (especially the first pair) have the larger heterochromatic blocks than the corresponding pairs of *O. huangensis*. Two large subtelocentric pairs of *O. alpina* also have the larger blocks of heterochromatin in comparison with the subtelocentric pairs of *O. huangensis*. The remaining three minor subtelocentric pairs of *O. alpina*, which contain the pericentromeric heterochromatin, have no analogues in the karyotype of *O. huangensis*. Besides, the X chromosome of *O. alpina* has no heterochromatin unlike *O. huangensis*. The Y chromosome of *O. alpina* is composed of heterochromatin entirely (Ivanitskaya 1991).

The molecular studies of the genus *Ochotona* (Yu et al. 2000, Formozov et al. personal communication) showed division of pikas for three superspecies groups: 1. *Pika* – northern pikas and Mongolian pika; 2. *Ochotona* – shrub-steppe pikas except Mongolian, Ladak and Kozlov's pikas; 3. *Conothoa* – mountain pikas with Ladak and Kozlov's pikas. At present, the statuses of subgenera are given for these groups of pikas (Hoffmann and Smith 2005). Formozov et al. (personal communication) suggested the existence of variation of the diploid chromosome number for each subgenus (group)

of pikas. The karyotypes of the subgenus *Pika* species have 38-42 chromosomes. The species of the subgenus *Ochotona* have the karyotypes with 46-50 chromosomes. The pikas of the subgenus *Conothoa* have 60-62 chromosomes in the karyotypes. Moreover, there are species with 2n=68 in each subgenus.

The position of *O. huangensis* is ambiguous in this system. According to the data of study of the cytochrome *b* and the ND4 gene (Yu et al. 2000), *O. huangensis* is very far distant from the group of shrub-steppe pikas. Also, *O. huangensis* is allocated to a separate independent group by analysis of the cytochrome *b* of 27 pikas species (Niu et al. 2004). At present, *O. huangensis* (2n=42) belongs to the subgenus *Ochotona* (Hoffmann and Smith 2005). If the view point of Hoffmann and Smith is true, our data extend the level of variation of the diploid chromosome number for the subgenus *Ochotona*. In this case, there is no border with the subgenus *Pika* by this indicator. Thus, *O. huangensis* is significantly diverging from the main group of the subgenus *Ochotona* by main karyotypic characteristics that corresponds to the data of mtDNA study (Yu et al. 2000, Niu et al. 2004). The recent cytogenetic study of *O. forresti* (2n=54) (Ye et al. 2011) also greatly expands karyotypic variability of the subgenus *Conothoa*. As yet, the karyotypes of eight species of the subgenus *Conothoa* and three species of the subgenus *Ochotona* are not investigated. It is not excluded that the new karyotypic data will changed the level of the diploid numbers variation between all subgenera of the genus. Thus, we assume that the karyotypic system of the genus *Ochotona* can not be constructed completely without studying cytogenetic characteristics of all species of pikas.

Acknowledgements

The authors express their sincere thanks to Dr A. P. Kryukov for establishing Russian-Chinese collaboration and attending the expedition to the Qinling, Dr M. V. Pavlenko for help in collecting of material, Dr I. V. Kartavtseva for valuable comments, advice and help in writing the manuscript. Special thanks to Dr N. A. Formozov for his help in molecular identifying of the *Ochotona huangensis* species.

This work was partially supported by Russian Foundation for Basic Research, Project no. 06-04-39015; Far East Branch Russian Acad. Sci., Project no. 09-II-CO-06-007 and grant "Complex expeditionary researches of environment of Amur river basin 2004-2007". The study was conducted with the technical support of laboratory microscopy of center for collective use "Biotechnology and Genetic Engineering" (Establishment of IBSS FEB RAS).

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CompCytogen 6(4): 359–369 (2012)
doi: 10.3897/CompCytogen.v6i4.3062
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

Discriminatory profile of rDNA sites and trend for acrocentric chromosome formation in the genus *Trachinotus* Lacépède, 1801 (Perciformes, Carangidae)

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Academic editor: *V. Gokhman* | Received 9 March 2012 | Accepted 29 May 2012 | Published 31 October 2012

Citation: Jacobina UP, Vicari MR, Bertollo LAC, Molina WF (2012) Discriminatory profile of rDNA sites and trend for acrocentric chromosome formation in the genus *Trachinotus* Lacépède, 1801 (Perciformes, Carangidae). Comparative Cytogenetics 6(4): 359–369. doi: 10.3897/CompCytogen.v6i4.3062

Abstract

Chromosomal traits have provided valuable information for phylogeny and taxonomy of several fish groups. Three Atlantic Carangidae species of the genus *Trachinotus* Lacépède, 1801 (*T. goodei* Jordan et Evermann, 1896, *T. carolinus* (Linnaeus, 1766) and *T. falcatus* (Linnaeus, 1758)) were investigated, having 2n=48 chromosomes but different chromosomal arms (FN number), i.e., 52, 56 and 58, respectively, in view of the different number of two-armed chromosomes found in their karyotypes. Thus, *T. goodei*, *T. carolinus* and *T. falcatus* present a progressive distance from the probable basal karyotype proposed for Perciformes (2n=48 acrocentrics, FN=48). At first sight, these findings do not agree with the phylogenetic hypothesis based on mitochondrial sequences, where *T. goodei* appear as the most derived species, followed by *T. falcatus* and *T. carolinus*, respectively. However, the chromosomal mapping of ribosomal DNAs was informative for clarifying this apparent conflict. Indeed, the multiple 5S and 18S rDNA sites found in *T. goodei* corroborate the most derived condition for this species. In this sense, the occurrence of the unexpected number of two-armed chromosomes and FN value for this species, as well as for *T. carolinus*, must be due to additional rounds of acrocentric formation in these species, modifying the macrostructure of their karyotypes.

Keywords
Carangidae, 18S rDNA, 5S rDNA, cytotaxonomic markers, evolutionary pathways

Introduction

The genus *Trachinotus* Lacépède, 1801, also known as pompanos, encompasses 20 species distributed in tropical and subtropical oceans (Cunha 1981). In the Eastern Atlantic, the species *Trachinotus carolinus* (Linnaeus, 1766), popular for both sport and commercial fishing, *T. falcatus* (Linnaeus, 1758), a game fish species, and *T. goodei* Jordan et Evermann, 1896, a species with a high potential for aquaculture and sport fishing, are the most widely distributed, occurring from the Southern United States to Northern Argentina (McMaster 1988, Lazo et al. 1998, Heilman and Spieler 1999). Recent data identified population differentiations in the number and positions of the ribosomal sites among the extensively distributed species, *T. falcatus* and *T. goodei* (Accioly et al. in press). Indeed, there is growing evidence that cytotaxonomic markers, particularly ribosomal sites, may reveal the genetic structure of marine fish populations (Motta-Neto et al. 2011a, Lima-Filho et al. in press).

In addition to their biological significance in commercial and sport fishing, representatives of the genus *Trachinotus* are considered potentially suitable for pisciculture purposes (Watanabe 1995, Weirich et al. 2006). *Trachinotus* species have very desirable biological characteristics, such as fast adaptation to confined environments, good tolerance to extreme environmental conditions and rapid growth (Jory et al. 1985). Nevertheless, genetic and cytogenetic foundations supporting their cultivation remain largely unknown.

Most species of the marine Perciformes exhibit a basal karyotype composed of 2n=48 acrocentric chromosomes, extensively conserved in several families (Molina 2007). Given the large number of species, most cytogenetic studies have focused on mapping biodiversity in this order, the largest of all living vertebrates. Among the family Carangidae, cytogenetic data have already been reported for a total of 27 species in 13 genera (e.g. Caputo et al. 1996, Sola et al. 1997, Rodrigues et al. 2007, Chai et al. 2009). Of these, few species occur exclusively in the Atlantic. The present cytogenetic study characterizes the species *Trachinotus carolinus*, *T. falcatus* and *T. goodei* through conventional staining, Ag-NOR detection, C-banding, CMA_3/DAPI fluorochrome staining, and mapping of the 18S and 5S rDNA sequences by dual-color FISH. Useful phylogenetic information was provided by ribosomal sequences mapping, indicating an intriguing scenario with additional acrocentrics formation in *T. goodei* and *T. carolinus*.

Material and methods

Samples of the species *Trachinotus carolinus* (N=5; 3 males. one female, one immature), *T. falcatus* (N=10; 4 males, 3 females, 3 immatures) and *T. goodei* (N=10; 6 males, 4 females) were obtained on the coast of Rio Grande do Norte state (05°05'26"S,

36°16'31"W), in Northeast Brazil. Prior to chromosomal preparations, specimens were submitted to *in vivo* mitotic stimulation for 24 hours, through intramuscular and intraperitoneal injection of complex antigens (Molina et al. 2010). Individuals were anesthetized with clove oil (Griffiths 2000) and sacrificed. Mitotic chromosomes were acquired from cell suspensions of anterior kidney fragments according to *in vitro* mitotic block (Gold et al. 1990). Cell suspensions were dripped onto slides coated with a film of distilled water heated to 60°C, and stained with 5% Giemsa diluted in a phosphate buffer pH 6.8. The material was analyzed under 1000× magnification and the best metaphases were photographed under an Olympus BX50® epifluorescence microscope, with an Olympus DP70® digital image capturing system. About 30 metaphases were analyzed for each individual in order to determine the diploid number for every species.

Chromosome nomenclature

Chromosomes were classified as metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a), based on the system proposed by Levan et al. (1964).

Chromosome banding

The heterochromatic and nucleolar organizer regions (Ag-NORs) were identified using techniques developed by Sumner (1972) and Howell and Black (1980) respectively. CMA_3/DAPI staining was applied in accordance with Barros-e-Silva and Guerra (2010).

Cytogenetic mapping protocols

Two probes were used: an 18S rDNA probe obtained from the nuclear DNA of *Prochilodus argenteus* Spix et Agassiz, 1829 (Hatanaka and Galetti 2004); a 5S rDNA probe isolated from the genomic DNA of *Leporinus elongatus* Valenciennes, 1850 (Martins and Galetti 1999); probes were labeled by polymerase chain reaction (PCR), using biotin-16-dUTP (Roche Applied Science®) for 18S rDNA or digoxigenin-11-dUTP (Roche Applied Science®) for 5S rDNA. PCR labeling for rDNA clones was performed with specific primers, using 20 ng of template DNA, 1X *Taq* reaction buffer (200 mM Tris pH 8.4, 500 mM KCl), 40 μM dATP, dGTP and dCTP, 28 μM of dTTP, 12 μM biotin-16-dUTP or digoxigenin-11-dUTP, 1 μM primers, 2 mM $MgCl_2$ and 2 U of *Taq* DNA Polymerase (Invitrogen®) under the following conditions: 5 min at 94°C; 35 cycles: 1 min at 90°C, 1 min 30 s at 52°C and 1 min 30 s at 72°C; and a final extension step at 72°C for 5 min.

The overall hybridization procedure followed the protocol described by Pinkel et al. (1986), under high stringency conditions (2.5 ng/μL from each probe, 50% deionized formamide, 10% dextran sulphate, 2XSSC, pH 7.0 – 7.2, at 37°C overnight).

After hybridization, slides were rinsed 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 (two times for 5 min each). Signal detection was performed using streptavidin-alexa fluor 488 (Molecular Probes®) for the 18S rDNA probe; and anti-digoxigenin-rhodamine (Roche Applied Science®) for 5S rDNA, which were detected by dual color FISH.

Results

All species analyzed exhibited 2n=48 chromosomes, however with a notable difference in the number of two-armed (bibrachial) elements.

The karyotype of *Trachinotus goodei* (Figure 1a, d, g) is composed of 4 m/sm and 44a (FN=52). The heterochromatic regions in this species are very reduced and restricted to small blocks in the chromosomal pericentromeric regions. The Ag-NORs/18S rDNA sites were identified near the centromeric region of two acrocentric pairs, tentatively No. 5 and 11 of the karyotype. These sites proved to be rich in GC base composition ($CMA^+/DAPI^-$) (Figure 1d). Hybridization signals with 5S rDNA probes were also identified on the terminal regions of the short arms of three acrocentric pairs, tentatively numbered as 9, 12 and 22 (Figure 1g).

The *T. carolinus* karyotype (Figures 1 b, e, h) consists of 8m/sm and 40a (FN=56). The content of heterochromatin is also poorly distributed in the pericentromeric regions of some chromosome pairs. Ag-NORs/18S rDNA sites were located on the short arm of only one acrocentric pair, identified as number 5. These sites are clearly hetero-

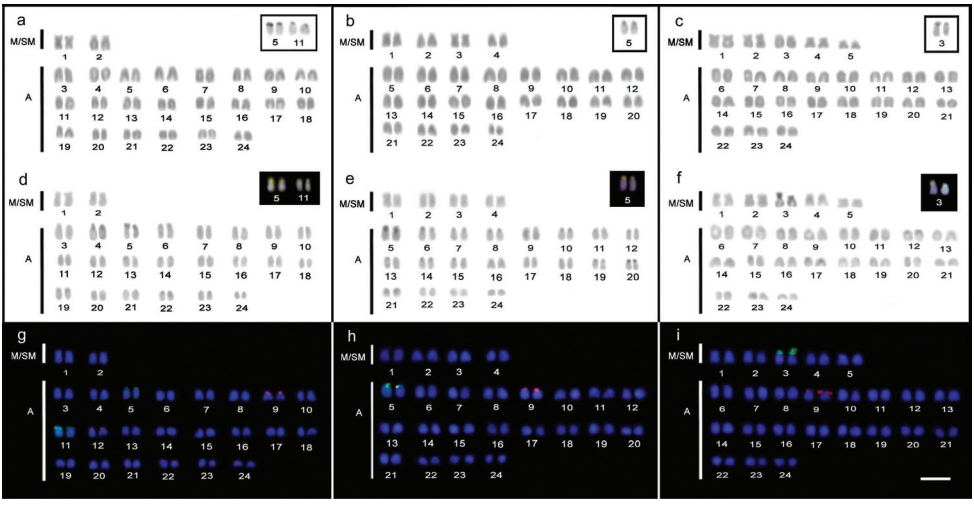

Figure 1. Karyotypes of *Trachinotus goodei* (**a, d, g**), *T. carolinus* (**b, e, h**) and *T. falcatus* (**c, f, i**). Conventional staining (**a, b, c**) highlighting the chromosomal pairs carrying Ag-NOR sites; C-banding (**d, e, f**); nucleolar organizer pairs are highlighted by staining with $CMA_3^+/DAPI^-$. Dual-color FISH (**f, g, h**) showing the chromosomal mapping of the 18S rDNA (green) and 5S rDNA (red) sites. Bar = 5 μm.

chromatic, with a $CMA^{+}/DAPI^{-}$ pattern. The 5S rDNA sites were mapped only on the short arm of the acrocentric chromosome 9.

The karyotype of *T. falcatus* (Figure 1c, f, i) has the largest number of bibrachial elements if compared to the other species, i.e., 10 m/sm and 38a (FN=58). As in the two previous species, small heterochromatic blocks are present in pericentromeric regions of the chromosomes. Ag-NORs/18S rDNA sites were situated in the terminal region of the short arm of the submetacentric chromosome pair 3, which also appears heterochromatic after C-banding, with a $CMA^{+}/DAPI^{-}$ pattern. The 5S rDNA sites were mapped exclusively on the short arms of the acrocentric pair 9.

Discussion

As in many species of Perciformes, the species analyzed displayed 2n=48 and large numbers of acrocentric chromosomes, although there were notable differences in karyotype macrostructure. This is particularly evident for the number of chromosome arms (FN) that varies between species. Thus, *T. goodei* exhibits FN=52, *T. carolinus* FN=56 and *T. falcatus* FN=58. Karyotypes similar to those presented here for *T. goodei* and *T. falcatus* were previously identified in other populations of this species on the Southeast and Northeast coasts of Brazil (Rodrigues et al. 2007, Accioly et al. in press).

Evolutionary karyotype modifications resulting from pericentric inversions are common in Perciformes. In fact, two-armed chromosomes have been found in approximately 30% of Carangidae species karyotyped to date (Chai et al. 2009). Furthermore, other kinds of chromosomal diversification have been identified for this family including Robertsonian translocations, transient in *Seriola* Cuvier, 1817 (Vitturi et al. 1986, Sola et al. 1997) or already established in *Selene setapinnis* (Mitchill, 1815) (Jacobina 2012).

Basing on morphological and molecular evidences, the genus *Trachinotus* is included in the tribe Trachinotini, which is considered one of the least diverse groups among carangids (Smith-Vaniz 1984, Gushiken 1988). Phylogenetic hypotheses based on mitochondrial sequences (Reed et al. 2002) suggest *T. carolinus* as the most basal species, followed by more derived *T. falcatus* and *T. goodei*, respectively. However, these phylogenetic relationships do not agree with the karyotypic characteristics presented by these species (Figure 2a).

Whereas the fully acrocentric karyotype with 2n=48 (FN=48) is considered basal for Perciformes, variations of this karyotypic formula can be interpreted as derived conditions. Thus, an increase in the number of two-armed chromosomes, as sequentially found in *T. carolinus* (eight two-armed chromosomes) and in *T. falcatus* (ten two-armed chromosomes), would be expected to represent derived cytogenetic characteristics. As such, *T. goodei,* showing only four two-armed chromosomes and, consequently, the largest number of acrocentric chromosomes, would be representing the species with the karyotype closer to the basal one.

Many closely related species of Perciformes show poorly varied or cryptic cytogenetic characteristics, hampering their application in phylogenetic inferences (Molina

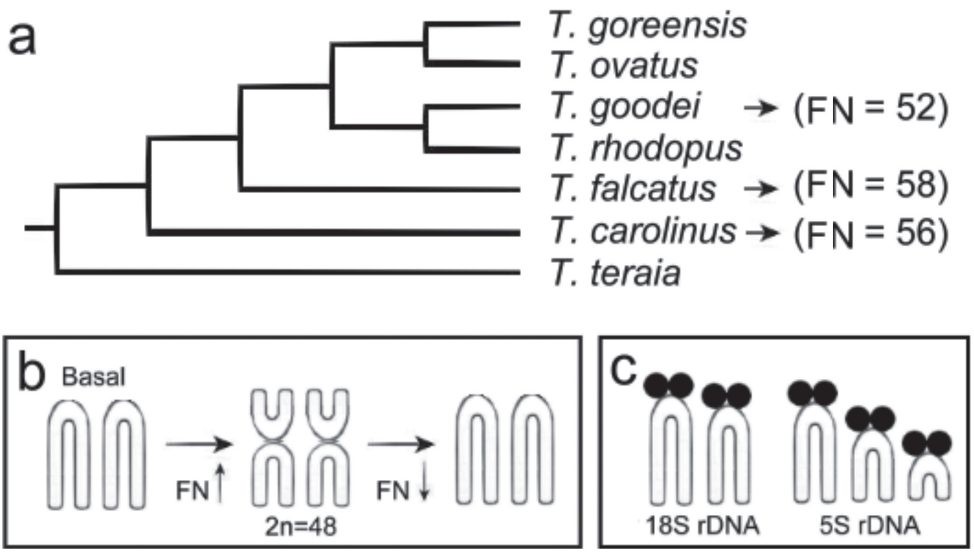

Figure 2. Phylogenetic tree from molecular data of some species of Trachinotini tribe (**a**), adapted from Reed et al. (2002). The molecular relationship is confronted with the chromosomal formula of the *Trachinotus* species analyzed. Schematic illustration shows the role of additional pericentric inversions leading to new acrocentric chromosomes and modification of the FN value (**b**), and the derived condition of multiple sites of 18S and 5S rDNAs in *T. goodei* (**c**).

2007, Motta-Neto et al. 2011a, b, c). Indeed, this is observed in the similar karyotype macrostructure or heterochromatic patterns, such as those found in *Trachinotus* species, where C-bands are inconspicuous and similarly located in the pericentromeric region of the chromosomes. A reduced amount of heterochromatin is also a common feature in other Perciformes, possibly resulting in lower karyotype evolution dynamics (Molina and Galetti 2004, Molina 2007). On the other hand, NORs were prominent characteristics, in lines with considerable karyotype variation between species. *Trachinotus carolinus* and *T. falcatus* displayed only one pair of chromosomes carrying ribosomal sites (Ag-NOR/18SrDNA/CMA$^+$/DAPI$^-$). This condition is considered basal and the most common for Carangidae (Caputo et al. 1996, Sola et al. 1997). As previously confirmed (Accioly et al. in press), the *T. goodei* population from Brazilian Northeastern coast exhibits a more derived condition, with two chromosomal pairs carrying ribosomal sites (pairs 5 and 11). Although multiple sites have not been identified in populations from the Southeastern coast (Rodrigues et al. 2007), the occurrence of more than one chromosome pair carrying NORs in *T. goodei* indicates some level of derivation in this species in relation to the others. Greater dynamic evolution of the ribosomal sites in this species is corroborated by the presence of three chromosomal pairs carrying 5S rDNA sequences (pairs 9, 12, 22), a condition not present in *T. carolinus* and *T. falcatus,* where these sites were mapped only in pair 9 (Figs 1, 2c). In addition, dual-color FISH showed no synteny between 18S and 5S rDNA sites in all the three species of *Trachinotus* analyzed here.

Simple ribosomal sites are considered an ancestral condition, most frequently found in carangids (Caputo et al. 1996, Sola et al. 1997), as well as among marine Perciformes (Galetti et al. 2000). Their location in distinct chromosomal pairs is an efficient cytotaxonomic marker of species and populations of *Trachinotus* (Accioly et al. in press). Indeed, Southeastern populations of *T. falcatus* and *T. goodei* are characterized by having simple Ag-NOR sites on the short arms of pair 18 and on the short arms of pair 3, respectively. The greater dynamic evolution of the 18S and 5S ribosomal sequences in *T. goodei* corroborates its more derived condition in relation to the other species (Figure 2), as suggested by molecular data (Reed et al. 2002). In turn, sharing of 5S rDNA sequences by a same chromosome pair, tentatively identified as no. 9, probably indicates homeologous chromosomes with similar syntenic content. The occurrence of three pairs carrying 5S rDNA sequences (pairs 9, 12 and 22) in *T. goodei* is uncommon among fish (Martins and Galetti 2000). The location of 5S and 18S rDNA sites in different chromosomes, and the functional divergence between 18S rDNA (transcribed by RNA polymerase I) and 5S rRNA genes (transcribed by RNA polymerase II) (Martins and Galetti 2000), supports the independent evolution of these multigene families due to specific selection pressures (Amarasinghe and Carlson 1998).

Variations in the number and location of NORs in some cases, are likely to be favored by a high and heterogeneous heterochromatic content, whereas the inverse seems to reduce the evolutionary dynamism of these regions (Molina 2007). Besides increasing the NORs' dynamics, there are also indications that heterochromatin may act as hotspots for chromosomal rearrangements (Almeida-Toledo et al. 1996; Jacobina 2012). However, there is currently no information that the heterochromatin may be exerting some role in the evolutionary dynamics of the rDNA in *T. goodei*. Dispersion of these sequences in the karyotype may occur via transposition events by mobile elements in the carrier genome, with subsequent amplification and formation of new repetitive DNA sites (Eickbush and Eickbush 1995; Almeida-Toledo et al. 1996). Indeed, a surprising chromosome spreading of associated transposable elements and ribosomal DNA (Rex3/5S rDNA) was demonstrated to occur in the freshwater fish *Erythrinus erythrinus* (Bloch et Schneider, 1801) (Erythrinidae), increasing the number of such rDNA sequences from 2 to 22 between distinct populations (Cioffi et al. 2010). Growing knowledge on the organization of repetitive DNAs also indicates that their evolution may be mediated by unequal crossover, transposition mediated by RNA and gene conversion (Dover 1986, Martins et al. 2006). Thus, different events may be associated with the serial repetition of the 5S rDNA multigene family in the genome of *T. goodei*, characterizing its more derived condition in relation to the other species, *T. falcatus* and *T. carolinus*.

The existing set of cytogenetic data for Carangidae suggests karyotype evolution strongly mediated by pericentric inversion events. Based on the basal karyotype for Perciformes (2n=48 acrocentrics, FN=48), the increase of FN indicates a derived condition. Thus, if *T. goodei* is the most derived species in respect to *T. falcatus* and *T. carolinus*, as indicated by mitochondrial sequences (Reed et al. 2002), and supported by the apomorphic features of its karyotype (multiple 18S and 5S rDNA sites), a par-

ticular evolutionary pathway provided by pericentric inversions must be considered for this species. Thus, the smaller number of two-armed chromosomes in *T. goodei* may indicate additional rounds of pericentric inversions on two-armed chromosomes, increasing the number of acrocentric chromosomes in the karyotype and, consequently, decreasing the FN value (Fig. 2b). The same could be also considered for *T. carolinus*, considering its more basal position in the phylogeny proposed for *Trachinotus* (Fig. 2a).

Our understanding of the karyotype evolution of Carangidae (including rDNA) was improved by the present findings. Our data demonstrate that, in addition to structural changes by pericentric inversions, rDNA sequences also acted as an important evolutionary indicator in *Trachinotus* karyotype. In this sense, the combined mapping of 18S and 5S rDNA sequences proved to be useful to clarify the relationships in this fish group.

Acknowledgements

We are grateful to the Coordination for the Improvement of Higher Education Teaching Personnel (CAPES) and the National Council for Scientific and Technological Development (CNPq) (Project No. 556793/2009-9) for their financial support. We also thank IBAMA (Process No. 19135/1) and José Garcia Júnior for the taxonomic identification of the species.

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CompCytogen 6(4): 371–377 (2012)
doi: 10.3897/CompCytogen.v6i4.3955
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

Does actually mean chromosome number increase with latitude in vascular plants? An answer from the comparison of Italian, Slovak and Polish floras

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Academic editor: *J. Daviña* | Received 6 September 2012 | Accepted 22 October 2012 | Published 19 November 2012

Citation: Peruzzi L, Góralski G, Joachimiak AJ, Bedini G (2012) Does actually mean chromosome number increase with latitude in vascular plants? An answer from the comparison of Italian, Slovak and Polish floras. Comparative Cytogenetics 6(4): 371–377. doi: 10.3897/CompCytogen.v6i4.3955

Abstract

We compared chromosome number (CN) variation among vascular floras of three different countries with increasing latitude in the Boreal hemisphere: Italy, Slovakia, Poland. Aim of the study was to verify whether the patterns of CN variation parallel the differences in latitudinal ranges. The three datasets comprised 3426 (Italy), 3493 (Slovakia) and 1870 (Poland) distinct cytotypes. Standard statistics (ANOVA, Kruskal–Wallis tests) evidenced significant differences among the three countries, mean CN increasing together with latitude. On the contrary, an inverse relation (r = -1) was evidenced among the frequency of odd CNs and latitude. Our results show that the hypothesis of a polyploid increase proportional with distance from the Equator seems to be confirmed, when territories from the same hemisphere are compared.

Keywords

Biogeography, chromosome number, cytogeography, cytotaxonomy, Europe, polyploidy

Introduction

Chromosome number is the most basic feature concerning the genome of a species, and it is also the easiest to obtain, technically. For this reason, since 1882 (Garbari et al. 2012), chromosome number data for many plant organisms have been accumulated worldwide accounting for about one third of plants being now known in this respect (Stace 2000). Although cytotaxonomy had become less popular in the end of twentieth

century (Guerra 2012), in the last years, a growing interest of scientific botanical community was devoted to plant chromosome number databases (Stuessy 2009), especially those in digitized format (Gacek et al. 2011; Bedini et al. 2012a, c).

As already pointed out by Peruzzi et al. (2011) and Bedini et al. (2012a, b, c), plant chromosome number databases are a useful tool for systematic comparisons of geographical or taxonomical groups of plants. In these studies, profound differences in chromosome number variation were evidenced for instance between Italian and antipodean New Zealand vascular flora, at various taxonomical scales (vascular plants as a whole, single orders), suggesting also possible different evolutionary dynamics among the two hemispheres (Peruzzi et al. 2011). Also just within Italian flora, a significant increase in mean chromosome number was evidenced to follow a bioclimatic/latitudinal gradient (Islands→southern peninsular Italy→northern Italy) (Bedini et al. 2012a) and specific orders and families where shown to be marked by peculiar chromosome number variation patterns (Bedini et al. 2012b).

A natural prosecution of the above mentioned studies, concerning geographical variation of mean chromosome number, was to extend the sample coverage, by selecting further countries (from the same hemisphere) to test the hypothesis that mean chromosome number in vascular plants tends to increase in parallel with latitude / cooler bioclimate. Accordingly, the aim of this study is to quantitatively evaluate chromosome number variation of vascular floras among three countries with increasing latitude and decreasing altitudinal range (Table 1): Italy, Slovakia and Poland.

Table 1. Range of latitudes (in degrees and in km) and altitudes for the considered countries.

	Degrees	Km	Altitude
Italy	35°29' to 47°05'N	1500	0–4810 m a.s.l.
Slovakia	47°40' to 49°35'N	200	94–2655 m a.s.l.
Poland	48°59' to 54°49'N	650	-2–2499 m a.s.l.

Methods

Data source

Chromosome numbers from the considered countries were taken from available online databases. *Chrobase.it* (Bedini et al. 2010 onwards) stores the available karyological information about Italian vascular flora, in terms of chromosome number (2n and/or n) and B-chromosome occurrence, along with main geographic-administrative data and literature references (Bedini et al. 2012a). The "Karyological database of ferns and flowering plants of Slovakia" (www.chromosomes.sav.sk/) stores the available karyological information about Slovak vascular flora, and was recently published also as hard-print book (Marhold et al. 2007). Finally "Chromosome number database – PLANTS" (Góralski et al. 2009 onwards) stores the available karyological information about Polish angiosperms. The latter database was also integrated by a recent survey on Polish

ferns (Ivanova and Piekos-Mirkova 2003). The total number of cytotypes retained for each dataset (ITA: Italy; SK: Slovakia; PL: Poland) was obtained by excluding counts in multiple copy (i.e. the same chromosome number for the same species). Eventual n counts (a minority in the three datasets) were transformed to 2n. Italian dataset coverage is about 35% of vascular plants (Bedini et al. 2012a), the Slovak dataset about 60% (Marhold et al. 2007), and the Polish one about 40% (Gacek et al. 2011). The families circumscription followed APG III (2009), Chase and Reveal (2011) and Christenhusz et al. (2011a–b).

Data analysis

Similarly to Bedini et al. (2012a, b), the following data were calculated for each dataset: mean chromosome number (CN hereafter), median, mode, Coefficient of Variation of CN (CV_{CN}), frequency of B-chromosomes occurrence (*f*B), frequency of odd CN (*f*OCN), not considering B-chromosomes. ANOVA was used to test statistical differences in CN among considered groups. If ANOVA was not applicable (Levene test), then the non-parametric U Mann-Whitney / H Kruskal-Wallis test was used.

Results

A total of 146 different CNs were found, ranging from 2n = 6 (in all datasets) to 2n = 304 (in the Slovak dataset only). The families included in the datasets were 107 for Italy, 123 for Slovakia and 114 for Poland. Of them, 82 were shared by all datasets. Most of the data (39–40% of each dataset) were concentrated in five families: Asteraceae, Brassicaceae, Fabaceae, Poaceae, Ranunculaceae (Table 2). CNs are apparently distributed in different proportions in the three geographical areas (Table 3; Figure 1). The most frequent (modal) CN in Italy is 2n = 18; in Slovakia it is 2n = 16 and in Poland 2n = 28. Despite this, mean CN is increasing from Italy, through Slovakia, to Poland (Table 1). This difference is supported by ANOVA ($F = 22.412$, $p < 0.000$), despite the absence of a significant distinction between Slovakia and Poland. On the contrary, the frequency of odd CNs (*f*OCN) tends to decrease from Italy to Poland (Spearman correlation between mean CN and *f*OCN: $r = -1.0$, $p < 0.01$), while the frequency of B-chromosomes is nearly 8-fold more frequent in Italy than in the other two countries. Indeed, B-chromosomes occur in 246 registered cytotypes (148 taxa) of the Italian vascular flora, in 65 cytotypes (27 taxa) of the Slovak flora and in 39 cytotypes (19 taxa) of Poland flora. Among the taxa showing B-chromosomes, their mean number is 2.03 ± 1.75 in Italy, 2.80 ± 1.99 in Slovakia and 1.95 ± 1.07 in Poland. Since the data on B-chromosome numbers did not follow a normal distribution, we performed the non-parametric Kruskal–Wallis test, which failed, however, to find significant differences between the number of B-chromosomes among the three geographical areas.

Table 2. Most represented families in the three datasets (> 100 registered cytotypes in at least one country).

	Italy		Slovakia		Poland	
	cytotypes	%	cytotypes	%	cytotypes	%
Amaryllidaceae	118	3.4	58	1.7	24	1.3
Asteraceae	579	16.9	573	16.4	275	14.7
Asparagaceae	135	3.9	53	1.5	21	1.1
Brassicaceae	193	5.6	238	6.8	80	4.3
Caryophyllaceae	133	3.9	145	4.2	56	3.0
Cyperaceae	56	1.6	110	3.1	36	1.9
Fabaceae	306	8.9	180	5.2	81	4.3
Juncaceae	9	0.3	103	2.9	18	1.0
Lamiaceae	111	3.2	127	3.6	62	3.3
Orchidaceae	158	4.6	62	1.8	30	1.6
Plumbaginaceae	128	3.7	4	0.1	1	0.1
Poaceae	166	4.8	251	7.2	209	11.2
Ranunculaceae	144	4.2	152	4.4	91	4.9
Rosaceae	27	0.8	187	5.4	118	6.3
other families	1163	33.9	1250	35.8	768	41.1

Table 3. Chromosome number parameters calculated for each country dataset.

	N°cytotypes	CN	± SD	median	mode	CV_{CN}	*f*B	*f*OCN
Italy	3426	30.560	22.060	24	18	72.186	0.071	0.087
Slovakia	3493	33.818	12.728	28	16	37.637	0.019	0.070
Poland	1870	33.820	23.386	28	28	69.149	0.021	0.044

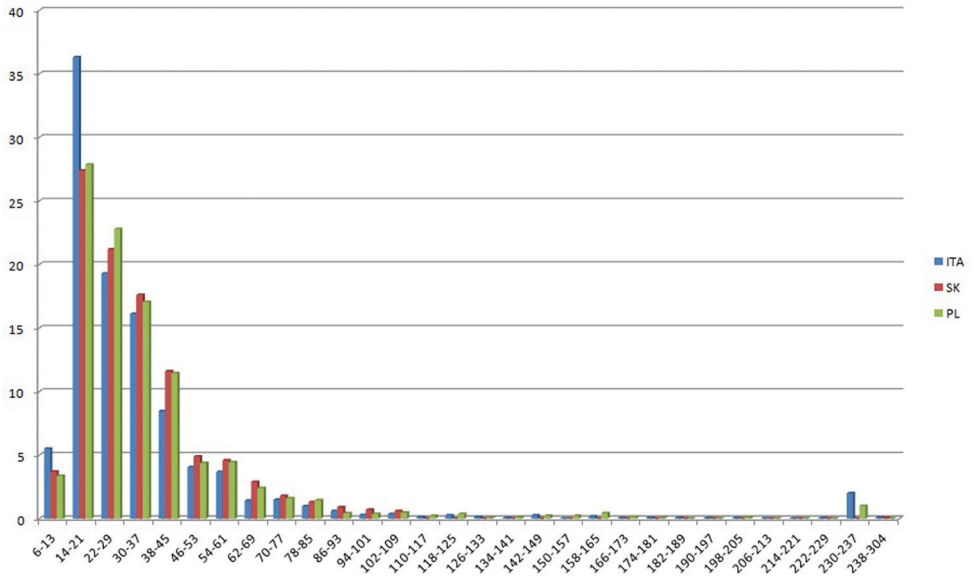

Figure 1. Histograms showing the percentage frequencies (y-axis) of 2n chromosome numbers, grouped in classes (x-axis) known for the Italian (ITA), Slovak (SK) and Polish (PL) vascular flora.

Discussion

According to our results, it was possible to confirm that, in Boreal hemisphere, mean CN in vascular plants tends to increase with increasing latitude, as already suggested by Bedini et al. (2012a) concerning Italy. Median and modal CN are less variable and not very useful to assess relationships among territories. Especially mode seems prone to reflect a casual abundance of certain taxa in the datasets. Indeed, Slovak and Polish floras, otherwise not statistically distinct, shows modal CNs 2n = 16 and 2n = 28, respectively. This is due to a number (62) of diploid Brassicaceae with $x = 8$ counted in the former country, and a number (56) of tetraploid *Rubus* Linnaeus, 1753 with x = 7 counted in the latter. The scarce differentiation between Slovakia and Poland could be easily explained by their shared administrative borders, with partial overlap of latitude range (cfr. Table 1). On the other hand, a possible influence of altitudinal range - in shaping CN variation among our datasets - cannot be ruled out too, since this parameter shows an exactly inverted variation trend respect with latitude variation (Table 1).

The idea that polyploidy tends to increase with latitude is not new (Löve and Löve 1957, Hanelt 1966, Hair 1966, Stebbins 1971, Levin 2002), but ploidy levels are not easy to assess on large datasets, with coverage comparable to that of current (either online or hard-printed) CN databases and atlases.

The use of mean CN as a proxy of polyploidy has the advantage to be easier to assess and more objective, albeit less precise. Indeed, CNs are unquestionable, while basic CNs are often subjective (see for instance the recent debate in Cusimano et al. 2012). Also the ancestral CN reconstructions are currently based on probabilistic models (Mayrose et al. 2010).

A further interesting point to address with further research is the seemingly different pattern of CN variation among the two hemispheres: Peruzzi et al. (2011) evidenced striking differences among Italy and New Zealand, two nearly antipodean countries. This could be due, to a certain degree, to the fully insular nature of the latter territory, where mean CN is about 2-fold. In order to positively verify whether the CN evolution dynamics in the Austral hemisphere are comparable to those in the Boreal one or not, it could be useful to compare different territories with increasing latitudes, for instance in the southern parts of America and Africa. Unfortunately, as far as we are aware, CN databases covering those territories are not available, or not significant in coverage of flora. Indeed, very recently an online cytogenetic database of Chilean plants was made available (Jara-Seguel and Urrutia 2011 onwards), but only 2.8% of Chilean angiosperm flora was karyologically studied (Jara-Seguel and Urrutia 2012). Similar degree of coverage exists for plants from Paraguay (Molero et al. 2001). Of course, the use of territories circumscribed by ecological and/or biogeographical criteria, instead of countries, could be even more useful to address these questions. Unfortunately, such kind of CN databases do not exist.

Contrary to what was observed for Italy (Bedini et al. 2012a), the frequency of B-chromosomes (*f*B) does not follow a geographical gradient, but in all the three considered countries values were higher than those reported for New Zealand (Peruzzi et

al. 2011). Indeed, the adaptive/ecological role of B-chromosomes is still a controversial issue (Jones 2012). Concerning the frequency of odd CNs (*f*OCN), it is clearly decreasing with increasing latitude, while New Zealand has a value intermediate between Slovakia and Poland (Peruzzi et al. 2011). Maybe the latter finding could be related with a different frequency of apomictic and/or holocentric species in the considered territories.

Acknowledgements

We are indebted to Prof. Karol Marhold (Bratislava) for having kindly provided an electronic version of the database for chromosome numbers of Slovakian flora.

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CompCytogen 6(4): 379–387 (2012)
doi: 10.3897/CompCytogen.v6i4.3422
www.pensoft.net/journals/compcytogen

RESEARCH ARTICLE

COMPARATIVE Cytogenetics
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Karyotype morphology suggests that the *Nyctibius griseus* (Gmelin, 1789) carries an ancestral ZW-chromosome pair to the order Caprimulgiformes (Aves)

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Academic editor: *N. Bulatova* | Received 26 May 2012 | Accepted 29 October 2012 | Published 30 November 2012

Citation: Nieto LM, Kretschmer R, Ledesma MA, Garnero ADV, Gunski RJ (2012) Karyotype morphology suggests that the *Nyctibius griseus* (Gmelin, 1789) carries an ancestral ZW-chromosome pair to the order Caprimulgiformes (Aves). Comparative Cytogenetics 6(4): 379–387. doi: 10.3897/CompCytogen.v6i4.3422

Abstract

Studies of karyotypes have been revealing important information on the taxonomic relationships and evolutionary patterns in various groups of birds. However, the order Caprimulgiformes is one of the least known in terms of its cytotaxonomy. So far, there are no cytogenetic data in the literature on birds belonging to 3 of 5 families of this order -Nyctibiidae, Steatornithidae and Aegothelidae. For this reason, the aim of our study was to describe the karyotype of *Nyctibius griseus* (Gmelin, 1789) (Aves, Nyctibiidae, Caprimulgiformes) and contribute with new data that could help to clarify the evolutionary relationships in this group. Bone marrow was cultured directly to obtain material for the chromosome study. C-banding was used to visualize the constitutive heterochromatin and Ag-NOR-banding to reveal nucleolus organizer regions. The diploid number observed was 2n=86±. Using sequential Giemsa/C-banding staining, we determined that the W chromosome was entirely C-band positive with the two most prominent markers in the interstitial and distal regions of the long arm. The nucleolus organizer regions showed a typical location in a pair of microchromosomes that exhibited Ag-NOR.The results obtained for *Nyctibius griseus* suggest that, of all the species studied in the references cited, it has the most ancestral sex chromosome composition of the order Caprimulgiformes.

Keywords
C and Ag-NOR bands, macrochromosomes, microchromosomes, ZW sex chromosomes, new karyotype

Introduction

At present, studies of the class Aves, which includes more than 9000 species, are fairly incomplete in regard to genetic and evolutionary studies (Pigozzi and Solari 2000). Cytogenetic studies have been conducted on less than 14% of the species content (Santos and Gunski 2006).

The species studied here, *Nyctibius griseus* (Gmelin, 1789) belongs to the order Caprimulgiformes, which includes the families Caprimulgidae, Nyctibiidae, Steatornithidae, Podargidae and Aegothelidae. *Nyctibius griseus* is found in South American territories from Costa Rica to Bolivia, Argentina, Uruguay and throughout Brazil, where it is common at the edges of forests, in fields with trees and on savannas. It feeds on nocturnal insects, mainly large moths, termites and beetles which it hunts in flight. It never lands on the ground, but always on branches, posts, fences and tree stumps, where it is easily camouflaged. This species has a form of adaptation unique among birds, known as the "magic eye" and consisting of two slits in the upper eyelid, which allows it to remain immobile for lengthy periods, watching its surroundings, even with its eyes closed. It lays one egg in a tree stump or branch cavity a few meters above ground level, where it is incubated for around 33 days (Accioly 2000). It is predominantly brownish in color, varying in tone from reddish to grayish, with streaks on its head and black markings on its breast. Its song consists of descending notes in the range of the human voice (Souza 2004).

Bird karyotypes generally consist of a diploid number of around 80 chromosomes, including eight macrochromosome pairs and 32 microchromosome pairs (Tegelström et al. 1983, De Lucca and Rocha 1992). According to Gunski et al. (2000), in species with a high number of microchromosomes, macrochromosomes with monobrachial or acrocentric morphology are prevalent. However, in species with a low number of microchromosomes, macrochromosomes with a bibrachial morphology are predominant, suggesting a process of karyotypic evolution through translocations between macro and microchromosomes, and also centric fusions of macrochromosomes.

In birds, a ZZ/ZW system determines gender, the male being the homogametic sex (the two sex chromosomes are homologous) and the female the heterogametic sex (the two sex chromosomes differ in size and morphology).

The members of the family Caprimulgidae are the most well-known of these birds, since they are found in all parts of the world, and consequently are better represented in terms of cytogenetic information. The references for the 40-year period of chromosome studies of this group of birds cited in Table 1 include descriptions of 8 karyotypes of Caprimulgidae: *Caprimulgus aegyptius arenicolor* (Lichtenstein, 1823), *Nyctidromus albicollis* (Gmelin, 1789), *Caprimulgus indicus* (Latham, 1790), *Hydropsalis brasiliana* (Gmelin, 1789), *Chordeiles pusillus* (Gould, 1861), *Caprimulgus parvulus*

Table 1. Diploid number and morphology of macrochromosomes (in the authors' original transcription) in Caprimulgiformes species already karyotyped.

Family/species	2n	Autosome pair number													Sex chromosomes		References
		1	2	3	4	5	6	7	8	9	10	11	12	13	Z	W	
Podargidae																	
Podargus strigoides	72	SM	T	T	T	T	T	T	T	T	T	T	T	T	_	_	Belterman and De Boer 1984
Caprimulgidae																	
Nictidromus albicollis	78	ST	ST	ST	ST	ST	ST	SM	T	T	T	T	T	T	M	M	De Lucca and Waldrigues 1986
Hidropsalis brasiliana	74	T	A	T	A	A	SM	SM	M	SM	M	T	T	T	A	M	Nieto and Gunski 1998
Chordeiles pusillus	68	A	T	T	SM	T	T	A	M	A	T	_	_	_	SM	T	Nieto and Gunski 1998
Caprimilgus aegyptius arenicolor	70	ST	A	A	A	A	SM	SM	ST	A	A	_	_	_	SM	_	Bulatova et al. 1971
Caprimilgus indicus	76	ST	T	T	ST	T	M	M	T	T	T	_	_	_	M	_	Bian et al. 1988
Caprimulgus parvulus	72	M	SM	SM	SM	M	M	SM	M	M	M	_	_	_	SM	T	Nieto and Gunski 1998
Caprimulgus rufus	78	A	A	T	A	A	A	A	M	A	A	_	_	_	M	M	Nieto and Gunski 1998
Lurocalis semitorquatus	82	SM	ST	T	ST	ST	SM	M	M	SM	M	SM	T	M	_	_	Francisco et al. 2006
Nyctibiidae																	
eusNyctibus gris	**86**	**ST**	**SM**	**A**	**A**	**SM**	**SM**	**ST**	**M**	**M**	**A**	**A**	**A**	**A**	**SM**	**SM**	this study

2n = diploid number, M = metacentric, SM = submetacentric, ST = subtelocentric, T = telocentric, A = acrocentric.

(Gould, 1837) and *Caprimulgus rufus* (Boddaert, 1783) and *Lurocalis semitorquatus* (Gmelin, 1789). Two families of the five are represented by one species each – Podargidae, *Podargus strigoides* (Latham, 1801) and Nyctibiidae, *Nyctibius griseus*. There were no previous description of the karyotypes in the latter family, and the aim of this work was to show some details characterizing the chromosome complement of this New World species and establish the C- and Ag-NOR-banding patterns which may be evolutionary informative for these birds.

Methods

Specimens were captured from dusk to nightfall, the period of greatest activity, using nets set up over tree stumps and branches of trees in the Misiones Province, Campo San Juan (Sta. Ana), Argentina.

Two specimens were analyzed, 1 male and 1 female. They were taxonomically identified by Professor Julio Contreras. The specimens were deposited at Bernardino Rivadavia Natural Sciences Museum Collection, under accession numbers 011578 (male) and 011577 (female).

Metaphases were obtained using the direct bone marrow culture technique (Garnero and Gunski 2000). The constitutive heterochromatin was identified using a modification of the method described by Ledesma et al. (2002) and the karyotypes were arranged according to the classification in Levan et al. (1964). The nucleolus organizer regions (AgNOR) were determined according to the silver–nitrate method described by Howell and Black (1980).

Results and discussion

This study presents the initial data on the number and morphology of chromosomes of *Nyctibius griseus* (Figs 1–4). The diploid number in specimens of both sexes is 2n=86±. The first chromosome pairs are the large subtelocentric, submetacentric, acrocentric and acrocentric, and the following 5 pairs reveal bibrachial constitution (submeta-, subtelo- or metacentrics). Lesser chromosomes look mainly acrocentrics. The sex chromosomes of this species are interesting, since the W chromosome has metacentric morphology and size similar to the Z chromosome, so externally the ZZ pair in a male and ZW in a female (Fig. 1) look the same.

Using Giemsa/C-banding re-staining, we determined that, in contrast to the C-negative Z chromosome with a centromeric C-band, the W chromosome (Fig. 2) looks entirely C-band positive with the two most prominent markers in the interstitial and distal region of the long arm, something that is not observed when the chromosome has a higher degree of condensation. The C-banding pattern shows that all macrochromosomes exhibit a centromeric C-band, except for pairs 8 and 9 that have an entirely C-band positive arm.

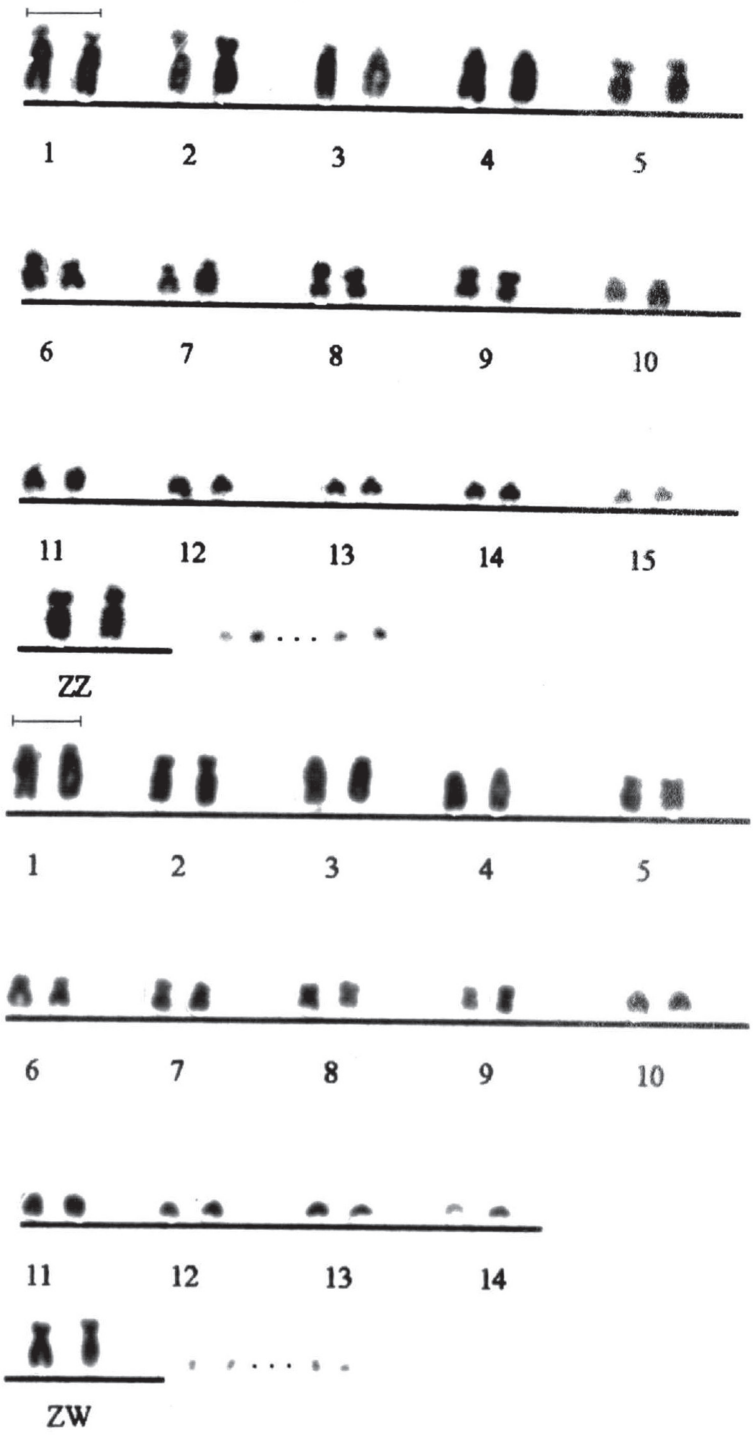
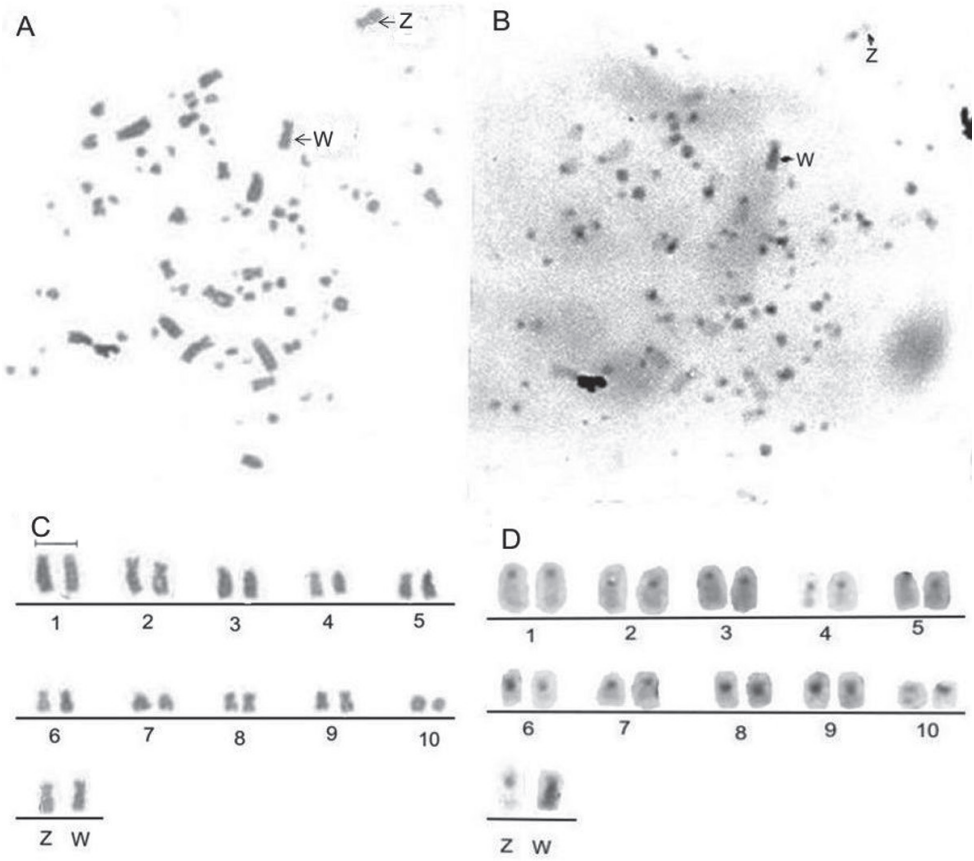


Figure 1. Male and female partial karyograms (without microchromosomes) of *Nyctibius griseus* (2n=86 ±). The similarity of the sex chromosomes ZZ and ZW is noticeable. Bar = 5 μm.



Figures 2 A–D. Metaphases and partial karyotype of female *Nyctibius griseus*: Giemsa (**A, C**) and C-banding (**B, D**) sequential staining. The arrows indicate the Z and W heterochromosomes. Among the autosomes, pairs 8 and 9 reveal an entirely heterochromatic short arm. Bar = 5 μm.

Figure 3 shows the Giemsa C-banding sequential staining for a male *Nyctibius griseus*, highlighting the positive C-band in the centromeric region of the two Z chromosomes. The nucleolus organizer regions (Fig. 4) show up in a microchromosome pair that exhibits a strong Ag-NOR-positive band, as it is common in many birds.

8 species belonging to the family Caprimulgidae (Table 1) exhibit a marked numeric variability, ranging from 2n=68 in *C. pusillus* to 2n=82 in *Lurocalis semitorquatus.* Thus, the newly described karyotype of *Nyctibius griseus* with its 86 chromosomes shows the highest 2n for the whole order Caprimulgiformes. Without data on chromosome homology, any suggestion on karyotypic rearrangements is unreliable, nevertheless, a monotonous size arrangement of the karyotype without sharp differences between one- and bi-brachial chromosomes does not support a proposition of fusion between macrochromosomes. Thus, the 2n variation observed might result from fusions of microchromosomes to macrochromosomes. The morphology and large size of the W chromosome of *N. griseus* represents one of the most important discoveries

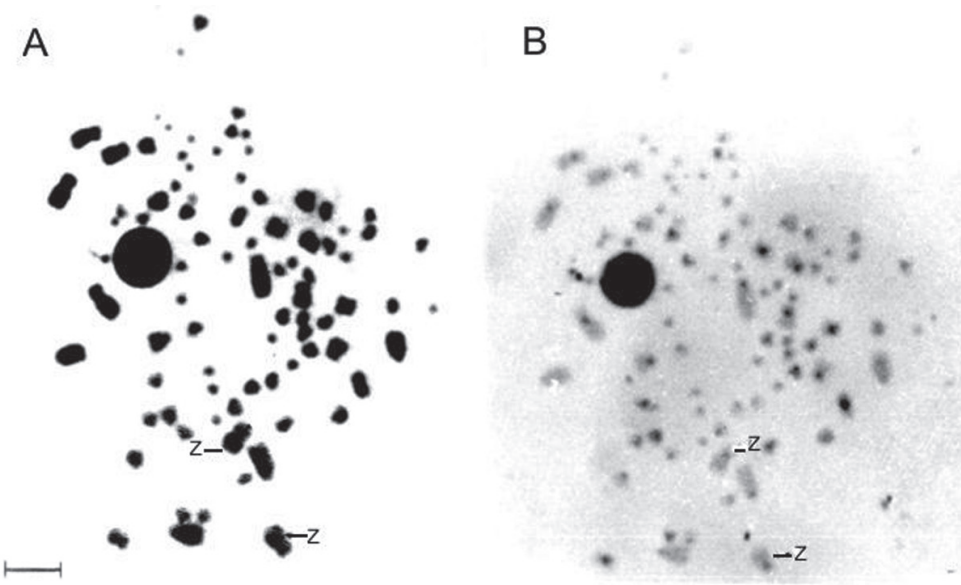


Figure 3. Routine Giemsa (**A**) and C-heterochromatin (**B**) sequential staining for a male *Nyctibius griseus*. The arrows indicate ZZ sex chromosomes. Bar = 5 μm.

in this study, and would lead us to infer that the species may be at a primitive stage of sex chromosome differentiation.

In the majority of bird species cytogenetically analyzed, the W chromosome is generally of a lesser size, close to the ninth or tenth pair, although there are cases like the one described by Christidis (1986) for *Neochmia phaeton* (Hombron & Jacquinot, 1841), a species in which the W chromosome is the third pair and is larger than the Z chromosome. Furthermore, in some species of Columbiformes (De Lucca and Aguiar 1976), Falconiformes (De Boer and Sinoo 1984), Passeriformes (Bulatova 1973) and Strigiformes (Renzoni and Vegni-Talluri 1966), the W chromosome was observed to be as large as the Z chromosome.

The W chromosome morphology in the family is also variable, ranging from metacentric to telocentric (Table 1). The wide variation in size and morphology of the W chromosome indicates different stages of differentiation, which shows that it has undergone greater changes in bird karyotype evolution than the Z chromosome.

In evolutionary terms, *Nyctibius griseus* may represent the first step in this differentiation, which according to Jones (1983), started from a homomorphic pair that acquired constitutive heterochromatin to become subsequently morphologically differentiated. These assumptions are in line with the distinct levels of heterochromatinization of the W chromosome of *N. griseus,* in which there is a general pattern of positive markers in the centromeric region, as well as an interstitial band and a telomeric region in the long arm.

The results we obtained for *Nyctibius griseus* lead us to assume that, of all the other Caprimulgiformes species studied, *N. griseus* exhibits the most ancestral sex chromsome composition.

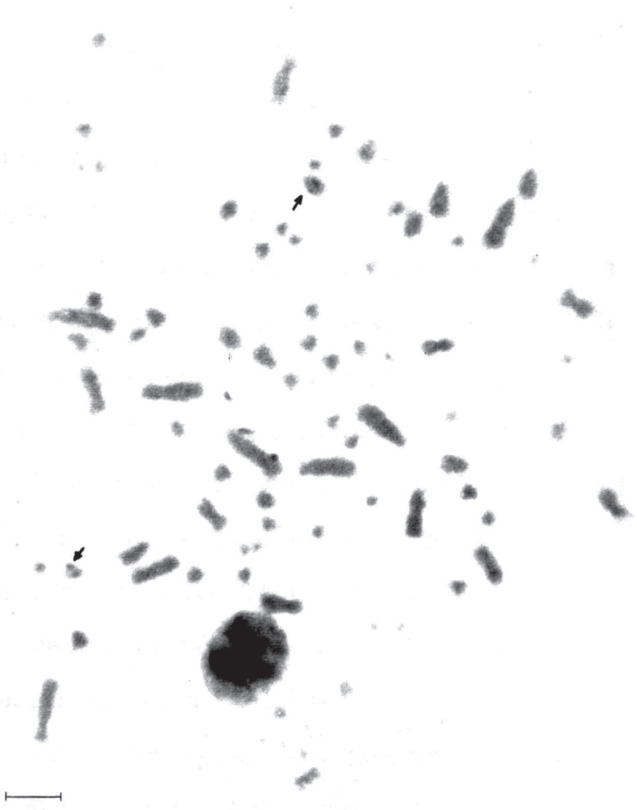


Figure 4. Nucleolus organizer regions in *Nyctibius griseus*. The arrows indicate NORs positioned in a strong secondary constriction probably of one pair of small one armed autosomes. Bar = 5 μm.

Acknowledgements

Rafael Kretschmer participate the Graduation Program - Masters in Biological Sciences - of the Federal University of Pampa (UNIPAMPA), Campus São Gabriel, RS, Brasil. Authors express their gratitude to the Refferee 2 for valuable criticism and commentary improving the initial text.

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CompCytogen 6(4): 389–395 (2012)
doi: 10.3897/CompCytogen.v6i4.4320
www.pensoft.net/journals/compcytogen

SHORT COMMUNICATIONS

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

A contribution to the taxonomy, cytogenetics and reproductive biology of the genus *Aclerda* Signoret (Homoptera, Coccinea, Aclerdidae)

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Academic editor: *V. Kuznetsova* | Received 15 November 2012 | Accepted 23 November 2012 | Published 30 November 2012

urn:lsid:zoobank.org:pub:C6EF0C47-F4FF-4335-9316-AF2FE610F70B

Citation: Gavrilov-Zimin IA (2012) A contribution to the taxonomy, cytogenetics and reproductive biology of the genus *Aclerda* Signoret (Homoptera, Coccinea, Aclerdidae). Comparative Cytogenetics 6(4): 389–395. doi: 10.3897/CompCytogen.v6i4.4320

Abstract

A new species of scale insects, *Aclerda pseudozoysiae* **sp. n.,** is described and illustrated. The karyotypes and some aspects of reproductive biology and cytogenetics of the new species species and *Aclerda takahashii* Kuwana, 1932 were studied, representing the first data for the genus *Aclerda* Signoret, 1874 and the family Aclerdidae as a whole. *A. pseudozoysiae* **sp. n.** has 2n=16, bisexual reproduction, and heterochromatinization of one haploid set of chromosomes in male stages of the life cycle, matching either a Lecanoid or a Comstockioid genetic system. *A. takahashii* demonstrates 2n=18 and unusual type of parthenogenesis with diploid and haploid embryos (inside each gravid female) without heterochromatinization. Both species are ovoviviparous; all stages of embryonic development occur inside the mother's body.

Keywords

Scale insects, Aclerdidae, *Aclerda takahashii, Aclerda pseudozoysiae*, taxonomy, morphology, new species

Introduction

The scale insect family Aclerdidae currently includes 5 genera with 58 species (ScaleNet <http://www.sel.barc.usda.gov/scalenet/scalenet.htm> – Ben-Dov et al. 2012) distributed mainly in hot and dry, often semi-desert regions of the world. Most of the species

are connected with grasses (Poaceae), inhabiting leaf sheathes, they demonstrate very specialized morphological characters such as the absence of legs and strong reduction of antennae, unique anal apparatus, unique invaginated setae, and others (see Fig. 1).

The species of the family have never been specially studied. However, Moharana (1990) noted 2n=18 for undetermined species of *Aclerda* Signoret, 1874 from India without photos or comments on karyotype and genetic system of this species. During an expedition to Indonesia in October – November 2011 I was able to collect two species of *Aclerda*. One of those species is suggested to be new for science and is described below; the second species, *Aclerda takahashii* Kuwana, 1932, is known to be widely distributed in tropical zones of the world. The study of the collected material has provided a possibility to present here some information on cytogenetics and reproductive biology of both discussed species.

Material and methods

Aclerda pseudozoysiae sp. n. K 884, Indonesia, New Guinea (Irian Jaya), vicinity of Jayapura city, slopes of Cyclop mountains above Entrop, dry primary forest interrupted by agricultural crops and sandy burrows, under the leaf sheaths of undetermined grass (Poaceae), 1.XI. 2011, Ilya Gavrilov-Zimin.

Aclerda takahashii. K 933, Indonesia, South-Eastern Sulawesi, vicinity of Kendari city near Haluoleo airport, chaotic agricultural plantations after recent deforestation, under the leaf sheaths of *Saccharum* sp., 12.XI.2011, Ilya Gavrilov-Zimin.

All material, including the types of the new species, is preserved in the Zoological Institute, Russian Academy of Sciences, St. Petersburg.

The chromosomal plates were prepared using a squash method in a drop of lactoacetorcein as previously described (Gavrilov and Trapeznikova 2007, 2008).

***Aclerda pseudozoysiae* sp. n.**
urn:lsid:zoobank.org:act:00A57BC1-DD95-4220-A601-C1554AFEBB87
http://species-id.net/wiki/Aclerda_pseudozoysiae
Fig. 1

Adult female. Body elongate oval, up to 7 mm long, slightly curved. Antennae small, 1-segmented, with several setae. Eyes and legs absent. Spiracles in two pairs; each with large and nearly circular and heavy sclerotized peritrema, covered by numerous quinquelocular pores. Posterior end of body heavily sclerotized on both surfaces even in very young females, abruptly narrowed and acutely pointed, ridged. Anal cleft short, about the same length as anal plate. Form of anal plate shown on the enlargement of Fig. 1. In general, the structure of anal complex is poorly visible because of heavy sclerotization of anal region of body, but it looks like anal complex in other species of the genus. Tubular ducts of 3 sizes: large tubular ducts about 18 μm long; medium-

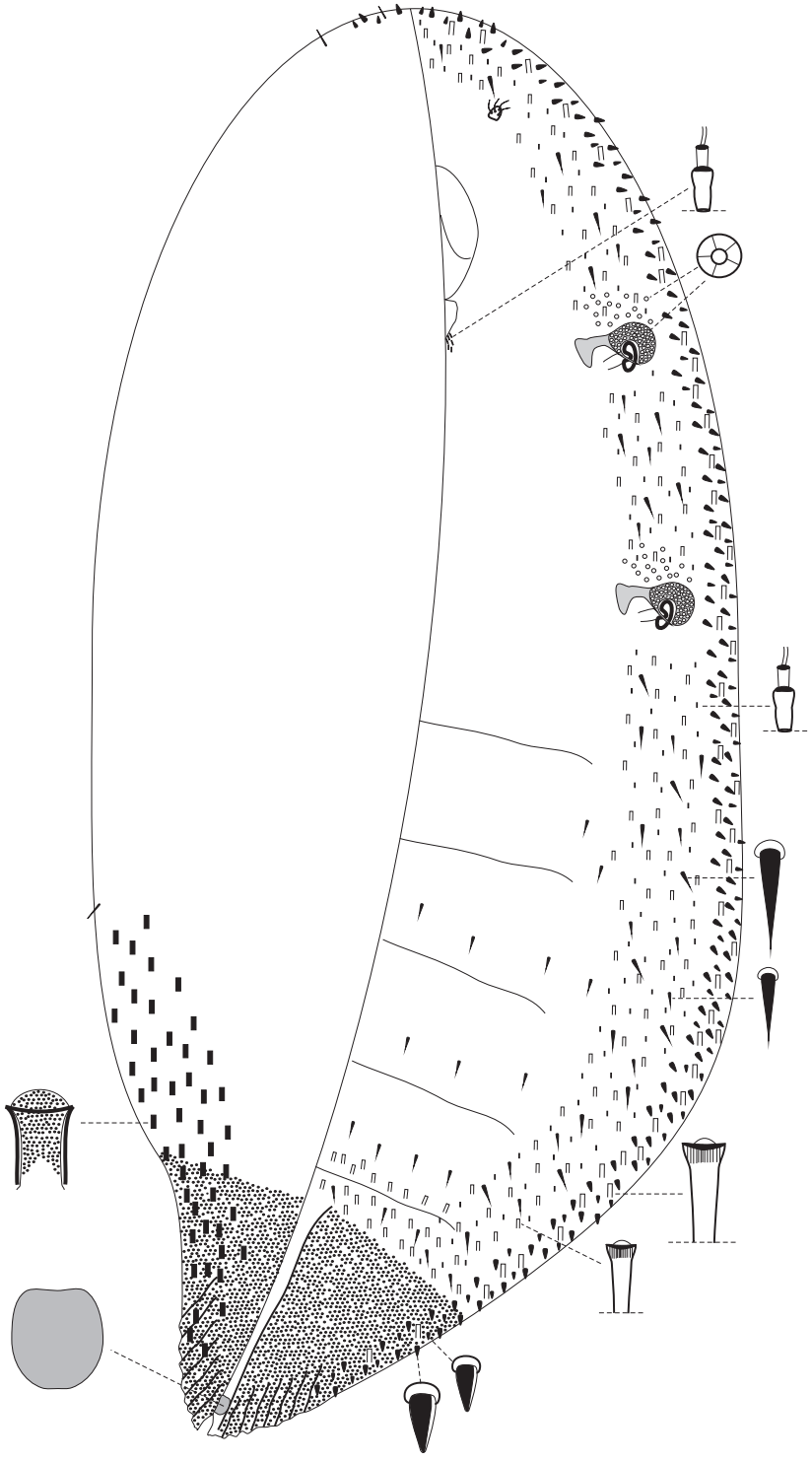


Figure 1. *Aclerda pseudozoysiae* sp. n., holotype.

sized ducts about 10 μm long; and microtubular ducts about 7–8 μm long. All 3 types of ducts form ventral submarginal band as shown in Fig. 1. Microtubular ducts form also a group near labium. Quinquelocular pores form small groups near spiracles (with about 10–20 pores in each group). Dorsal invaginated setae (about 12–15 μm long) arranged along submarginal area of abdomen.

Taxonomic notes. The large and widely distributed genus *Aclerda* was comprehensively revised by McConnell (1953). After this review no new *Aclerda* species have been described from Australasian or Indomalasian regions and in view of this I consider the McConnell' s identification key as correct until now. Based on McConnell's key, figures and descriptions, *Aclerda pseudozoysiae* sp. n. is similar to *A. zoysiae* McConnell, 1953 which was described from the Philippine Islands, but differs in the presence of 3 types of tubular ducts which are all located on the ventrum only in contrast to *A. zoysiae* having two types of ducts only (microtubular and macrotubular) distributed on both surfaces of the body.

Material. Holotype: female, K 884, vicinity of Jayapura, under the leaf sheath of undetermined grass (Poaceae), 1.XI. 2011, specimen in a black circle. Paratypes: 1 female on the same slide; 3 females on other slides and series of unmounted females and larvae in acet-ethanol; all with the same collecting data as holotype.

Etymology. The species name "*pseudozoysiae*" is composed of *pseudo* (false) and "*zoysiae*", and is intended to show its similarity to the related species, *A. zoysiae*.

Cytogenetics and reproductive biology

Both species are ovoviviparous; all stages of embryonic development occur inside the mother's body. In view of the absence of any notes on ovisacs in other species of *Aclerda* in the coccidological literature, I suppose that the genus as a whole is ovoviviparous.

Both species have a spermatheca, attached medially between two lateral oviducts (Fig. 2).

Unexpectedly, the mode of reproduction is found to be absolutely different in these two species.

Aclerda pseudozoysiae has bisexual reproduction, with the presence of male stages of the life cycle in the analyzed population. The studied male ultimonymphs contained bundles of sperms in their testicles (Fig. 3). Specimens with meiotic divisions were not collected. Male larvae and nymphs, and about 50% of the embryos inside each of the four dissected adult females demonstrated a heterochromatinization of one haploid set of chromosomes (Fig. 4), that is common for the majority of cytogenetically studied groups of the superfamily Coccoidea (see, for example, the review of Gavrilov 2007). According to the special experimental studies, elaborated on different genera of Coccoidea (see Brown and Nelson-Rees 1961) the presence of heterochromatinized haploid set characterizes male developmental stages only and moreover, the heterochromatinized set is usually of the paternal origin. Based on this heterochromatinization, *A. pseudozoysiae* is suggested to have either a Lecanoid or a Comstockioid genetic system,

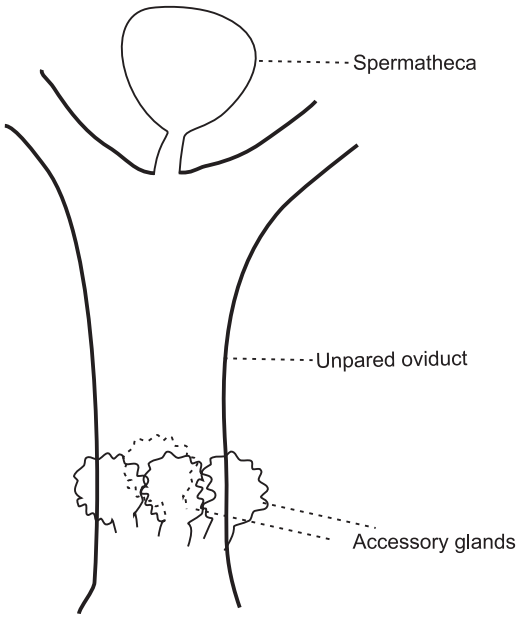
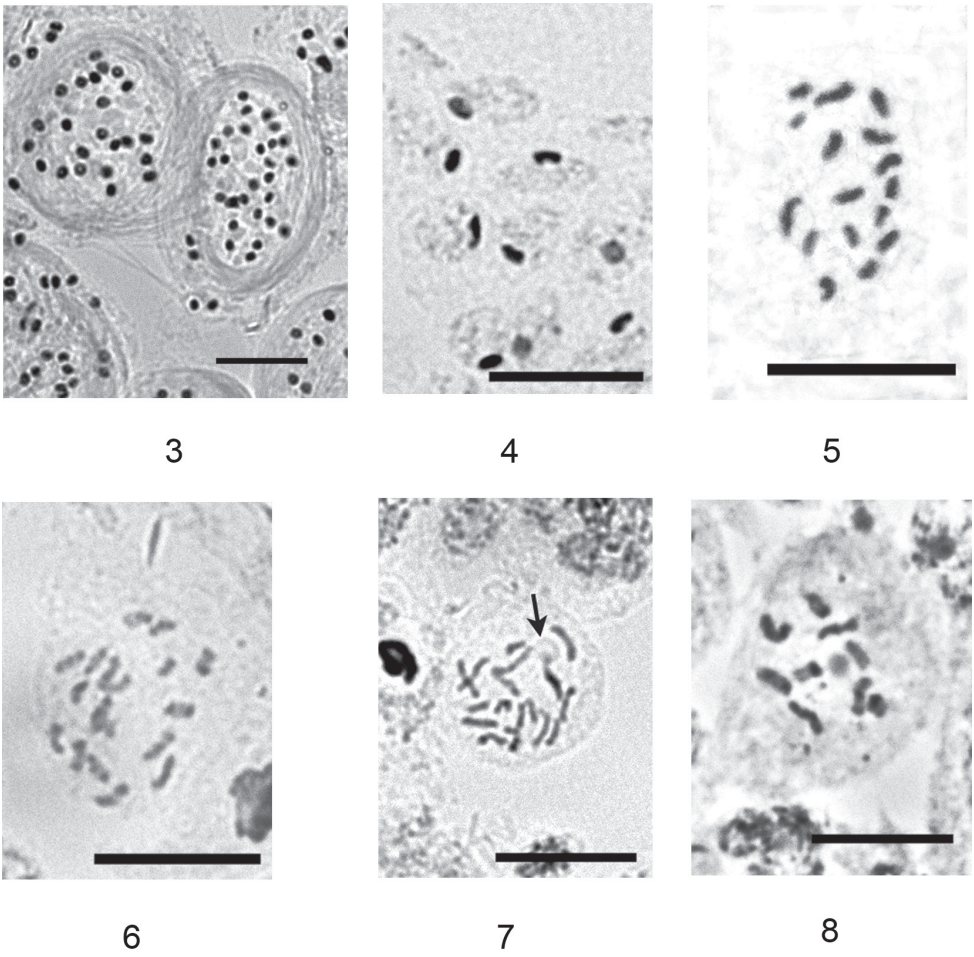


Figure 2. Schematic drawing of oviducts and spermatheca of studied *Aclerda* spp.

these systems being difficult to distinguish without special analysis of male meiosis (see, for example, Nur 1980). The diploid karyotype of *A. pseudozoysiae* includes 16 chromosomes forming gradual size series (Fig. 5).

On the contrary, in the studied population of *Aclerda takahashii*, no male stages of the life cycle were found and adult females did not have sperms and their spermathecae and oviducts. So, the species demonstrates a parthenogenetic form of reproduction. The diploid chromosomal number of *A. takahashii* was found to be 18 (Fig. 6, 7) with chromosomes forming more or less gradual size series. Some of the cells showed a nucleolus located at the end of one of the longer chromosomes (Fig. 7) (the localization of NORs in scale insects was discussed earlier by Gavrilov and Trapeznikova 2007). The heterochromatinization of one haploid set of chromosomes was not found in any of about 150 studied embryos from 4 females and, so, theoretically, all these embryos must be female embryos. However, only about 50 % of the embryos inside each studied female were diploid, and the others demonstrated haploid number (n=9) of chromosomes in each of the cells (Fig. 8). This sudden form of parthenogenesis seems to be unknown in scale insects. Usual haplo-diploidy is inherent in different species of Iceryini scale insects (superfamily Orthezioidea), but diploid progeny are characteristically produced by fertilized Iceryini females only (Hughes-Schrader 1948). In the superfamily Coccoidea, parthenogenesis with different ways of diploidy restoration is known in different families, but in all studied cases of deuterotoky and arrhenotoky, haploid embryos are not produced, diploidy is restored in all embryos and the heterochromatinization of one haploid set of chromosomes marks male embryos (Nur 1971, 1980). Probably, in *A. takahashii* the parthenogenetic diploidy res-



Figures 3–8. 3–5 *Aclerda pseudozoysiae* sp. n.: **3** bundles of sperms, **4** heterochromatinization of one haploid set of chromosomes (black bodies inside the cells), **5** karyotype **6–8** *Aclerda takahashii*: **6** diploid karyotype, **7** diploid karyotype with nucleolus (arrowed) **8** haploid karyotype. Bar = 10 μm.

toration takes place in a part of embryos only. The question whether haploid embryos are able to produce viable males/ females can not be answered without additional observations in the field and laboratory experiments.

Acknowledgements

The work was partly supported by the RFBR grants 11-04-00734-a and 12-04-31797. The collection of Zoological Institute of Russian Academy of Sciences was financially supported by the Ministry of Education and Science of the Russian Federation (project no. 16.518.11.7070).

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CompCytogen 6(4): 397–407 (2012)
doi: 10.3897/CompCytogen.v6i4.3722
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RESEARCH ARTICLE

COMPARATIVE Cytogenetics
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Chromosomes of *Lepidochitona caprearum* (Scacchi, 1836) (Polyplacophora, Acanthochitonina, Tonicellidae) provide insights into Acanthochitonina karyological evolution

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Academic editor: *V. Stunzenas* | Received 24 July 2012 | Accepted 22 October 2012 | Published 12 December 2012

Citation: Petraccioli A, Maio N, Odierna G (2012) Chromosomes of *Lepidochitona caprearum* (Scacchi, 1836) (Polyplacophora, Acanthochitonina, Tonicellidae) provide insights into Acanthochitonina karyological evolution. Comparative Cytogenetics 6(4): 397–407. doi: 10.3897/CompCytogen.v6i4.3722

Abstract

We describe the karyotype, location of nucleolus-organizing regions (NORs) and heterochromatin composition and distribution in *Lepidochitona caprearum* (Scacchi, 1836). The examined specimens had 2n=24 chromosomes; the elements of pairs 1–4 were metacentric, subtelocentric those of the fifth pair, telocentric the elements of other pairs. NOR-FISH, Ag-NOR- and CMA_3 banding showed NORs localized on pericentromeric regions of a medium small sized, telocentric chromosome pair. After C-banding or digestions with restriction enzyme NOR associate heterochromatin only was cytologically evident, resulting CMA_3 positive. The comparison with chromosome data of other chitons, other than to evidence a karyotypic similarity of *L. caprearum* to species of suborder Acanthochitonina, allows us to infer that chromosome evolution in the suborder mainly occurred via reduction of the number of the chromosomes by centric fusions, which took place repeatedly and independently in the different lineages of Acanthochitonina.

Keywords

Chromosome evolution, chromosome banding, heterochromatin, chitons, *Lepidochitona caprearum*, Acanthochitonina

Introduction

Polyplacophora, known also as chitons, includes about 900 living species, exclusively marine, distributed worldwide, mostly from the intertidal to the sub-littoral zone (Slieker 2000). These mollusks are scarcely investigated from a karyological point of view: data are available for only 21 species, all of the order Chitonida (*sensu* Sirenko 2006), namely ten of the suborder Chitonina (six species of the family Chitonidae and four of Ischnochitonidae) and eleven of the suborder Acanthochitonina (seven species of Acanthochitonidae, three of Mopaliidae and one of Tonicellidae) (Table 1). Though few, the karyological data have provided valuable information for systematics and phylogeny of chitons (Odierna et al. 2008). In order to increase karyological data on this class of mollusks we performed a chromosomal analysis using both conventional and banding staining methods and in situ hybridization (NOR-FISH) on *Lepidochitona caprearum* (Scacchi, 1836). For this chiton karyological data concern the chromosome number of 2n=24 and some details on morphology of eight large elements (meta- or sub-metacentric) (Vitturi et al. 1982). Systematic and phylogenetic relationships of this species are debated. In addition, *L. caprearum* has been the subject of several nomenclatural and taxonomic revisions. First Scacchi (1836) described this common Mediterranean chiton as *Chiton caprearum* Scacchi, 1836 (pag. 9); later, it was described by Reeve (1848) as *Chiton corrugatus* Reeve, 1848 (Plate 28, figure 185). Dall (1882) created the genus *Middendorffia* Dall, 1882 for it, and, successively, Kaas (1957) synonymised *Middendorffia caprearum* (Scacchi, 1836) with *Chiton corrugatus*. Successively, Kaas and van Belle (1981) carried out a systematic revision of perimediterranean and Atlantic species of the genus *Lepidochitona* Gray, 1821 and considered the taxon *Middendorffia* as synonym of the genus *Lepidochitona*. Finally, on the basis of the classification priority criterion, nomenclatural validity of the Scacchian taxon was demonstrated by Piani (1983) and a few years later by Gaglini (1985).

Table 1. Chomosome data of the chitons studied to date, classified according to Sirenko (2006). n= haploid number; FN = Fundamental number (arm number), M= metacentric, SM= Submetacentric, ST=subtelocentric; T=telocentric.

Order	Suborder	Family	Species	n	Haploid chr. for.	FN	Chitonida
Chitonida	Chitonina	Chitonidae	*Acanthopleura gemmata* (Blainville, 1825)	13	10 M, 3 SM	26	Yassen et al. (1995)
			Chiton granosus Frembly, 1827	12	6 M, 6 SM	24	Northland-Leppe et al. (2010)
			Chiton kurodai Is. & Iw. Taki, 1929	12	7 M, 4 SM, 1 ST	24	Yum and Choe (1996)
			Chiton olivaceus Spengler, 1797	13	12M, 1 SM	26	Vitturi et al. (1982)
			Liolophura japonica (Lischke, 1873)	12	12 M/SM	24	Nishikawa and Ishida (1969), Kawai (1976)
			Onithochiton hirasei Pilsbry, 1901	12			Nishikawa and Ishida (1969)

Order	Suborder	Family	Species	n	Haploid chr. for.	FN	Chitonida
		Ischnochitonidae	*Ischnochiton boninensis* Bergenhayn, 1933	12			Nishikawa and Ishida (1969)
			Ischnochiton comptus (Gould, 1859)	12			Nishikawa and Ishida (1969)
			Lepidozona albrechtii (von Schrenck, 1862) [= *Tripoplax albrechtii* (von Schrenck, 1862)]	12	10 M, 1M/SM, 1 SM	24	Choe et al. (1995), Yum and Choe (1996)
			Lepidozona coreanica (Reeve, 1847)	12	8 M, 1 M/SM, 3 SM	24	Nishikawa and Ishida (1969), Yum and Choe (1996)
	Acanthochitonina	Acanthochitonidae	*Acanthochitona achates* (Gould, 1859)	8	5 M, 1 SM, 2 ST	16	Rho et al. (1998)
			Acanthochitoa circellata (A. Adams & Reeve MS, Reeve, 1847)	8	1 M, 4 SM, 2 ST, 1 T	15	Rho et al. (1998)
			Acanthochitona communis (Risso, 1826) [= A. fascicularis (Linnaeus, 1767)]	12	2M, 5T, ?	undefined	Vitturi et al. (1982)
			Acanthochitona crinita (Pennant, 1777)	9	5 M, 2 SM, 2 ST	18	Colombera and Tagliaferri (1983)
			Acanthochitona defilippii (Tapparone Canefri, 1874)	8	3 M, 3 SM, 1 ST, 1 T	15	Nishikawa and Ishida (1969),Kawai (1976), Rho et al. (1998)
			Acanthochitona discrepans (Brown, 1827)	9	7 M, 1 St, 1 T	17	Certain (1951) in Nishikawa and Ishida (1969)
			Acanthochitona rubrolineata (Lischke, 1873)	8	5 M, 1 SM, 1 SM/ST, 1 ST	15	Nishikawa and Ishida (1969), Rho et al. (1998)
		Mopaliidae	*Katharina tunica* (Wood, 1815)	6	4 M, 2 T	10	Dolph and Humphrey (1970)
			Nuttallochiton mirandus (E. A. Smith MS, Thiele, 1906)	16	1M, 1SM, 14T	18	Odierna et al. (2008)
			Placiphorella stimpsoni (Gould, 1859)	12	6 M, 1 ST, 5 T	19	Nishikawa and Ishida (1969), Yum and Choe (1996)
		Tonicellidae	*Lepidochitona caprearum* (Scacchi, 1836)	12	4M/SM, ?	undefined	Vitturi et al. (1982)
				12	4 M, 1 ST, 7 T	17	present paper

Material and methods

We studied 4 males and 3 females of *L. caprearum* from Seiano (Naples, Italy) and 3 males and two females from Gaeta (Latina, Italy).

Gonads of each individual were excised and incubated for two hours in 1 ml of calf serum, previously heat inactivated at 56°C for 30 min, containing 50 ml of colcemid at 10 mg/ml. Then, the gonads were incubated for 30 min in hypotonic solution (KCl 0.075 M + sodium citrate 0.5%, 1:1) and fixed for 15 min in methanol + acetic acid, 3:1. After that, cell dissociations of gonads were made on a tea steel sieve and 20 µl of cell suspensions were dropped on clean slides (Petraccioli et al. 2010).

Standard chromosome staining was performed by using 5% Giemsa, pH 7.0. The following chromosome banding techniques also were used: Ag-NOR staining of Nucleolus Organizer Regions (Ag-NORs), chromomycin A_3 (CMA_3)/ methyl green staining, quinacrine (Q) banding, DA/DAPI, C-banding and sequential staining of C-banding+CMA_3+DAPI (details in Odierna et al. 2008), conducting the incubation in $Ba(OH)_2$ for 2 min and at room temperature. Karyotypes were constructed from seven Giemsa-stained mitotic metaphase plates and used to measure chromosome centromeric index (CI) and relative length (RL) according to the nomenclature by Levan et al. (1964).

NOR-FISH was performed as described by Petraccioli et al. (2010), with slight modifications, using as probe PCR amplified and biotinaled 18S rRNA gene sequence units of the pectenid *Adamussium colbecki* (Smith, 1902). Slides were aged for a week at room temperature and two hours a 60°C, and then incubated for 30 min in Rnase at 100 mg/ml in Tris-HCl pH 6.5. Slides were washed two min for each ethanol 50, 70, 90 and 100% and air dried. Chromosomes and probe were denatured at 72°C with the hybridization mixture (10 ng/ml biotinylated 16 dUTP probe + 0.1 mg/ml shared *E. coli* DNA in 2xSSC with 50% formamide) for 2 min. The hybridizations were carried over-night at 40 °C. After washing in 1xSSC at 72°C for 5 min and at RT for 2 min in blocking solution (dry milk 2% + 0,1% of Tween 20 in 4xSSC), cytochemical detection was performed by incubating slides for 1 h with monoclonal anti-biotin (Sigma cod. B7653) diluted 1:500 in PTB (1 ml PTB= 5 µl of Tween 20% + 0.01 g of Dry milk + in 1 ml of PBS 0,2 M), washing in 1xPBS and incubating for 30 min in anti-anti-biotin diluted 1:50 in PTB. After washing in PBS, slides were counterstained with 5 µg/ml propidium iodide (PI) in 1xPBS for 15 min at room temperature and, finally, mounted with antifade (DABCO, Sigma). The hybridization signals were detected and recorded under an epifluorescent microscope (Axioscope Zeiss) equipped with a digital camera.

Results

Twelve bivalents, four larger than the other eight ones resulted present in 25 examined male, diakinetic, meiotic figures (Fig. 1). The diploid number of 2n=24 chromosomes was confirmed by the examination of 15 spermatogonial and ten oogonial metaphase plates. Independently of sex and provenance, karyotypes consisted of four pairs (1–4)

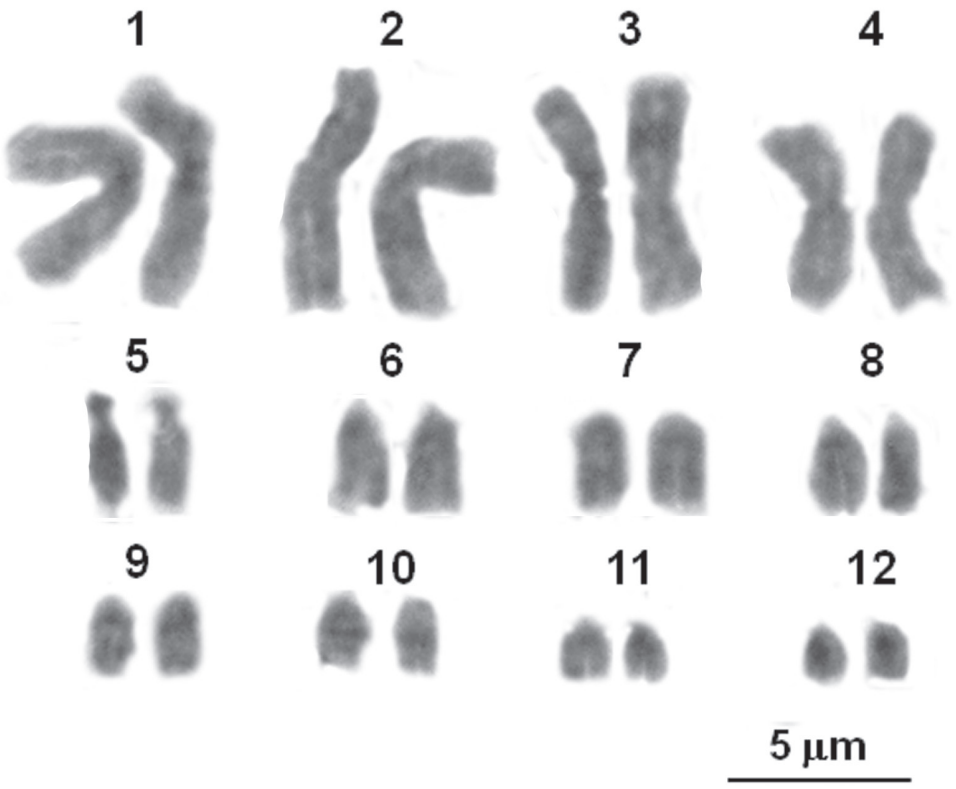

Figure 1. Giemsa stained karyotype of a male of *L. caprearum* from Seiano (Naples, Italy).

Table 2. Chromosome morphometric parameters of *L. caprearum*, according to Levan et al. (1964); M= metacentric, ST= subtelocentric, T= telocentric.

Chromosome	Relative Length (RL) mean ± SD	Centromeric index (CI) mean ± SD	Chromosome type
1	18.2 ± 0.5	48.3 ± 3.0	M
2	17.0 ± 0.7	39.9 ± 2.8	M
3	15.2 ± 0.4	49.0 ± 3.1	M
4	12.8 ± 0.6	39.1 ± 2.9	M
5	7.7 ± 0.5	18.2 ± 2.0	ST
6	6.2 ± 0.4	0	T
7	5.3 ± 0.3	0	T
8	4.0 ± 0.5	0	T
9	3.9 ± 0.6	0	T
10	3.8 ± 0.4	0	T
11	3.2 ± 0.5	0	T
12	2.7 ± 0.4	0	T

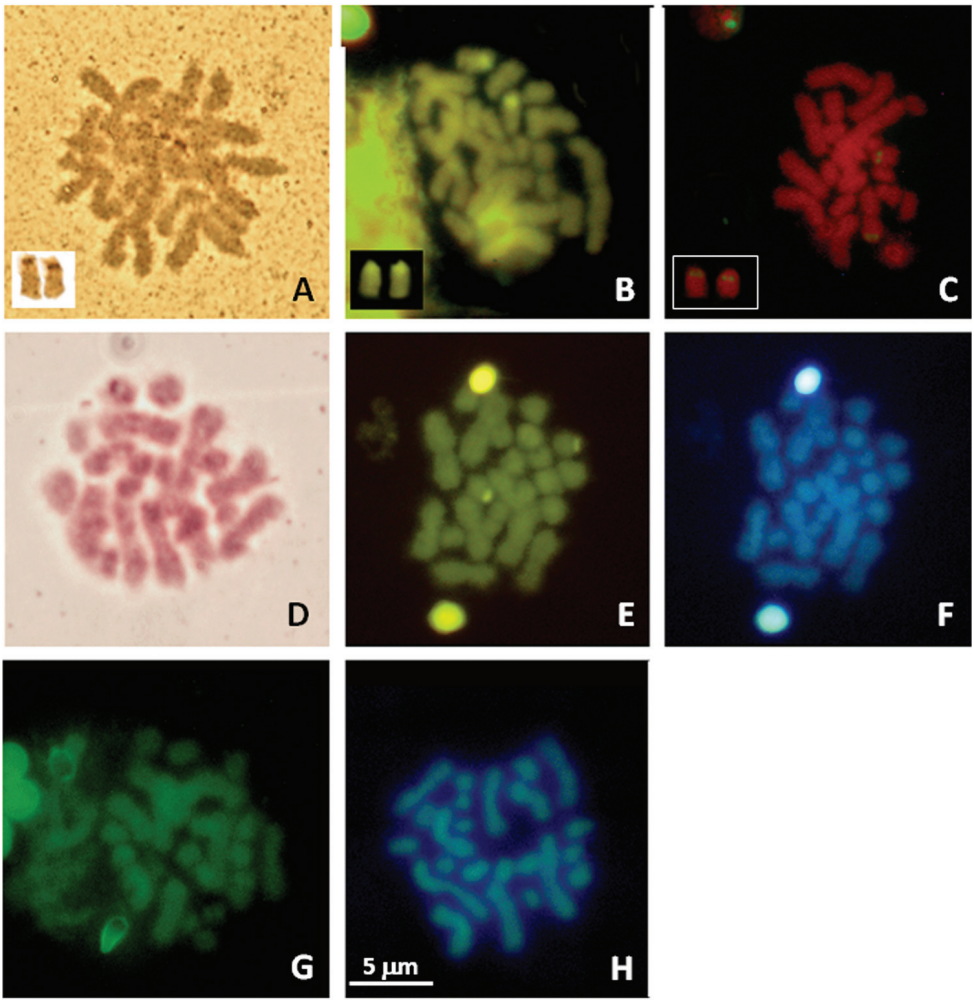

Figure 2. Male (**A**, **C**, **E**, **F** and **G**) from Seiano, Naples, Italy, and female, from Gaeta, Latina, Italy, (**B**, **D** and **H**) metaphase plates of *L. caprearum*, stained with Ag-NOR banding (**A**),CMA_3 banding (**B**), NOR-FISH (**C**), C-banding + Giemsa (**D**); C+banding + CMA_3 (**E**)+DAPI (**F**), Quinacrine (**G**) and DA/DAPI (**H**). Panels in A, B and C include their relative NOR bearing chromosome pair. Scale bar in **H** refers all images.

with metacentric elements, a pair (the fifth) with subtelocentric chromosomes, the remaining pairs (6–12) included telocentric elements (haploid chromosome formula: 4M, 1ST, 7T; Arm number, FN=17 (Table 2; Fig. 1). One NOR bearing pair resulted evidenced after staining with Ag-NOR-, CMA_3 banding and NOR-FISH; loci NORs were on pericentromeric regions of two medium sized telocentric chromosomes, tentatively the pair eight or nine (Fig. 2 A, B and C). After C-banding staining or digestions with Restriction enzyme *Alu*I, NOR associated heterochromatin only was well evident, resulting CMA_3 positive and DAPI negative (Fig. 2 D, E and F). Quinacrine and DA/DAPI banding uniformly stained the chromosomes (Fig. 2 G and H).

Discussion

According to the classification by Kaas and Van Belle (1998) species of genus *Lepidochitona* belong to the family Ischnochitonidae Dall, 1989, suborder Chitonina. In contrast, Sirenko (2006), in his classification, included *Lepidochitona* in the family Tonicellidae Simroth, 1894, suborder Acanthochitonina. In agreement with Vitturi et al. (1982) we find that *L. caprearum* possesses 2n=24 chromosomes. This chromosome number is also displayed by all the so far studied species of Ischnochitonidae, namely two species of *Ischnochiton* Gray, 1847 and two of *Lepidozona* Pilsbry,1892 (Nishikawa and Ishida 1969, Choe et al. 1995, Yum and Choe 1996). Only for the two *Lepidozona* species the chromosome morphology is given (Yum and Choe 1996), and in both cases the elements only are metacentric or submetacentric. This kind of chromosome sets can be ranked more or less symmetric (White 1978), that is karyotypes only including a series of elements gradually decreasing and with chromosome arms of almost equal length. Interestingly, the other so far investigated species of the suborder Chitonina possess karyotypes of 2n=24 or 26 elements metacentric or submetacentric, (see Table 1), excluding *Chiton kurodai* Is. & Iw. Taky, 1929, which has a karyotype with a pair of subtelocentric elements (Yum and Choe 1996). In contrast, even if possessing 2n=24 elements, the karyotype of *L. caprearum* strongly deviates from those of Chitonina species. In fact, other than biarmed chromosomes, its karyotype includes also subtelocentric and telocentric elements. Interestingly, a similar karyotype is also displayed from all Acanthochitonina species (see Table 1), to which, then, *L. caprearum* is karyologically related. Molecular phylogenetic study on chitons by Okusu et al. (2003) suggests a close relationship between *Lepidochitona* and the mopaliid species, *Katharina tunicata* (Wood, 1815), which, according to Dolph and Humphrey (1970), possesses 2n=12 chromosomes with a chromosome formula of 8M+4ST. However, both molecular relationship and chromosome record for *K. tunicata* have to be considered with caution. In fact, Mopaliidae in the molecular phylogeny by Okusu et al. (2003), appear polyphyletic, a state not considered in the systematic revision by Sirenko (2006), where Mopaliidae are monophyletic. Concerning chromosome data of *K. tunicata*, the record by Dolph and Humphrey (1970) needs confirmation, because from examination of the figure provided by the authors, all chromosome pairs are unpaired (each pair contains elements differing in length and/or shape). However, among Acanthochitonina a set with 2n=24 elements is shown by two species: one of the family of Acanthochitonidae, namely *Acanthochitona communis* (Risso, 1826) [= *A. fascicularis* (Linnaeus, 1767)], but with the chromosome formula not completely resolved (Vitturi et al. 1982); the second species of the family of Mopaliidae, namely, *Placiphorella stimpson*i (Gould, 1859), which has a chromosome formula of 6M, 1ST, 5T (Yum and Choe 1996) (Table 1). However, the karyotypes of *P. stimpsoni* and *L. caprearum* are strongly divergent (see Table 1). In fact their chromosome sets differ both in the number of metacentric and telocentric elements and because in the set of *P. stimpsoni* the first two pairs are markedly longer than the other pairs, while in *L. caprearum* are four the pairs clearly longer than the other ones (see Fig. 3 for a compari-

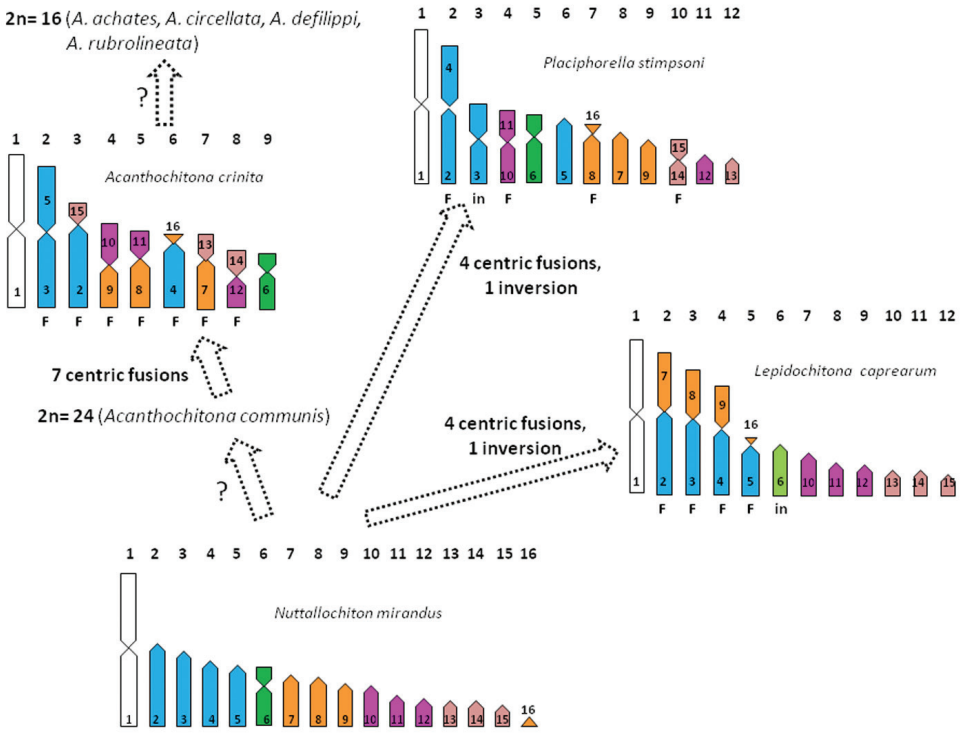

Figure 3. Hypothesis on the derivation of the karytotypes of *L. caprearum*, *P. stimpsoni* and *A. crinita* from that of *N. mirandus*. Haploid chromosome ideograms have been depicted according to the relative length and centromeric indexes given by Yum and Choe (1996) for *P. stimpsoni*, Colombera and Tagliaferri (1970) for *A. crinita*, Odierna et al. (2008) for *N. mirandus* and the present paper for *L. caprearum*. The numbers included in the chromosomes refer to those of *N. mirandus* supposed involved in the chromosome changes.

son). So, multiple and complex chromosome rearrangements occur for the transition between karyotypes of *L. caprearum* and *P. stimpsoni*. A possible, alternative scenario for the origin of their chromosome set is given in Fig. 3. The scenario is based on the hypothesis, that we advanced in our previous study (Odierna et al. 2008), according to which a karyotype like that of *Nuttallochiton mirandus* (E. A. Smith MS, Thiele, 1906), of 2n=32 elements with a chromosome formula of 1M, 1SM, 14T, is primitive and the karyotypes with lesser chromosome number derived from it, mainly by a series of Robertsonian fusions. Accordingly, the karyotype of *L. caprearum* could have arisen from a *N. mirandus* like karyotype by four centric fusions plus one inversion (see Fig. 3). Similarly, one inversion and four centric fusions also could give rise to the karyotype of the *P. stimpsoni* from one *N. mirandus* like. In addition, a derivation from a karyotype *N. mirandus* like could also be supposed for that one of 2n=18 chromosomes of the Acanthochitonid species, *Acanthochitona crinita* (Pennant, 1777), (Colombera and Tagliaferri 1983): in fact, seven centric fusions occur for the transition from *N. miran-*

dus like karyotype to that of *A. crinita* (see Fig. 3). Moreover, in this genus a further reduction to 2n=16 chromosomes also occurred; since this chromosome number is showed by *Acanthochitona achates* (Gould, 1859), *Acanthochitona circellata* (A. Adams & Reeve MS, Reeve, 1847) *Acanthochitona defilippi* (Tapparone Canefri, 1874), and *Acanthochitona rubrolineata* (Lischke, 1873) (see Table 1). Interestingly, in this genus the reduction of chromosomes number to 2n=18 or 16 an intermediate step of 2n=24 could not be ruled out, as suggested by the karyotype of *A. communis*, which has 2n=24 elements (Vitturi et al. 1982). It should be noted that for the derivation of the chromosome set of *L. caprearum*, *P. stimpsoni* and *A. crinita*, different elements of the karyotype like that of *N. mirandu*s have supposedly been involved both in the centric fusions and inversions, meaning that these rearrangements have occurred repeatedly and independently in the diverse lineages of suborder Acanthochitonina. This hypothesis on the chromosome evolution in Acanthochitonina is also the most parsimonious and supports the inclusion of *Lepidochitona* in the suborder Acanthochitonina operated by Sirenko (2006) in his chiton systematic revision.

Studies on NOR localization and heterochromatin distribution and composition proved to be valuable in providing taxonomic, systematic and evolutionary information in several taxa, including bivalves (Thiriot-Quievreux 2002, Wang and Guo 2004) and gastropods (Thiriot-Quievreux 2003, Odierna et al. 2006 a, b). Conversely, comparable data on NOR loci and heterochromatin distribution and composition in chitons are only available for *N. mirandus* (Odierna et al. 2008). Two chitons species display quite different patterns of those chromatinic markers. In fact, in *L. caprearum* NORs are on the pericentromeric regions of a single pair and in at least three pairs in *N. mirandus*, karyological characters considered, respectively, a primitive and derivate in several taxa, including mollusks (Thiriot-Quievreux 2002, 2003, Wang and Guo 2004, Odierna et al. 2006 a, b). Heterochromatin in *L. caprearum* is very scarce and with a uniform constitution with the exclusion of that associated with the NOR, which is CMA_3 positive, then GC rich, as usually observed in several taxa, including mollusks (Odierna et al. 2006 a, b, Petraccioli et al. 2010). In contrast heterochromatin in *N. mirandus* is abundant and has a compound composition with clusters AT and GC rich (Odierna et al. 2008). Further studies on localization of NORs and/or heterochromatin composition and distribution in other chitons could provide useful taxonomic and systematic information on this class of mollusks.

Acknowledgements

This study was carried out in accordance with the guidelines for the care and use of experimental animals of the University of Study of Naples. We thank Giuseppe Fasulo and Paolo Crovato which helped us in the specific taxonomic attribution of the samples to *L. caprearum*, Peter N. Psomadakis and Boris Sirenko for their suggenstions and language revision.

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CompCytogen 6(4): 409–423 (2012)
doi: 10.3897/CompCytogen.v6i4.3945
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 seven species of the tribe Lophiohylini (Hylinae, Hylidae, Anura), with conventional and molecular cytogenetic techniques

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Academic editor: *L. Kupriyanova* | Received 3 September 2012 | Accepted 21 November 2012 | Published 3 December 2012

Citation: Gruber SL, Haddad CFB, Kasahara S (2012) Karyotype analysis of seven species of the tribe Lophiohylini (Hylinae, Hylidae, Anura), with conventional and molecular cytogenetic techniques. Comparative Cytogenetics 6(4): 409–423. doi: 10.3897/CompCytogen.v6i4.3945

Abstract

Few species of the tribe Lophiohylini have been karyotyped so far, and earlier analyses were performed mainly with standard staining. Based on the analysis of seven species with use of routine banding and molecular cytogenetic techniques, the karyotypes were compared and the cytogenetic data were evaluated in the light of the current phylogenies. A karyotype with 2n = 24 and NOR in the chromosome 10 detected by Ag-impregnation and FISH with an rDNA probe was shared by *Aparasphenodon bokermanni* Miranda-Ribeiro, 1920, *Itapotihyla langsdorffii* (Duméril and Bibron, 1841), *Trachycephalus* sp., *T. mesophaeus* (Hensel, 1867), and *T. typhonius* (Linnaeus, 1758). *Phyllodytes edelmoi* Peixoto, Caramaschi et Freire, 2003 and *P. luteolus* (Wied-Neuwied, 1824) had reduced the diploid number from 2n = 24 to 2n = 22 with one of the small-sized pairs clearly missing, and NOR in the large chromosome 2, but the karyotypes were distinct regarding the morphology of chromosome pairs 4 and 6. Based on the cytogenetic and phylogenetic data, it was presumed that the chromosome evolution occurred from an ancestral type with 2n = 24, in which a small chromosome had been translocated to one or more unidentified chromosomes. Whichever hypothesis is more probable, other rearrangements should have occurred later, to explain the karyotype differences between the two species of *Phyllodytes* Wagler, 1830. The majority of the species presented a small amount of centromeric C-banded heterochromatin and these regions were GC-rich. The FISH technique using a telomeric probe identified the chromosome ends and possibly $(TTAGGG)_n$-like sequences in the repetitive DNA out of the telomeres in *I. langsdorffii* and *P. edelmoi*. The data herein obtained represent an important contribution for characterizing the karyotype variability within the tribe Lophiohylini scarcely analysed so far.

Keywords
Amphibian cytogenetics, Ag-NOR, C-banding, rDNA probe, telomeric probe, fluorochrome staining

Introduction

The hylids of the subfamily Hylinae Rafinesque, 1815 are grouped into four large tribes: Cophomantini, Dendropsophini, Hylini, and Lophiohylini (Faivovich et al. 2005, Wiens et al. 2010). In the tribe Lophiohylini 11 genera are assigned and the majority of them included the known casque-headed frogs which are distributed throughout Central and South America. According to Faivovich et al. (2005), despite the phylogenetic review based mainly on molecular gene sequencing, few morphological synapomorphies support the current taxonomy of the tribe Lophiohylini and many unresolved questions still remain. Recently, the separate genus *Phytotriades* Jowers, Downieb & Cohen, 2009 was erected for the species *Phyllodytes auratus* (Boulenger, 1917) based on analysis of mitochondrial rDNA sequences.

About 70 species are recognised in the tribe Lophiohylini (Frost 2011), but only a dozen of them from seven genera have been karyotyped (Catroli and Kasahara 2009). Earlier analyses, performed exclusively with standard staining, were conducted during the 1960s and 1970s in the species *Aparasphenodon brunoi* Miranda-Ribeiro, 1920, *Itapotihyla langsdorffii* (Duméril & Bibron, 1841), *Osteopilus septentrionalis* (Duméril & Bibron, 1841), *Trachycephalus mesophaeus* (Hensel, 1867), and *T. typhonius* (Linnaeus, 1758), all of them with 2n = 24, and *Osteopilus brunneus* Trueb and Tyler, 1974 with 2n = 34 (Duellman and Cole 1965, Rabello 1970, Bogart and Bogart 1971, Foresti 1972, Bogart 1973, Cole 1974). Subsequently studies were carried out with use of banding and FISH techniques on some of these species (*A. brunoi*, *I. langsdorffii*, *O. septentrionalis*, and *O. brunneus*) and also in *Argenteohyla siemersi* (Mertens, 1937), *Corythomantis greeningi* Boulenger, 1896, *Osteocephalus taurinus* Steindachner, 1862, *O. dominicensis* (Tschudi, 1838), and *O. marianae* (Dunn, 1926), all of them with 2n = 24, and in *O. wilderi* (Dunn, 1925) with 2n = 28 (Schmid 1978, 1980, Anderson 1996, Morand and Hernando 1996, Kasahara et al. 2003, Nunes and Fagundes 2008). The species of the Lophiohylini genera *Nyctimantis* Boulenger, 1882, *Tepuihyla* Ayarzagüena, Señaris and Gorzula, 1993, *Phyllodytes* Wagler, 1830, and *Phytotriades* Jowers, Downieb et Cohen, 2008 have never been karyotyped.

The present paper deals with the chromosome analysis of *A. bokermanni* Pombal, 1993, *I. langsdorffii*, *P. edelmoi* Peixoto, Caramaschi et Freire, 2003, *P. luteolus* (Wied-Neuwied, 1824), *T. mesophaeus*, *T. typhonius*, and *Trachycephalus* sp. (probably an undescribed species) with use of routine and molecular cytogenetic techniques. The aim was to analyze species never karyotyped before and to improve the cytogenetic data from some other species, in order to better characterizing the karyotype variability within the tribe Lophiohylini and to carry out a more comprehensive comparative analysis in the light of the current phylogeny.

Material and methods

Cytogenetic analyses were performed with specimens of *Aparasphenodon* Miranda-Ribeiro, 1920, *Itapotihyla* Faivovich, Haddad, Garcia, Frost, Campbell, et Wheeler, 2005, *Phyllodytes*, and *Trachycephalus* Tschudi, 1838 (Table 1) collected in the Brazilian states of Alagoas (AL), Bahia (BA), Espírito Santo (ES), Mato Grosso (MS), and São Paulo (SP). The voucher specimens were deposited in the amphibian collection Célio Fernando Baptista Haddad (CFBH), housed in the Departamento de Zoologia, UNESP, Rio Claro, SP, Brazil.

Direct cytological suspensions of bone marrow, liver, and testes were prepared according to the procedures described in Baldissera et al. (1993), and from the intestinal epithelium according to the method of Schmid (1978). The slides were subjected to standard Giemsa staining and to the techniques of Ag-NOR (Howell and Black 1980), C-banding (Sumner 1972), and double staining with the fluorochromes AT-specific DAPI and GC-specific CMA_3 (Christian et al. 1998). Fluorescent in situ hybridisation (FISH) (Pinkel et al. 1986) was carried out using the ribosomal probe HM123 (Meunier-Rotival et al. 1979) and a telomeric probe $(TTAGGG)_n$ according to the DAKO kit instructions (Denmark). The Ag-NOR technique was frequently performed using the same slide after Giemsa staining or FISH technique with the HM123 probe. In both cases, the slides were washed with xylol to remove the immersion oil and then submitted to the technique for obtaining Ag-NOR as usual but decreasing the time of incubation in all steps of the procedure. Chromosomal images were captured with an Olympus digital camera D71 with use of the DP Controller program. The bi-armed chromosomes were classified as metacentric, submetacentric or subtelocentric according to the nomenclature proposed by Green and Sessions (1991, 2007).

Table 1. Species, number of individuals, sex, voucher numbers, and collection locations in Brazil.

species	number	sex	voucher numbers CFBH	collection locations
Aparasphenodon bokermanni	1	male	22575	Cananéia, SP (25°01'19"S; 47°55'41"W)
Itapotihyla langsdorffii	2	males	22369, 22370	Ilhéus, BA (14°47'29"S; 39°02'41"W)
	1	female	30973	Rio Claro, SP (22°25'20"S; 47°34'23"W)
Phyllodytes edelmoi	2	females	22583, 22584	Maceió, AL (09°40'06"S; 35°43'59"W)
	1	male	22585	Maceió, AL (09°40'06"S; 35°43'59"W)
Phyllodytes luteolus	2	males	22462, 22463	Guaraparí, ES (20°39'01"S; 40°29'10"W)
Trachycephalus sp.	1	male	20664	Paranaíta, MT (09°40'56"S; 56°28'50"W)
Trachycephalus mesophaeus	3	males	22366, 22367, 22368	Ilhéus, BA (14°47'29"S; 39°02'41"W)
	2	females	22371, 22372	Ilhéus, BA (14°47'29"S; 39°02'41"W)
	1	juvenile	22484	Ubatuba, SP (23°26'19"S; 45°05'25"W)
	1	male	24222	Biritiba Mirim, SP (23°34'17"S; 46°02'15"W)
Trachycephalus typhonius	1	female	22365	Porto Primavera, MS (22°26'01"S, 52°58'11"W)
	1	male	10033	Rio Claro, SP (22°25'20"S; 47°34'23"W)

CFBH - Célio Fernando Baptista Haddad Collection, UNESP, Rio Claro, SP, Brazil.

Results

Specimens of *Aparasphenodon bokermanni*, *Itapotihyla langsdorffii*, *Trachycephalus* sp., *T. mesophaeus*, and *T. typhonius* had a diploid number of 2n = 24 (Fig. 1a–e) and a fundamental number FN = 48 and *Phyllodytes edelmoi* and *P. luteolus* had 2n = 22, FN = 44 (Fig. 1f–g). The Table 2 presents the relative length (RL), centromeric index (CI), and the centromeric position (CP) with morphologic classification of the chromosomes of the seven species.

The technique of Ag-NOR was carried out in all species. In the 2n = 24 karyotypes the Ag-NORs were located on chromosome 10, at the terminal long arm in the case of *A. bokermanni*, *Trachycephalus* sp., *T. mesophaeus*, and *T. typhonius* (Fig. 1a, c–e), or at the interstitial short arm in *I. langsdorffii* (Fig. 1b). In *P. edelmoi* and *P. luteolus* Ag-NOR was located at the terminal long arm of chromosome 2 (Fig. 1f–g). The Ag-impregnation occurred in the sites of the secondary constriction, although this marker was not always visualised in the standard stained chromosomes. In *A. bokermanni* and *Trachycephalus* sp. and in some individuals of the remaining species, there was variation in the pattern of Ag-NOR labelling. Within the same individual, metaphases exhibited Ag-NORs with conspicuous or slight difference in size or carried two Ag-

Table 2. Relative length (RL), centromeric index (CI), and nomenclature for centromeric position (CP) on mitotic chromosomes according to Green and Sessions (1991, 2007).

Species		Chromosome number											
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A. bokermanni	RL	15.57	12.93	10.65	9.63	9.48	7.48	5.68	6.78	6.27	6.5	4.12	3.83
	CI	0.479	0.459	0.396	0.263	0.344	0.286	0.321	0.464	0.487	0.284	0.420	0.465
	CP	**m**	**m**	**m**	**sm**	**sm**	**sm**	**sm**	**m**	**m**	**sm**	**m**	**m**
I. langsdorffii	RL	15.06	13.52	11.50	10.41	9.82	7.68	6.59	6.35	5.17	5.00	5.02	3.90
	CI	0.460	0.421	0.355	0.241	0.361	0.225	0.391	0.483	0.472	0.472	0.460	0.467
	CP	**m**	**m**	**sm**	**st**	**sm**	**st**	**m**	**m**	**m**	**m**	**m**	**m**
Trachycephalus sp.	RL	14.57	11.79	11.57	9.95	9.18	7.81	6.75	6.06	4.43	5.15	4.65	4.30
	CI	0.430	0.429	0.383	0.257	0.319	0.261	0.344	0.453	0.456	0.301	0.443	0.461
	CP	**m**	**m**	**m**	**sm**	**sm**	**sm**	**sm**	**m**	**m**	**sm**	**m**	**m**
T. mesophaeus	RL	14.33	13.57	10.66	10.47	9.04	7.98	6.76	6.35	5.94	6.97	4.72	3.83
	CI	0.457	0.435	0.366	0.268	0.370	0.224	0.338	0.481	0.424	0.351	0.353	0.414
	CP	**m**	**m**	**sm**	**sm**	**sm**	**st**	**sm**	**m**	**m**	**sm**	**sm**	**m**
T. typhonius	RL	15.63	12.80	11.05	10.51	10.06	8.16	7.07	6.02	5.02	5.23	4.59	4.10
	CI	0.462	0.397	0.364	0.236	0314	0.200	0.317	0.424	0.444	0.304	0.461	0.485
	CP	**m**	**m**	**sm**	**st**	**sm**	**st**	**sm**	**m**	**m**	**sm**	**m**	**m**
P. edelmoi	RL	18.38	13.74	12.88	9.90	9.74	7.73	6.86	6.77	4.88	4.06	3.74	--
	CI	0.453	0.403	0.335	0.430	0.341	0.414	0.367	0.404	0.440	0.444	0.472	--
	CP	**m**	**m**	**sm**	**m**	**sm**	**m**	**sm**	**m**	**m**	**m**	**m**	**--**
P. luteolus	RL	16.62	12.56	11.11	10.65	9.57	8.72	8.38	7.05	5.38	4.80	4.56	--
	CI	0.450	0.422	0.370	0.249	0.352	0.237	0.336	0.354	0.472	0.430	0.443	--
	CP	**m**	**m**	**sm**	**st**	**sm**	**st**	**sm**	**sm**	**m**	**m**	**m**	**--**

m = metacentric; sm = submetacentric; st = subtelocentric.

Figure 1. Giemsa-stained karyotypes. **a** *Aparasphenodon bokermanni*, male, 2n = 24 **b** *Itapotihyla langsdorffii*, male, 2n = 24 **c** *Trachycephalus* sp., male, 2n = 24 **d** *T. mesophaeus*, male, 2n = 24 **e** *T. typhonius*, male, 2n = 24; f. *Phyllodytes edelmoi*, male, 2n = 22 **g** *P. luteolus*, male, 2n = 22. The insets show the chromosome pairs with Ag-NOR and FISH using the rDNA probe. Bar = 10 mm.

NORs with equivalent sizes; occasionally a single Ag-NOR per metaphase was also observed in the same cytological preparation. FISH with an rDNA probe was performed in six species, with exception of *P. edelmoi*. Two fluorescent signals were observed in all analysed metaphases (Fig. 1a–e, g). In the species *Trachycephalus* sp. and *T. mesophaeus* the hybridisation signals always presented the same size and in *A. bokermanni*, *I. langsdorffii*, *T. typhonius*, and *P. luteolus* the labelling was heteromorphic in all metaphases.

The C-banding in *A. bokermanni*, *I. langsdorffii*, *Trachycephalus* sp., *T. mesophaeus*, and *T. typhonius* showed heterochromatin distribution in the pericentromeric regions of all chromosomes (Fig. 2). In *I. langsdorffii* additional C-bands were noticed at terminal (chromosomes 1 and 4) and interstitial (chromosome 5) regions. This technique was carried out in mitotic and meiotic cytological preparations of *P. edelmoi* and *P. luteolus*, but no C-banded region was demonstrated in the chromosomes of these species. The NOR site in all species was brilliant with CMA_3, as well as the chromosome pericentromeric region (Fig. 3a, c–h). The pericentromeric fluorescence was in general faint and not visualised in all chromosomes. In *A. bokermanni* the centromeric signals were particularly prominent in size and brightness (Fig. 3a). No brilliant labelling was observed after DAPI staining in any species, except in *A. bokermanni* which showed

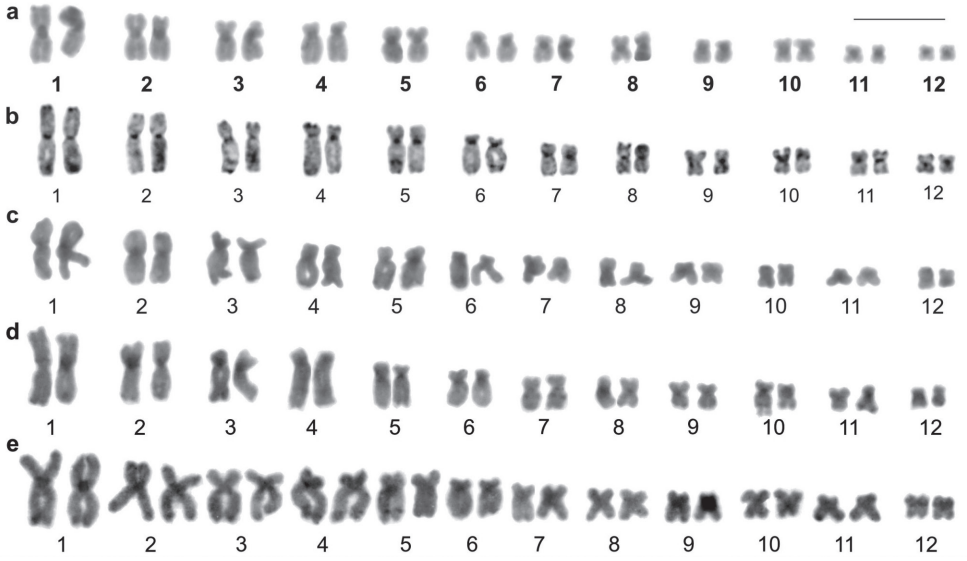

Figure 2. C-banded karyotypes. **a** *Aparasphenodon bokermanni* **b** *Itapotihyla langsdorffii* **c** *Trachycephalus* sp. **d** *T. mesophaeus* **e** *T. typhonius*. Bar = 10 mm.

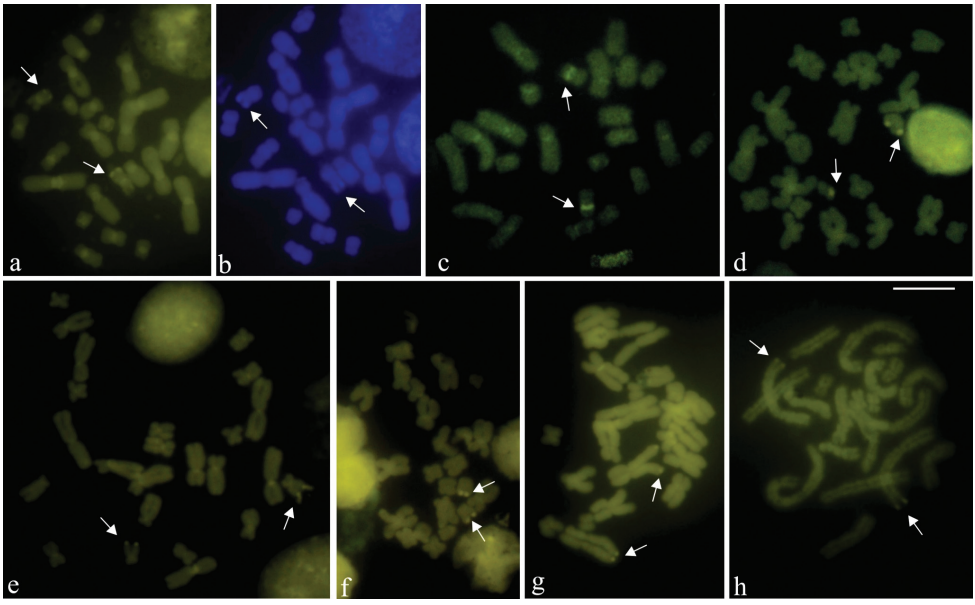

Figure 3. Fluorochrome-stained metaphases. **a, c-h** CMA_3 **b** DAPI **a–b** *Aparasphenodon bokermanni* **c** *I. langsdorffii* **d** *Trachycephalus* sp. **e** *T. mesophaeus* **f** *T. typhonius* **g** *Phyllodytes edelmoi* **h** *P. luteolus*. Bright DAPI fluorescence at the terminal short arms of chromosomes 10 (arrows) and the negative centromeric region are shown in **a**. CMA_3 fluorescent labelling of the NOR site (arrows) and in the centromeric region of chromosomes in **a**, **c–h**. Bar = 10 mm.

slight fluorescence at the terminal short arm of chromosome 10 (Fig. 3b). The chromosome pericentromeric region of this species was DAPI-negative.

The telomeric probe hybridized on the chromosome ends in six of the species, excepting in *P. luteolus* without cytological material available for the FISH technique. Figure 4a–e showed metaphases of *A. bokermanni*, *I. langsdorffii*, *Trachycephalus* sp., *T. mesophaeus*, and *T. typhonius* with probe labelling at the chromosome ends and, in the case of *I. langsdorffii* (Fig. 4b), also in the pericentromeric region. In *P. edelmoi* no good metaphases were obtained, but the chromosomes showed telomeric labelling. In one metaphase of this species, however, the large-sized chromosome pair 1 and 2 had probe hybridization at the proximal short and long arms (Fig. 4f).

No sex-chromosome pairs were detected in male or female specimens of *I. langsdorffii*, *T. mesophaeus*, *T. typhonius*, and *P. edelmoi*. In the remaining three species only males were karyotyped with no evidence of sex related heteromorphism. Meiotic analysis confirmed the diploid number in all species (Fig. 5a–g). *Aparasphenodon bokermanni*, *I. langsdorffii*, *Trachycephalus* sp., *T. mesophaeus*, and *T. typhonius* showed 12 bivalents. *Phyllodytes edelmoi* and *P. luteolus* showed 11 bivalents.

The main cytogenetic data obtained in the present study are summarized in the Table 3.

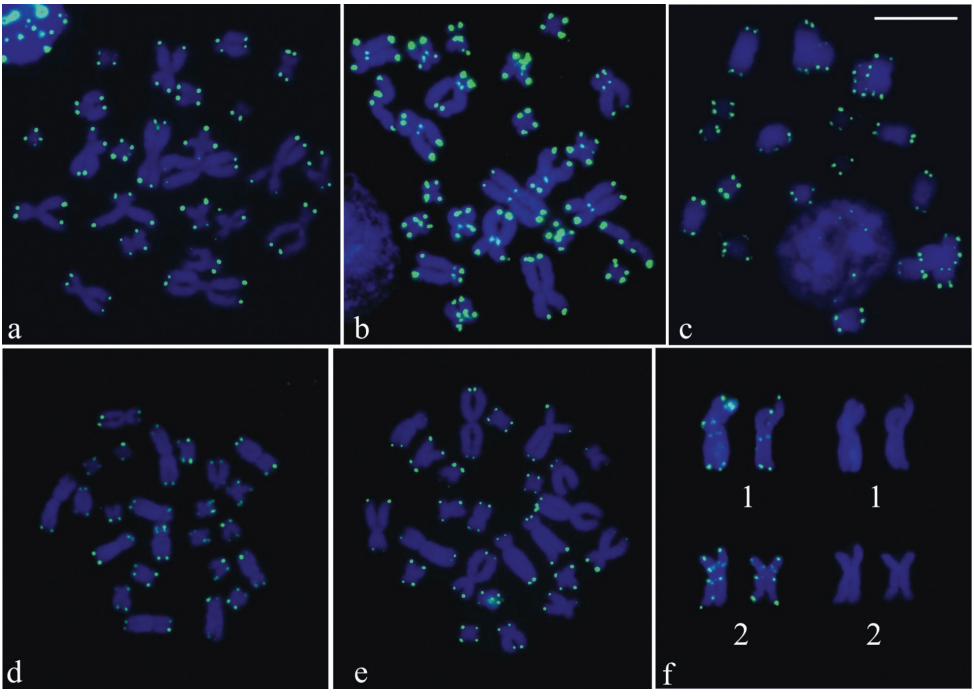

Figure 4. FISH using a telomeric probe. **a** *Aparasphenodon bokermanni* **b** *Itapotihyla langsdorffii* **c** *Trachycephalus* sp. **d,** *T. mesophaeus* **e** *T. typhonius* **f** *P. edelmoi*. In **b** hybridisation labelling is visible in the centromeric region of the chromosomes and in **f,** at the proximal short and long arms of chromosomes 1 and 2 observed with telomeric probe hybridisation (left) and with DAPI staining (right). Bar = 10 mm.

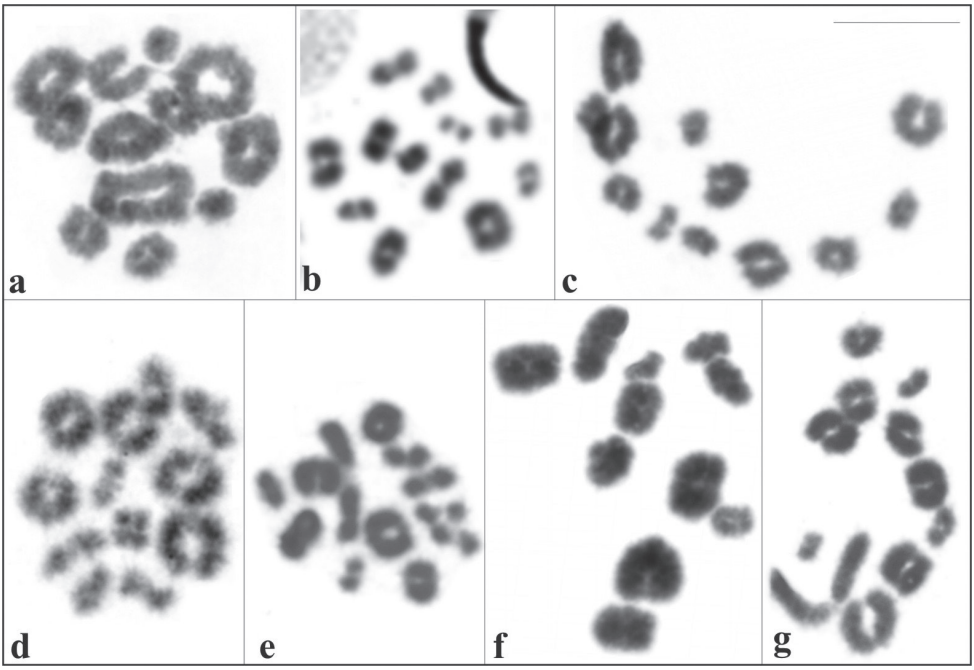

Figure 5. Giemsa-stained diakinesis and metaphases I cells. **a** *Aparasphenodon bokermanni*, 2n = 24 **b** *Itapotihyla langsdorffii*, 2n = 24 **c** *Trachycephalus* sp., 2n = 24 **d** *T. mesophaeus*, 2n = 24 **e** *T. typhonius*, 2n = 24 **f** *Phyllodytes edelmoi*, 2n = 22 **g** *P. luteolus*, 2n = 22. Bar = 10 mm.

Table 3. Data on chromosome number, chromosome formula, NOR and telomeric sequence localization, C-band distribution and molecular content of repetitive DNA sequences of studied species.

species	2n	fomula	NOR	Tel	C bands	DAPI	CMA_3
A. bokermanni	24	7m+5sm	11qt	T	C+NOR	10pt	C*+NOR
I. langsdorffii	24	8m+2sm+2st	11pi	T+C	C+NOR	--	C+NOR
Trachycephalus sp.	24	7m+5sm	11qt	T	C+NOR	--	C+NOR
T. mesophaeus	24	5m+6sm+1st	11qt	T	C+NOR	--	C+NOR
T. typhonius	24	6m+4sm+2st	11qt	T	C+NOR	--	C+NOR
P. edelmoi	22	8m+3sm	2qt	T+C	C+NOR	--	C+NOR
P. luteolus	22	5m+4sm+2st	2qt	---	C+NOR	--	C+NOR

m = metacentric; sm = submetacentric; st = subtelocentric; p = short chromosome arm; q = long chromosome arm; i = interstitial region; t = terminal region; T = telomere; C = centromeric/ pericentromeric region; * intense mark.

Discussion

The species of the tribe Lophiohylini *A. bokermanni*, *I. langsdorffii*, *Trachycephalus* sp., *T. mesophaeus*, and *T. typhonius* with 2n = 24 shared indistinguishable karyotypes even though there was discrepancy in morphological classification shown in Table 2 for some chromosomes, as the chromosome 3 of the species, due to slight differences in

the chromosome arm proportion. No evidence of population karyotype difference was observed for *I. langsdorffii*, *T. mesophaeus*, and *T. typhonius* sampled in distinct locations. Considering previous data for these three species (Rabello 1970, Foresti 1972, Bogart 1973, Kasahara et al. 2003, Nunes and Fagundes 2008), no difference was noticeable in the karyotypes, although the morphological classification of chromosomes and the ordering of the pairs in the distinct karyograms were not the same.

The chromosome constitution with 2n = 24 herein described is the same as found for the remaining eight species of Lophiohylini analysed so far, corresponding to *Aparasphenodon brunoi*, *Argenteohyla siemersi*, *Corythomantis greeningi*, *Osteocephalus taurinus*, *Osteopilus dominicensis*, *O. marianae*, *O. septentrionalis*, and an unidentified species of *Trachycephalus* (see Catroli and Kasahara 2009 for references). This finding suggests a high degree of karyotype conservation within the tribe. Actually, a detailed comparative analysis of the replication banding obtained by BrdU incorporation had shown unequivocal homeology at least among the chromosomes of *A. brunoi*, *C. greeningi*, and *I. langsdorffii* (Kasahara et al. 2003). It is important to emphasise that this conservative pattern of chromosome constitution has been observed in representatives of Hylinae and, according to the molecular phylogeny of Faivovich et al. (2005), a karyotype with 2n = 24 could be a synapomorphic condition within this subfamily. Another karyotype characteristic shared by the majority of the Lophiohylini species with 2n = 24 is the NOR site in a small-sized chromosome (Schmid 1978, 1980, Anderson 1996, Kasahara et al. 2003, Nunes and Fagundes 2008), with the exception of *Argenteohyla siemersi* (Morand and Hernando 1996) with NOR in the chromosome pair 4.

Phyllodytes edelmoi and *P. luteolus,* the first two species of the genus that were analysed so far, had reduced the diploid numbers from 2n = 24 to 2n = 22 and the NOR site was in the large-sized chromosome 2. Nevertheless, the karyotypes of these two species were distinct regarding the morphology of pairs 4 and 6, that is, in *P. edelmoi* these pairs were metacentric and in *P. luteolus* they were subtelocentric, as it has been usually observed in Hylinae species with 2n = 24. The discrepancy in the morphology of the chromosome pairs 4 and 6 was supported by the chromosome measurements and the mechanism responsible for these differences might be, for example, a pericentric inversion or another type of chromosome rearrangement, but this could not be determined at least with the cytogenetic techniques used here.

Within the sub-family Hylinae, variation as resulted of fusion events from an ancestral karyotype with 24 chromosomes was described for *Hypsiboas albopunctatus* (Spix, 1824) (2n = 22) and for species of the genus *Aplastodiscus* (2n = 18, 20, 22) (Gruber et al. 2007, Gruber et al. 2012). Although the chromosomes involved in the rearrangements could not be recognized with certainty in neither case, the derived chromosomes in *H. albopunctatus* and in *Aplastodiscus* species were tentatively identified by their altered morphology regarding the presumed ancestral. The reduction in the diploid number to 2n = 22 in *Phyllodytes* might also be due to fusion rearrangement of end-to-end or centric type from the ancestral 2n = 24 karyotype. Taking into account that the two analysed species presented four small pairs instead of five and the NOR was on large-sized pair, the fusion, at first sight, occurred between a small

NOR-bearing chromosome and chromosome 2. Nevertheless, the NOR-bearing chromosome 2 of *Phyllodytes* had no noticeable relative size differences regarding the chromosome 2, not carrying NOR, of the 2n = 24 species. Another possibility is the translocation of one of the smallest chromosomes to chromosome 1, since this element in the *Phyllodytes* species has a larger relative length when compared to the chromosome 1 of 2n = 24 karyotypes. The translocation of a small pair to more than one unidentified chromosomes, leading to the reduction in the diploid number to 2n = 22 could not be discarded. Whichever of the hypotheses is more probable, other rearrangements should have occurred later, to explain the differences observed between the karyotypes of the two species of *Phyllodytes.* Certainly, additional cytogenetic analyses within the genus are necessary to outline the events occurred during the chromosome evolution.

In males and females of *I. langsdorffii*, *P. edelmoi*, *T mesophaeus*, and *T. typhonius* and in males of *A. bokermanni*, *Trachycephalus* sp., and *P. luteolus* heteromorphic sex chromosomes were not observed. Nevertheless in females of these three latter species sex chromosomes could not be discarded. Anurans, in general, do not present cytological sex chromosome differentiation and both male or female heterogamety has been described in some species (Schmid et al. 2010).

A single NOR pair located in a small-sized chromosome (Schmid 1978, Green and Sessions 2007, Schmid et al. 2010) is a shared characteristic for the majority of the Lophiohylini species and this condition has also been frequently observed in other Hylinae of the genera *Bokermannohyla* Faivovich, Haddad, Garcia, Frost, Campbell et Wheeler, 2005, *Hyla* Laurenti, 1768, *Hypsiboas* Wagler, 1830, and *Scinax* Wagler, 1830 (clade *S. ruber*) (Catroli et al. 2011, Cardozo et al. 2011). Although the NOR-bearing pair has been referred in the literature to as chromosome pairs 10, 11, or 12, most probably we are dealing with the same pair. In fact, Kasahara et al. (2003) demonstrated close correspondence in the replication banding patterns between the NOR-bearing chromosomes 10 of the Lophiohylini *A. brunoi*, *C. greeningi*, and *I. langsdorffii* and the NOR-bearing chromosomes 12 of the Dendropsophini *Scinax fuscovarius* (Lutz, 1925). As stressed by Cardozo et al. (2011), NOR in a small-sized chromosome is considered a plesiomorphy within the subfamily, wherefore NOR location out of small element, as observed in *Argenteohyla siemersi* and in *Phyllodytes*, is a derived condition.

The NOR marker chromosome in our species of Lophiohylini with 2n = 24 was considered as the 10 and the rDNA sequences were at the interstitial short arm or at the terminal long arm, but no major differences were observed in the morphology of the chromosomes 10 among distinct species. Therefore, the mechanism that changed the NOR sites apparently was not a gross rearrangement, but minute structural rearrangements or transposition by means of mobile elements could not be discarded. If the movement of the NOR from chromosome 10 to chromosome 2 in *Phyllodytes* species was not a direct consequence of the rearrangement which reduced the diploid number in the genus, one of the two mentioned mechanisms would also be a reasonable explanation for the discrepant NOR site, in *P. edelmoi* and in *P. luteolus*.

The technique of Ag-impregnation showed large variation in the Ag-NOR pattern within the same individual. Nevertheless, the FISH with an rDNA probe revealed that

the NOR labelling in each individual had either equivalent or distinct size in all the analysed cells. Such data allowed us to conclude that most probably the Ag-NOR variation was a result of differential activity of ribosomal gene in *Trachycephalus* sp. and *T. mesophaeus* because the hybridization labelling had the same size in both homologues; on the other hand, different amounts of repetitive rDNA units would be responsible for the observed Ag-NOR variation in *A. bokermanni*, *I. langsdorffii*, and *T. typhonius* because hybridization labelling had distinct sizes in both homologues. The single Ag-NOR seen occasionally in some metaphases could be attributed to the lacking or insufficient amount of the non-histone proteins available for the Ag-impregnation.

The chromosomes of the species herein analysed produced C-banding results only after over treatment of the distinct steps of the technique. However, it was undoubtedly demonstrated that heterochromatin was distributed mainly in the centromeric regions. A similar centromeric C-banding pattern had been described in *A. brunoi*, *C. greeningi*, and *I. langsdorffii* (Kasahara et al. 2003) besides some interstitial and terminal additional C-bands in the latter species. The lack of C-bands in the chromosomes of *P. edelmoi* and *P. luteolus* might be due to the absence of repetitive DNA identifiable by means of C-banding technique. Nevertheless, it will be important to confirm such possibility or if we are dealing with some technical difficulty, since CMA_3 staining at the centromeres in both species, albeit with faint fluorescence, confirmed the presence of repetitive sequences in these regions.

Surprisingly, in spite of the low amount of C-banded heterochromatin, *A. bokermanni* showed conspicuous bright fluorescence at the centromeres, similar to that observed in *A. brunoi* (Kasahara et al. 2003). This result and the corresponding DAPI-negative fluorescence in both species indicated presence of a particular repetitive DNA characteristic of the genus *Aparasphenodon* with an exceptional GC-content. Besides the centromere, each of these two species had own fluorescent markers in other chromosome regions: *Aparasphenodon brunoi* exhibited a bright CMA_3 site in the long arm of chromosome 5 (Kasahara et al. 2003), whereas *A. bokermanni* had bright CMA_3 site in the long arm of chromosome 10 and bright DAPI site in the short arm of the same chromosome 10. *Itapotihyla langsdorffii* and the species of *Trachycephalus* and *Phyllodytes* showed faint centromeric fluorescence with CMA_3 indicating that the GC-content was not high.

Although the FISH with the telomeric probe is primarily designed for identification of chromosome-ends, this procedure may provide information about the molecular nature of some repetitive sequences. As far as it has been shown, distinct organisms, including frogs (Meyne et al. 1990, Wiley et al. 1992, Nanda et al. 2008, Gruber et al. 2012), disclosed hybridization out of the telomeres, even in the cases without evidence of chromosome rearrangements. This would mean presence of telomere-like sequences $(TTAGGG)_n$ in sites of repetitive DNA and it seems to explain the labelling out the telomere sites in *I. langsdorffii* and *P. edelmoi*. These data reinforce the importance of the FISH with the telomeric probe used in combination with base-specific fluorochrome staining and C-banding for obtaining information on the content of distinct repetitive regions.

The interstitial hybridization signals of telomeric probe could correspond to vestiges of true telomeres, as reported in rodents (Fagundes et al. 1997, Ventura et al. 2006),

but in our sampled species, there was no evidence of telomere remnants resulted probably from chromosome rearrangements. Despite presumed fission and fusion during the chromosome evolution, Anderson (1996) noticed no hybridisation interstitial labelling in the Lophiohylini *Osteopilus septentrionalis* (2n=24) and *O. brunneus* (2n=34).

Based on the data of 22 species, a phylogenetic tree of the Lophiohylini was provided by Faivovich et al. (2005). Later, Jowers et al. (2008) added the molecular information of *Phytotriades auratus* and, more recently, the phylogeny of Lophiohylini was expanded by Wiens et al. (2010) for a total of 35 representatives. All these trees support the monophyly of the tribe, although the relationships of the distinct genera remain unclear. In the phylogeny of Faivovich et al. (2005) *Phyllodytes* appears in an isolated clade at a basal position. In the phylogeny of Wiens et al. (2010) the representatives of Lophiohylini are grouped into two major sister-clades and the species of *Phyllodytes* and *Osteopilus* are included in one of these clades, along with the species with 2n =24. Regardless of which of the two phylogenetic hypotheses is most accurate, it is clear that 2n = 22 exhibited by the species of *Phyllodytes* is a derived condition.

The present study showed that in spite of the high similarity of the chromosome constitution and of the NOR pattern among the species of Lophiohylini with 2n = 24, the karyotypes could be recognized by the nature of the repetitive sequences, as differentiated through C-banding, base-specific fluorochrome staining, and, in a certain extension, by FISH with telomeric probe. Cytogenetic information on the tribe is still minimal, but the analyses of the available data in light of the phylogeny allowed for visualization of the occurrence of karyotypic variations restricted to the clades of the genera *Phyllodytes* and *Osteopilus*. It would be interesting to enlighten the chromosome evolution with other accurate technical approaches and to extend the karyotyping to other species of Lophiohylini, especially new representatives of *Phyllodytes* and *Phytotriades auratus*.

Acknowledgements

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The authors thank to Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) for providing the collection permits to SLG and CFBH. The authors are grateful to Akio Miyoshi, Carlos Jared, Edson Zefa, Hideki Narimatsu, João Luiz Gasparini, Juliana Zina, Katyuscia de Araujo Vieira, and Olívia Araújo, for help during field work.

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CompCytogen 6(4): 425–441 (2012)
doi: 10.3897/CompCytogen.v6i4.3740
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 *Panax ginseng* C.A.Meyer, 1843 (Araliaceae) based on rDNA loci and DAPI band distribution

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Academic editor: *L. Peruzzi* | Received 30 July 2012 | Accepted 26 October 2012 | Published 15 December 2012

Citation: Waminal NE, Park HM, Ryu KB, Kim JH, Yang T-J, Kim HH (2012) Karyotype analysis of *Panax ginseng* C.A.Meyer, 1843 (Araliaceae) based on rDNA loci and DAPI band distribution. Comparative Cytogenetics 6(4): 425–441. doi: 10.3897/CompCytogen.v6i4.3740

Abstract

Ginseng has long been considered a valuable plant owing to its medicinal properties; however, genomic information based on chromosome characterization and physical mapping of cytogenetic markers has been very limited. Dual-color FISH karyotype and DAPI banding analyses of *Panax ginseng* C. A. Meyer, 1843 were conducted using 5S and 45S rDNA probes. The somatic chromosome complement was $2n$=48 with lengths from 3.3 μm to 6.3 μm. The karyotype was composed of 12 metacentric, 9 submetacentric, and 3 subtelocentric pairs. The 5S rDNA probe localized to the intercalary region of the short arm of pair 11, while the 45S rDNA was located at the secondary constriction of the subtelocentric satellited chromosome 14. DAPI bands were clearly observed for most chromosomes, with various signal intensities and chromosomal distributions that consequently improved chromosome identification. As a result, all 24 chromosomes could be distinguished and numbers were assigned to each chromosome for the first time. The results presented here will be useful for the on-going ginseng genome sequencing and further molecular-cytogenetic studies and breeding programs of ginseng.

Keywords

Panax ginseng, FISH, 5SrDNA, 45S rDNA, DAPI band, Araliaceae

Introduction

Ginseng (*Panax ginseng* C.A.Meyer, 1843) is highly valued owing to its medicinal properties (Zhang et al. 2011), and the ginsenosides found in the plant contribute greatly to its pharmacological value (Court 2000, Leung and Wong 2010, Yuan et al. 2010). Along with 15–17 other species, ginseng belongs to the genus *Panax* in the family Araliaceae (Ho and Leung 2002, Yi et al. 2004). This genus is only one of the approximately 120 genera of angiosperms with a disjunct distribution pattern between eastern North America and eastern Asia (Wen and Zimmer 1996). Most of the species of *Panax* are geographically distributed in eastern Asia, but two (*P. trifolius* Linnaeus, 1753 and *P. quinquefolius* Linnaeus, 1753) are isolated in eastern North America (Ho and Leung 2002). American ginseng (*P. quinquefolius*) is morphologically similar to ginseng (Ngan et al. 1999), and both are regarded as polyploid (Court 2000); however, their origin (auto- vs. allopolyploidy) is not yet fully understood (Yi et al. 2004, Choi et al. 2009). Cytogenetic data have been employed in an attempt to explain the possible origins of their disjunct distribution (Yang 1981, Wen and Zimmer 1996, Yi et al. 2004), but these did not sufficiently resolve the question. Apparently, more research is needed to fully understand their phylogenetic relationship.

Information regarding the chromosome number of ginseng has been available since 1936 (Darlington and Wylie 1956, Yi et al. 2004). However, data reported by different researchers have been inconsistent. For example, Graham (1966) and Yang (1981) reported $2n$=44, while Ko et al. (1993) and Choi et al. (2009) reported a complement of $2n$=48. Regardless of whether or not the discrepancy in the reported chromosome number is caused by intraspecific variation (Blair 1975), it is essential to establish a detailed karyotype for ginseng.

The translocation of DNA blocks in some plants have been observed through cytogenetic investigations (e.g. Han et al. 2009, Huang et al. 2009, Topp et al. 2009), and helped us to understand the genomic relationships among several plants (Leflon et al. 2006, Snowdon 2007, Xiong and Pires 2011, Chester et al. 2012), making cytogenetics an essential tool to the overall understanding of a genome. Moreover, fluorescence *in situ* hybridization (FISH) is an excellent technique for use in plant cytogenetics (Sadder and Weber 2001, Capdeville et al. 2008, Vasconcelos et al. 2010) because it allows physical mapping of a particular DNA sequence along the chromosome complement. Examples include the repetitive sequences of ribosomal RNA genes (rDNA), centromeric and telomeric repeats (e.g. Kato et al. 2004, Lim et al. 2005), and single-copy genes (e.g. Fransz et al. 1996, Kharb et al. 2001). Owing to their sequence conservation among eukaryotic genomes despite the repeating unit copy number, loci number, and distribution pattern variations, the multiple tandem repeats of the 5S and 45S rDNA are the most widely used probes in molecular cytogenetic analyses (e.g. Chen et al. 1999, Hwang et al. 2009, Waminal et al. 2011). Indeed, these cytogenetic markers are invaluable in cytogenetic studies such as karyotyping, investigations of chromosomal organizational changes, and physical mapping of DNA sequences (Huang et al. 2009, Park et al. 2012).

Probes labeled with different fluorophores for simultaneous detection have been widely employed in rDNA loci distribution analyses and dual-color FISH karyotyping (e.g. Ali et al. 2005, Lan and Albert 2011, Xiang-Hui 2011, Waminal and Kim 2012). Choi et al. (2009) recently reported the number of rDNA loci in ginseng using dual-color FISH; however, no detailed karyotype or chromosome characterization was presented. To date, molecular cytogenetic information pertaining to ginseng, despite recent development of molecular markers (Choi et al. 2011, Kim et al. 2012a, 2012b), has been very limited causing the slow progress of genomic studies.

Here, we used dual-color FISH to analyze the distribution of rDNA loci in *P. ginseng*. In addition, we used the DAPI banding pattern to pair homologous chromosomes. Collectively, this made numbering of the chromosome of *P. ginseng* possible for the first time. These data will be useful for future cytogenetic analyses and should enable a better understanding of the genomic history of ginseng, and can be used for subsequent distribution analyses of repeat sequences, retrotransposons, and chromosome-specific cytogenetic markers. Consequently, the results presented here will make a significant contribution to studies related to the on-going ginseng genome sequencing and the overall understanding of the *P. ginseng* genome.

Material and methods

Root sample preparation

Stratified seeds of three ginseng cultivars 'Sunun', 'Chunpoong', 'Gopoong', and a local landrace 'Hwangsook' were provided by the Korea Ginseng Corporation (KGC) Natural Resources Research Institute (Daejeon, Korea). Stratified seeds were allowed to germinate in petri dishes with wet filter papers at 10–15°C. The root meristems were then excised (about 2 cm from the root tips), pretreated with 0.002M 8-hydroxyquinoline for 5 hours at 18°C, fixed in 90% acetic acid for 15 min at room temperature (RT, ~24°C), and then stored in 70% ethanol until use.

Chromosome spread preparation

Somatic chromosome spreads were obtained using a modified version of the technique described by Kato et al. (2004). After thorough washing with distilled water, the meristematic regions of the fixed root tips (~2 mm) were excised and digested in a pectolytic enzyme mix [2% cellulase (MB Cell, Korea), 1.5% macerozyme (Maxim Bio, USA) and 1% pectolyase (Sigma, Japan) in 150 mM Citrate Buffer, pH 4.5] for 75 min at 37°C. The digested meristems were then pipetted into a petri dish with chilled distilled water and incubated on ice for 15 min to wash out the enzymes. Using a stereomicroscope, the root epidermis was removed, and the protoplasts were gently pipetted into a 1.5 ml tube containing 40 µl chilled Carnoy's fixative. The

protoplasts were then suspended by gently vortexing the tube for 30 sec at room temperature, after which the sample was centrifuged at 4,000 ×*g* for 3 min and the pellet was resuspended in acetic acid-ethanol (9:1) solution. Finally, the protoplast suspension was pipette-mounted onto ethanol cleaned glass slides, which were placed in a humid chamber to facilitate spreading of the chromosomes and allowed to dry.

Probe labeling

A 9-kb fragment of 45S rDNA (18S-5.8S-25S) (Gerlach and Bedbrook 1979) was labeled with biotin-16-dUTP (Roche, Germany) by nick translation. The 5S rDNA was obtained according to the procedure described by Hwang et al. (2009) and then labeled with digoxigenin-11-dUTP (Roche, Germany) by nick translation. Labeled DNA fragments within the range of 200–500 bp were used as probes.

Fluorescence *in situ* hybridization

Slide pretreatment. To remove contaminating RNA, the slides were treated with RNase A buffer (RNase A final conc. 100 μg ml^{-1} in 2× SSC) for 1 hr at 37°C. The slides were then incubated in 0.01 M HCl for two minutes, followed by subsequent treatment in pepsin buffer [stock: 10% (w/v) pepsin in dH_2O, working: 1:100 dilution in 0.01 M HCl] for 10 min at 37°C to lyse endogenous proteins that could cause background signals. Next, the chromosomes were fixed by treating the slides with 4% paraformaldehyde in 2× SSC. Finally, the slides were dehydrated in ethanol series (70%, 90%, 100%, 3 min each) and air-dried. The slides were washed in 2× SSC for 5 min (3×) between each step. All incubation steps at 37°C were conducted in a humidified chamber.

Probe hybridization. The hybridization mixture contained 50% formamide, 10% dextran sulfate, 2× SSC, 5 ng μl^{-1} salmon sperm DNA and 500 ng μl^{-1} of each probe DNA adjusted with DNase- and RNase-free water (Sigma, USA, #W4502) to a total volume of 40 μl/slide. The mixture was denatured at 90°C for 10 min and immediately kept on ice for at least 5 min prior to mounting on slides. After covering with a glass cover slip, the chromosomes were denatured at 80°C for 3–5 min on a hot plate. The slides were then immediately transferred into a humid chamber preset at 37°C and incubated overnight (~16 hr). The following day, the slides were washed in 2× SSC (15 min at RT), 0.1× SSC (35 min at 42°C), and finally 2×SSC (30 min at RT).

Signal detection. The slides were treated with TNB [0.1 M Tris-HCl, 0.15 M NaCl, 1% (w/v) blocking reagent] at RT for 5 min, after which they were subjected to antibody detection. Briefly, biotinylated 45S rDNA probe was detected with streptavidin-Cy3 conjugate (Zymed, USA), while digoxigenin-labeled 5S rDNA probe was detected using anti-digoxigenin-FITC conjugate (Sigma, USA). Both antibodies were

diluted in TNB to a ratio of 1:100, and the slides were then incubated at 37°C for one hour. Excess reagents were subsequently washed off in TNT [0.1 M Tris-HCl, 0.15 M NaCl, 0.2% (v/v) Tween-20] at 37°C for 5 min (3×), after which they were subjected to dehydration in ethanol series (70%, 90%, 100%, 3 min each) and air-dried. Chromosomes were then counterstained with a premixed DAPI solution [1 μg ml^{-1} DAPI in Vectashield (Vector Laboratories, USA)].

Karyotyping

Image capture and measurement. Well-spread chromosomes with well-preserved chromosome morphology were observed and captured using an Olympus BX51 fluorescence microscope equipped with a CCD camera (CoolSNAP™ cf) and filters for DAPI, FITC, and Cy3. The captured FISH images were analyzed, after which each homologue was measured 3–7 times using Genus™ version 3.1 (Applied Imaging, USA) to obtain the mean values. Raw images for each probe were saved separately and a pseudo-colored image of the merged signals was obtained for each chromosome spread. The sharpness value in Genus™ was set to 7 to enhance the details and texture of the chromosomes. Final images were edited using Adobe Photoshop CS3.

Chromosome numbering and pairing. Chromosome number assignment was based on the decreasing order of chromosome lengths, while homologous chromosome pairing was achieved according to the centromeric position (Levan et al. 1964), DAPI band and rDNA loci distribution. Chromosomes were grouped according to the number of DAPI bands in each arm. As demonstrated by Costa Silva et al. (2011), the estimated DNA content in each chromosome was calculated by distributing the 1C DNA content of *P. ginseng* (3.12×10^3 Mb, Hong et al. 2004) relative to the length of each chromosome.

Results

Chromosome complement composition and rDNA localization

The three cultivars and one landrace of *P. ginseng* evaluated in this study were all confirmed to have a chromosome complement of $2n$=48 (Fig. 1). With reference to the centromere position (i.e. arm ratio), the complement comprised 12 metacentric (1–7, 11–13, 15, and 18), 9 submetacentric (8–10, 16–17, 19, and 22–24), and 3 subtelocentric (14 and 20–21) homologous pairs with a karyotype formula of 24m+18sm+6st. The chromosome lengths ranged from 3.27 to 6.30 μm (Table 1).

Only one pair of satellited chromosomes (pair 14) was observed, and the only locus of 45S rDNA in the genome was localized at the secondary constriction of this subtelocentric chromosome (Figs 2, 3 and Table 2). Moreover, one locus of 5S rDNA signal was

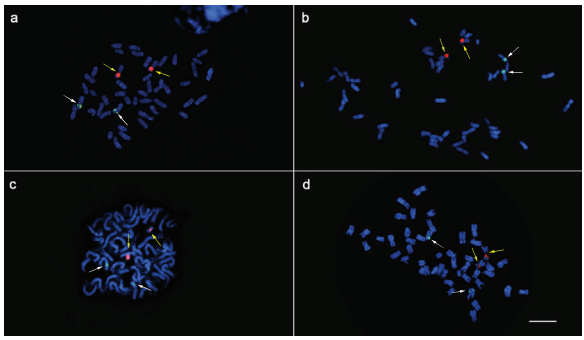

Figure 1. Chromosome complement of three *P. ginseng* cultivars, 'Sunun' (**a**), 'Gopoong' (**b**), 'Chunpoong' (**c**), and one local landrace, 'Hwangsook' (**d**) showing $2n$=48. One pair of 45S rDNA (red signals, yellow arrows) and one pair of 5S rDNA (green signals, white arrows) was observed among the four samples. Bar = 10 μm.

detected at the intercalary region of the short arm of chromosome 11. This locus was flanked by two DAPI bands. There was no variation in the number of rDNA loci among the three cultivars and one landrace of *P. ginseng* investigated in this study (Fig. 1).

DAPI band distribution

Numerous DAPI-binding heterochromatic regions were dispersed along all chromosomes and were visible as DAPI dots. These dots, similar to those in chromosomes 5 and 8, did not form distinct DAPI bands. Both the DAPI dots and bands were made more easily visible by inverting the images (Fig. 2c and d).

In addition to the rDNA loci, the presence of several observable DAPI bands along the chromosome complement made identification of homologous pairs possible. The number of the observed bands further increased as the resolution increased after subsequent enhancement of the image sharpness in Genus™. A total of 32 DAPI bands were initially observed in the sharpness-enhanced DAPI images, but six additional DAPI bands were observed after using the inverse tool of Genus™ with adjustment to the brightness and contrast, resulting in a total of 38 bands (Fig. 2b and d).

Twelve of the observed DAPI bands were localized on the short arms, while 26 were on the long arms (Table 2). Among the 24 chromosomes, four had no band (5, 8, 15, and 23), six had one band (2, 3, 9, and 12–14), 11 had two bands (1, 4, 7, 16–22, 24), two had three bands (6 and 10), and one had four bands (11). Furthermore, chromosomes were grouped according to the presence or absence of DAPI bands on each arm (Fig. 3). Group A had no band on either arm (pairs 5, 8, 15, and 23), group B had no band on the short arm, but one band on the long arm (2, 3, 9, and 12), group C had no band on the short arm, but two bands on the long arm (7, 17, and 21), group D had no band on the short arm, but three bands on the long

Table 1. Chromosome analyses of *P. ginseng* based on chromosome length and centromeric position.

Chr. no.	Chr. length (μm)			Arm ratio (q/p)	Type
	Short arm (p)	Long arm (q)	Total		
1	3.16 ± 0.12	3.17 ± 0.11	6.30 ± 0.22	1.002	m
2	2.64 ± 0.08	3.27 ± 0.12	6.05 ± 0.06	1.237	m
3	2.52 ± 0.23	3.61 ± 0.11	5.88 ± 0.18	1.434	m
4	2.54 ± 0.20	3.27 ± 0.22	5.64 ± 0.07	1.289	m
5	2.23 ± 0.27	3.35 ± 0.12	5.41 ± 0.17	1.506	m
6	2.05 ± 0.11	3.30 ± 0.06	5.31 ± 0.23	1.609	m
7	2.09 ± 0.07	3.35 ± 0.22	5.30 ± 0.14	1.605	m
8	1.54 ± 0.32	3.66 ± 0.13	5.23 ± 0.40	2.378	sm
9	1.52 ± 0.19	3.82 ± 0.13	5.08 ± 0.21	2.515	sm
10	1.77 ± 0.04	3.49 ± 0.06	5.04 ± 0.28	1.965	sm
11†	2.13 ± 0.12	2.91 ± 0.13	4.94 ± 0.12	1.363	m
12	1.96 ± 0.07	3.03 ± 0.12	4.83 ± 0.28	1.547	m
13	2.04 ± 0.05	3.05 ± 0.04	4.82 ± 0.07	1.492	m
14‡	1.99[§] ± 0.21	3.21 ± 0.14	4.80 ± 0.31	1.612[|]	st
15	2.26 ± 0.17	2.58 ± 0.28	4.73 ± 0.49	1.143	m
16	1.55 ± 0.09	3.33 ± 0.10	4.72 ± 0.08	2.157	sm
17	1.59 ± 0.15	3.05 ± 0.07	4.50 ± 0.11	1.919	sm
18	2.09 ± 0.25	2.54 ± 0.19	4.50 ± 0.06	1.214	m
19	1.39 ± 0.12	2.78 ± 0.17	4.11 ± 0.21	1.998	sm
20	1.05 ± 0.04	3.24 ± 0.07	4.09 ± 0.06	3.067	st
21	0.90 ± 0.05	3.02 ± 0.21	3.80 ± 0.13	3.355	st
22	1.32 ± 0.06	2.32 ± 0.10	3.56 ± 0.09	1.761	sm
23	1.25 ± 0.11	2.30 ± 0.22	3.38 ± 0.09	1.836	sm
24	1.13 ± 0.25	2.08 ± 0.24	3.27 ± 0.10	1.840	sm

†5S rDNA, ‡45S rDNA, [§]satellite length, [|]value obtained using satellite instead of short arm, m: metacentric, sm: submetacentric, st: subtelocentric (Levan et al. 1964)

arm (6 and 10), group E had one band on the short arm, but none on the long arm (13 and 14), group F had two bands on both arms (11), and group G had one band on each arm (1, 4, 16, 18–20, 22, and 24).

Chromosome characterization

In addition to the chromosome length, centromeric position, and rDNA loci distribution, we utilized the observed DAPI bands to characterize the chromosomes. Collectively, these DAPI bands could be very useful in identifying homologues for further cytogenetic analyses, especially of the *P. ginseng* genome, which comprises a large number of chromosomes with mostly similar sizes. The distinguishing features of each chromosome are presented in Table 2.

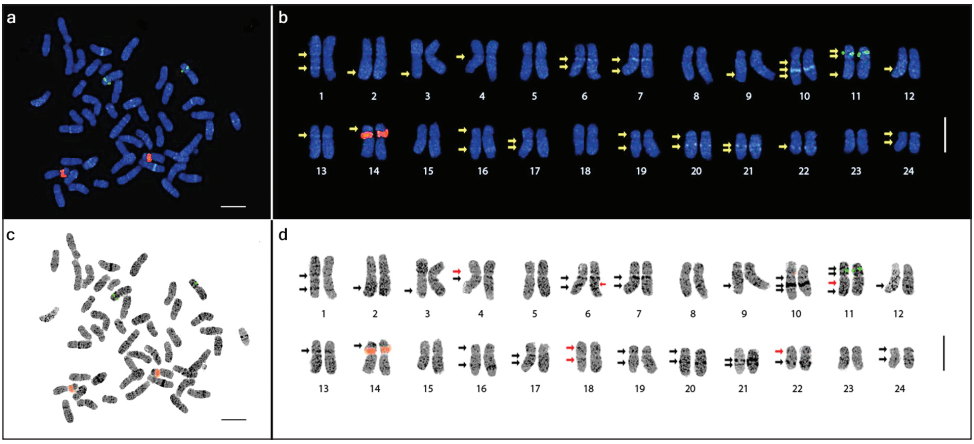

Figure 2. Metaphase spread of *P. ginseng* 2*n*=48 chromosomes (**a** and **c**) and the karyotype idiogram showing 24 homologous pairs (enlarged; **b** and **d**) arranged in decreasing lengths. The 5S and 45S rDNA loci are shown as green and red signals, respectively. DAPI bands (arrows) were detected in various intensities and inverse images (**c** and **d**) were obtained to emphasize these DAPI bands. Note the heterochromatic dots (dark dots in **d**). The red arrows in **d** indicate the six bands observed after inversing the image. Bar=5 μm.

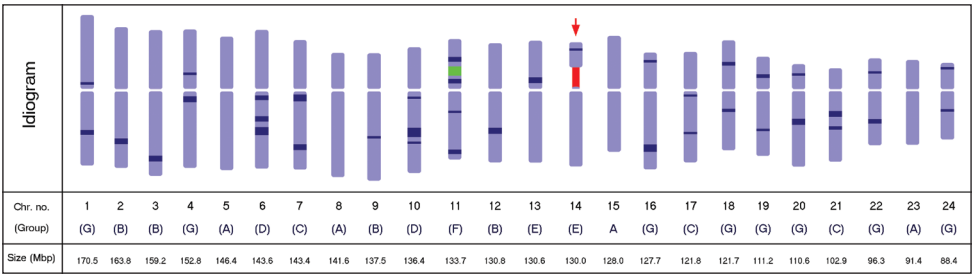

Figure 3. Diagrammatic idiogram of the *P. ginseng* karyotype showing the 5S (green) and the 45S (red) rDNA loci, and the 38 observed DAPI bands (dark blue), 12 on the short arm and 26 on the long arm. The satellited chromosome is indicated by the red arrow. DAPI band depths indicate relative intensities. Chromosomes were grouped according to the DAPI band pattern on each arm. The estimated relative size of each chromosome is presented in mega base-pairs.

Discussion

There is currently not much genomic or cytogenetic information available for ginseng. Consequently, there are no established cytogenetic markers for the identification of homologous chromosomes. This lack of data has limited our understanding of the karyotype of ginseng and therefore its phylogenetic relationship with other species in the genus *Panax*. In this study, we exploited the usefulness of the 5S and 45S rDNA and the DAPI-binding heterochromatins as molecular cytogenetic markers in pairing homologous chromosomes by analyzing their distribution in the *P. ginseng* genome.

Table 2. Summary of the rDNA and DAPI band distribution patterns.

Chr. no.	rDNA distribution		DAPI band distribution		Remarks
	5S	45S	Short arm	Long arm	
1	-	-	1	1	Pericentric on short arm, more intense intercalary on long arm
2	-	-	-	1	Dispersed, weak,subtelomeric
3	-	-	-	1	Subtelomeric, average intensity
4	-	-	1	1	Pericentric on both arms. Weaker on short arm
5	-	-	-	-	
6	-	-	-	3	One intense pericentric, two intercalary with weaker proximal
7	-	-	-	2	intense pericentric, weak distal
8	-	-	-	-	
9	-	-	-	1	Weak, intercalary
10	-	-	-	3	Weak pericentric, two intercalary with very intense middle and weak distal
11	1	-	2	2	Two moderate intensity flanking 5S rDNA on short arm, one weak intercalary and one weak subtelomeric on long arm. 5S rDNA moderate intensity
12	-	-	-	1	Intercalary, moderate intensity
13	-	-	1	-	Pericentric, weak
14†	-	1	1	-	Subtelomeric on satellite, weak; intense 45S rDNA
15	-	-	-	-	
16	-	-	1	1	Weak subtelomeric on short arm, more intense intercalary on long arm
17	-	-	-	2	Weak pericentric, weak intercalary
18	-	-	1	1	Weak intercalary on short arm, weak pericentric on long arm
19	-	-	1	1	Intercalary on both arms, more intense on short arm
20	-	-	1	1	Intercalary on both arms, more intense on long arm
21	-	-	-	2	Intercalary, proximal more intense than distal
22	-	-	1	1	Intercalary on both arms, more intense on long arm, long arm signal more intense than that on chromosome 20 long arm
23	-	-	-	-	
24	-	-	1	1	Weak subtelomeric on short arm, more intense intercalary on long arm
Total	1	1	12	26	

†satellited chromosome

Ribosomal DNA and DAPI-binding heterochromatin distribution

We detected only one locus each for 5S and 45S rDNA, which is in agreement with the results reported by Choi et al. (2009). However, the 45S rDNA signal was more intense than the 5S rDNA signal. Owing to the semi-quantitative nature of FISH (Maluszynska and Heslop-Harrison 1991), this could indicate that the 45S rDNA has more repeating units than the 5S rDNA in the ginseng genome.

Localization of the rDNA resulted in our only being able to easily pair two out of the 24 homologues. However, the existence of several DAPI bands distributed along most of the chromosomes greatly facilitated the identification of the other homologous pairs, which otherwise would have been challenging owing to the very low size difference among most ginseng chromosomes. As a result, DAPI banding, which has been utilized in several previously conducted studies (e.g. Schweizer 1976, Heng and Tsui 1993, Costa Silva et al. 2011),was found to also be an excellent cytogenetic marker in ginseng. Further analysis of the chromosomes based on the DAPI banding pattern on each arm enabled us to categorize them into seven groups (Fig. 3). This technique, which utilizes the presence or absence of DAPI-binding heterochromatin, has the potential for use in future karyotype analyses of ginseng varieties and other *Panax* species.

Chromosomal DAPI bands are caused by the preferential binding of DAPI to AT-rich heterochromatic DNA segments (Schweizer 1976, Eriksson et al. 1993, Heng and Tsui 1993, Kubota et al. 2000) that are long enough to be seen using a fluorescence microscope, suggesting that these DAPI-intense heterochromatic regions in ginseng are AT-rich DNA segments. This information should be useful in the ongoing ginseng genome sequencing because it enables identification of possible characteristics of heterochromatin types present in its genome. Nevertheless, further molecular and cytogenetic analyses are necessary to quantify the AT content of these regions and isolate DNA sequences specific to these heterochromatic bands, like the DAPI-intense signal of the 180-bp knob-specific satellite repeat in maize (Lamb et al. 2007), which is about 56% AT (Peacock et al. 1981, Ananiev et al. 1998).

The use of the rDNA loci number and distribution pattern of other *Panax* species can be useful in deducing the phylogenetic relationship among these species. Choi et al. (2009) showed that wild ginseng and American ginseng (*P. quinquefolius*), although geographically isolated, have equal numbers of 5S and 45S rDNA loci (2 and 1, respectively), while the cultivated ginseng, although found in the same geographic area with the wild ginseng has only one locus of each rDNA. Although further research is needed to confirm the possible phylogenetic significance of this report, we found only one locus for each type of rDNA in all three cultivars and one local landrace of *P. ginseng*.

Ginseng karyotype and ploidy

Karyotype data are essential to understanding the phylogenetic relationships among species belonging to the same family (Heslop-Harrison and Schwarzacher 2011,

Mendes et al. 2011), making them useful to cyto-taxonomic studies (Pinto et al. 2012). Additionally, comparative cytogenetics provide knowledge regarding the cytogenetic relationships between diploid species and their polyploid cytotypes, as well as between allopolyploids and their ancestral genomes (Kovarik et al. 2005, Leflon et al. 2006, Snowdon 2007, Wang et al. 2007, Kolano et al. 2008, Xiong and Pires 2011).

Most species belonging to the family Araliaceae are $2n$=24 or $2n$=48, except for a few genera that have little chromosomal number variation (Yi et al. 2004). In a review of the chromosomal evolution of the family Araliaceae, Yi et al. (2004) discussed that, although the actual basic chromosome number of the family was thought to be x=12, some species were $2n$=36. These species would be triploids if the basic chromosome number 12 is considered, but triploids are genetically unstable. This caused a challenge in establishing the basic chromosome number of the family. The x=12 hypothesis was further challenged after the genus *Hydrocotyle* which has several taxa with $2n$=18, 36, and 60 were moved into Araliaceae form Apiaceae, giving an alternative basic chromosome number x=9 and x=6. Nevertheless, x=12 is generally accepted as the basic chromosome number in the family, but this does not eliminate the possible ancestral x=6 (Yi et al. 2004). One hypothesis cannot easily rule out the other but further phylogenetic and karyotype analyses in the family are necessary to resolve these competing hypotheses.

Considering a basic chromosome number of 12 or 6, ginseng would be considered a tetraploid or octoploid, respectively; the latter having a more ancient nature. Recently, Choi et al. (2011) showed the high replication of homologous genes in ginseng using SSR markers and suggested that the polyploidy could range from tetra- to octoploidy. Nevertheless, in practice, *Panax ginseng* is regarded as a tetraploid species with a basic chromosome number of 12 (Wen and Zimmer 1996, Court 2000, Yi et al. 2004, Choi et al. 2009).

Our data showed a somatic cell chromosome complement of $2n$=48, supporting previously reported chromosome numbers (Ko et al. 1993, Choi et al. 2009) and polyploidy (Wen and Zimmer 1996, Court 2000, Yi et al. 2004, Choi et al. 2009). However, evaluation of the rDNA loci number revealed only one locus for each 5S and 45S rDNA, despite its polyploid nature. This reduction of rDNA loci may be explained by the non-additive nature of rDNA loci and other genomic DNA segments after polyploidization (Snowdon et al. 1997, Ozkan et al. 2003, Yoshikazu et al. 2006). Moreover, loss of the duplicate loci may be brought about by single-generation or rapid genome/chromosomal reorganization (Wendel 2000, Heslop-Harrison and Schwarzacher 2011), or from the gradual action of concerted evolution after genome duplication or alloploidization (Kovarik et al. 2005). In the former case, it would be difficult to tell whether ginseng is an ancient polyploid, while in the latter, the loss of these loci would provide obvious evidence of an ancient polyploidization event. However, some species do not really reflect a correlation between the rDNA loci number and the level of ploidy; in fact, polyploids can even have half the number of rDNA signals than their diploid counterparts (Yoshikazu et al. 2006). This rDNA reduction phenomenon has been well-documented in the *Artemisia* species (Pellicer et al. 2010).

Additionally, based on localization of the 45S rDNA near the centromere area and the intercalary position of the 5S rDNA, it is just as likely that these loci were favored to survive locus loss from non-additive recombination over their duplicated counterparts, which probably would have been in more distal positions, or epigenetically silenced (Kovarik et al. 2008).

Conclusion

The first report of *P. ginseng* karyotype using ribosomal DNA and DAPI bands as cytogenetic markers is presented here. The presence of long stretches of DAPI-binding heterochromatin was useful in the detailed karyotyping. The results presented here will be useful in further cytogenetic analyses and the on-going genome sequencing of ginseng. More cytogenetic research is needed to understand the cytogenetic history of ginseng and other species in the genus *Panax*. Further comparative cytogenetic analyses among its close relatives will provide more insight, and further genomic analyses of the heterochromatin distribution will enhance our knowledge of its genomic history.

Acknowledgments

This work was supported by the Next-Generation BioGreen21 Program (No. PJ008202), Rural Development Administration, Republic of Korea, and by the Sahmyook University Research Fund (No. RI2011039).

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CompCytogen 6(4): 443–452 (2012)
doi: 10.3897/CompCytogen.v6i4.4028
www.pensoft.net/journals/compcytogen

SHORT COMMUNICATIONS

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

Karyotypic diversity and evolutionary trends in the Neotropical catfish genus *Hypostomus* Lacépède, 1803 (Teleostei, Siluriformes, Loricariidae)

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Academic editor: *N. Golub* | Received 24 September 2012 | Accepted 21 November 2012 | Published 15 December 2012

Citation: Alves AL, Borba RS, Oliveira C, Nirchio M, Granado A, Foresti F (2012) Karyotypic diversity and evolutionary trends in the Neotropical catfish genus *Hypostomus* Lacépède, 1803 (Teleostei, Siluriformes, Loricariidae). Comparative Cytogenetics 6(4): 443–452. doi: 10.3897/CompCytogen.v6i4.4028

Abstract

The family Loricariidae with 813 nominal species is one of the largest fish families of the world. Hypostominae, its more complex subfamily, was recently divided into five tribes. The tribe Hypostomini is composed of a single genus, *Hypostomus* Lacépède, 1803, which exhibits the largest karyotypic diversity in the family Loricariidae. With the main objective of contributing to a better understanding of the relationship and the patterns of evolution among the karyotypes of *Hypostomus* species, cytogenetic studies were conducted in six species of the genus from Brazil and Venezuela. The results show a great chromosome variety with diploid numbers ranging from 2n=68 to 2n=76, with a clear predominance of acrocentric chromosomes. The Ag-NORs are located in terminal position in all species analyzed. Three species have single Ag-NORs (*Hypostomus albopunctatus* (Regan, 1908), *H.* prope *plecostomus* (Linnaeus, 1758), and *H.* prope *paulinus* (Ihering, 1905)) and three have multiple Ag-NORs (*H. ancistroides* (Ihering, 1911), *H.* prope *iheringi* (Regan, 1908), and *H. strigaticeps* (Regan, 1908)). In the process of karyotype evolution of the group, the main type of chromosome rearrangements was possibly centric fissions, which may have been facilitated by the putative tetraploid origin of *Hypostomus* species. The relationship between the karyotype changes and the evolution in the genus is discussed.

Keywords

Armoured-catfish, Loricariidae, *Hypostomus*, karyotype evolution, Ag-NORs, centric fission, polyploidy

Introduction

The subfamily Hypostominae with about 386 species (Reis et al. 2006) is the largest one in the family Loricariidae. The subfamily Hypostominae can only be recognized as monophyletic with the inclusion of the old subfamily Ancistrinae and the exclusion of some genera more related to the subfamily Neoplecostominae (Armbruster 2004). This subfamily is divided into five tribes: Corymbophanini, Rhinelepini, Hypostomini, Ancistrini, and Pterygoplichithini (Armbruster 2004) (Fig. 1). The tribe Hypostomini, with the only genus *Hypostomus*, has the greatest number of Hypostominae species (Reis et al. 2003).

The genus *Hypostomus* is the most representative in the family (Weber 2003, Hollanda Carvalho et al. 2010) with 126 species distributed from Central America to southern South America (Zawadzki et al. 2010). Species of the genus display phenotypic plasticity that makes difficult to obtain diagnostic characters for the group (Armbruster 2004).

Recent studies suggested that the genus *Hypostomus* might be composed of some monophyletic groups (Muller and Weber 1992, Montoya-Burgos 2003, Armbruster 2004, Zawadzki et al. 2004, Alves et al. 2006). This suggestion is confirmed by extensive morphological variation in the genus combined with a largest variety of diploid numbers and karyotype formulae in Loricariidae (Artoni and Bertollo 1996, Alves et al. 2006), with diploid numbers ranging from 2n=52 in *Hypostomus emarginatus* (Valenciennes, 1840) (Artoni 1996) to 2n=84 in *Hypostomus* sp. 2 (Cereali et al. 2008) (Table 1).

Cytogenetic studies in *Hypostomus* are relatively well documented (Table 1). In a review of genus cytogenetic data by Bueno et al. (2011) the relations between diploid number and karyotypic formulae of genus were established. However, several problems were not yet solved, including the pattern of karyotype evolution in Hypostomini. In the present study, six species of *Hypostomus* were karyotyped and the results employed to discuss the karyotype evolution of the genus.

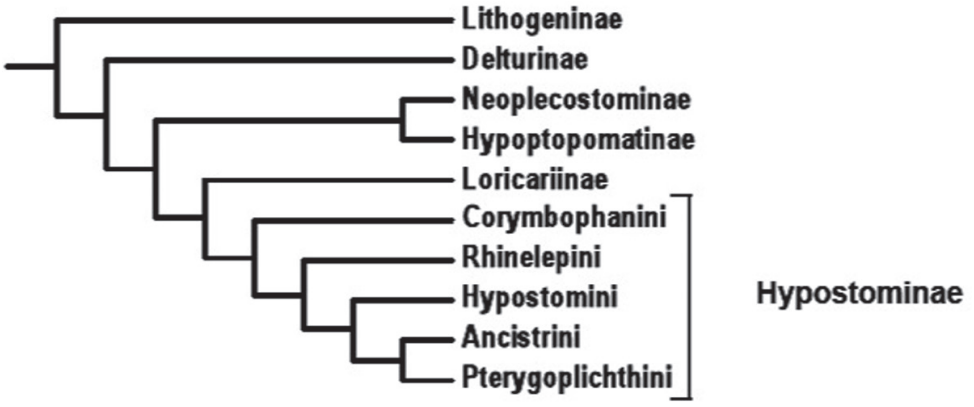

Figure 1. Phylogeny of the family Loricariidae proposed by Armbruster (2004).

Table 1. A summary of the cytogenetic data available for the genus *Hypostomus*. 2n = diploid number; M = metacentric; SM = submetacentric; ST = subtelocentric; A = acrocentric.

Species	Locality	2n	Karyotypic formulae	References
Hypostomus affinis (Steindachner, 1877)	Paraitinga River, São Paulo, Brazil	66	14M, 14SM, 12ST, 26A	Kavalco et al. (2004)
	Jacuí stream (SP)	66	14M, 14SM, 12ST, 26A	Fenerich et al. (2004)
H. albopunctalus (Regan, 1908)	Mogi-Guaçu River, São Paulo, Brazil	74	10M, 20SM, 44ST/A	Artoni and Bertollo (1996)
	Corumbataí River, São Paulo, Brazil	74	10M, 20M, 16ST, 28A	Present study
H. ancistroides (Ihering, 1911)	--	68	10M, 28SM, 30ST/A	Michele et al. (1977)
	Araquá River, São Paulo, Brazil	68	18M, 10SM, 12ST, 28A	Alves et al. (2006)
	Corumbataí River, São Paulo, Brazil	68	16M, 4SM, 16ST, 32A	Present study
	Mogi-Guaçu River, São Paulo, Brazil	68	16M, 18SM, 34ST/A	Artoni and Bertollo (1996)
	Paranapanema River, São Paulo, Brazil	68	10M, 26SM, 32ST/A	Rubert et al. (2011)
H. prope *auroguttatus* (Kner, 1854)	Mogi-Guaçu River, São Paulo, Brazil	76	8M, 30SM, 38ST/A	Artoni and Bertollo (1996)
Hypostomus cochliodon (Kner, 1854)	Salobra river and Salobrinha stream (MS)	64	16M, 20SM, 28ST-A (male)/ 16M, 19SM, 27ST-A (female)	Cereali (2006)
H. emarginatus (Valenciennes, 1840)	Araguaia River, Mato Grosso, Brazil	52	16M, 30SM, 6ST	Artoni (1996)
H. goyazensis (Regan, 1908)	Vermelho River, Goiás, Brazil	72	10M, 16SM, 10ST, 36A	Alves et al. (2006)
H. prope *iheringi* (Regan, 1908)	Corumbataí River, São Paulo, Brazil	74	10M, 14M, 20ST, 30A	Present study
H. macrops (Eigenmann & Eigenmann, 1888)	--	68	10M, 14SM, 44ST/A	Michelle et al. (1977)
H. nigromaculatus (Schubart, 1964)	Tibagi River, Paraná, Brazil.	76	6M, 20SM, 50ST/A	Rubert et al. (2008)
	Mogi-Guaçu River, São Paulo, Brazil	76	8M, 20SM, 48ST/A	Rubert et al. (2008)
H. paulinus (Ihering, 1905)	--	74	10M, 20SM, 44ST/A	Michele et al. (1977)
H. prope *paulinus* (Ihering, 1905)	Corumbataí River, São Paulo, Brazil	76	6M, 18M, 12ST, 40A	Present study
H. prope *paulinus* (Ihering, 1905)	Corumbataí River, São Paulo, Brazil	76	6M, 18M, 12ST, 40A	Present study
H. plecostomus (Linnaeus, 1758)	--	54	24M/SM, 12ST, 18A	Muramoto et al. (1968)
H. prope *plecostomus* (Linnaeus, 1758)	Orinoco River, Bolivar, Venezuela	68	12M, 16M, 12ST, 28A	Present study
H. regani (Ihering, 1905)	Mogi-Guaçu River, São Paulo, Brazil	72	10M, 20SM, 42ST/A	Artoni and Bertollo (1996)
	Paranapanema River, São Paulo, Brazil	72	10M, 18SM, 44ST/A	Rubert et al. 2011
	Araguá River, São Paulo, Brazil	72	12M, 18SM, 26ST, 16A	Alves et al. (2006)

Species	Locality	2n	Karyotypic formulae	References
H. strigaticeps (Regan, 1908)	Corumbataí River, São Paulo, Brazil	74	10M, 14M, 14ST, 36A	Present study
	Mogi-Guaçu River, São Paulo, Brazil	74	8M, 4SM, 62ST/A	Michele et al. (1977)
	Paranapanema River, São Paulo, Brazil	72	10M, 16SM, 46ST/A	Rubert et al. (2011)
Hypostomus sp. 2	Salobrinha stream, Mato Grosso do Sul, Brazil	84	6M, 16SM, 62ST/A	Cereali et al. (2008)
Hypostomus sp. 3	Perdido River, Mato Grosso do Sul, Brazil	82–84	6M, 16SM, 64ST/A - 6M, 12SM, 66ST/A	Cereali et al. (2008)
Hypostomus sp. A	Rincão Stream, São Paulo, Brazil	70	18M, 14SM, 38ST/A	Artoni and Bertollo (1996)
Hypostomus sp. B	Mogi-Guaçu River, São Paulo, Brazil	72	12M, 18SM, 42ST/A	Artoni and Bertollo (1996)
Hypostomus sp. C	Mogi-Guaçu River, São Paulo, Brazil	68	10M, 18SM, 40ST/A	Artoni and Bertollo (1996)
Hypostomus sp. D1	Mogi-Guaçu River, São Paulo, Brazil	72	10M, 26SM, 36ST/A	Artoni and Bertollo (1996)
Hypostomus sp. D2	Mogi-Guaçu River, São Paulo, Brazil	72	14M, 20SM, 38ST/A	Artoni and Bertollo (1996)
Hypostomus sp. E	Mogi-Guaçu River, São Paulo, Brazil	80	8M, 16SM, 56ST/A	Artoni and Bertollo (1996)
Hypostomus sp. F	São Francisco River, Minas Gerais, Brazil	76	10M, 16SM, 50ST/A	Artoni (1996)
Hypostomus sp. G	Araguaia River, Mato Grosso, Brazil	64	14M, 24SM, 26ST/A	Artoni (1996)
Hypostomus sp. Xingu-1	Xingu River, Pará, Brazil	64	32M/SM, 32ST/A	Milhomem et al. (2010)
Hypostomus sp. Xingu-2	Xingu River, Pará, Brazil	66	32M/SM, 34ST/A	Milhomem et al. (2010)
Hypostomus sp. Xingu-3	Xingu River, Pará, Brazil	65	38M/SM, 26ST/A, 1b	Milhomem et al. (2010)

Material and methods

Cytogenetic analyses were performed on chromosomal preparations obtained from six species. Five species were collected in the Corumbataí River, São Paulo, Brazil: *Hypostomus ancistroides* (Ihering, 1911) (6 males and 4 females) (LBP 2544), *H. albopunctatus* (Regan, 1908) (5 males and 6 females) (LBP 2547), *H. strigaticeps* (Regan, 1908) (6 males and 7 females) (LBP 2545), *H.* prope *iheringi* (Regan, 1908) (5 males and 4 females) (LBP 1674), and *H.* prope *paulinus* (Ihering, 1905) (5 males and 6 females) (LBP 2548). One species of *H.* prope *plecostomus* (Linnaeus, 1758) (3 males and 2 females) (LBP 2198) was collected in the Orinoco River, Bolivar, Venezuela. Vouchers were deposited in the fish collection of Laboratório de Biologia e Genética de Peixes (LBP), UNESP, Botucatu, São Paulo, Brazil.

Chromosome preparations were obtained from kidney tissues using the technique described by Foresti et al. (1993). Silver staining of the nucleolus organizer regions (Ag-NORs) was performed according to the technique proposed by Howell and Black

(1980). Chromosome morphology was determined on the basis of arm ratio, as proposed by Levan et al. (1964) and the chromosomes were classified as metacentrics (M), submetacentrics (SM), subtelocentrics (ST) and acrocentrics (A).

Results and discussion

Hypostomus ancistroides has karyotype with 2n=68 (16M, 4SM, 16ST, 32A) and terminal Ag-NORs on the short arm of the chromosome pair 1 (M) and pair 10 (SM) (Fig. 2a).

H. albopunctatus has 2n=74 (10M, 20SM, 16ST, 28A) and terminal Ag-NORs on the short arm of the chromosome pair 15 (SM) (Fig. 2b).

H. prope *iheringi* has 2n=74 (10M, 14SM, 20ST, 30A) and terminal Ag-NORs on the long arms of the chromosome pairs 23, 24, 25, 30 (A) (Fig. 3a).

H. prope *paulinus* has 2n=76 (6M, 18SM, 12ST, 40A) and terminal Ag-NORs on the long arm of the chromosome pair 20 (A) (Fig. 4b).

H. prope *plecostomus* has 2n=68 (12M, 16SM, 12ST, 28A) and terminal Ag-NORs on the short arm of the chromosome pair 16 (ST) (Fig. 4a).

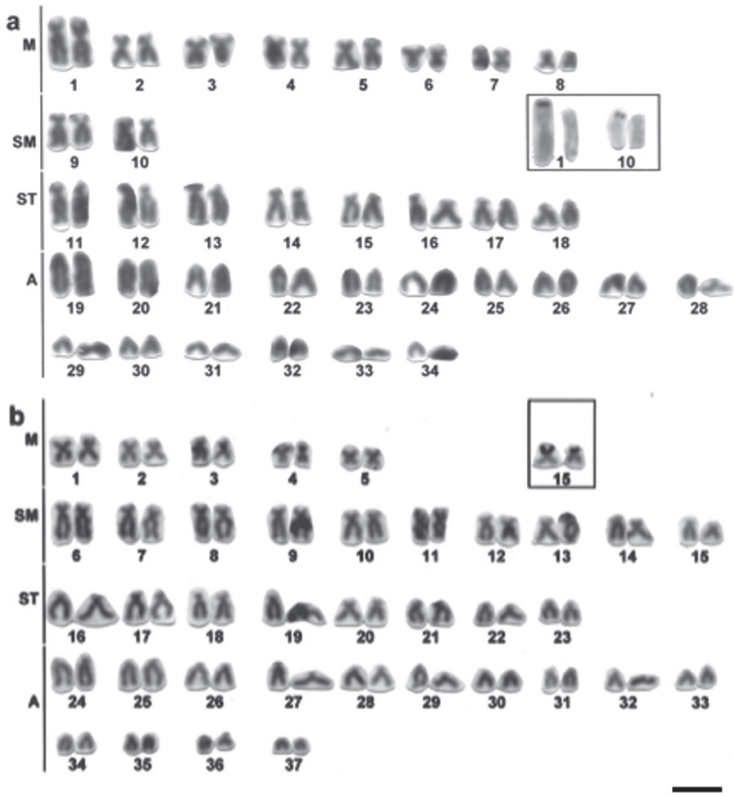

Figure 2. Giemsa stained karyotypes of *Hypostomus* **a** *H. ancistroides*, 2n=68 **b** *H. albopunctatus*, 2n=74. Ag-NOR-bearing chromosome pairs in the insets. Bar = 10μm.

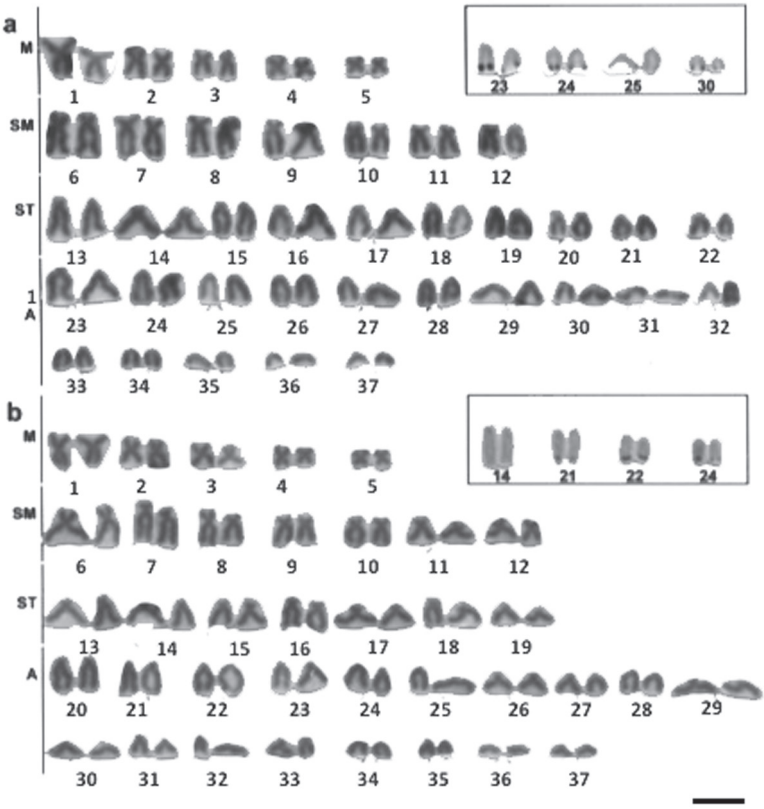

Figure 3. Giemsa stained karyotypes of *Hypostomus* **a** *H*. prope *iheringi*, 2n=74 **b** *H. strigaticeps*, 2n=74. Ag-NOR-bearing chromosome pairs in the insets. Bar = 10μm.

H. strigaticeps has 2n=74 (10M, 14SM, 14ST, 36A) and terminal Ag-NORs on the short arm of the chromosome pair 14 (ST) and on the long arm of the chromosome pairs 21, 22 e 24 (A) (Fig. 3b).

The genus *Hypostomus* seems to be the karyotypically most derived genus in Loricariidae (Rubert et al. 2011), the variation of diploid number observed in the six species of *Hypostomus* analyzed (2n=68 to 2n=76) confirms this hypothesis. All species analyzed exhibited a large number of acrocentric chromosomes, reinforcing the hypothesis that higher diploid numbers are positively related to higher number of acrocentric chromosomes in *Hypostomus* (Artoni and Bertollo 2001). According to Oliveira and Gosztonyi (2000), high diploid numbers may represent a derived characteristic in siluriforms.

Three species had single Ag-NORs (*H. albopunctatus*, *H.* prope *plecostomus*, and *H.* prope *paulinus*); and the three others had multiple Ag-NORs (*H. ancistroides*, *H.* prope *iheringi*, and *H. strigaticeps*). All species presented terminal Ag-NORs, a marked characteristic of the species of this genus. The occurrence of multiple Ag-NORs is the most common characteristic among the Hypostomini, however, this phenotype is

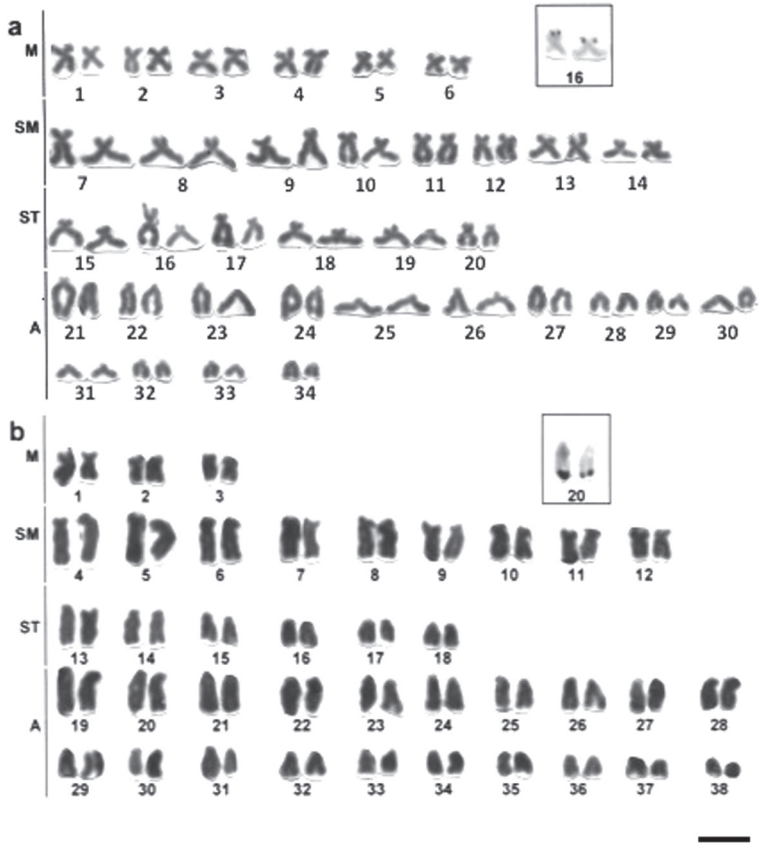

Figure 4. Giemsa stained karyotypes of *Hypostomus* **a** *H*. prope *plecostomus*, 2n=68 **b** *H*. prope *paulinus*, 2n=76. Ag-NOR-bearing chromosome pairs in the insets. Bar = 10μm.

considered a derived characteristic among siluriforms (Oliveira and Gosztonyi 2000), which usually predominate single Ag-NORs.

Differences in the karyotype formulae or in the number and position of Ag-NORs are common in species that do not present extensive migration behaviour, since isolated populations are more commonly involved in inbreeding processes, which makes the fixation of chromosome rearrangements easier (Almeida-Toledo et al. 2000). This kind of phenomenon has been extensively documented in fishes as in *Astyanax scabripinnis* (Jenyns, 1842) (Moreira-Filho and Bertollo 1991, Maistro et al. 1998, Alves and Martins-Santos 2002). On the other hand one of the most important problems associated with the study of the genus *Hypostomus* is the correct species identification due to the large number of species as well as the close morphological similarity among species (Armbruster 2004). Thus, Table 1 shows many samples identified as *Hypostomus* sp., which reflects our poor taxonomic knowledge of the group. Among the *Hypostomus* species, the high diploid number is coincident with a high the number of uniarmed chromosomes (Table 1), suggesting the

occurrence of a large number of centric fissions in the karyotypic evolution of the group (Artoni and Bertollo 1996). This hypothesis is reinforced considering that the species of Rhinelepini, the sister group of Hypostomini, has 2n=54 chromosomes (Alves et al. 2003, Alves et al. 2005, Alves et al. 2006). The occurrence of a polyploidy event in the origin of the tribe Hypostomini may explain the existence of duplicated centromeres and telomeres that could have been activated in the centric fissions rearrangements.

Thus, in the ancestor of Hypostomini an extensive process of chromosome fusions should have occurred changing a putative original karyotype with 2n=108 chromosomes into a karyotype with 2n=54 chromosomes. The alternative hypothesis that species of *Hypostomus* with high diploid numbers are the most primitive, suggesting that new chromosome fusions are reducing the diploid numbers in the genus, is not corroborated by the phylogenies available for the genus (Montoya-Burgos 2003, Armbruster 2004). Considering that the available phylogenies for the genus Hypostomus are very limited regarding the number of species and precise fish identification, further phylogenetic studies including karyotyped fishes are fundamental for a better understanding of the chromosome evolution in *Hypostomus*.

Acknowledgments

The authors are grateful to Renato Devidé for his technical assistance and to C.H. Zawadzki for the taxonomic identification of the species. This research was supported by the Brazilian agencies FAPESP (Fundação de Apoio à Pesquisa do Estado de São Paulo) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico).

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CompCytogen 6(4): 453–465 (2012)
doi: 10.3897/CompCytogen.v6i4.3461
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 karyomorphological study of some Indian *Cymbidium* Swartz, 1799 (Cymbidieae, Orchidaceae)

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Academic editor: *Joan Vallès* | Received 6 July 2012 | Accepted 20 August 2012 | Published 27 December 2012

Citation: Sharma SK, Kumaria S, Tandon P, Satyawada RR (2012) Comparative karyomorphological study of some Indian *Cymbidium* Swartz, 1799 (Cymbidieae, Orchidaceae). Comparative Cytogenetics 6(4): 453–465. doi: 10.3897/CompCytogen.v6i4.3461

Abstract

Understanding the genetic resources and diversity is very important for the breeding programs and improvement of several economically important orchids like *Cymbidium.* Karyomorphological studies have been carried out on seven *Cymbidium* species*, C. aloifolium* (Linnaeus, 1753), *C. devonianum* Paxton,1843, *C. elegans* Lindley, 1828, *C. iridioides* D. Don, 1825, *C. lowianum* Rchb. f.,1877, *C. tigrinum* Parish ex Hook. f., 1864, and *C. tracyanum* L. Castle,1890, most of them endangered/threatened in their natural habitat. As reported earlier, the somatic chromosome number (2n = 40) has been observed in all the seven species. Distinct inter-specific variation was recorded in the arm ratio of few homologous pairs in the complements. Symmetrical or almost symmetrical karyotypes were prevalent; however significant asymmetry was reported in *C. iridioides* and *C. tracyanum*. The significance of karyotypic variation in speciation of the genus *Cymbidium* has been discussed. This study provides useful chromosome landmarks and evidence about genome evolution, heteromorphic chromosomes based heterozygosity, basic chromosome number and ploidy level in the genus *Cymbidium*.

Keywords

Orchidaceae, mitosis, karyotype, heteromorphism, symmetry

Introduction

Cymbidium, or boat orchid, is a myriad orchid with evergreen foliage and arching sprays of delicately colored and waxy flowers, comprising of 52 evergreen species in the subtribe Cyrtopodiinae of tribe Cymbidieae (Orchidaceae). Cymbidiums are renowned for an abundance of morpho-types, with a seemingly unending array of strange and often impressive variations, and represent a highly advanced terminal line of floral evolution in the family. The genus is characterized by a broad geographical distribution encompassing tropical and subtropical Asia, South of Papua, New Guinea and Northern Australia, and exhibits a tremendous diversity in growth habits. It comprises several such representatives capable of occupying almost every conceivable ecological situation, apart from marine environments and habitats characterized by extreme cold throughout the year. Inter-generic compatibility is giving rise to hybrid groups, which are characterized by both greater size and hybrid vigor vis-à-vis their putative parental species. Therefore, characterization of genetic resources and diversity is a clue for framing meaningful breeding programs of economically important orchids like *Cymbidium* (Wang et al. 2009, Sharma et al. 2010, 2011, 2012a, b, c, d).

A number of workers from Asiatic regions especially China and Japan focused on cytogenetical aspects of several *Cymbidium* species: *C. cyperifolium* Lindly, 1833*, C. faberi* Rolfe, 1896*, C. goeringii* Rchb. f., 1852*, C. kanran* Makino, 1902*, C. longibracteatum* Y.S. Wu et S.C. Chen, 1966*, C. qiubeiense* K.M. Feng et Li, 1980 and *C. serratum* Schlechter, 1919 (Aoyama and Tanaka 1988, Li et al. 2002a, 2002b, 2003, Long et al. 2000), and reported extensive details on chromosome counts in somatic as well as gametic cells, presence of B-chromosomes and aneuploidy/polyploidy. Conversely, data on Indian cymbidiums mostly restrict to chromosome counts (Mehra and Yashpal 1961, Mehra and Bawa 1962, Chennaveeraiah and Jorapur 1966, Singh 1984, Mehra and Kashyap 1983, 1984a, b, c, d). Vij and Shekhar (1987) did an enormous investigation on cytogenetical aspects of Indian cymbidiums. Recently, our group reported the karyomorphological characterization of three species of Asiatic cymbidiums: *C. eburneum*, *C. hookerianum* and *C. mastersii* (Sharma et al. 2010), as well as endomitotic events in tapetal cells of some *Cymbidium* species (Sharma et al. 2012c). The unequivocal species differentiation on the base of karyological has been hampered by almost identical chromosome numbers (2n = 40), minute differences in chromosome morphology and low heteromorphism with no clear indications for morphologically distinct satellite chromosomes.

The karyomorphological details of Indian representatives of *Cymbidium* are still ambiguous, which make it difficult to correctly estimate ploidy levels vis-à-vis karyological evolution. In addition to our earlier efforts (Sharma et al. 2010), the present study focuses on seven more *Cymbidium* species, most of them are endangered/threatened in their natural habitat namely *C. aloifolium*, *C. devonianum*, *C. elegans*, *C. iridioides*, C. *lowianum*, *C. tigrinum*, and *C. tracyanum*, found in India, are expected to provide valuable baseline genetic data of the genus *Cymbidium*.

Cytological data on the Indian orchid flora are available for relatively few genera and most of them are restricted to chromosome counts only (Arora 1960, Sharma and Chat-

terji 1961, Mehra and Yashpal 1961, Mehra and Bawa 1962, Chennaveeraiah and Jorapur 1966). Sharma and Chatterji (1966), from their investigations encompassing 35 species of orchids belonging to 17 genera, reported the occurrence of a wide spectrum of basic numbers within each tribe and genus of family Orchidaceae. The genus *Cymbidium* has attracted a number of biologists from time to time to study a range of genetic aspects. However, from a cytogenetical and karyological point of view, scant reports are available (Singh 1984, Vij 1985, Vij and Shekhar 1987, Sharma et al. 2010). This genus has not found favor with cytogeneticists, perhaps owing to restricted geographical distribution, rarity of the plants in nature and difficulties in maintaining them under cultivation. Thus, the present investigation is an attempt to record karyomorphological details in more precise manner with prime objective of chromosome based genetic variation analysis in seven species of *Cymbidium.*

Methods

The young plants belonging to seven species of *Cymbidium* were collected mainly from Arunachal Pradesh, Meghalaya and Sikkim provinces of Northeastern region of India. The plants were grown in greenhouses of North-Eastern Hill University, Shillong. For each species, a minimum of five individuals belonging to more than one population were studied. Details regarding collection of root tips, staining, chromosome complement preparation and their analysis are as described than described in Sharma et al. (2010). A minimum of five chromosome plates were analyzed per individual of the species. The standard method of chromosome classification (Battaglia 1955) of median (V), submedian (L), subtelocentric (J) and telocentric (I) based on the arm ratio of 1:1, >1:1<1:3, >1:3<1:0 and 1:0 respectively, was used for comparison. The degree of symmetry was estimated as per the scheme proposed by Stebbins (1971). The karyotype asymmetry indices were calculated following Paszko (2006) method considering the parameters: (1) shortest (SC) and longest (LC) chromosome length; (2) ratio of longest to shortest chromosome (LC/SC); (3) mean long arm length (p); (4) mean of short (q) and of total chromosome length (CL); (5) mean centromeric index (CI = 100xlength of short arm/total chromosome length); and (6) coefficient of variation in terms of chromosome length (CV_{CL}) and (7) centromeric index (CV_{CI}). The karyotype asymmetry index (AI) defined as the product of coefficient of variations (both CV_{CL} and CV_{CI}) traduces the heterogeneity of chromosome length and/or centromeric index in a studied karyotype. As higher gets the AI index so does karyotype asymmetry, and inversely.

Results

Chromosome complement

The seven *Cymbidium* species presently investigated show the diploid number of 2n = 40 chromosomes in root tip cells, which were clearly resolved into 20 pairs forming a series

from the longest to shortest pair within the complements. The details of karyomorphological aspects including pair-wise arm ratio, karyotypic formula, number of subtelocentric chromosome and/or heteromorphic pairs are illustrated in Tables 1–2 and Figs 1–14. One notable feature was the lack of distinct nucleolar chromosomes in any of the seven species investigated. Variation was recorded with respect to the number of metacentric and submetacentric chromosomes, presence or absence of heteromorphic pairs in the chromosome complements of all the seven species of *Cymbidium* (Table 1). This study revealed that the plants belonging to *C. tracyanum* are peculiar in presenting metacentric and/or submetacentric chromosomes with one pair of distinct subtelocentric chromosomes in the complement. On the other hand, all the other cymbidiums are characterized by having exclusively submetacentric and/or metacentic chromosome pairs in karyotypes and are devoid of any subtelocentrics (Table 1).The chromosome morphology with regard to a particular pair in the karyotype has shown significant variation at inter-specific level (Table 2, Figs 8–14). For example, the third pair of *C. lowianum* and *C. tracyanum* is metacentric whereas all other cymbidiums have sub-metacentric chromosomes for this particular pair. The fifth pair in *C. tracyanum*, is found to be subtelocentric whereas, in other cymbidiums it is either metacentric or sub-metacentric. Such observation can be extended even too other pairs (i.e. IV-VIII, XIII, XVII, and XX) as well. Except for these, the rest of the pairs were found to be exclusively submetacentric (Table 1). Chromosome pairs VI, IX, XIV, XV, XVI, XVII, XVIII and XIX are found to be heteromorphic in *C. aloifoium, C. devonianum, C. elegans, C. lowianum, C. mastersii*, *C. tigrinum* and *C. tracyanum,* respectively. The highest number of heteromorphic pairs i.e. three (XV, XVII, and XVIII) are recorded in *C. elegans,* (Fig. 11) followed by *C. tracyanum* (Fig. 14) which had two heteromorphic pairs (XVII and XIX). Alternatively, not a single pair of the chromosome was found to be heteromorphic in *C. iridioides* (Table 1 and Fig. 13).

Asymmetry

Following the classification of Stebbins (1971), the karyotypes of five species of *Cymbidium* (*C. aloifolium, C. devonianum, C. elegans, C. lowianum* and *C. tigrinum*) were resolved into 2B category while 2C and 3B types were recorded in *C. iridioides* and *C. tracyanum* , respectively (Table 2). On the other hand, asymmetry indices estimated on the basis of chromosomal statistical data (Paszko 2006) resolved the *Cymbidium* karyotypes into the range of symmetrical to lowest asymmetrical values. On the one hand, *Cymbidium devonianum* had lowest value of AI (2.26), while *C. tracyanum* showed highest asymmetry having highest AI value (5.39) (Table 2). Karyotype asymmetry also depends on both the relative variation in chromosome length (CV_{CL}) and the relative variation in centromeric index (CV_{CI}). *Cymbidium tracyanum* was characterized by the highest value of both CV_{CL} and CV_{CI}, and then followed by *C. aloifolium* and *C. iridioides*. Remaining species of *Cymbidium* were characterized by much lower values of both CV_{CL} and CV_{CI} (Table 2).

Table 1. Karyomorphology and arm ratio in *Cymbidium* species.

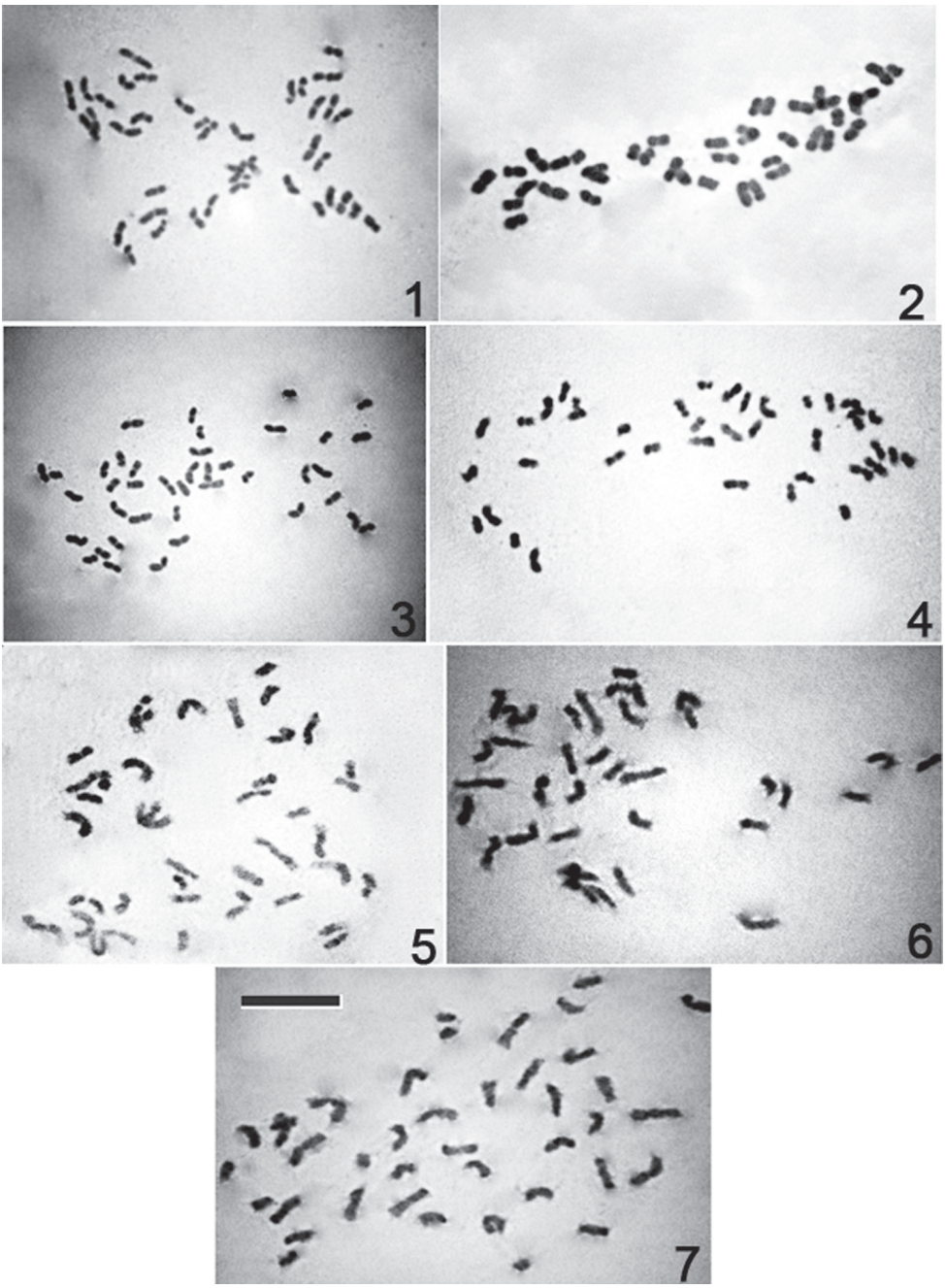
Taxa	2n	r-index in different chromosomes																			
		I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**	**IX**	**X**	**XI**	**XII**	**XIII**	**XIV**	**XV**	**XVI**	**XVII**	**XVIII**	**XIX**	**XX**
Cymbidium tigrinum	40	1.21 L	1.21 L	1.21 L	1.04 V	1.04 V	1.46 L	1.2 L	1.33 L	2.54 L	1.18 L	1.84 L	1.36 L	1.29 L	1.18 L	1.45 L	2.03 L	1.43 L	1.27 L	1.48 L	1.5 L
C. lowianum	40	1.44 L	1.12 L	1.09 V	1.23 L	1.16 L	1.14 L	1.24 L	1.6 L	1.2 L	1.29 L	1.37 L	1.14 L	1.19 L	1.57 L	1.34 L	1.12 L	1.08 V	1.38 L	2.26 L	1.06 V
C. devonianum	40	1.12 L	1.25 L	1.20 L	1.44 L	1.35 L	1.27 L	1.06 V	1.7 L	1.32 L	1.33 L	1.34 L	1.33 L	1.7 L	1.48 L	1.25 L	1.16 L	1.62 L	1.17 L	1.55 L	1.35 L
C. elegans	40	1.24 L	1.37 L	1.1 L	2.67 L	1.21 L	1.09 V	1.25 L	1.22 L	1.46 L	1.24 L	1.25 L	1.59 L	1.09 V	1.32 L	1.54 L	1.26 L	1.43 L	1.15 L	1.33 L	1.23 L
C. aloifolium	40	1.63 L	1.33 L	2.46 L	1.97 L	1.36 L	2.5 L	1.06 V	1.08 V	1.9 L	2.2 L	1.67 L	1.3 L	1.08 V	1.13 L	1.22 L	1.19 L	1.16 L	1.35 L	1.72 L	1.32 L
C. iridioides	40	1.22 L	1.29 L	1.46 L	1.44 L	1.07 V	1.11 L	1.06 V	1.52 L	1.27 L	2.27 L	1.7 L	1.02 L	1.18 L	1.31 L	1.26 L	1.28 L	1.64 L	1.15 L	2.2 L	1.25 L
C. tracyanum	40	1.64 L	1.2 L	1.02 V	1.08 V	3.24 J	1.05 V	1.16 L	1.61 L	1.25 L	1.8 L	1.18 L	1.06 V	1.58 L	1.67 L	2.11 L	2.3 L	2.17 L	1.47 L	1.21 L	1.23 L

Underlined values are showing heteromorphic pairs of chromosomes.

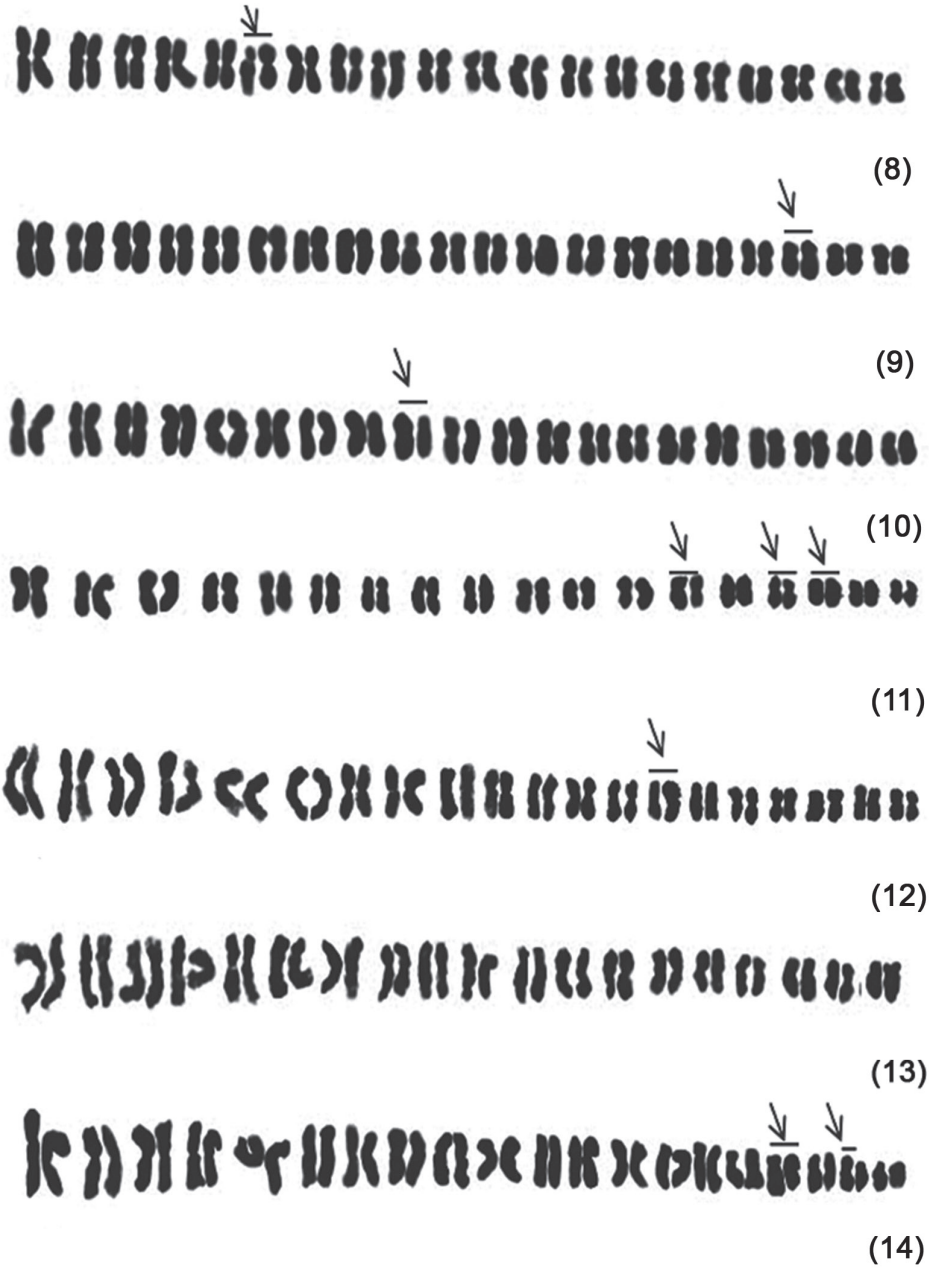
Table 2. Chromosome characteristics in various *Cymbidium* species.

Taxa	Range SC-LC (µm)	Ratio LC/SC	p (µm) Mean (±SD)	q (µm) Mean (±SD)	CL (µm) Mean (±SD)	CI Mean (±SD)	CV_{CL}	CV_{CI}	AI (Paszko 2006)	Category of symmetry (Stebbins 1971)	Karyotypic formula (Battaglia 1955)
Cymbidium tigrinum	2.04–4.97	2.43	1.91 (±0.343)	1.43 (±0.373)	3.35 (±0.612)	42.46 (±5.843)	18.26	13.76	2.51	2B	4V+36L
C. lowianum	1.76–4.44	2.52	1.82 (±0.358)	1.47 (±0.278)	3.29 (±0.584)	44.88 (±3.913)	17.75	8.71	1.54	2B	6V+34L
C. devonianum	1.87–3.80	2.03	1.60 (±0.311)	1.20 (±0.279)	2.81 (±0.505)	42.84 (±5.413)	17.97	12.63	2.26	2B	2V+38L
C. elegans	1.56–4.29	2.75	1.74 (±0.436)	1.32 (±0.331)	3.07 (±0.672)	43.39 (±5.352)	21.88	12.33	2.69	2B	4V+36L
C. aloifolium	1.89–4.87	2.57	1.81 (±0.607)	1.24 (±0.364)	3.05 (±0.830)	41.18 (±7.397)	27.21	17.96	4.88	2B	6V+34L
C. iridioides	2.62–7.25	2.76	2.46 (±0.617)	1.88 (±0.569)	4.34 (±1.073)	43.01 (±6.260)	24.72	14.55	3.59	3B	4V+36L
C. tracyanum	2.01–8.81	4.38	3.09 (±1.462)	2.18 (±0.782)	5.28 (±1.462)	41.21 (±8.030)	27.68	19.48	5.39	2C	8V+30L+2J

Abbreviations: (SC) shortest and (LC) longest chromosome length; (p) mean long arm length; (q) mean of short chromosome length; (CL) mean of total chromosome length; (CI) mean centromeric index; (CV_{CL}) coefficient of variation in terms of chromosome length; (CV_{CI}) coefficient of variation in terms of centromeric index; (AI) karyotype asymmetry index .



Figures 1–7. Mitotic complements of *Cymbidium* species. **1** *C. tigrinum* **2** *C. lowianum* **3** *C. devonianum* **4** *C. elegans* **5** *C. aloifolium* **6** *C. iridioides* **7** *C. tracyanum.* Bar = 10 μm.



Figures 8–14. Karyotypes of *Cymbidium* species. **8** *C. tigrinum* **9** *C. lowianum* **10** *C. devonianum* **11** *C. elegans* **12** *C. aloifolium* **13** *C. iridioides* **14** *C. tracyanum*. Heteromorphic pair marked by arrows above the short arm.

Discussion

Felix and Guerra (2000) have published some excellent cytogenetical details on orchids especially on members of cymbidioid phylad. About 44 species belonging to cymbidioid genera were cytogenetically characterized and the pattern of karyological evolution within the group was reported. The chromosome variability reported by them ranges from 2n = 10 (*Psygmorchis pusilla* (Linnaeus, 1752) to 2n = 168 (*Oncidium* Swartz,1800 species). They have also investigated various sub-tribes for chromosome counts and recorded variation both within and between sub-tribes, which was quite remarkable. They were of the opinion that orchids in general and cymbidioid phylad in particular have extensively benefited by the occurrence of variable base numbers followed by attainment of higher ploidy levels. From the review of published chromosome counts of *Cymbidium* and allied species from various parts of the world namely Brazil (Felix and Guerra 2000), China (Li et al. 2002a, 2002b, 2003) and Japan (Aoyama and Tanaka 1988, Aoyama 1989), it can be observed that barring few exceptions, the genus *Cymbidium* showed x = 10 as the basic number and therefore majority of the species revealed somatic chromosome number 2n = 40. The present investigation on cymbidiums also supports the earlier views with regard to x = 10 as true basic number of the genus *Cymbidium*. The genus *Cymbidium* is known for consistency in somatic chromosome numbers (2n = 40). However, certain deviant chromosome counts of 2n = 32, 38, 42 and 52 in species like *C. aloifolium*, *C. bicolor*, *C. eburneum*, *C. hookerianum*, *C. iridioides* and *C. tigrinum* are also reported (Vij and Shekhar 1987, Aoyama and Tanaka 1988, Aoyama 1989, Felix and Guerra 2000). Besides these unique observations on chromosome counts, they have also reported the occurrence of significant numbers of B-chromosomes in various *Cymbidium* species, whose number ranged from 1-5 in *C. lancifolium* and *C. javanicum*. The occasional occurrence of triploid cytotypes was another novel finding reported by Aoyoma and Tanaka (1988). In the present investigation, we do not come across such deviations in any of the materials investigated from north-east India. The absence of deviant chromosome numbers and overall symmetry also suggests that the diversification at inter-specific level has occurred without any significant numerical changes. However, one important point emerging out of the present study is that *C. tigrinum,* which is also considered as one of the Indian miniature cymbidiums exhibited somatic chromosome number of 2n = 40. Such observations differ from results of Vij and Shekhar (1987) who reported 2n = 38 (x = 19) originated through loss of one pair of chromosomes for this species.

In the present study, characteristic differences have been recorded in karyotypes at inter-specific level of the genus *Cymbidium.* In general, nine pairs out of twenty i.e. I-II, IX-X, XIV-XVI and XVIII to XIX, showed uniformity with respect to the chromosome morphology at inter-specific level while moderate to greater degree of variation was recorded in the remaining eleven pairs of the chromosome complements pattern. Such observations indicate the high degree of gene/genome stability in the genus. In general, it is predicted that orchid seeds, which are very small and light weight,

can be wind-dispersed over long distances (Dressler 1993, Ackerman and Ward 1999, Chung et al. 2004), promoting genetic homogeneity among populations.

The karyotypes in most of the species investigated were found to be symmetrical according to Stebbins (1971) classification. The relative variation in chromosome length and centromeric position also provides a measure of the heterogeneity in a given karyotype. The karyotype of *C. tracyanum* was found to be most asymmetric by having 2C type of symmetry along with highest values of relative variation in chromosome length (CV_{CL}), centromeric position (CV_{CI}) as well as asymmetry index (AI). On the other hand, *C. lowianum* revealed least asymmetric having lowest values of CV_{CL} and CV_{CI} (17.75 and 8.71 respectively) as well as lowest AI (1.54), thereby confirming the high degree of genome stability with symmetric karyotypes. Further, the ratio of longest and shortest chromosome ranged from 2.03 in *C. devonianum* and 4.38 in *C. tracyanum*. The absence of nucleolus organizers in the chromosomes and deviant chromosome number (barring few cells in *C. aloifolium* and *C. tigrinum*) accompanied by lack of any numerical and structural changes in chromosomes suggests a more or less stabilized genome of *Cymbidium* as evident in various species presently investigated. Most of the species (~70%) of the Orchidaceae are epiphytic (Dressler, 1993) including those of *Cymbidium*. All the available data on genetic diversity is biased towards terrestrial species and suggests that the gene flow of epiphytes could be more susceptible to environmental changes than other species due to the habitat, patchy distribution and specific pollination strategies (González-Astorga et al. 2004, Trapnell et al. 2004). The heteromorphic pairs recorded in *C. aloifolium, C. devonianum, C. elegans, C. lowianum, C. tigrinum* and *C. tracyanum* are indicative of heterogeneity and exhibit less genomic stability ultimately leading to help the species to attempt structural alterations as means of speciation. It is also opined that the chromosome re-patterning through either loss or gain of chromatin matter has also played a significant role in the evolution of the genus *Cymbidium* (Vij and Shekhar 1987). Not a single pair of nucleolus organizers has been observed in the form of a secondary constriction in any of the species investigated. However, physical localization of 45S rDNA in eight species of *Cymbidium* using fluorescent *in situ* hybridization (FISH) has confirmed the nucleolar nature of the chromosomes (Sharma et al. 2012a). *Cymbidium aloifolium, C. tigrinum* and *C. tracyanum* showed decondensed, dispersed, extended form of hybridization signals of rDNA as dots of fluorescence (transcriptionally active), whereas rest of the cymbidiums revealed condensed (non-active) forms, the genus hence showing a certain degree of heteromorphism in the size, intensities and appearance of rDNA signal. This phenomenon was earlier advocated by Nagl (1977) in case of *Cymbidium*, stating that it is unique among monocots having AT rich regions in genome. ITS sequence data have also determined the phylogeny of Asiatic cymbidiums with high bootstrap values and all three proposed subgenera could be distinguished clearly (Sharma et al. 2012b). Thus, it is opined that the genomic distribution pattern of 45S rDNA is very similar in most of the *Cymbidium* species, however, *C. aloifolium, C. tigrinum* and *C. tracyanum* did show variation and are consistently distinguished from other cymbidiums both at chromosome and molecular levels (Sharma et al. 2012a, b, c, d).

Concluding remarks

Karyotype similarities between *Cymbidium* species traduce the high degree of gene stability in the genus at inter-specific level and indicate lack of chromosome structural rearrangements during speciation in *Cymbidium*. The present investigation may also provide useful information on chromosome markers including heteromorphic chromosomes based speciation, basic chromosome number and ploidy level vis-à-vis genome evolution; which is more or less poorly known in the family Orchidaceae and especially in the genus *Cymbidium*.

Acknowledgements

The present work was supported by a grant from University Grants Commission, Government of India, New Delhi, through University with Potential for Excellence (UPE) – Bioscience program. The authors are thankful to Head, Department of Biotechnology and Bioinformatics, NEHU for providing facilities. Sincere thanks are due to Prof. M.S. Bisht, Dr. C.S. Rao and all members of Plant Biotechnology Laboratories of Department of Botany and Department of Biotechnology and Bioinformatics, NEHU, Shillong, for their constant encouragement and help.

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