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Male meiosis, morphometric analysis and distribution pattern of 2× and 4× cytotypes of *Ranunculus hirtellus* Royle, 1834 (Ranunculaceae) from the cold regions of northwest Himalayas (India)

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Abstract

In this study, we examined the chromosome number, detailed male meiosis, microsporogenesis, pollen fertility and morphological features and distribution of 2× and 4× cytotypes of *Ranunculus hirtellus* Royle, 1834. The majority of the populations scored now from cold regions of the northwest Himalayas showed tetraploid (n=16) meiotic chromosome count and one of the populations studied from the Manimahesh hills existed at diploid level (n=8). The individuals of diploid cytotype exhibited perfectly normal meiotic course resulting in 100% pollen fertility and pollen grains of uniform sizes. On the other hand, the plants of the tetraploid cytotype from all the populations in spite of showing normal bivalent formation and equal distribution to the opposite poles at anaphases showed various meiotic abnormalities. The most prominent among these meiotic abnormalities was the cytomixis which involved inter PMC (pollen mother cell) chromatin material transfer at different stages of meiosis-I. The phenomenon of cytomixis induced various meiotic abnormalities which include chromatin stickiness, pycnotic chromatin, laggards and chromatin bridges, out of plate bivalents at metaphase-I, disoriented chromatin material at anaphase/telophase and micronuclei. Consequently, these populations exhibited varying percentages of pollen sterility (24 - 77 %) and pollen grains of heterogeneous sizes. Analysis of various morphometric features including the stomata in 2× and 4× cytotypes showed that increase in ploidy level in the species is correlated with gigantism of vegetative and floral characters and the two cytotypes can be distinguished from each other on the basis of morphological characters. The distribution patterns of the 2× and 4× cytotypes now detected and 2×, 3×, 4× cytotypes detected earlier by workers from other regions of the Indian Himalayas have also been discussed.

Keywords
chromosome number, cytotype, cytomixis, Lahaul-Spiti, Manali hills, Manimahesh hills, meiotic abnormalities, stomata

Introduction

Ranunculus hirtellus Royle, 1834 (Ranunculaceae), a perennial erect or decumbent herb, distinctly pubescent with fibrous and shortly fusiform root stock is endemic to Himalayas, and distributed in the temperate, sub-alpine and alpine slopes in North-West to North-East Himalaya, temperate to subalpine slopes at 2000 – 4500 m in the states of Jammu & Kashmir, Himachal Pradesh, Uttarakhand, Sikkim, and Arunachal Pradesh and also in Afghanistan, Pakistan, Nepal and Tibet (Sharma et al. 1993, Srivastava 2010). The species is medicinally important as the paste made by crushing roots in cow's urine is used to cure the swellings of testes by the tribal communities of Chhota Bhangal area of Kangra district in Himachal Pradesh (Uniyal et al. 2006). Besides, the species is also used to cure skin diseases and wounds, and as a vermifacient, cooling agent and anthelmintic in other parts of the Himalayas in India (Jain 1991, Iyer 1992, Kumar 2010, Pharswan et al. 2010).

The species is highly variable with respect to habit, plant size, shape and hairiness of leaves and sepals, hairiness of pedicels, and size of flowers (Aswal and Mehrotra 1994). The information gathered from various Indexes to Plant Chromosome Numbers (Goldblatt 1981, 1984, 1985, 1988, Kumar and Subramanian 1986, Khatoon and Ali 1993) also revealed that the species is equally variable in terms of chromosome number (2n=14, 16, 24, 28, 32) and level of ploidy (2×, 3×, 4×). Earlier chromosomal studies in the species were confined to merely counting the chromosome number and no attempt has been made to correlate the extent of morphological variability among different cytotypes and their relative distribution patterns in the Himalayas.

Our research group has been engaged in the study of cytological aspects of plants of cold deserts of India through male meiosis since 2006. Male meiosis of more than 300 species from the cold desert areas of Chamba, Lahaul-Spiti and Kinnaur districts of Himachal Pradesh has been studied and various aberrations were detected during male meiosis in *Caltha palustris* Linnaeus, 1753 (Kumar and Singhal 2008), *Meconopsis aculeata* Royle, 1834 (Singhal and Kumar 2008a), *Hippophae rhamnoides* Linnaeus, 1753 (Singhal et al. 2008), *Papaver dubium* Linnaeus, 1753 (Singhal and Kaur 2009), *Anemone rivularis* Buch.-Ham. ex DC., 1817 (Singhal et al. 2009a), *Inula cuspidata* (DC.) C. B. Clarke, 1876 (Kaur et al. 2010), *Clematis orientalis* Linnaeus, 1753 (Kumar et al. 2010), *Ranunculus laetus* Wall. ex Royle, 1839 (Kumar et al. 2011), *Thalictrum foetidum* Linnaeus, 1753 (Singhal et al. 2011), *Dianthus angulatus* Royle ex Benth., 1835 (Kumar et al. in press) and *Lindelofia longiflora* Baill., 1890 (Singhal et al. in press).

The aim of the present research was to study the male meiosis in detail and to find the impact of chromatin transfer in inducing meiotic aberrations and their consequent effect on pollen fertility and pollen size. The purpose of the present study was also to differentiate the 2× and 4× individuals growing wild and also to find out the distribution patterns of different cytotypes in the Indian Himalayas.

Material and methods

Plant material and identification – Material for male meiotic studies were collected from the wild plants growing in different localities of Lahaul-Spiti, Manimahesh hills and Manali hills of Himachal Pradesh, India in the months of May - July during the years 2008 and 2009 (Table 1, Fig. 1). The identification of the taxon was done by consulting the various floras of the region such as, Flora of Lahaul-Spiti (Aswal and Mehrotra 1994), Flora of Kullu district (Dhaliwal and Sharma 1999) and Flora of Chamba district (Singh and Sharma 2006). Besides, the plant specimens were also compared with the samples in the Herbaria of the Department of Botany, Punjabi University, Patiala (PUN), Botanical Survey of India, Dehra Dun (BSI), and Forest Research Institute, Dehra Dun (FRI). The voucher specimens are deposited in the Herbarium, Department of Botany, Punjabi University, Patiala. The young developing floral buds from healthy plants were fixed in freshly prepared Carnoy's fixative (6 Ethanol: 3 Chloroform: 1 Glacial acetic acid v; v; v) for 24 hours and subsequently stored in 70% ethanol until analysis.

Chromosome counts and male meiotic analysis – Developing anthers from floral buds were squashed in 1% acetocarmine and preparations were studied for chromosome counts, and detailed meiotic behavior in pollen mother cells (PMCs) at early prophase-I, metaphase-I (MI), anaphases-I/II (AI/II), telophases-I/II (TI/II) and sporad stage. In populations with normal meiotic course, a total of 10–30 PMCs were examined for determining the chromosome counts while in cytologically abnormal populations 20–50 slides prepared from different anthers/flowers (with 100–200 PMCs) were analyzed in each case.

Pollen fertility – Pollen fertility was estimated through stainability tests for which anthers of mature flowers were squashed in glyceroacetocarmine mixture (1:1) or 1% aniline blue dye. 200–500 pollen grains were analyzed in each case for pollen fertility and pollen size. Well-filled pollen grains with uniformly darkly stained cytoplasm were scored as fertile/viable while shrivelled pollen with unstained or poorly stained cytoplasm were counted as sterile/unviable. Pollen fertility was expressed as an average percentage of the stained pollen grains/total pollen grains analyzed. Size of stained pollen grains was measured with occulomicrometer.

Photomicrographs – Chromosome spreads were analyzed with Olympus light microscope and the best plates of chromosome counts, meiotic abnormalities, sporads and pollen grains (fertile, sterile) were photographed from the temporary mounts with Nikon Eclipse 80*i* microscope.

Table 1. List of specimen number/s, meiotic chromosome number, and places of collection with district, province, habitat, latitude and longitude, altitude and habitat of different populations of the diploid ($2n$ = 2× = 16) and tetraploid ($2n$ = 4× = 32) cytotypes of *R. hirtellus*. *Herbarium code as per "Index Herbariorum" by Holmgren and Holmgren (1998).

Cytotype	Specimen number (PUN*)	Meiotic chromosome number (n)	Places of collection with district, province, habitat, latitude and longitude and altitude in meters (Alt. m)
Diploid	51801	8	Gauri Kund, Manimahesh hills, Chamba, Himachal Pradesh, alpine moist slopes, 32°24.11'N; 76°38.25'E, Alt.: 3930 m
Tetraploid	51370	16	Manimahesh Lake, Manimahesh hills, Chamba, Himachal Pradesh, alpine moist slopes, 32°23.91'N; 76°38.30'E, Alt.: 4300 m
	51356	16	Dhancho, Manimahesh hills, Chamba, Himachal Pradesh, along water course, 32°25.18'N; 76°36.53'E, Alt.: 3030 m
	51360	16	Jalori Pass, Manali hills in Kullu, Himachal Pradesh, moist slopes in Oak forest, 31°31.95'N; 77°23.87'E, Alt.: 3140 m
	51364	16	Rohtang Pass, Manali hills in Kullu, Himachal Pradesh, alpine moist slopes, 32°21.84'N; 77°14.59'E, Alt.: 3980 m
	51138	16	Shashur, Lahaul Valley in Lahaul-Spiti, Himachal Pradesh, open and moist grassy slopes among scattered trees of *Salix* and *Juniperus* 32°34.56'N; 77°1.54'E, Alt.: 3340 m
	51374	16	Keylong, Lahaul Valley in Lahaul-Spiti, Himachal Pradesh, growing under *Salix* trees in moist conditions, 32°34.18'N; 77°2.01'E, Alt.: 3340 m

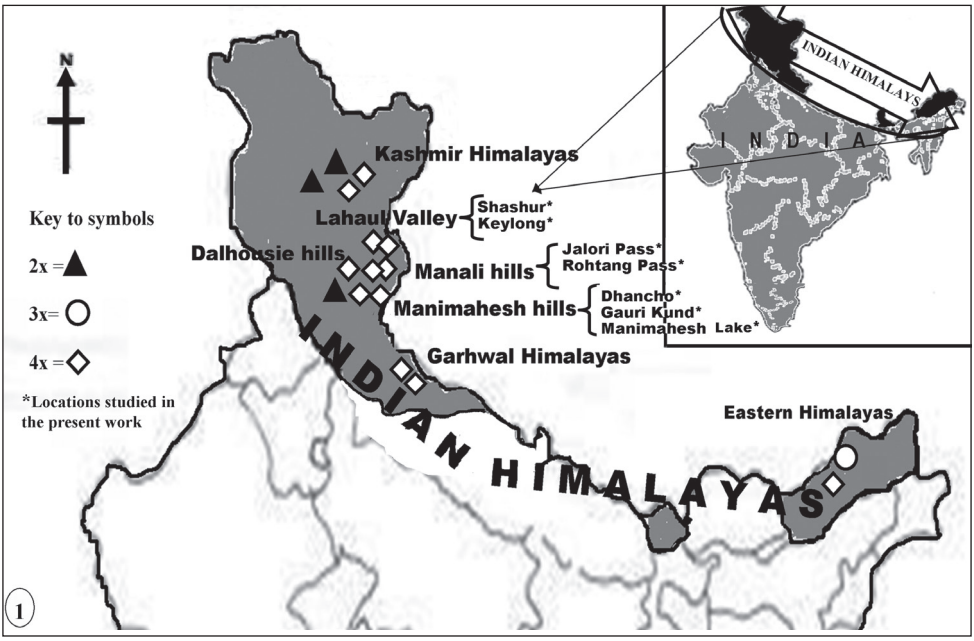

Figure 1. Map showing the distribution pattern of the 2× and 4× cytotypes reported here (marked with asterisks) and the 2×, 3×, 4× cytotypes detected by workers from other regions of the Indian Himalayas.

Results

R. hirtellus has been worked out for male meiosis and morphometric analysis from seven different localities of Manimahesh and Manali hills, and Lahaul-Spiti (Table 1). Two intraspecific cytotypes (Fig. 2, A & B), the diploid (n=8) and the tetraploid (n=16) have been detected in the species. The population scored from the Manimahesh hills was found to be diploid while rest of the six populations studied from Kullu, Chamba and Lahaul-Spiti districts existed at tetraploid level. Cytological and morphometric analysis have been performed on both the cytotypes and data regarding the micro- and macroscopic characters are provided in Table 2.

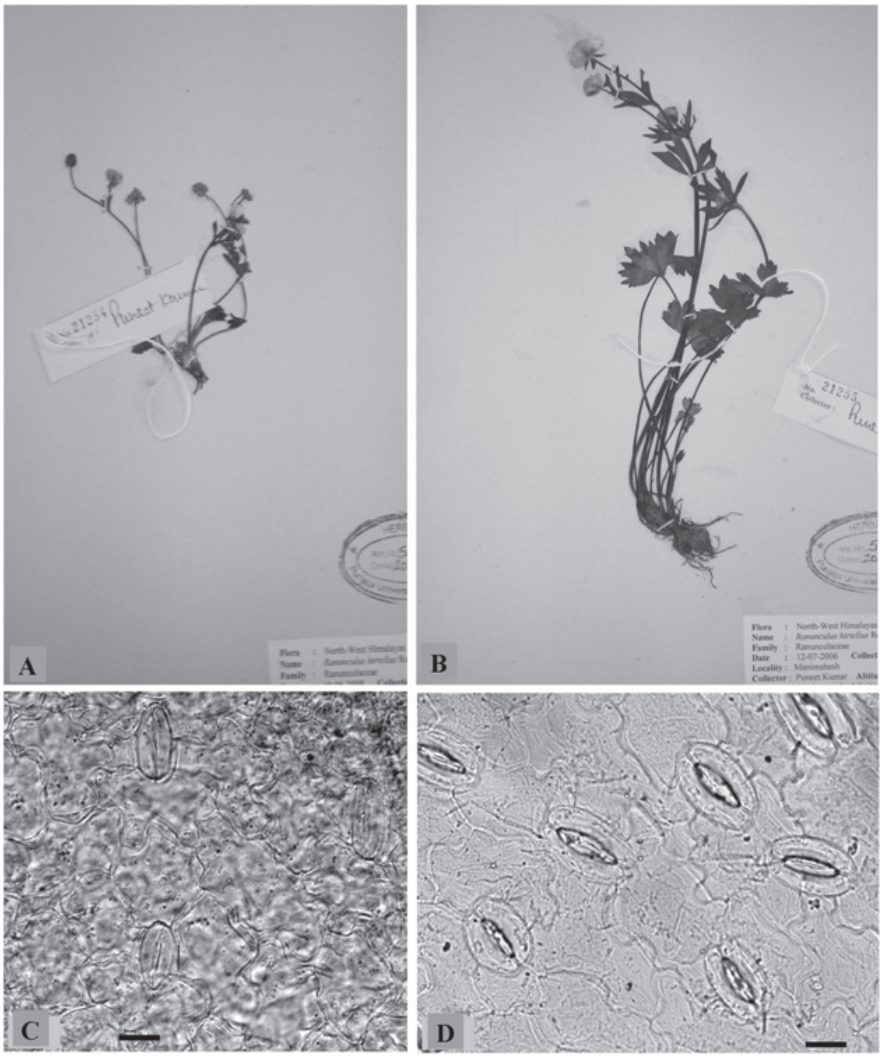

Figure 2A–D. Individuals of *R. hirtellus* **A** 2× **B** 4× cytotype. Stomata **C** 2× and **D** 4× cytotype. Scale bars = 20 μm.

Table 2. Comparison of micro- and macroscopic characters of the diploid ($2n$ = 2× = 16) and tetraploid ($2n$ = 4× = 32) cytotypes of *R. hirtellus* (Figures in the parentheses represent the mean ± standard deviation). *4× populations from Manimahesh Lake and Keylong

S. No.	Characters	Cytotype	
		Diploid	**Tetraploid**
1.	Meiotic chromosome number (n)	8	16
2.	Plant height (cm)	21.20–23.50 (22.41±0.89)	34.80–37.20 (35.78±0.90)
3.	Radical leaf length (cm)	6.80–10.28 (8.03±1.45)	16.80–22.40 (18.58 ±2.71)
4.	Number of flowers/plant	15–21 (16 ±2.11)	18–27 (25.3±2.7)
5.	Stomatal size (μm)	29.54–39.29 × 17.08–27.10 (34.04±2.47) (21.14±2.56)	34.55–45.46 × 23.56–28.41 (39.15±3.23) (26.98±1.77)
6.	Stomatal density/mm^2	63–127	90–137
7.	Stomatal index	11.47	24.94
8.	Pollen grain size (μm)	24.52 - 24.85 × 25.13 - 26.55 (24. 63±0.35) (25.95±1.26)	24.52 × 25.13* 24.85 × 26.55*

Morphometric analysis

Morphometric analysis involves both macro- and microscopic characters (Table 2.) Macroscopic characters including plant height, radical leaf length and number of flowers per plant were studied from all the populations of the tetraploid and one population of the diploid cytotype. The tetraploid plants measured in height were much taller than the diploid. Also the radical leaves were noticed to be much larger in the tetraploid cytotype compared with the diploid. The number of flowers was more in the tetraploid compared to the diploid. Stomata were analysed from 2× population collected from Manimahesh Lake, 4300 m and the 4× population from Keylong, 3340 m (Fig. 2, C & D). The values for stomatal size, density and index were found to be more in the tetraploid compared to the diploid (Table 2). Pollen grains in the diploid cytotype were almost uniform sized whereas in the tetraploid cytotype pollen grains were of variable sizes except for two populations (Table 3).

Cytological analysis

The diploid (n=8) cytotype

Only one population growing on the moist alpine slopes of Gauri Kund (3930 m) in the Manimahesh hills (Chamba district) existed at diploid level (based on x=8) as confirmed from the presence of 8 medium sized bivalents in the PMCs at MI (Fig. 3, A). These bivalents showed regular segregation during AI. Further meiotic course was also regular resulting into normal tetrad formation, nearly cent per cent pollen fertility and uniform sized pollen grains.

Table 3. Pollen grain size, relative frequency of variable sized pollen grains and pollen sterility in diploid 2× and tetraploid 4× cytotypes of *R. hirtellus* (Figures in the parentheses represent the mean ± standard deviation). Rf = relative frequency of variable sized pollen grains.

S. No.	Populations	Pollen grains size (µm)		Rf % age	Pollen sterility % age
		Diploid	Tetraploid		
1.	Gauri Kund	24.52 - 24.85 × 25.13 - 26.55 (24. 63±0.35) (25.95±1.26)		100	00
2.	Dhancho		59.96 × 59.96 40.04 × 40.04 32.76-36.40 × 29.12 - 36.40 (35.25±4.21) (33.72±5.13) 21.84 - 29.12 × 21.84 - 25.48 (24.66±2.03) (23.66±1.97)	3.51 28.07 35.09 33.33	64
3.	Manimahesh Lake		24.52 × 25.13	100	26
4.	Jalori Pass		36.40-40.04 × 36.40 (37.44±1.71) 32.76 × 25.48-32.76 (31.08±2.83) 25.48 × 25.48 21.84 × 21.84 10.92 × 10.92	1.58 39.10 37.50 20.63 1.19	77
5.	Rohtang Pass		19.27-23.85 × 20.64-27.52 (21.73±1.43) (25.05±1.48) 16.05-16.51 × 17.89 - 18.35 (16.28±0.19) (18.05±0.31)	53.99 46.01	56
6.	Keylong		24.85 × 26.55	100	24
7.	Shashur		21.10 × 19.26 16.13 × 16.13	86.03 13.97	70

The tetraploid (n=16) cytotype

The tetraploid cytotype has been found to be more common as confirmed from the presence of meiotic chromosome number of n=16 in six out of the seven populations scored presently from the different localities in the Himalayas. These tetraploid individuals in all the populations unequivocally showed the presence of 16 bivalents in the PMCs (Fig. 3, B). In spite of normal bivalent/s formation and their equal distribution during anaphases, PMCs showed various meiotic abnormalities which include PMCs involved in chromatin transfer at different stages (Fig. 3, C-L), out of plate bivalent/s (Fig. 3, M), chromatin stickiness (Fig. 3, N), pycnotic chromatin (Fig. 3, D), extra chromatin in PMCs (Fig. 4, A), supernumerary nucleoli (Fig. 4, B, I), laggards and chromatin bridges (Fig. 4, C-E), micronuclei (Fig. 4, I, J) and disoriented chromatin material at anaphases/telophases (Fig. 4, F-H). Consequent to these meiotic abnormalities, abnormal sporads (Fig. 4, J -L) were produced which lead to varying percentages of pollen sterility (24 - 77 %) and pollen grains of heterogeneous sizes (Fig. 4, M & N). The data on cytomixis, meiotic course, microsporogenesis and pollen sterility and pollen size in each population of the tetraploid cytotype are provided in the Tables 3, 4.

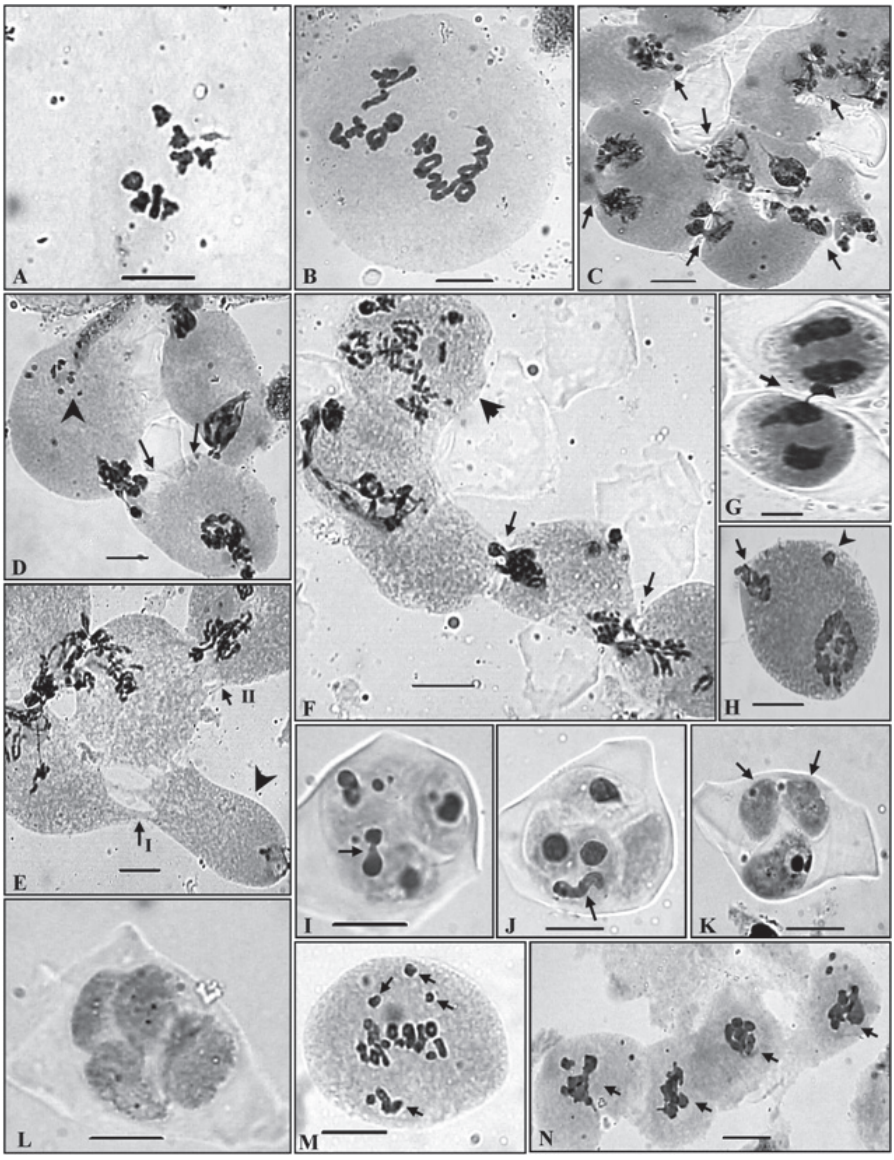

Figure 3A–N. PMCs showing meiotic chromosome number and abnormal meiotic behaviour in *R. hirtellus*. **A** 2× cytotype, n=8 at MI **B** 4× cytotype, n=16 at diakinesis **C** A group of PMCs involved in the transfer of chromatin material at early prophase-I (arrowed) **D** Two PMCs (arrowed) showing simultaneous transfer of chromatin material and pycnotic chromatin material (arrowhead) **E** A group of PMCs showing narrow and broad cytoplasmic connections (arrowed) and an almost enucleated PMC (arrowhead) **F** A group of PMCs showing transfer of chromatin material (arrowed) and a hyperploid PMC (arrowhead) **G** Two PMCs involved in chromatin material transfer at TI (arrowed) **H** A PMC at TI showing broken chromatin strand at one pole (arrowed) and a laggard (arrowhead) **I, J** Microspores showing transfer of chromatin material within the sporads (arrowed) **K** Two empty microspores (arrowed) without any chromatin material in a sporad **L** Completely empty microspores in a sporad **M** Out of metaphase plate bivalents (arrowed) **N** A group of PMCs showing chromatin stickiness (arrowed).

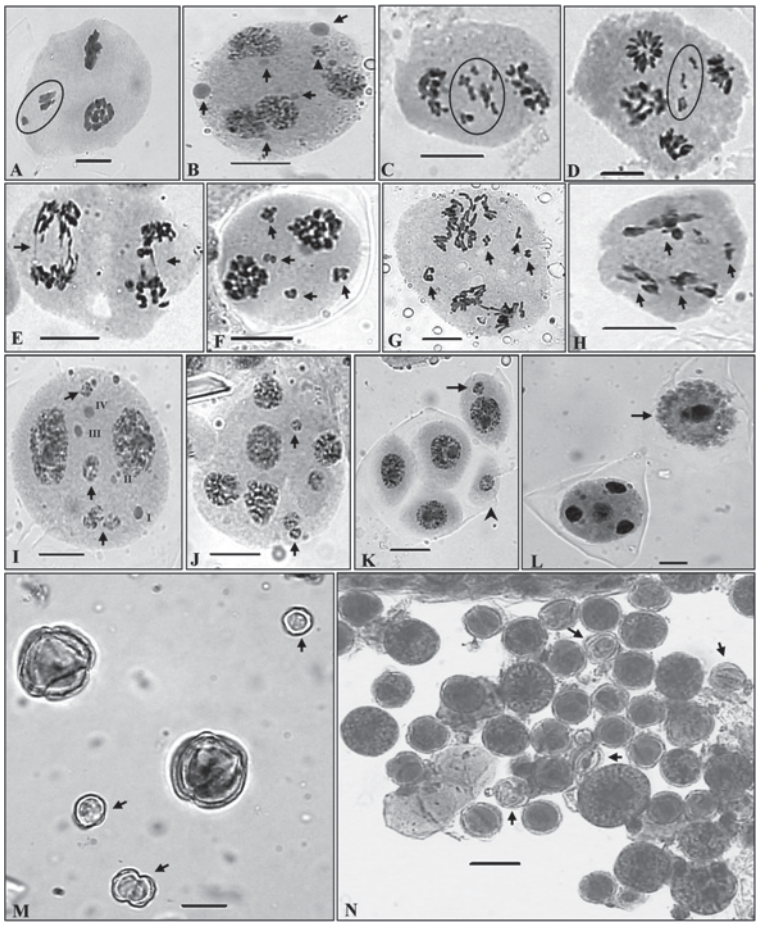

Figure 4A–N. Meiotic abnormalities and pollen grains. **A** A PMC showing extra chromatin material (encircled) **B** A PMC showing unequal sized supernumerary nucleoli (arrowed) and micronuclei (arrowhead) **C, D** Laggards at late AI/II (encircled) **E** Chromatin bridges at AII (arrowed) **F–H** PMCs showing disoriented chromosomes in multiple groups (arrowed) **I** A PMC with micronuclei (arrowed) and supernumerary nucleoli (I–IV) **J** A polyad with micronuclei (arrowed) **K** Sporad with included micronuclei in microspore (arrowed) and a microcyte (arrowhead) **L** A monad (arrowed) and a normal PMC with four haploid nuclei at TII **M** Very small sized sterile/unstained (arrowed) and large lightly stained pollen grains **N** Stained apparently fertile heterogeneous sized and sterile/unstained (arrowed) pollen grains. Scale bars = 10 μm, except micrograph N=20 μm

Characteristics of meiotic abnormalities

Cytomixis involving inter PMC transfer of chromatin material was observed only during the meiotic stages of meiosis-I (Table 4). The chromatin transfer which occurred through narrow as well as broad cytomictic channels among 2–5 proximate PMCs leads to the formation of PMCs with extra chromatin material (Fig. 3, C-H & 4, A). Transfer of chromatin material among PMCs was observed to be both unidirectional

Table 4. Cytomixis, meiotic course and microsporogenesis in the 4× cytotype of *R. hirtellus.* PMC = pollen mother cell; M-I = metaphase-I; P-I = Prophase-I; AI/TI= anaphase-I/telophase-I; AII/TII = anaphase-II/telophase-II;

Populations	Cytomixis			Meiotic course				Microsporogenesis
	% age of PMCs involved	No. of PMCs involved	Meiotic stage/s	PMCs with chromosome stickiness (%)	PMCs with laggards at AI/TI, AII/TII (%)	PMCs with bridges at AI/TI, AII /TII (%)	PMCs with unoriented chromatin material (%)	Abnormal sporads (tetrads and polyads with and without micronuclei)
Dhancho	5.33	2-3	M-I	18.10	35.90	1.93	---	---
Manimahesh Lake	15.95	2-3	P-I	---	6.83	---	---	---
Jalori Pass	26.40	2-5	P-I, M-I	---	11.40	2.50	---	---
Rohtang Pass	22.85	2-4	M-I	---	53.85	---	30.80	---
Keylong	26.47	2-3	M-I	---	5.03	---	---	15.55
Shashur	26.17	2-4	P-I, M-I, T-I	---	26.53	---	---	44.49

as well as bidirectional forming 1–2 chromatin strands. Hypo-, hyperploid and enucleated PMCs were resulted due to partial and complete transfer of chromatin material (Fig. 3, E, F). Interestingly in few instances transfer of chromatin material occurred simultaneously from two PMCs to a single recipient PMC (Fig. 3, D). In some cases remnants of chromatin strands which existed between proximate PMCs during cytomixis were seen as broken chromatin strands (Fig. 3, H). Out of plate bivalent/s at MI was also noticed in a few PMCs (Fig. 3, M). Chromatin stickiness mostly existed in the meiocytes at MI (Fig. 3, N, Table 4). Pycnotic chromatin formed due to chromatin stickiness was also noticed at earlier stages of prophase-I (Fig. 3, D). Some PMCs also showed the presence of supernumerary nucleoli which were of unequal sizes (Fig. 4, B, I). Other most prominent meiotic anomalies noticed were the occurrence of laggards (1–7) at anaphases/ telophases (Fig. 4, C & D, Table 3) and disorientation of chromosomes during anaphases owing to spindle irregularities (Fig. 4, F-H, Table 4). These laggards and unoriented chromatin material failed to get included at poles during telophases, and constituted micronuclei, multipolar PMCs and microcytes (small sized microspore) during sporad formation (Fig. 4, K). The number of such micronuclei in PMCs varied from 1–4 (Fig. 4, I & J). During microsporogenesis these micronuclei were observed to present either freely in the sporads along with four microspores (1–3 micronuclei as separate units) or as included in microspores (Fig. 4, K). Polyads with 1–2 micronuclei and without micronuclei were also noticed. Chromatin bridges were also observed during late AII/TII stages Fig. 4, E, Table 4). Another interesting observation in the population collected from Dhancho (3,030 m) was the occurrence of sporads with empty microspores i.e. microspores without any chromatin material in 6.40 % of cases (Fig. 3, K). Sporads with all the microspores without any chromatin material were also observed (Fig. 3, L). Transfer of chromatin within the sporad units has also been observed in some cases (Fig. 3, I & J). Besides, monads were also recorded

in 2.4 % of the observed cases in the same population (Fig.4, L). Chromatin transfer coupled with associated meiotic abnormalities and consequent abnormal microsporogenesis resulted into high pollen sterility (Table 3) and heterogeneous sized pollen grains (Fig. 4, L, Table 4).

Discussion

Chromosomal status, comparison of 2× and 4× cytotypes and their distributional pattern in Indian Himalayas

The present diploid (n=8) and tetraploid (n=16) chromosome counts for the species from this region of the Himalayas, explored for the first time, agree with the earlier reports from other regions of Indian Himalayas. Both the diploid, 2n=2×=16 (Mehra and Remanandan 1972, Gulmarg in Kashmir, Jammu and Kashmir) and tetraploid, 2n=4×=32 cytotype (Jee et al. 1983a, b, Kashmir, Jammu and Kashmir, Bir and Thakur 1984, Bir et al. 1987, Valley of Flowers', Garhwal Himalayas, Uttarakhand, Kaur et al. 2010, Dalhousie hills, Chamba district, Himachal Pradesh) have been recorded from Indian Himalayas (Fig. 1). A triploid cytotype (2n=24) had also been recorded from eastern Himalayas in India by Sharma (1970) and Roy and Sharma (1971). Based on x=7 another proposed basic number for the genus *Ranunculus* (Darlington and Wylie 1955) a diploid (2n=14) from Gulmarg in Kashmir Himalayas (Koul and Gohil 1973) and tetraploid (2n=28) cytotype from other parts of Indian Himalayas (Mehra and Kaur 1963) have also been reported. It is thus apparent that the species exhibits considerable degree of variability in chromosome number and morphological characters in the Indian Himalayas. In addition to the presence of intraspecific polyploid cytotypes (2×, 3×, 4× at x=8), the species also showed the existence of diploid and tetraploid chromosomal races (2n=14, 28) at basic number of x=7.

The two intraspecific cytotypes (2×, 4× at x=8) here recorded in *R. hirtellus* from the Northwest Himalayas showed variation in vegetative and reproductive characters. Analysis of various macro- and microscopic characters in individuals with 2x and 4x cytotypes revealed that increase in ploidy level is correlated with gigantism for vegetative (plant height, radical leaf length), stomatal (density, size and index) characters and number of flowers/plant. Consequently, the individuals of 2× and 4× cytotypes of *R. hirtellus* can be distinguished from each other in the field. The 4× plants were much taller in size, and had large leaves. It is thus apparent that morphological characters in the intraspecific 2× and 4× cytotypes of *R. hirtellus* are directly correlated with the increase in ploidy level as had been reported earlier in *Capsella bursa-pastoris* (L.) Medik., 1792 (Svensson 1983), *Andropogon gerardii* Vitman, 1792 (Keeler and Davis 1999), *Centaurea jacea* Linnaeus, 1753 (Hardy et al. 2000), *Stevia rebaudiana* (Bertoni) Bertoni, 1905 (de Oliveira et al. 2004), *Parasenecio auriculata* (DC.) J.R. Grant, 1993 (Nakagawa 2006), *Dactylis* Linnaeus, 1753 (Amirouche and Misset 2007), *Centaurea phrygia* Linnaeus, 1753 (Koutecký 2007), *Rorippa amphibia* Linnaeus, 1753

(Luttikhuizen et al. 2007), *Centaurea stoebe* Linnaeus, 1753 (Španiel et al. 2008, Mráz et al. 2011), *Ranunculus parnassifolius* Linnaeus, 1753 (Cires et al. 2009) and number of woody species (Singhal et al. 2007). There is no significant difference in the pollen grain size of the 2× cytotype and in the two populations (Keylong and Manimahesh Lake, Table 3) of the 4× cytotype where the typical pollen grains (normal) were of the same size as that of the 2× cytotype. So, the increase in the ploidy level has not affected the pollen grain size in the 4× cytotype. Different sized pollen grains in other populations of the 4× cytotypes are the product of various meiotic abnormalities (hypo- and hyperploid PMCs) and abnormal microsporogenesis (monads, polyads and sporads with microsporocytes). Generally polyploid plants are considered to have delayed flowering but in the presently studied species the flowering period among 2× and 4× cytotypes has been observed to be nearly the same. As far as the distribution of the two cytotypes are concerned, the 4× cytotype is widely distributed in different geographical areas of the Manimahesh hills, Manali hills and Lahaul Valley compared to the 2× cytotype which is restricted to the Manimahesh hills. Furthermore, on the basis of overall information gathered from the works of other Indian workers from Himalayas it becomes more clear that the 4× cytotype is widely distributed in the Kashmir Himalayas (Jee et al. 1983a, b), Garhwal Himalayas (Bir and Thakur 1984, Bir et al. 1987) and other regions of the Indian Himalayas. On the other hand, the 2× cytotype has been recorded earlier only from Gulmarg (Kashmir Himalaya) by Mehra and Remanandan (1972) and Koul and Gohil (1973).

Meiotic course

The male meiotic course in the meiocytes was perfectly normal in the diploid cytotype resulting into cent percent pollen fertility. However, all the individuals of the 4× cytotype showed the phenomenon of cytomixis involving chromatin transfer among proximate PMCs and various other associated meiotic abnormalities. Consequently very high pollen sterility and fertile pollen grains of two heterogeneous sizes were resulted. The phenomenon of cytomixis is reported here for the first time in the species.

Cytomixis in the PMCs of tetraploid cytotype

Transfer of chromatin material between the adjacent PMCs occurred through cytomictic channels and these cytoplasmic channels originating from the pre-existing connections of plasmodesmata formed within the anther tissues. As meiosis progress these connections get obstructed by the callose plugs. However, in some cases they may exist till the later stages of meiosis and their size may increase to form conspicuous inter-PMC cytomictic channels through which transfer of chromatin or chromosomes may take place (Falistocco et al. 1995, Haroun 1995, Singhal and Kumar 2008a, b, 2010, Kumar et al. in press, Shabrangi et al. 2010, Mursalimov and Deineko 2011).

Chromatin transfer was reported for the first time in gymnosperms by Arnoldy (1900) and subsequently by Koernicke (1901). However, it was Gates who coined the term cytomixis after eleven years in 1911. Since that time it has been reported in a large number of plants. Occurrence of cytomixis only in the tetraploid cytotype and not in the diploid individuals in *R. hirtellus* confirms the view of other workers that the phenomenon is more prevalent in polyploids than their diploid counterparts (Kamra 1960, Semyarkhina and Kuptsou, 1974, Basavaiah and Murthy 1987, Sheidai and Attaei 2005).

Chromatin transfer occurred through variable sized cytoplasmic channels forming 1–2 chromatin strands involving 2–5 PMCs and the percentage of meiocytes involved in cytomixis ranged between 5.53–26.47%. The chromatin material transfer was observed only during the early stages of the meiosis-I, which confirmed the view of other workers that earlier stages of meiosis-I are more favourable for cytomixis (Maheshwari 1950, Kundu and Sharma 1988, Sen and Bhattacharya 1988, Haroun 1995, Singhal and Kumar 2010). Some of the PMCs were also directly fused to facilitate the chromatin transfer. In some cases, cytomixis may lead to the migration of the whole chromatin material among the neighbouring meiocytes and lead to the formation of unreduced gametes. Hypo-, hyperploid and enucleated meiocytes observed at different meiotic stages were the result of partial or complete chromatin transfer between meiocytes. And the products of such PMCs in these individuals yield variable sized apparently fertile and sterile pollen grains. Various workers who considered cytomixis to be of considerable significance, the most probable consequence of cytomixis is the formation of hypo-, hyperploid and enucleated PMCs, aberrant microspore tetrads and pollen sterility (Haroun 1995, Caetano-Pereira and Pagliarini 1997, Malallah and Attia 2003, Haroun et al. 2004, Singhal et al. 2007, 2008, 2009a, b, 2010, Kumar and Singhal 2008, Singhal and Kumar 2008a, b).

Another rare and interesting observation recorded during the meiotic course of *R. hirtellus* was the occurrence of sporads with empty microspores. In some cases sporads were devoid of any chromatin material. One of the possible explanations for the presence of empty microspores in a sporad is the transfer of chromatin within the sporad. Completely empty sporads might have resulted due to the transfer of chromatin between the units of two different sporads. To the best of our knowledge this is the first report of the occurrence of empty microspore units in sporads which were devoid of chromatin material due to complete transfer of chromatin material among microspores of sporads.

Other meiotic abnormalities

The other most frequently observed meiotic abnormalities included laggards and bridges at anaphase/telophase, chromatin stickiness, pycnotic chromatin and aberrant spindle activity in the PMCs which possibly have been induced by cytomixis (Kumar and Singhal 2008, Singhal and Kumar 2008a, b, 2010, Kumar et al. 2008a, 2010).

These laggards when failing to get included in telophase nuclei resulted in the formation of micronuclei at late telophase and sporad stage. The presence of extra chromatin material in the recipient meiocytes due to chromatin transfer also contributed to the formation of micronuclei at late telophase and sporad stage. Chromosome stickiness also resulted in the formation of pycnotic chromatin at earlier stages of prophase-I and chromatin bridges at anaphase/telophase. The normal functioning of spindle apparatus is crucial for chromosome alignment during metaphase and correct segregation of chromosomes to poles (Shabrangi et al. 2010). Disturbed spindle apparatus orientation may have resulted in scattered and disoriented chromosomes in the meiocytes. Spindle irregularity in *R. hirtellus* resulted in unoriented chromatin material at anaphase/telophase which led to the formation of laggards, and these laggards subsequently failed to be included in the telophase haploid nuclei and yielded micronuclei at late telophases and in the sporads.

The phenomenon of cytomixis has been reported a large number of angiospermic plants, and many workers consider cytomixis to be of considerable evolutionary significance (Falistocco et al. 1995, Morikawa and Leggett 1996, Malallah and Attia 2003, Singhal and Kumar 2010). But so far no consensus regarding its fate, cause and importance has been developed due to different opinions and explanations. Some of the possible causes and explanations put forth by the earlier workers include the effect of fixation (Haroun 1995), pathological changes (Morisset 1978), physiological control (Bahl and Tyagi 1988), chemical and herbicides (Haroun 1995), environmental stress and pollution (Haroun et al. 2004), temperature (Kumar and Tripathi 2008), stress factors and genetic control (Malallah and Attia 2003), pressure difference (Morisset 1978) and clumped chromatin bridges during premeiotic anaphase (Mendes and Rijo 1951). The impact of cytomixis and chromatin transfer in inducing various meiotic abnormalities in *R. hirtellus* resulting into abnormal sporad formation, and some pollen malformation seem to be under some genetic factors (Singhal and Gill 1985, Bellucci et al. 2003, Haroun et al. 2004, Lattoo et al. 2006, Singhal et al. 2007, 2008, 2009a, b, 2010, in press, Kumar and Singhal 2008, Singhal and Kumar 2008a, b, 2010 Kumar et al. 2008a, b 2010, in press, Himshikha et al. 2010) or the genetic imbalance in the 4× cytotype, high altitude and low temperature stress conditions prevailing in the cold deserts, where temperature during the months of May - July dips to below freezing, the time the plants enters the reproductive/ flowering bud stage.

Conclusion

On the basis of morphological characters both the 2× and 4× cytotypes are distinguishable in the field from each other. The 4× cytotype has a wider distribution in the Indian Himalayas compared to 2× and 3× cytotypes. And the occurrence of various meiotic abnormalities in the 4× cytotype may be attributed to the genetic imbalance in the 4× cytotype, high altitude and low temperature stress conditions prevailing in the cold deserts.

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RESEARCH ARTICLE

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Cytogenetic and molecular analysis of the holocentric chromosomes of the potato aphid *Macrosiphum euphorbiae* (Thomas, 1878)

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Abstract

Cytogenetic and molecular investigations on the holocentric chromosomes of the aphid *Macrosiphum euphorbiae* (Thomas, 1878) have been carried out using silver staining and C-banding (followed by chromomycin A_3 and DAPI staining) in order to improve our knowledge about the structure of aphid chromosomes. The C-banding pattern is peculiar since only the two X chromosomes and a single pair of autosomes presented heterochromatic bands. Silver staining and FISH with the 28S rDNA probe localized the rDNA genes on one telomere of each X chromosome that were also brightly fluorescent after chromomycin A_3 staining of C-banded chromosomes, whereas all other heterochromatic bands were DAPI positive. Interestingly, a remarkable nucleolar organizing region (NOR) heteromorphism was present making the two X chromosomes easily distinguishable. Southern blotting and FISH assessed the presence of the $(TTAGG)_n$ repeat at the ends of all the *M. euphorbiae* chromosomes. Karyotype analysis showed that all males possessed the X chromosome with the larger amount of rDNA suggesting a non-Mendelian inheritance of the two X chromosomes.

Keywords

aphid, holocentric chromosomes, telomeres, heterochromatin, NOR heteromorphism

Introduction

In the last decades classic and molecular cytogenetics provided an integrated approach for the structural, functional and evolutionary analysis of aphid holocentric chromosomes (Blackman 1980, Manicardi et al. 1998, Mandrioli et al. 1999a, 1999b, Manicardi et al. 2002, Mandrioli et al. 2011).

Interest in aphid cytogenetics is mostly due to the holocentric/holokinetic structure of their chromosomes that present a diffused centromeric activity (Wrensch et al. 1994). This particular type of chromatin organization has been described in almost all the eukaryotic taxa examined so far, with the exception of echinoderms and chordates (Wrensch et al. 1994).

Aphids, in view of the ease with which mitotic chromosome can be obtained from embryonic tissues, represent an ideal model to better understand the architecture of holocentric chromosomes, and to work out the differences/similarities with monocentric ones (Manicardi et al. 2002). At the same time, a cytogenetic analysis of aphids is very helpful since the description of species-specific chromosomal markers could make easier the identification of species that is, at present, quite difficult (Rakauskas 1998, Blackman 1980).

Aphid X chromosomes have been studied with great attention since they present several structural constraints (Manicardi et al. 2002). In particular, the X chromosomes showed a large amount of heterochromatin and possessed the rDNA cluster located at one telomere in almost all the species studied at a cytogenetic level (Mandrioli et al. 1999a, b, Manicardi et al. 2002). Furthermore, aphid X chromosomes seem to be more stable than autosomes since, when fragmentations occur, they mostly affected autosomes leaving X chromosomes usually as the longest in aphid karyotype (Khuda-Buksh and Datta 1981, Blackman 1987, Hales 1989).

In order to better understand X chromosome evolution in aphids, we decided to carry out a cytogenetic analysis of the holocentric chromosomes of the aphid *Macrosiphum euphorbiae* (Thomas, 1878), an important pest of several crops, belonging to a genus that has been up to date scarcely studied at a cytogenetic level (Blackman 1980).

Material and methods

Specimens of *Macrosiphum euphorbiae* were collected on *Bellis perennis* (Linnaeus, 1753) in Modena (Italy) and maintained at 22°C with 16:8 hours light/darkness on *B. perennis* plants. Male aphids were obtained by exposing parthenogenetic females to short photoperiods (8:16 hours light/darkness) according to Crema (1979).

Chromosome preparations from 150 parthenogenetic females were made by spreading embryo cells, as described by Manicardi et al. (1996). Male chromosomes have been obtained by squash preparation of 30 embryos as reported by Manicardi et al. (1991).

C banding treatment was performed according to the technique of Sumner (1972). C banded chromosomes were stained with DAPI according to Donlon and Magenis (1983) and with chromomycin A_3 (CMA_3) as described in Schweizer (1976). NOR regions were labelled by silver staining following the technique of Howell and Black (1980).

DNA extraction from aphid embryos was performed as described in Mandrioli et al. (1999a).

The 28S rDNA probe was obtained by PCR amplification of a 400 bp long fragment of the 28S rDNA gene using the two primers, F (5'-AACAAACAACCGATACGTTCCG) and R (5'-CTCTGTCCGTTTACAACCGAGC), designed according to the insect 28S rRNA sequences available in GenBank. The amplification mix contained 100 ng genomic DNA, 1 mM of each primer, 200 mM dNTPs and 2 U of DyNAZyme II polymerase (Finnzymes Oy). Amplification was performed using a Hybaid thermal-cycler at an annealing temperature of 60°C for 1 min with an extension time of 1 min at 72°C.

In order to test the presence of the telomeric $(TTAGG)_n$ repeat, a probe was obtained by PCR amplification using the two primers F $(TTAGG)_5$ and R $(CCTAA)_5$ in the absence of template, as described by Ijdo et al. (1991).

The telomeric and 28S rDNA probes were labelled using the PCR DIG labelling mix (Roche) according to the Roche protocols.

Southern blotting and fluorescent *in situ* hybridization (FISH) were made as described by Mandrioli et al. (1999a). FISH slides were observed using a Zeiss Axioplan epifluorescence microscope equipped with a 100 W mercury light source. Photographs of the fluorescent images were taken using a CCD camera (Spot, Digital Instrument, Madison, USA) and using the Spot software supplied with the camera and processed using Adobe Photoshop (Adobe Systems, Mountain View, CA).

Results

The parthenogenetic females of *M. euphorbiae* showed a chromosome number of 2n=10 (Fig. 1). C banding followed by CMA_3 staining showed a bright fluorescence exclusively limited to one telomere of the two longest chromosomes (Fig. 1a) that, on the basis of the comparison with male karyotype, have been identified as X chromosomes. DAPI staining showed a large heterochromatic band at the opposite end of the X chromosomes in respect to the GC-rich CMA_3-stained telomere. A second heterochromatic band was observed at one telomere of the autosome pair 2 (Fig. 1b).

The overlapping between CMA_3 areas and rDNA genes has been confirmed by both silver staining (Fig. 1c) and FISH with the 28S rDNA probe (Fig. 1d) showing an exclusive localization of the rDNA genes on *M. euphorbiae* X chromosomes. Silver staining, FISH and CMA_3 staining demonstrated the remarkable occurrence of heteromorphism between homologous NORs on X chromosomes (Figs. 1a, c, d) allowing us to distinguish the two X chromosomes. The same heteromorphism was also evident in interphase nuclei (Fig. 1a).

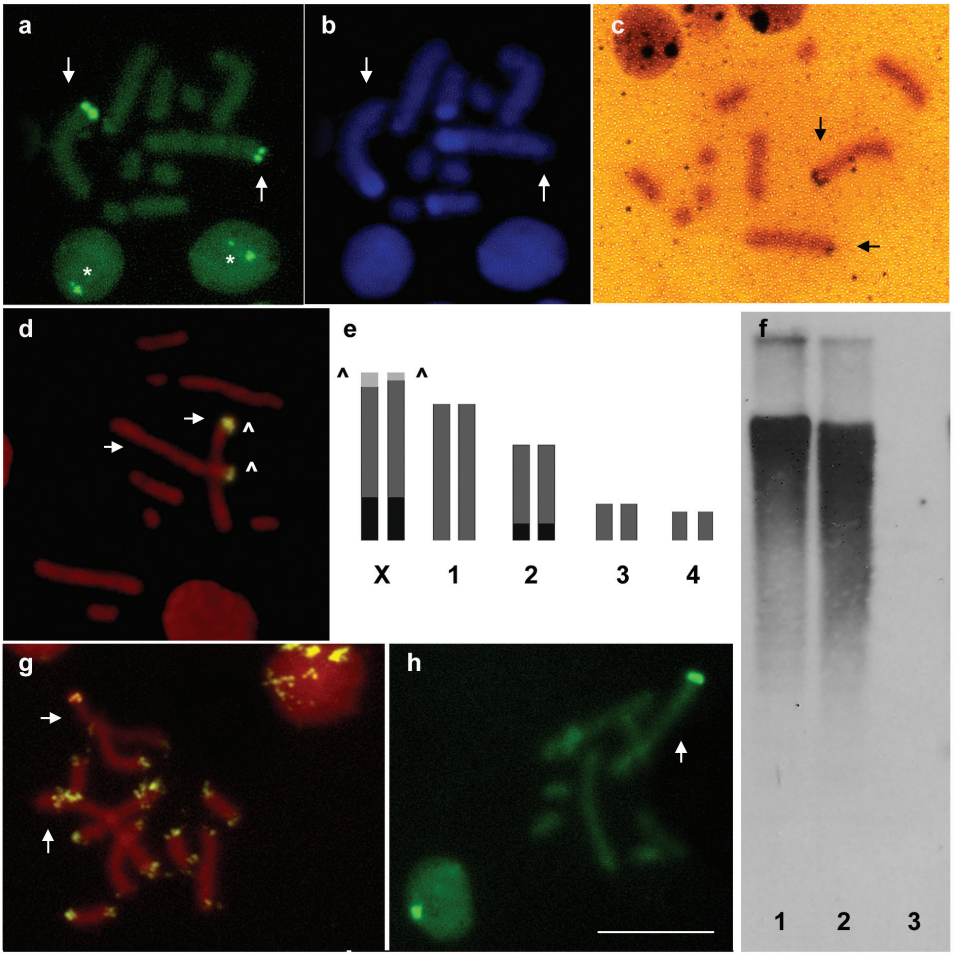

Figure 1a–h. *M. euphorbiae* chromosomes, stained with CMA_3 **(a)** and DAPI **(b)** after C banding, showing heterochromatin on one telomere of the two X chromosomes and on autosome pair 2. Silver staining **(c)** and FISH with a 28S rDNA probe **(d)** evidenced heteromorphic NORs located at one telomere of each X chromosome that was also fluorescent after CMA_3 staining, as summarized in the panel **(e)** Southern blotting **(f)** after digestion with *Xho*I of DNA samples of *A. pisum* **1** *M. euphorbiae* **2** and *D. melanogaster* **3** together with FISH **(g)** assessed that the telomeric sequence $(TTAGG)_n$ constitute each chromosomal end of the *M. euphorbiae* chromosomes. CMA_3 staining of male chromosomes showed that all the male plates present the X chromosome with the larger NOR **(h)** Arrows indicate X chromosomes. Arrowheads indicate NORs. Asterisks evidence the presence of heteromorphic nucleoli in the *M. euphorbiae* nuclei. Bar = 10 μm.

The presence of the $(TTAGG)_n$ repeat has been evaluated by Southern blotting and FISH. Southern blotting revealed a diffuse smear of hybridization (Fig. 1f), whereas FISH experiments with the telomeric $(TTAGG)_n$ probe showed bright FITC-fluorescent spots at the ends of all chromosomes (Fig. 1g). In the interphase nuclei of *M. euphorbiae*, telomeres clustered in few highly fluorescent foci (Fig. 1g).

The male karyotype consisted of 8 autosomes and one X chromosome only (Fig. 1h). Interestingly, all the analysed males possessed the X chromosome with the larger NOR.

Discussion

Currently, more than 4000 aphid species have been described, but the chromosome number has been reported only for about 500 of them (Blackman 1980). Furthermore, less than 10% of the described aphid karyotypes have been deeply studied at a cytogenetic level, even if aphids are intriguing model in animal cytogenetics in view of the holocentric nature of their chromosomes (Manicardi et al. 2002).

According to our results, *M. euphorbiae* has a chromosome number of 2n=10, which represents the typical diploid chromosome number reported in the literature for species of this genus (Blackman 1980).

C banding carried out on *M. euphorbiae* mitotic chromosomes revealed that heterochromatin was not equilocated on each chromosome, but limited to telomeric regions of the two X chromosomes and to autosome pair 2. A preferential storage of heterochromatin on X chromosomes has been previously observed in almost all the aphid species cytogenetically studied to date with the exception of *Diuraphis noxia* (Mordvilko ex Kurdjumov, 1913) (Novotna et al. 2011), but C-bands on a single autosome pair were reported only in *Acyrthosiphon pisum* (Harris, 1776) (Bizzaro et al. 2000) and *Brevicoryne brassicae* (Linnaeus, 1758) (Giannini et al. 2003).

The different responses to CMA_3 and DAPI staining after C banding point out a DNA composition heterogeneity of *M. euphorbiae* heterochromatin. Indeed, GC-rich NOR-associated heterochromatin differs from all other heterochromatic bands that are made by AT-rich DNAs. This pattern of heterochromatin heterogeneity seems to be a general characteristic of aphid chromatin since it has been described in all species investigated so far at a cytogenetic level (Manicardi et al. 2002).

Contrary to the protocol followed for several aphid species, in order to induce a clear-cut banding pattern on *M. euphorbiae* chromosomes, we modified the usual C-banding procedure making a 7 minutes long barium hydroxide treatment. Difficulties in obtaining clear-cut C-banding have been recently reported in the aphid *D. noxia* (Novotna et al. 2011). Interestingly, both these species belong to the tribe Macrosiphini, whereas most of the species with a clear cut banding were Aphidini. Further studies on the tribe Macrosiphini could shed light on the peculiarities of their chromatin organization, which are at the basis of this different banding propensity.

Southern blot experiments with the $(TTAGG)_n$ probe showed smears in *M. euphorbiae* genome suggesting that its telomeres are composed of TTAGG repeats. This result has been confirmed by FISH experiments that clearly showed a hybridization signal on each telomere, whereas no evidence of any interstitial labelling has been observed demonstrating that the TTAGG repeats are restricted to the terminal regions of all aphid chromosomes. The presence of the $(TTAGG)_n$ repeat in *M. euphorbiae* further support the hypothesis that this telomeric sequence is common in aphids (Bizzaro

et al. 2000, Monti et al. 2011), so that its absence in *D. noxia* (Novotna et al. 2011) could be considered an exception, as previously reported in other insects (Frydrychovà et al. 2004, Lukhtanov and Kuznetsova 2010).

In interphase nuclei of most organisms the telomeric regions are situated in an ordered fashion with an association to the nuclear matrix and clustering at least in some stage of cell life (Palladino et al. 1993, Luderus et al. 1996, Pryde et al. 1997). Accordingly to previous results in aphids (Monti et al. 2011), telomeres appeared clustered into few foci in the *M. euphorbiae* nuclei and were not located mainly near the nuclear periphery as reported in other insects such as *Drosophila melanogaster* (Meigen, 1830) (Hochstrasser et al. 1986) and the cabbage moth *Mamestra brassicae* (Linnaeus, 1758) (Mandrioli 2002).

NOR number and position have been frequently reported as highly variable in insects, where rDNA genes have been frequently found also on autosomes or only on autosomes as generally reported in Lepidoptera and Psylloidea (Maryańska-Nadachowska et al. 1992, Maryańska-Nadachowska and Grozeva 2001, Nguyen et al. 2010), including several species with multiple NORs (e.g. Postiglioni and Brum-Zorrilla 1988, Juan et al. 1993). Silver staining of *M. euphorbiae* mitotic metaphases revealed two dots located on one telomere of each X chromosome. This seems to be a highly conservative characteristic of aphid chromosomes, since the same NOR localization has been described in almost all the aphids species studied to date (Kuznetsova and Gandrabur 1991, Kuznetsova and Maryańska-Nadachowska 1993, Blackman and Spence 1996, Manicardi et al. 1998, 2002), with the unique exception of *Schoutedenia ralumensis* (Rübsaamen, 1905) and *Maculolachnus submacula* (Walker, 1848) that present autosomal NORs and *Amphorophora idaei* (Borner, 1839) showing interstitial NORs on X chromosomes (Blackman 1987).

In several aphid species silver staining revealed the occurrence of an appreciable level of heteromorphism between homologous NORs due to a different distribution of rDNA genes between the two X chromosomes, but, in all the previous studies, different levels of heteromorphism have been observed both at inter- and intra-individual levels (Blackman and Spence 1996, Mandrioli et al. 1999b; Manicardi et al. 1998, 2002). Contrarily to previous observations, all the observed *M. euphorbiae* plates presented an X chromosome with a NOR region larger than the homologue allowing us to clearly differentiate the two sex chromosomes. In view of this stable chromosomal marker we performed experiments of male determination in order to evaluate if during this process both the X chromosomes have the same chances of being inherited in males.

All the parthenogenetic eggs during the prophase present two X chromosomes linked by NORs (Schrader 1940, Orlando 1974, Hales and Mitler 1983, Blackman and Hales 1986). However, in eggs developing as females, the connection is quickly lost, but in male generating eggs the X chromosomes remain attached by sticky NORs and undergo a sort of non-canonical reductional division (Blackman and Hales 1986). At the end of this peculiar division the egg has one X chromosome only and it is determined as a male.

The observation that all the *M. euphorbiae* male metaphases had an X chromosome with a large NOR evidenced a selective bias favouring X chromosomes with larger number of rDNA genes. This fact could be due by a non-random elimination of one X chromosome during male determination process or by the early abortion of embryos containing an X chromosome with few rDNA genes. Contrarily to what has been observed in *Megoura viciae* (Buckton, 1876) (Crema 1981), we never observed the presence of aborted eggs among the developing male embryos of *M. euphorbiae* so that we hypothesized that the bias occurred during the male determination process which ended with a selective elimination of the X chromosome bearing few rDNA genes.

The phenomenon of biased inheritance of X chromosomes in aphids seems to be controversial among aphids since observations on *Sitobion fragariae* (Walker, 1848), using an X-linked polymorphic microsatellite marker, suggested that X chromosome loss during male determination was random (Wilson et al. 1997), whereas the presence of strong biases in the transmission of sex chromosomes has been reported in *Rhopalosiphum padi* (Linnaeus, 1758) (Franz et al., 2005)

On the basis of our results on *M. euphorbiae,* we suggest that the presence of an unequal distribution of rDNA between the two X chromosomes could affect the attachment of the X chromosome by sticky NORs favouring the loss of the X chromosome with few rDNA genes and a not random inheritance of the X chromosomes. On the contrary, aphid specimens with an equal distribution of rDNA genes could undergo a random loss of the X chromosomes during male determination. On the basis of our hypothesis, therefore, NOR heteromorphism does not inhibit male determination (as previously reported by Blackman and Spence 1996), but affects the inheritance of the X chromosomes making it non random.

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RESEARCH ARTICLE

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A karyosystematic analysis of some water beetles related to *Deronectes* Sharp (Coleoptera, Dytiscidae)

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Abstract

An account is given of the karyotypes of five species and one additional subspecies of *Deronectes* Sharp, 1882, three species of *Stictotarsus* Zimmermann, 1919, one species of *Trichonectes* Guignot, 1941, four species of *Scarodytes* Gozis, 1914 and 17 species of *Nebrioporus* Régimbart, 1906. *Deronectes* species are characterised by a neo-Xy system of sex chromosomes and autosome numbers ranging from 60 (*D. ferrugineus* Fery et Brancucci, 1987 and *D. wewalkai* Fery et Fresneda, 1988) through 48 (*D. latus* (Stephens, 1829), *D. angusi* Fery et Brancucci, 1990) to 28 (*D. costipennis* Brancucci, 1983, *D. costipennis gignouxi* Fery et Brancucci, 1989 and *D. platynotus* (Germar, 1834)). The three species of *Stictotarsus*, *S. duodecimpustulatus* (Fabricius, 1792), *S. procerus* (Aubé, 1838) and *S. bertrandi* (Legros, 1956), all belonging to the *S. duodecimpustulatus* group of species, have karyotypes comprising 54 autosomes and neo-Xy sex chromosomes. *Trichonectes otini* Guignot, 1941 has 48 autosomes and an X0 system of sex chromosomes, an arrangement shared with the 17 species of *Nebrioporus* Régimbart. The four *Scarodytes* species, *S. halensis* (Fabricius, 1787), *S. nigriventris* (Zimmermann, 1919), *S. fuscitarsis* (Aubé, 1838) and *S. malicky*i Wewalka, 1997, all have 54 autosomes and X0 sex chromosomes. The karyotypes of the various species are found to be distinctive and to support separation of these species from one another. In two cases (*Nebrioporus martinii* (Fairmaire, 1858) and *N. sardus* (Gemminger et Harold, 1868), and *Scarodytes halensis* and *S. fuscitarsis*) the karyotypes require the recognition of the taxa as full species, not subspecies. The implications of these data for the generic classification are considered. The data are found to be compatible with the DNA-based phylogeny proposed by Ribera (2003), where the enlarged *Stictotarsus* proposed by Nilsson and Angus (1992) is found to be unsatisfactory.

Keywords

Chromosomes, karyotypes, Dytiscidae, *Deronectes*, *Stictotarsus*, *Scarodytes*, *Trichonectes*, *Nebrioporus*, species, phylogeny

Introduction

The *Deronectes* group of genera comprise small diving beetles (Dytiscidae) which belong to the subfamily Hydroporinae, tribe Hydroporini. They typically inhabit stony or gravelly rivers or lakes, and often have a characteristic, quite flattened appearance. Beyond that, it is not easy to formulate a set of characteristics which delimit the group, and in a number of cases the scope and arrangement of the included genera are not clear. Nilsson and Angus (1992) sought to clarify the situation, and in particular to separate the mainly northern hemisphere *Deronectes* Sharp, 1882 group from various superficially similar southern hemisphere genera. They separated *Deronectes* itself as having a more or less uniformly coloured upper surface, parameres without an apical hook, and larvae without extra swimming-hairs on the legs. The other genera, all of whose larvae have extra swimming-hairs on the legs and whose upper surfaces are patterned, were divided into those whose parameres had an apical hook and those without such a hook. The species that possessed hooked parameres were placed in the genera *Nebrioporus* Regimbart, 1906 and *Scarodytes* Gozis, 1914, while those without them were placed in an expanded *Stictotarsus* Zimmermann, 1919. This expanded *Stictotarsus* group has not been favourably received. The genus *Trichonectes* Guignot, 1941, which was included in *Stictotarsus* by Nilsson and Angus, was reinstated by Ribera (2003) because DNA analysis showed it to be only distantly related to other *Stictotarsus*, and Angus (2010b) erected the genus *Boreonectes* for the *Stictotarsus griseostriatus* group of Nilsson and Angus.

Nilsson and Angus reported some chromosomal data, which indicated a considerable diversity of chromosome number and sex-determining mechanisms within the group, with the only notable generic uniformity being shown by *Nebrioporus*, whose studied species all had 2n = 48 + X0 (♂), XX (♀). So far karyotypes have been published for six species of *Nebrioporus* – *N. carinatus* (Aubé, 1838), *N. fabressei* (Régimbart, 1901) and *N. croceus* Angus, Fresneda et Fery, 1992, all Spanish species (Angus et al., 1992); and three Egyptian species, *N. crotchi* (Preudhomme de Borre, 1871), *N. insignis* (Klug, 1833) and *N. lanceolatus* (Walker, 1871) (Saleh Ahmed et al. 2000).

Of the other genera, *Scarodytes halensis* (Fabricius, 1787) was reported to have 27 pairs of autosomes and an X0 sex chromosome system, while three *Stictotarsus* (*duodecimpustulatus* group) species have 27 pairs of autosomes and a neo-XY sex chromosome system (*S. duodecimpustulatus* (Fabricius, 1792), *S. procerus* (Aubé, 1838) and *S. bertrandi* (Legros, 1956)). *Boreonectes* species have chromosome numbers ranging from 50–60 autosomes and an X0 sex chromosome system (Dutton and Angus 2007; Angus, 2008, 2010a, b). The remaining genus for which some chromosome data have been reported is *Deronectes*. Here autosome numbers ranging from 14 to 30 pairs have been observed, with a neo-XY sex chromosome system in all cases. Considerable variation in the sizes of both autosomes and sex chromosomes was also noted.

The main aims of the present work are to present karyotypes from species of these genera for which such information is not yet available, and to see to what extent, if any, these other genera show generic patterns of karyotype, such as that shown by *Nebrioporus*.

The chromosomal features available for analysis are limited to their size, given as the length of each chromosome expressed as a percentage of the total haploid autosome length in the nucleus (relative chromosome length (RCL)) – which compensates for differing degrees of chromosome condensation in different preparations, the centromere position, given as the percentage of the total length of a chromosome occupied by its shorter arm (centromere index (CI)), heterochromatin development and distribution (indicated by C-banding, in this case only available for *Stictotarsus*), and any obvious secondary constrictions on the chromosomes. Detailed G-banding, which allows accurate identification of individual chromosome arms or even portions of arms, in mammals, is not possible with insects, and no attempt was made to use DNA probes on these nuclei. The centromere indices of the chromosomes are most conveniently expressed as the standard terms for centromere position. Based on Sumner (2003), these are: metacentric (median centromere), CI 50–46; submetacentric (centromere clearly not quite median), CI 45–26; subacrocentric (centromere almost at one end of the chromosome), CI 25–16; acrocentric (including telocentric), CI < 16.

One result of the limited range of features available is that in some of these karyotypes, particularly those involving fairly large numbers of chromosomes similar in size and centromere position, there is inevitably a degree of ambiguity. However, even when comparison is limited to clearly expressed differences, many of the species are seen to have distinctive karyotypes.

Material and methods

The material used in this study is archive photographs of mitotic chromosomes of various species, accumulated by R.B. Angus. The details – species, localities, collector and date, and number of specimens analysed – are given in Table 1. These preparations have been accumulated over more than 20 years, and in many cases only a few successful preparations were obtained, even though large numbers of beetles were used in attempts. The number analysed given in the table refers to the number of beetles from which successful preparations were obtained.

The methods of chromosome preparation are outlined by Dutton and Angus (2007). Photographs were printed at a magnification of X 3000. Chromosomes were cut from the photographs, paired up and plated as karyotypes. They were then scanned into a computer for analysis using Adobe Photoshop. This facilitated necessary rearrangement of various karyotypes, as well as bringing them together on single plates, for comparison of species and populations. In addition to this, the photographs were "cleaned up" by adjustment of contrast and brightness (to allow for different backgrounds), and extraneous blemishes were removed.

The chromosomes were measured, so that the relative chromosome length (RCL) and centromere index (CI) of the chromosomes could be calculated. Because of the scarcity of replicates no statistical analysis has been attempted with these data, and the RCL and CI values are given as a rough guide only.

Table 1. Material analysed.

Species	Locality	Collector, date	Number analysed
Deronectes ferrugineus Fery et Brancucci	Portugal, Distr. Guarda, Serra do Estrela	H. Fery 1990	2 ♂ 1 ♀
D. wewalkai Fery et Fresneda	Spain, Provincia de Avila, Sierra de Gredos	H. Fery 1990	2 ♂ 1 ♀
D. latus (Stephens)	England, Hampshire, New Forest	R.B. Angus 1990	3 ♂ 1 ♀
D. angusi Fery et Brancucci	Spain, Provincia de Burgos, Sierra de Arlanzon	H. Fery 1990	1 ♂ 1 ♀
D. costipennis costipennis Brancucci	Portugal, Distr. Guarda, Serra do Estrela	H. Fery 1990	2 ♂ 1 ♀
D. c. gignouxi Fery et Brancucci	Spain, Provincia de Leon, Posada de Valdeon	H. Fery 1990	4 ♂♂
D. platynotus (Germar)	Bulgaria, Pindus Mountains	D. Bilton, 2006	1 ♂
	Germany, Saxonia, Weisseritz Kreis	L. Hendrich 2006	2 ♀♀
Stictotarsus duodecimpustulatus (Fabricius)	Scotland, Kirkcudbrightshire Clatteringshaws Loch	G.N. Foster 1990	1 ♂
	England, Hampshire, New Forest	R.B. Angus 1990	1 ♀
	Spain, Provincia de La Coruña, Esclavitud	R.B. Angus 1990	1 ♂ 1 ♀
	Spain, Provincia de Caceres, Abadia	R.B. Angus 1990	1 ♀
S. procerus (Aubé)	Corsica, Haute Corse, Solenzara	R.B. Angus 1993	3 ♂ 1 ♀
	Corsica, Haute Corse, R.Casaluna, Pont de Lano	R.B. Angus 1993	2 ♂♂ 1 ♀
	Sardinia, Provincia de Nuoro, Posada	R.B. Angus 1994	2 ♂ 1 ♀
S. bertrandi (Legros)	Spain, Provincia de Lugo. Rio Landro	J. Diaz Pazos 1990	1 ♂ 1 ♀
Trichonectes otini (Guignot)	Spain, Provincia de Cordoba, Salinas de la Maturra, Baena	M. Baena 1993	4 ♂♂
	Spain, Provincia de Sevilla, S of Osuna	H. Fery 1993	2 ♂ 1 ♀
Scarodytes halensis (Fabricius)	England, Oxfordshire, Stanton Harcourt	R.B. Angus 1990	2 ♂♂ 1 ♀
	France, Alpes Maritimes, Menton	H. Fery 1990	1 ♀
S. nigriventris (Zimmermann)	Corsica, Haute Corse, Solenzara	R.B. Angus 1993	1 ♂
	Corsica, Haute Corse, R.Casaluna, Pont de Lano	R.B. Angus 1993	1 ♂ 1 ♀
S. fuscitarsis (Aubé)	Sardinia, Provincia de Nuoro, Budoni	R.B. Angus 1994	3 ♂ 1 ♀
S. malickyi Wewalka	Crete, Nomos Rethymnou. Spili – Gerakari	R.B. Angus 1996	2 ♂♂
Nebrioporus ceresyi (Aubé)	Cyprus, Akrotiri, Zakaki marshes	R.B. Angus 1995	2 ♂♂
	Sardinia, Provincia di Oristano, Sinis Peninsula	R.B. Angus 1994	4 ♂♂
N. baeticus (Schaum)	Spain, Provincia de Sevilla, S of Osuna	H. Fery 1993	2 ♂♂
	Provincia de Cordoba, Castro del Rio	M. Baena 1993	1 ♂
N. canaliculatus (Lacordaire)	Spain, Provincia de Burgos, 4 km SSW of Sasamon	H. Fery 1994	1 ♂
	Sweden	A.N. Nilsson, 1991	1 ♂
N. bucheti (Régimbart)	France, Alpes Maritimes, Menton	H. Fery 1990	1 ♂, 1 ♀
N. martinii (Fairmaire)	Corsica, Haute-Corse, Solenzara	R.B. Angus 1993	2 ♂♂
N. sardus (Gemminger et Harold)	Sardinia, Provincia di Nuoro, Budoni	R.B. Angus 1994	1 ♂
N. depressus (F.)	England, Cumbria, Talkin Tarn	R.B. Angus 1987	1 ♂
Nebrioporus depressus-elegans intermediate	Scotland, Kirkcudbrightshire, Clatteringshaws Loch	G.N. Foster 1987	2 ♂♂
N. elegans (Panzer)	England, Oxfordshire, Cassington	R.B. Angus 1987	2 ♂♂
N. assimilis (Paykull)	England, Cumbria, Grasmere	R.B. Angus 1987, 1990	3♂♂

Species	Locality	Collector, date	Number analysed
N. carinatus (Aubé)	Spain, Provincias de La Coruña, Lugo & Palencia	(Angus et al. 1992)	3 ♂♂, 1 ♀
N. fabressei (Régimbart)	France, Pyrenées Orientales; Spain, Provincia de Segovia	(Angus et al. 1992)	4 ♂♂
N. croceus Angus, Fery et Fresneda	Spain, Provincia de Soria.	(Angus et al. 1992)	5 ♂♂
N. amicorum Toledo	Crete, Nomos Rethymnou. Spili – Gerakari	R. B. Angus 1996	1 ♂
N. lanceolatus (Walker)	Egypt, Sinai, St Katherine & El Gharandal	(Saleh Ahmed et al. 2000)	3 ♂♂
N. insignis (Klug)	Egypt, Sinai, St Katherine	(Saleh Ahmed et al. 2000)	1 ♂
N. crotchi (Preudhomme de Borre)	Egypt, Sinai, St Katherine	(Saleh Ahmed et al. 2000)	2 ♂♂
N. canariensis (Bedel)	Canary Islands, Tenerife	A.N. Nilsson 1991	2 ♂♂

Results

Deronectes Sharp, 1882

Fig. 1, a – o, shows mitotic chromosomes from mid-gut cells, of the six *Deronectes* species (and one subspecies) studied. The diploid numbers of autosomes range from 60 (*D. ferrugineus* and *D. wewalkai*) through 48 (*D. latus* and *D. angusi*) to 28 (*D. costipennis*, *D. costipennis gignouxi* and *D. platynotus*). The sex chromosomes are XY or Xy (♂), XX (♀). In *D. ferrugineus*, *D. wewalkai*, *D. latus* and *D. angusi*, the y-chromosomes are dot-like, but in *D. costipennis*, *D. costipennis gignouxi* and *D. platynotus* the Y chromosomes are rather larger, and match the short arms of the X chromosome, strongly suggesting that this is a neo-XY system. The features of the various species are discussed below.

D. ferrugineus Fery et Brancucci, 1987. Fig. 1 a (♂), Fig. 1 b (♀). 2 n = 60 + Xy (♂), XX (♀). Pair 1 is clearly the longest (RCL about 14), with pairs 2–4 only slightly smaller (RCL about 6.5–10) placed together as they cannot be distinguished from one another with certainty). These four pairs of chromosomes have the long arm much longer than the short arm (CI about 30–40), and although no C-banding is available, the way the chromatids of these long arms lie closely applied to another suggests that they are largely heterochromatic (comparing the heterochromatic long arms of some of the *Stictotarsus* chromosomes shown in Fig. 2). Pairs 5 and 6 are about half the length of pairs 2–4 (RCL about 4.5), and are almost metacentric. Pairs 7–12 show a gradual decrease in RCL, from about 4 to about 3. Pairs 7, 9 and 10 are subacrocentric, while pairs 8, 11 and 12 are submetacentric. Pairs 13–30 are acrocentric and their RCLs range from 2.5–0.9. The X chromosome (RCL about 2.6) is submetacentric, similar in length to autosomes 5 and 6, but appearing a little more dense. The y chromosome is dot-like, thus is too small for the RCL to be measured.

D. wewalkai Fery et Fresneda, 1988. Fig. 1 c (♂), Fig. 1 d (♀). 2 n = 60 + Xy (♂), XX (♀). The karyotype of this species shows no detectable difference from that of *D. ferrugineus*. One peculiar feature of the female karyotype is the loss of the long arm of one replicate of autosome 4. This appears to be a one-off deletion from this nucleus, but as this was the only platable nucleus obtained from this beetle it is impossible to say whether other cells showed the same deletion. If these long arms are heterochromatic, as suggested for *D. ferrugineus,* the genetic consequences of the deletion would be minimal.

D. latus (Stephens, 1829). Fig. 1 e, f (♂), Fig. 1 g (♀). 2n = 48 + Xy (♂), XX (♀). Autosome pairs 1–13 are all about the same size (RCLs range from about 6 to about 5), and are all more or less metacentric. Pairs 14–16 are slightly smaller, RCL about 4.5, and are subacrocentric. Pair 17 is acrocentric and markedly smaller than pair 16 (RCL about 3), and pairs 17–25 are all similar in shape, with a gradual decrease in RCL from 3 to 2.3. The X chromosome is subacrocentric, and similar in size to autosome 16. The y chromosome is dot-like.

D. angusi Fery et Brancucci, 1990. Fig. 1 h (♂), Fig. 1 i (♀). 2n = 48 + Xy (♂), XX (♀). The karyotype of this species is very similar to that of *D. latus*, with the exception of autosome 16, which is similar to autosome 17, unlike that of *D. latus*, which is similar to autosome 15. This species was originally considered as a somewhat peculiar Spanish form of *D. latus*, but the discovery of the chromosomal difference convinced H. Fery (pers. comm.)that it was in fact a good species.

D. costipennis costipennis Brancucci, 1983. Fig. 1 j (♂), Fig. 1 k (♀). 2n = 28 + XY (♂), XX (♀). Autosome pairs 1–3 are conspicuously large, RCLs about 12.5–8.5. Pair 2 is metacentric, while pairs 1 and 3 are submetacentric, towards the subacrocentric end of the range. Pairs 4–14 are more less metacentric and their RCLs range from about 7.8–3, with a gradual size decrease along the row. The X chromosome is large, RCL about 14.8, and submetacentric, CI about 30. The y chromosome, RCL about 5.5, is acrocentric and matches the short arm of the X chromosome – a typical neo-XY configuration.

D. costipennis gignouxi Fery et Brancucci, 1989. Fig. 1 l (♂). 2n = 28 + XY (♂), XX (♀). This karyotype is almost certainly indistinguishable from that of *D. c. costipennis.* The X chromosome (RCL) appears slightly shorter, but all the chromosomes appear more condensed than in the *costipennis* nuclei, and this is probably sufficient to account for the apparent difference. In any event, a lot more material would be needed to demonstrate so small a difference.

D. platynotus (Germar, 1834). Fig. 1 m (♂), Fig. 1 n, o (♀). 2n = 28 + XY (♂), XX (♀). The karyotype appears very similar to that of *D. costipennis*, but chromosome 1 is more metacentric and chromosome 2 has the distinct secondary constriction in

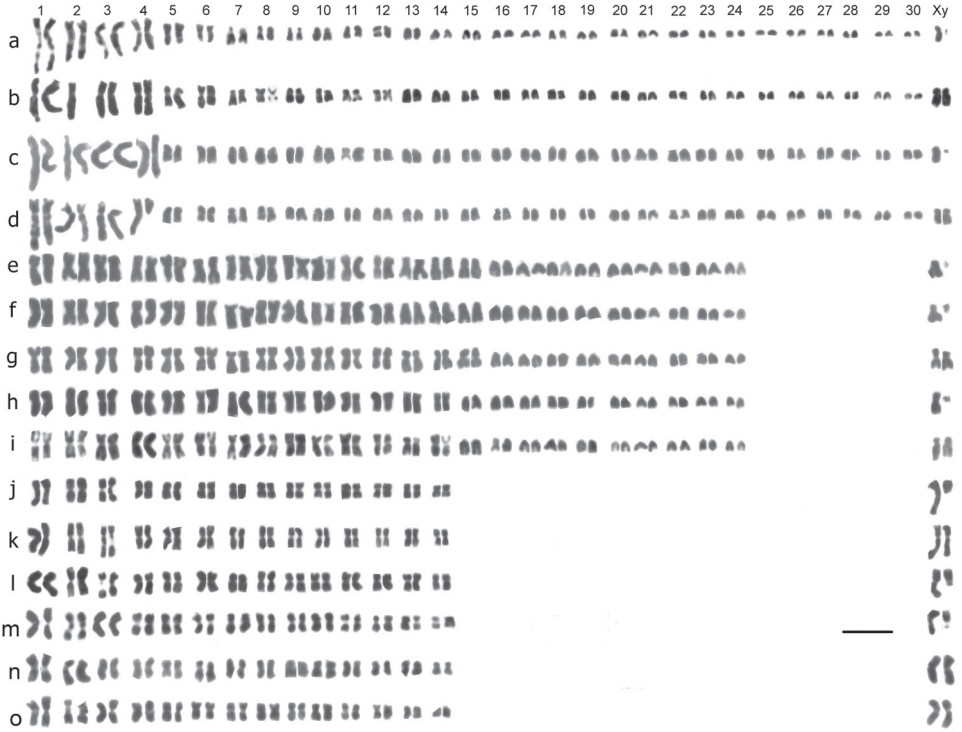

Figure 1. Mitotic chromosomes from mid-gut cells of *Deronectes* species, arranged as karyotypes. **a** *D. ferrugineus*, ♂ **b** *D. ferrugineus*, ♀ **c** *D. wewalkai*, ♂ **d** *D. wewalkai*, ♀ **e, f** *D. latus*, ♂ **g** *D. latus*, ♀ **h** *D. angusi*, ♂ **i** *D. angusi*, ♀ **j** *D. costipennis costipennis*, ♂ **k** *D. c. costipennis*, ♀ **l** *D. costipennis gignouxi*, ♂, **m** *D. platynotus*, ♂, Bulgaria **n, o** *D. platynotus*, ♀, Germany. Bar = 5μm.

this species, as against chromosome 3 in *D. costipennis*. Although the RCLs of various chromosomes can be affected by irregularities of condensation, this difference appears consistent. It is worth mentioning that the Bulgarian male has the pointed aedeagus characteristic of *D. platynotus platynotus*, not the truncated one found in the Greek *D. p. mazzoldii*.

Trichonectes Guignot, 1941

T. otini (Guignot, 1941). Fig. 2 a – c (♂), Fig. 2 d (♀). 2n = 48 + X0 (♂), XX (♀). The RCLs of the autosomes decrease rather evenly from about 6.5–2, and most of the autosomes are metacentric or submetacentric apart from pairs 7, 8, 10, 14–18, which are more or less subacrocentric and pair 24 which is acrocentric and, although small, not dot-like. The short arms of pairs 7, 10 and 12 appear to have secondary constrictions. The X chromosome is submetacentric, with a RCL of about 5.

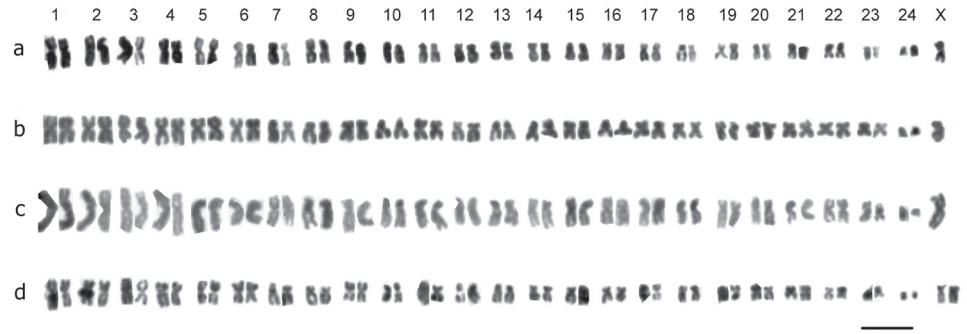

Figure 2. Mitotic chromosomes from mid-gut cells of *Trichonectes otini*, arranged as karyotypes. **a** ♂, Cordoba **b, c** ♂♂, Sevilla **d** ♀, Sevilla. Bar = 5μm.

Nebrioporus Régimbart, 1906

Chromosome data are now available for 17 species out of the 57 listed by Toledo (2009), with this number increased to 58 in the present work. The species are arranged according to the groupings suggested by Toledo (2009). In all cases the diploid number is 48 + X0 (♂), XX (♀).

The *N. ceresyi* group

N. ceresyi (Aubé 1838). Fig. 3 a, b (♂). The RCLs of autosome pairs 1–18 decrease fairly evenly from about 8 to 3.5. Pairs 19–21 have RCLs of about 2.5 while pairs 22–24 are almost dot-like, RCLs about 1.5. The X chromosome is metacentric, RCL about 5. Most of the autosomes are metacentric, with pairs 4, 7, 9, 11, 13, 14, 16, 17 and 19 more or less subacrocentric, some probably with secondary constrictions, though these cannot be seen in the preparations available.

N. baeticus (Schaum, 1864). Fig. 3 c, d (♂). The karyotype is very similar to that of *N. ceresyi*, but with some clear differences. The larger autosomes, in this case pairs 1–19, have RCLs decreasing evenly from about 8 to 3, with an abrupt decrease to pair 20, RCL about 2. Pairs 21–24 are all very small, RCLs about 1.5–1. The X chromosome is metacentric, RCL about 4. Most of the autosomes are more or less metacentric, with the obvious subacrocentrics being pairs 7, 9–13, 15–17 and 19.

The *N. canaliculatus* group

N. canaliculatus (Lacordaire, 1835). Fig. 3 e, f (♂). The RCLs of the autosomes decrease evenly from about 5.5–2.2, with no dot-like chromosomes. The X chromosome is metacentric, RCL about 4. Most of the autosomes are more or less metacentric, with

pairs 5, 9, 11, 14, 17, 19 and 21 either submetacentric or subacrocentric, and pairs 19 and 21 with secondary constrictions (Fig. 3 e).

The *N. sansii* group

N. bucheti (Regimbart, 1898). Fig. 3 g (♂), h (♀). The RCLs of autosome pairs 1–23 decrease evenly from about 7.5–1.9. The RCL of pair 24 is about 1.6 and this pair can appear dot-like. The X chromosome is submetacentric, RCL about 4.6. Most autosomes are either metacentric or submetacentric, with pairs 7, 8, 10, 18–23 the most obvious subacrocentrics. The material belongs to the nominate subspecies, *N. bucheti bucheti.*

N. martinii (Fairmaire, 1858). Fig. 3 i (♂). The RCLs of the autosomes decreasing rather evenly from about 7.5–1.8. The X chromosome is metacentric, RCL about 4.5, similar to autosomes 11–12. Autosomes 1, 3, 10, 11, 12, 14 and 17 are clearly submetacentric and pairs 7, 8 and 18–24 are clearly subacrocentric, with the others more or less metacentric.

N. sardus (Gemminger et Harold, 1868), stat. n. Fig. 3 j (♂). A more condensed preparation than the *N. martinii* shown in Fig. 3 i. The RCLs of the autosomes decrease rather evenly from about 6–2, and the X chromosome is metacentric, RCL about 5, comparable with autosome 6. Autosomes 1, 3, 7, 8 , 12, 14, 15 and16 are clearly submetacentric, pairs 18–24 appear acrocentric to subacrocentric, and the rest are metacentric. The chromosome array presented here, although condensed, shows a number of clear differences from that of *N. martinii*. Thus pair 8 is submetacentric in *N. sardus* but almost acrocentric in *N. martinii*, and pairs 10 and 11 are clearly metacentric in *N. sardus* but submetacentric in *N. martinii*. These differences cannot be resolved by slight rearrangement of the karyotype of either species, and strongly suggest that there has been chromosomal rearrangement since the two taxa separated. For this reason *N. sardus* is here elevated to species rank.

The *N. depressus* group

N. depressus (Fabricius, 1775) and *N. elegans* (Panzer, 1794). Fig. 3 k, l, m (♂). *N. depressus* and *N. elegans* are listed as separate species of a *N. depressus* complex by Toledo, and as the regional occurrence of intermediate specimens is well documented (Balfour-Browne, 1919, 1934; Franck, 1935; Shirt, 1983) they are considered together. Fig. 3 k is a good *N. depressus*, Fig. 3 l is a Scottish intermediate specimen, and Fig. 3 m is an English *N. elegans*. The three karyotypes show no differences from one another. The RCLs of autosome pairs 1–21 decrease evenly from about 7.5–2.2, while the three smallest pairs verge on dot-like and are thus more or less impossible to measure. The X chromosome is a small acrocentric, RCL about 2.8. Autosomes 8, 9 and 18–23 are subacrocentric to acrocentric, and the remainder are metacentric to submetacentric.

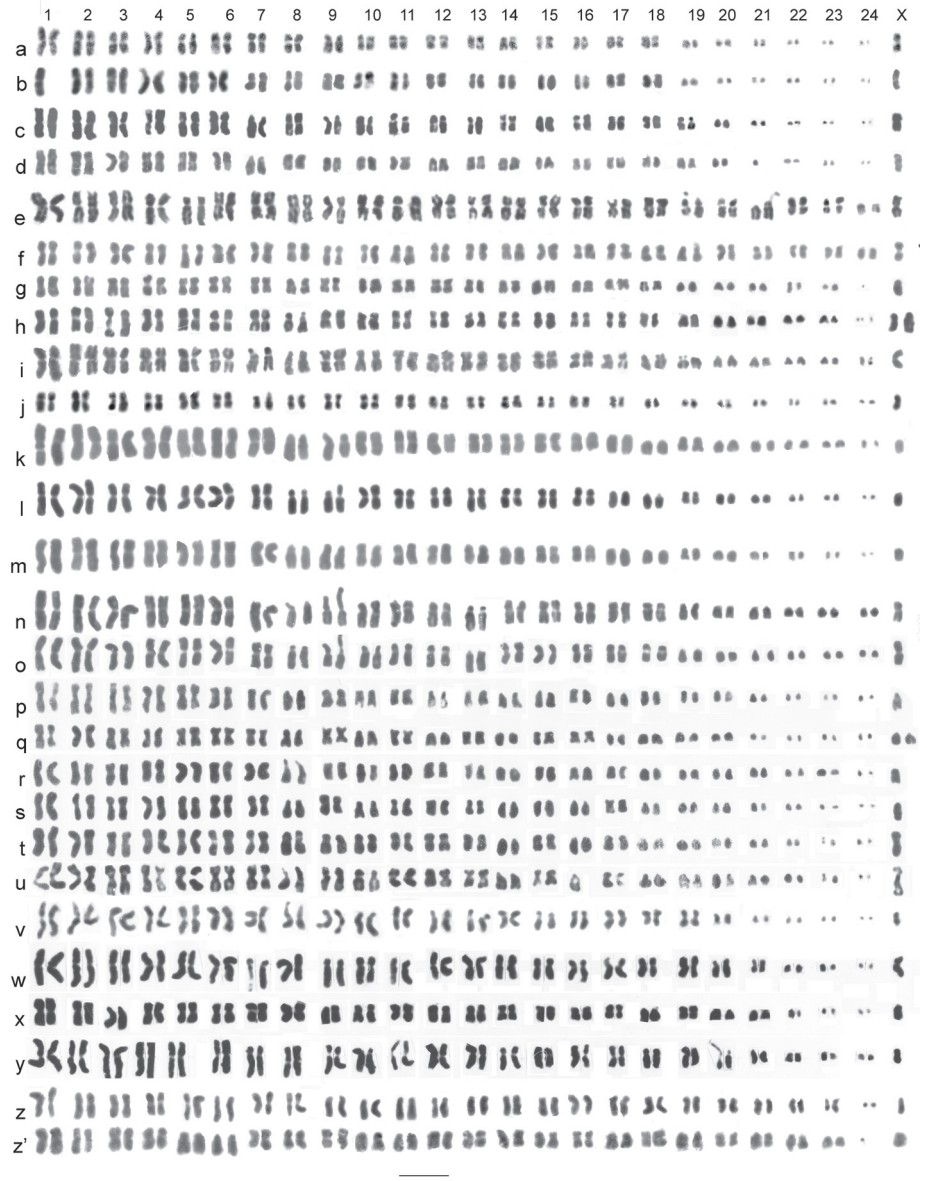

Figure 3. Mitotic chromosomes of *Nebrioporus* species, arranged as karyotypes. **a, b** *N. ceresyi*, ♂, mid-gut, **a** Cyprus **b** Sardinia; **c, d** *N. baeticus*, ♂, mid-gut, **c** Castro del Rio **d** S of Osuna; **e, f** *N. canaliculatus*, ♂, mid-gut; **e** Sasamon **f** Sweden; **g, h** *N. bucheti*, mid-gut, Menton, **g** ♂ **h** ♀; **i** *N. martinii*, ♂, mid-gut, Corsica **j** *N. sardus*, ♂, mid-gut, Sardinia **k** *N. depressus*, ♂, mid-gut, Talkin Tarn **l** *N. depressus-elegans* intermediate, ♂, mid-gut, Clatteringshaws Loch **m** *N. elegans*, ♂, mid-gut, Cassington **n, o** *N. assimilis*, ♂, mid-gut, Grasmere **p, q** *N. carinatus*, mid-gut **p** ♂ **q** ♀; **r, s** *N. fabressei*, ♂, testis **r** France **s** Spain; **t, u** *N. croceus*, ♂ paratypes **t** testis **u** mid-gut **v** *N. amicorum*, ♂, mid-gut, Crete **w** *N. lanceolatus*, ♂, testis, St Katherine **x** *N. insignis*, ♂, mid-gut, St Katherine **y** *N. crotchi*, ♂, mid-gut, St Katherine **z, z'** *N. canariensis*, ♂, mid-gut, Bar = 5 μm.

N. assimilis (Paykull, 1798). Fig. 3 n, o (♂). The RCLs of the autosomes decrease evenly from about 6.5–1.8, with no dot-like autosomes. The X chromosome is metacentric, RCL about 4, comparable with autosome 11. Autosomes 8–12, and 18–24 are subacrocentric to acrocentric, and pairs 3, 7, 11, 12 and 14–16 are submetacentric. The rest are more or less metacentric. Pairs 8 and 9 have secondary constrictions on their short arms.

N. carinatus (Aubé, 1838), Fig. 3 p (♂), 3 q (♀), *N. fabressei* (Régimbart, 1901), Fig. 3 r, s (♂) and *N. croceus* Angus, Fresneda and Fery, 1992, Fig. 3 t, u (♂) were figured and discussed by Angus, Fresneda and Fery (1992) and are shown here for completeness and to permit comparison with the other species.

The *N. laeviventris* group

N. amicorum Toledo, 2009, Fig. 3 v (♂). The RCLs of autosomes 1–19 decrease fairly evenly from about 7.5–3.2. There is then an abrupt decrease to pair 20, RCL about 2, and a further drop to the four smallest pairs, which are more or less dot-like, RCLs 1.5–0.6. The X chromosome metacentric, RCL about 2.7, intermediate in size between autosomes 19 and 20. Autosomes 8, 10, 12, 13, 16, 17 and 20–23 are subacrocentric to acrocentric, and the rest are more or less metacentric. Pair 9 is submetacentric, with a secondary constriction in its long arm.

N. lanceolatus (Walker, 1871), Fig. 3 w (♂), *N. insignis* (Klug, 1833) Fig. 3 x (♂) and *N. crotchi* (Preudhomme de Borre, 1871), Fig. 3 y (♂) are Egyptian species and were discussed and figured by Saleh Ahmed et al., 2000. There are figured here, with some slight rearrangement of karyotypes, for completeness and to facilitate comparison with the other species. Apart from the differences among themselves, they differ from *N. amicorum* in the smaller number (1 or 2 pairs) of dot-like autosomes.

***Nebrioporus* incertae sedis**

N. canariensis (Bedel, 1881) Fig. 3 z, z' (♂). The RCLs of autosome pairs 1–23 decrease evenly from about 6.4–2.9. Pair 24 is dot-like, RCL about 1. The X chromosome is submetacentric, RCL about 3.5, comparable with autosome 19. Autosomes 2–4, 10–12, 14, 15, 17, 19 and 21–23 are subacrocentric to acrocentric, and the rest are more on less metacentric.

***Stictotarsus* Zimmermann, 1919**

Fig. 4, a – i and Fig. 5 show mitotic chromosomes of the three species studied. All have 27 pairs of autosomes, some of which may be subacrocentric with heterochromatic long arms, X chromosomes with heterochromatic long arms, and a neo-XY system of

sex chromosomes, with the Y chromosome resembling the short (euchromatic) arm of the X chromosome.

S. duodecimpustulatus (Fabricius, 1792). Fig. 4 a (♂), 4 b – d (♀). 2n = 54 + XY (♂), XX (♀). Autosome pairs 1–5, 7–9 and 16 are subacrocentric, and their long arms are entirely heterochromatic (Fig. 4 d). Pairs 1–8 have RCLs ranging from about 7.5–5.7, and this includes pair 6, which is metacentric with a small centromeric C-band. Pair 9, is similar in form to pair 8, but is smaller, RCL about 4.7. Pairs 10–14 show an even decrease in RCL from about 4.2–3.8. There is then an abrupt decrease in RCL with pair 15 having a value of about 3, and pairs 15–21 showing an even decrease in RCL to about 2. The remaining small autosomes show a decrease in RCL from about 1.9–0.9. Pairs 10, 11, 13 and 14 are metacentric with small centromeric C-bands. Pair 12 is subacrocentric and apparently fairly extensively heterochromatic (Fig. 4 d), though without a clear heavily heterochromatic long arm. Pair 17 is acrocentric with a heavy centromeric C-band, and pairs 18–20 are submetacentric with very weak centromeric C-bands. The remaining autosomes are acrocentric, with only pair 22 having a strong centromeric C-band. The X chromosome, RCL about 8, is slightly larger than, and very similar in form to the longest autosomes, and, like them, has a heavily heterochromatic long arm. The y chromosome RCL about 2, is acrocentric and matches the euchromatic short arm of the X chromosome.

S. procerus (Aubé, 1838). Fig. 4 e, g (♂), Fig. 4 f (♀).2n = 54 + XY (♂), XX (♀). The karyotype is broadly similar to that of *S. duodecimpustulatus*, but with some clear differences. Autosome 1, RCL about 6.2, is subacrocentric with a heavily heterochromatic long arm, but autosome 2 has the heterochromatin apparently confined to the basal half of the long arm (Fig. 4 g).

Autosome 3 is similar in size to pairs 1 and 2, but is metacentric and the middle half of the chromosome is heterochromatic. Autosome 4 is slightly smaller, RCL about 5, but similar in form to pair 1. Autosome 5 is similar in size to pair 4 and is metacentric with a small centromeric C-band, as in *S. duodecimpustulatus* autosome 6. Autosomes 6–20 show a steady decrease in RCL from about 5 to about 3.5, with pairs 12–20 all having RCLs of about 3.5. Pair 6 is submetacentric and the short arm is heterochromatic, while pair 7 is subacrocentric with the long arm heterochromatic. Pair 8 is acrocentric without conspicuous C-banding, while pair 9 is almost metacentric with one arm heterochromatic. Pairs 10–13 are metacentric or almost so, with heavy C-bands, which extend onto the shorter arms of pair 12. Pairs 14 and 16 are subacrocentric with heterochromatic short arms. Pairs 15 and 17 are metacentric with centromeric C-bands, and pairs 18 and 19 are metacentric, with a heterochromatic arm in pair 18. Pair 20 is acrocentric with a heavy centromeric C-band. Pairs 21–24 are metacentric, and pair 24 has a heterochromatic arm. Pairs 21 and 22 have RCLs of about 2.6, pairs 23–25 are slightly smaller, RCL about 2.3. Pair 25 is acrocentric with a heavy centromeric C-band. Pairs 26 is smaller again, RCL about 1.3, and is submetacentric with a

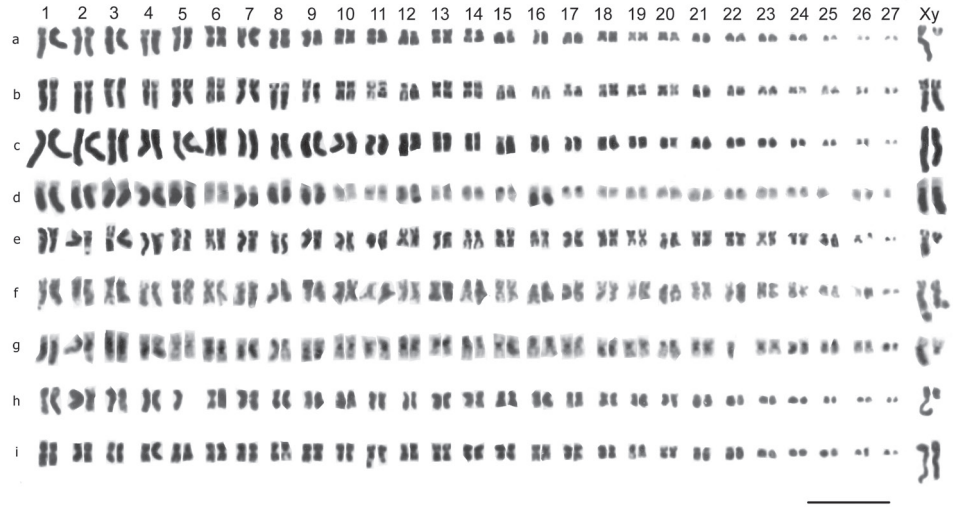

Figure 4. Mitotic chromosomes from mid-gut cells of *Stictotarsus* species, arranged as karyotypes. **a** *S. duodecimpustulatus*, ♂, Clatteringshaws Loch, plain; **b, c** *S. duodecimpustulatus*, ♀, plain, **b** New Forest **c** Abadia **d** *S. duodecimpustulatus*, ♀, C-banded, Esclavitud **e** *S. procerus*, ♂, Corsica, Solenzara, plain **f** *S. procerus* ♀, Sardinia, Posada, plain **g** *S. procerus*, ♂, Corsica, Casaluna, C-banded **h** *S. bertrandi*, ♂, pain **i** *S. bertrandi*, ♀, plain. Bar = 5μm.

centromeric C-band, and pair 27 is a small acrocentric, RCL 0.9 or less. The X chromosome, RCL about 5.4 is similar in form to autosome 1. The y chromosome, RCL about 2.2, is acrocentric and matches the euchromatic short arm of the X chromosome.

S. bertrandi (Legros, 1956). Fig. 4 h (♂), Fig. 4 i (♀), Fig. 5 (♀). 2n = 54 + XY (♂), XX (♀). Although the chromosome number and sex determining mechanism are the same as in *S. duodecimpustulatus* and *S. procerus*, the general appearance of the karyotype is rather different, with only the X chromosome conspicuously long (RCL about 10.5), subacrocentric and with the long arm heterochromatic. Fig. 5 shows a C-banded preparation of a female mitotic nucleus and although it is not possible to prepare a karyotype from this preparation, the two heterochromatic long arms of the X chromosomes are very distinct. Autosome pairs 1–22 are metacentric or submetacentric, and their RCLs show a gradual decrease from about 6.3 to 3. Autosome 1 is clearly submetacentric, and its longer arm appears to have the chromatids closely applied to one another, suggesting that it is heterochromatic. However, C-banding does not bear this out. It may, however, be the site of a secondary constriction. It may be similar in its structure to the long arm of autosome 2 of *S. procerus*, but the material available is not adequate to show this. Autosome pairs 23–27 are acrocentric, and their RCLs range from about 2 to about 1, with pair 27 often appearing dot-like. The Y chromosome, RCL about 2, is acrocentric and matches the short arm of the X chromosome, as is typical of a neo-XY system.

Scarodytes Gozis, 1914

Mitotic chromosomes of the four species analysed are shown in Fig. 6, a – i. All the species have 27 pairs of autosomes and sex chromosomes which are X0 (♂), XX (♀).

S. halensis (Fabricius, 1787). Fig. 6 a, b (♂), Fig. 6 c (♀). 2n = 54 + X0 (♂), XX (♀). The RCLs of the autosomes decrease rather evenly from about 9 to just over 0.5, with pair 27 dot-like. Pairs 1–9, 14, 17 and 18 are metacentric or submetacentric, pairs 10–12 are subacrocentric. Pairs 13, 15, 16 and 19–27 are acrocentric. It is not possible to detect any secondary constrictions in these preparations. The female karyotype, from the French Alpes Maritimes, shows no detectable difference from those of British material.

S. nigriventris (Zimmermann, 1919). Fig. 6 d (♂), Fig. 6 e (♀). 2n = 54 + X0 (♂), XX (♀). The RCLs of the autosomes decrease fairly evenly from about 5.7 to 1.5, and the smallest pair (pair 27) is clearly not dot-like. Pairs 1–3, 5–9, are metacentric to submetacentric, and pair 4 is subacrocentric with a well-marked secondary constriction in its short arm. Pair 10 is acrocentric, 11–14, 17, 18, 24, 25 and 27 are subacrocentric, while the remaining autosomes are submetacentric. The X chromosome, RCL about 6, is submetacentric, similar in appearance to aurosome1. Although *S. nigriventris* has in the past been placed as a subspecies of *S. halensis*, its karyotype is clearly different from that of *S. halensis*, with only about half the range of RCLs, and no dot-like autosomes.

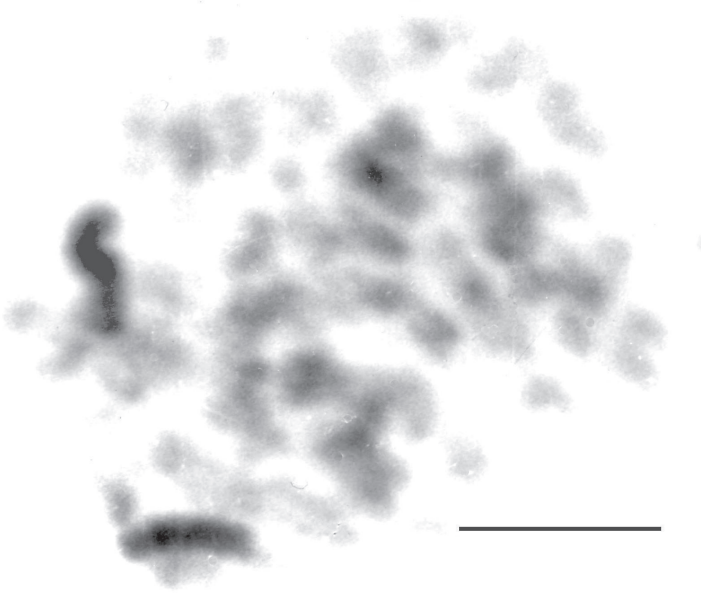

Figure 5. *Stictotarsus bertrandi*, ♀, C-banded nucleus from mid-gut, showing the long heterochromatic arms on the two X chromosomes. Bar = 5μm.

This is in complete agreement with the conclusions drawn by Ribera (2003) from his DNA data.

S. fuscitarsis (Aubé, 1838), stat. n. Fig. 6 f (♂), Fig.6 g (♀). 2n = 54 + X0 (♂), XX (♀). This name is used for Sardinian material which differs from Corsican *S. nigriventris* in lacking a tooth on the ventral margin of the inner anterior tarsal claw, as well as in having a rather broader body form. This identification has been confirmed by Dr H. Fery (*in litt*. 12.xii.2008). *S. fuscitarsis*, described from Sardinia, is currently listed as a subspecies of *S. halensis* (Nilsson, 2001, 2011). *S. fuscitarsis* is described as having the underside and legs brownish, but in the present material they are black. The general appearance of the karyotype is very similar to that of *S. nigriventris*, including the secondary constriction on the short arm of autosome 4. It is clearly unlike that of *S. halensis*, indicating that *S. fuscitarsis* cannot be a subspecies of it. The range of RCLs is very similar to that shown by *S. nigriventris*, but the X chromosome appears shorter, RCL about 5.2, and smaller than autosome 1. The morphology of *S. fuscitarsis* (size and shape of the aedeagus, lack of a tooth on the anterior tarsal claws of the male, size and shape of the beetles) is clearly distinct from *S. nigriventris*.

S. malickyi Wewalka, 1997. Fig. 6 h, i (♂). 2n = 54 + X0 (♂). The two preparations shown here are difficult to work with because a number of the chromosomes

Figure 6. Mitotic chromosomes from mid-gut (**a, c–i**) and testis (**b**) cells of *Scarodytes* species, arranged as karyotypes. **a, b** *S. halensis*, ♂, Stanton Harcourt **c** *S. halensis*, ♀, Menton **d** *S. nigriventris*, ♂, Solenzara **e** *S. nigriventris*, ♀, Casaluna **f** *S. fuscitarsis*, ♂, Sardinia **g** *S. fuscitarsis*, ♀, Sardinia **h, i** *S. malickyi*, ♂, Crete. Bar = 5μm.

have distinct gaps which appear to be stretched centromeres rather than secondary constrictions (e.g. Fig. 5 h, autosome pair 16. The RCLs of the autosomes range from about 7–0.6, with pairs 17–27 all acrocentric. In both the karyotypes shown here one replicate of autosome 27 is dot-like, but the other clearly is not. More material of this species is needed. Autosomes 1, 2 and 5 are submetacentric, autosome 3 is subacrocentric, while autosome 4, which has the secondary constriction in its short arm, appears submetacentric. Pairs 6–9 are on the border between submetacentric and subacrocentric, and autosome 10 is submetacentric with the short arm appearing rather faint. Autosomes 11–16 are subacrocentric, with pair 16 appearing normal (without the centromeric gap) in Fig. 5 i. The X chromosome is submetacentric, and about as long as autosome 1 (RCL about 6.5).

Discussion

Two major features emerge from the results presented here: the different species almost all have distinct karyotypes, and within the genera the species tend to have broadly similar karyotypes; and the karyotypes of the different genera are easily reconciled with the phylogram obtained by Ribera (2003, Fig. 3).

In *Deronectes* there is a progressive reduction in the number of autosomes, with an associated increase in their size. In all the species studied the sex chromosome system appears to be neo-Xy.

The karyotypes of *Trichonectes* and *Nebrioporus* agree with one another in having 24 pairs of autosomes and X0/XX sex chromosomes. Relevant interspecies comparisons of *Nebrioporus* chromosomes are given with the results.

The karyotypes of the three *Stictotarsus* species studied here are particularly interesting, not only because of their broad agreement with one another (27 pairs of autosomes and a neo-XY sex chromosome system with the X chromosome having a heterochromatic long arm), but also because the two sibling species (*S. duodecimpustulatus* and *S. procerus*) have very closely similar karyotypes, while that of *S. bertrandi* is more distinct.

The *Scarodytes* karyotypes all have 27 pairs of autosomes and X0/XX sex chromosomes. Those of Corsican *S. nigriventris* and Sardinian *S. fuscitarsis* are scarcely different from one another, but the other two species are clearly distinct.

The phylogram given by Ribera (2003) includes all the genera discussed here, plus *Boreonectes* Angus, 2010 and the "*Stictotarsus*" *roffii* group (as "*Stictotarsus griseostriatus-roffii* group") and *Oreodytes* Seidlitz , 1887, with *Laccornis* Gozis, 1914 as an outgroup. This phylogram shows two major groupings. The first comprises two main, well-separated lineages, *Deronectes* and an assemblage including *Boreonectes*, *Oreodytes* and the "*Stictotarsus" roffii* group. The karyotypes of the *Deronectes* species differ from the others mentioned in having a neo-XY sex chromosome system. The karyotypes of the *Boreonectes griseostriatus* species complex are by now well known (Dutton and Angus 2007, Angus 2008, 2010a & b), but those of the other genera remain unknown.

The second major grouping has *Trichonectes* at its base, then branches into a *Stictotarsus* (*duodecimpustulatus* group) plus *Scarodytes* clade and a *Nebrioporus* clade.

The basal position of *Trichonectes* in this second group is very interesting. Its karyotype, with 24 pairs of autosomes plus X0/XX sex chromosome resembles those of all species of *Nebrioporus* for which data are available. This would suggest that the karyotypes of the *Stictotarsus* are highly apomorphic, having evolved not only their own autosome number, but also a common neo-Xy sex chromosome system. In this phylogram *Scarodytes* is placed as sister genus to *Stictotarsus* (*duodecimpustulatus* group), with these two genera as the sister group to *Nebrioporus*. The karyotypes of the four *Scarodytes* species considered here resemble those of *Nebrioporus* in having an X0/XX sex chromosome system, but differ in having three more pairs of autosomes (27 pairs instead of 24).

It can thus be seen that the karyotypes of the genera in this second group could agree with the phylogeny suggested by Ribera. *Trichonectes* would have a karyotype ancestral for the group as a whole, and this would be retained along the *Nebrioporus* clade. However, in the *Stictotarsus* – *Scarodytes* clade two major changes would have occurred; first an increase in the number of autosomes by three pairs (as shown by *Scarodytes*), and second a change in the sex chromosomes to neo-Xy as shown by *Stictotarsus*. Interestingly these *Stictotarsus* resemble *Scarodytes* in the fact that they have 27 pairs of autosomes, but presumably they once had 28 pairs, in order to give an original autosome which could fuse with the original X chromosome to give a neo-X.

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"Darwin's butterflies"? DNA barcoding and the radiation of the endemic Caribbean butterfly genus *Calisto* (Lepidoptera, Nymphalidae, Satyrinae)

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Abstract

The genus *Calisto* Hübner, 1823 is the only member of the diverse, global subfamily Satyrinae found in the West Indies, and by far the richest endemic Caribbean butterfly radiation. *Calisto* species occupy an extremely diverse array of habitats, suggestive of adaptive radiation on the scale of other classic examples such as the Galápagos or Darwin's finches. However, a reliable species classification is a key requisite before further evolutionary or ecological research. An analysis of 111 DNA 'barcodes' (655 bp of the mitochondrial gene COI) from 29 putative *Calisto* species represented by 31 putative taxa was therefore conducted to elucidate taxonomic relationships among these often highly cryptic and confusing taxa. The sympatric, morphologically and ecologically similar taxa *C. confusa* Lathy, 1899 and *C. confusa debarriera* Clench, 1943 proved to be extremely divergent, and we therefore recognize *Calisto debarriera* **stat. n.** as a distinct species, with *Calisto neiba* Schwartz et Gali, 1984 as a junior synonym **syn. n.** Species status of certain allopatric, morphologically similar sister species has been confirmed: *Calisto hysius* (Godart, 1824) (including its subspecies *C. hysius aleucosticha* Correa et Schwartz, 1986, **stat. n.**), and its former subspecies *C. batesi* Michener, 1943 showed a high degree of divergence (above 6%) and should be considered separate species. *Calisto lyceius* Bates, 1935/*C. crypta* Gali, 1985/*C. franciscoi* Gali, 1985 complex, also showed a high degree of divergence (above 6%), confirming the species status of these taxa. In contrast, our data suggest that the *Calisto grannus* Bates, 1939 species complex (including *Calisto*

grannus dilemma González, 1987, *C. grannus amazona* González, 1987, **stat. n.**, *C. grannus micrommata* Schwartz et Gali, 1984, **stat. n.**, *C. grannus dystacta* González, 1987, **stat. n.**, *C. grannus phoinix* González, 1987, **stat. n.**, *C. grannus sommeri* Schwartz et Gali, 1984, **stat. n.**, and *C. grannus micheneri* Clench, 1944, **stat. n.**) should be treated as a single polytypic species, as genetic divergence among sampled populations representing these taxa is low (and stable morphological apomorphies are absent). A widely-distributed pest of sugar cane, *Calisto pulchella* Lathy, 1899 showed higher diversification among isolated populations (3.5%) than expected, hence supporting former separation of this species into two taxa (*pulchella* and *darlingtoni* Clench, 1943), of which the latter might prove to be a separate species rather than subspecies. The taxonomic revisions presented here result in *Calisto* now containing 34 species and 17 subspecies. Three species endemic to islands other than Hispaniola appear to be derived lineages of various Hispaniolan clades, indicating ancient dispersal events from Hispaniola to Puerto Rico, Cuba, and Jamaica. Overall, the degree of intrageneric and intraspecific divergence within *Calisto* suggests a long and continuous diversification period of 4–8 Myr. The maximum divergence within the genus (ca. 13.3%) is almost equivalent to the maximum divergence of *Calisto* from the distant pronophiline relative *Auca* Hayward, 1953 from the southern Andes (14.1%) and from the presumed closest relative *Eretris* Thieme, 1905 (14.4%), suggesting that the genus began to diversify soon after its split from its continental sister taxon. In general, this 'barcode' divergence corresponds to the high degree of morphological and ecological variation found among major lineages within the genus.

Keywords

COI, biogeography, DNA barcoding, islands, intraspecific variation, Lepidoptera, Nymphalidae, Satyrinae, speciation, taxonomy

Introduction

The genus *Calisto* Hübner, 1823 is endemic to the West Indies, and, until the present revision, comprised 54 named taxa (Lamas et al. 2004) of small to medium sized butterflies in the subfamily Satyrinae, a diverse global radiation including ca. 2,200 described species. *Calisto* is considered a member of the neotropical subtribe Pronophilina, but while many *Calisto* are lowland dwellers, occurring as low as sea level, almost all other pronophilines are exclusively montane and/or temperate. Most of the extant described species of *Calisto* are found on the island of Hispaniola, with a single species on Jamaica, one on Puerto Rico, two species on the Bahama Islands, one on Anegada, and two on Cuba (Smith et al.1994).

Though *Calisto* are neither visually spectacular nor economically important (with the exception of *C. pulchella* Lathy, 1899 , which is a pest of sugar cane), a significant amount of information is available on the distribution of the more common species on Hispaniola from the general survey of the island's butterflies by Schwartz (1989). However, phylogenetic relationships of the genus are unclear and affinities to both South American and African taxa have been proposed based on adult morphology (Riley 1975, Miller and Miller 1989), although the most recent taxonomic treatments of the tribe (e. g., Viloria 1998; Lamas et al. 2004) kept *Calisto* in Pronophilina. The

montane neotropical genus *Eretris* Thieme, 1905 has been considered one of the closest relatives of *Calisto* by some (Miller 1968, De Vries 1987, Peña et al. 2011).

The morphology of immature stages has been utilized extensively in phylogenetic studies of butterflies (e. g., Kitching 1985, Murray 2001, Penz and DeVries 2001, Freitas and Brown 2004, Willmott and Freitas 2006), however this has been mostly at higher taxonomic levels. *Calisto* is one of the few satyrine genera for which the immature stages have been studied in detail at least for most major species groups, providing insights into a high degree of morphological diversification in the egg and larvae, atypical for other satyrine genera (Sourakov 1996, 2000). Structures that normally show little variation in the Satyrinae intragenerically, such as male and female genitalia, are also remarkably diverse in *Calisto* (Sourakov 1997). Until now, however, it has been unclear whether this spectacular morphological variation results from an ancient history of divergence, or from strong disruptive selection on traits potentially involved with fitness and reproductive isolation.

Many species of *Calisto* were described only recently, towards the end of the 20th century (e.g., Schwartz and Gali 1984, Gali 1985, Johnson and Hedges 1998) and are still known only from the type series. Small numbers of specimens, in conjunction with reliance on wing pattern elements alone, which often seem to be variable in better known taxa, makes the status of many of these recent names difficult to determine. For example, additional eyespots were used to define the species *C. neiba* Schwartz et Gali, 1984 and *C. amazona* González, 1987. Many of these names might thus prove to be synonyms, or, conversely, represent a formerly unexplored array of cryptic species that are only just being recognized. The taxonomic confusion is evident in Smith et al.'s (1994) comprehensive treatment of Caribbean butterflies; they listed all the described taxa, but for many species avoided illustrating them and provided inconclusive comments on the validity of many taxa. For instance, they did not illustrate *Calisto montana* Clench, 1943 for which only the male holotype is known, and of which even the precise collecting locality is uncertain. For *Calisto neiba*, Smith et al. (1994) stated that it has additional wing ocelli (which are, however, a variable character in many Satyrinae (e. g., Sourakov 1995, Kooi et al. 1996), repeatedly appearing within all species of *Calisto*, usually as an occasional aberration), and concluded that "the final estimate of the affinities of *C. neiba* cannot yet be made." Another un-illustrated species, *C. aleucosticha* Correa et Schwartz, 1986, described from a couple of individuals that could represent aberrant *C. batesi* Michener, 1943 females, was assessed as "very close to *C. hysius* (Godart, 1824), and discovery of the male may well clarify its status." The illustration of *Calisto micheneri* Clench, 1944 represents a taxon similar to our concept of *C. grannus dilemma* González, 1987, a taxon not illustrated by Smith et al. (1994) and said to be known "from a single specimen only. It is readily confused with other common species, and may well be more frequent than the rather sparse records would suggest." Also not illustrated were *Calisto phoinix* González, 1987, of which Smith et al. (1994) said that "there seems little doubt that this species is not conspecific with *C. grannus*, but their relationships remains to be established," and *C. dystacta* González, 1987, which "occurs at lower altitude than *C. phoinix*. The two are very similar and

may be conspecific." We examined type specimens and the original descriptions of Schwartz and Gali (1984) and González (1987) and could only conclude that these names most likely represent variants of *Calisto grannus* Bates, 1939 found at unusual elevations and slopes, and hence exhibiting slightly different phenotypes from typical specimens of the latter taxon.

A different issue is presented by the taxa that are clearly allopatric (and probably remained in isolation for a long time), but which are so morphologically similar that one must question the extent of diversification between them. For instance, Smith et al. (1994) treat *Calisto batesi* as a separate species, following treatment by Schwartz (1989), yet state that "this insect has generally been considered a subspecies of *C. hysius*." Originally, *batesi* was described as a subspecies of *hysius* and Smith et al. (1994) chose to illustrate *C. batesi*, but did not illustrate *C. hysius*, because, we presume, the main difference between these taxa aside from their distribution is their size (*batesi* 13–15 mm; *hysius* 16.5–17.5mm), while the wing patterns are identical.

We find allopatric similar taxa within other major species groups, such as *C. chrysaoros* Bates, 1935 (names include *Calisto galii* Schwartz, 1983 and *galii choneupsilon* Schwartz, 1985) and *C. lyceius* Bates, 1935 (names include *Calisto crypta* Gali, 1985 and *Calisto franciscoi* Gali, 1985). In the *Calisto confusa* Lathy, 1899 complex, the name *C. confusa debarriera* Clench, 1943 has been attributed to a form with reduced white discal and extradiscal bands on the underside, which is found throughout the geographic range of *C. confusa confusa* and is occasionally sympatric, though frequently replaces typical *C. confusa* phenotypes at higher elevations. *Calisto montana* Clench, 1943 was described from the same group based on a single very worn specimen which had an unusual double-pupiled eye-spot on the underside of its forewing (Fig. 7) – a character found occasionally throughout *Calisto*. Other taxa within *Calisto confusa* species complex have also been described, such as *C. gonzalezi* Schwartz, 1988 for which Smith et al. (1994) state that "the exact relationship between this species and *C. confusa* remains to be clarified should new populations of *C. gonzalezi* be discovered."

The above confusion over the recently described taxa is perhaps partly due to sole reliance of the authors on wing characteristics combined with distribution data in their approach to delineating new species, partly due to limited series and quality of specimens, and partly due to the exercising of the typological approach in its extreme form, with a disregard for interspecific variation. A possible solution to the problem is to use a new set of characters such as molecular sequence data. The technique of 'DNA barcoding' is based on the analysis of short, standardized gene regions; in the case of animals, this is a 655-bp segment of mitochondrial cytochrome oxidase subunit I (COI). DNA barcoding potentially provides an efficient method for species identification as well as for solving species-level taxonomical problems. Although the DNA barcode region can vary intraspecifically on a geographic scale as well as within populations (e. g., Lukhtanov et al. 2009, DeWalt 2011), and has shown varying degrees of success in species delimitation (e.g., Wiemers and Fiedler 2007), it has overall proved to be an excellent tool for species identification as illustrated in several large Holarctic

Lepidoptera groups (Hebert et al. 2010, Lukhtanov et al. 2009). In the present study, therefore, we explore the potential for DNA barcode data to attempt to answer long-standing questions concerning interspecific and intraspecific relationships within the genus by studying 21 species of *Calisto* (representing almost all of the major species groups). We examine a number of questionable taxa, such as representatives of *C. grannus* and *C. confusa*, and the *C. lyceius* species complex. Furthermore, this study allows us to examine the utility of the DNA barcoding method for species delimitation using a group, which, unlike the Holarctic fauna, probably underwent continuous diversification for a prolonged period without the major climatic stresses of glaciations. The results of this study should also add to our understanding of the extent to which DNA-barcode divergence correlates with morphological and ecological divergence. Prior to further phylogenetic work based on morphological, molecular or combined characters, it is key to establish species boundaries and the alpha taxonomy of the genus. In this study, we therefore use DNA barcodes to test the current species classification based on traditional characters, and to try to resolve the taxonomic status of a number of problematic phenotypes and populations.

Methods

A total of 110 *Calisto* specimens representing 31 putative taxa were sampled (Table 1). All specimens were collected in 1994–1999 by the first author. None of the specimens were subjected to any chemical treatment before desiccation. The climate of the regions ensured quick drying of specimens, which were stored at a room temperature (18–25°C) for over 10 years. DNA was extracted from a single leg removed from each specimen. Specimens were mostly unprepared (papered), with the exception of several individuals.

We amplified a 655-bp segment of mitochondrial cytochrome oxidase subunit I, from the *COI* barcode region. All polymerase chain reactions and DNA sequencing were carried out following standard DNA barcoding procedures for Lepidoptera as described previously (Hajibabaei et al. 2006, deWaard et al. 2008). Photographs of all specimens used in the analysis as well as specimen collection data and sequences are available in the Barcode of Life Data System (BOLD) at http://www.barcodinglife.org/ as well as in GenBank (accession numbers JN197297--JN197406). All voucher specimens are deposited at the McGuire Center for Lepidoptera and Biodiversity (Florida Museum of Natural History, University of Florida).

We chose two genera as outgroups: *Eretris*, which Miller (1968) thought to be *Calisto*'s closest relative on the mainland, based on wing shape and relative proximity to the Caribbean, and the southern Andean genus *Auca* Hayward, 1953 (Satyrinae: Pronophilina), which we have observed to be morphologically and behaviorally similar to *Calisto* (e. g., *Auca*'s association with bunch grass in arid lowland habitats is very similar to species in the *Calisto lyceius* complex) (Sourakov pers. obs.). Though geographically distant from *Calisto*, the inclusion of such a Pronophilina member from the southern Andes could provide insight into the origin of *Calisto* should the genus prove to be

non-monophyletic and also provides an additional point of comparison for the pairwise divergence analysis. Hence, we obtained five additional sequences from GenBank (table 1), including two species of *Auca*, *A. coctei* (GenBank number DQ338833) and *A. barrosi* (GenBank number DQ338832) (Peña et al. 2011), and two species of *Eretris*, *Eretris* sp. (GenBank number GQ357229) and *Eretris* sp.2 (GenBank number GQ864764) (Peña et al. 2006). We also obtained one additional sequence of *Calisto pulchella* (GenBank number GQ357225) (Peña et al. 2011).

Sequences were aligned using BioEdit software (Hall 1999) and manually edited. Sequence information was entered into the Barcode of Life Data System (http://www.barcodinglife.org) along with an image and collateral information for each voucher specimen. Detailed specimen records and sequence information, including trace files, are available in the LOWA project file in the BOLD website. All sequences are also available through GenBank.

Sequence data were analyzed using Bayesian inference (BI), as implemented in Mr Bayes 3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003). A GTR substitution model with gamma-distributed rate variation across sites and a proportion of invariable sites was specified before running the program for 5,000,000 generations with default settings. The first 2500 trees (out of 10000) were discarded prior to computing a consensus phylogeny and posterior probabilities.

Maximum parsimony (MP) analysis was performed using a heuristic search as implemented in MEGA4 (Tamura et al. 2007). We used the close-neighbor-interchange algorithm with search level 3 (Nei and Kumar 2000) in which the initial trees were obtained by random addition of sequences (100 replicates). We used nonparametric bootstrap values (Felsenstein 1985) to estimate branch support on the recovered tree, with the bootstrap consensus tree inferred from 1000 replicates (MP tree is provided as Supplementary file). The Kimura 2-parameter model of base substitution was used to calculate genetic distances in MEGA4 software (Tamura et al. 2007). Dendroscope (Huson et al. 2007) was used to edit trees for publication.

Results

Fig. 1 shows the results of the Bayesian Inference analysis (BI). The maximum parsimony analysis revealed a similar topology, but deeper nodes were not strongly supported (bootstrap value < 0.5). Bootstrap values higher than 0.5 are shown on the MP tree (see Supplementary file). For the further analysis and discussion of results we refer to the BI tree. The BI analysis of the tree topology and the Kimura 2-parameter model estimation of genetic distances showed the following results:

1. The sympatric, superficially similar widespread species *Calisto confusa*, *C. obscura* and *C. batesi*, which frequently share the same habitat, proved to be extremely divergent. *Calisto confusa* appear to be related to the morphologically highly derived *C. arcas* Bates, 1939 Fig. 3 (Clades A, B). *Calisto obscura*, which is found throughout the lowlands and mid-elevations proved to be related to the *C. grannus* species group

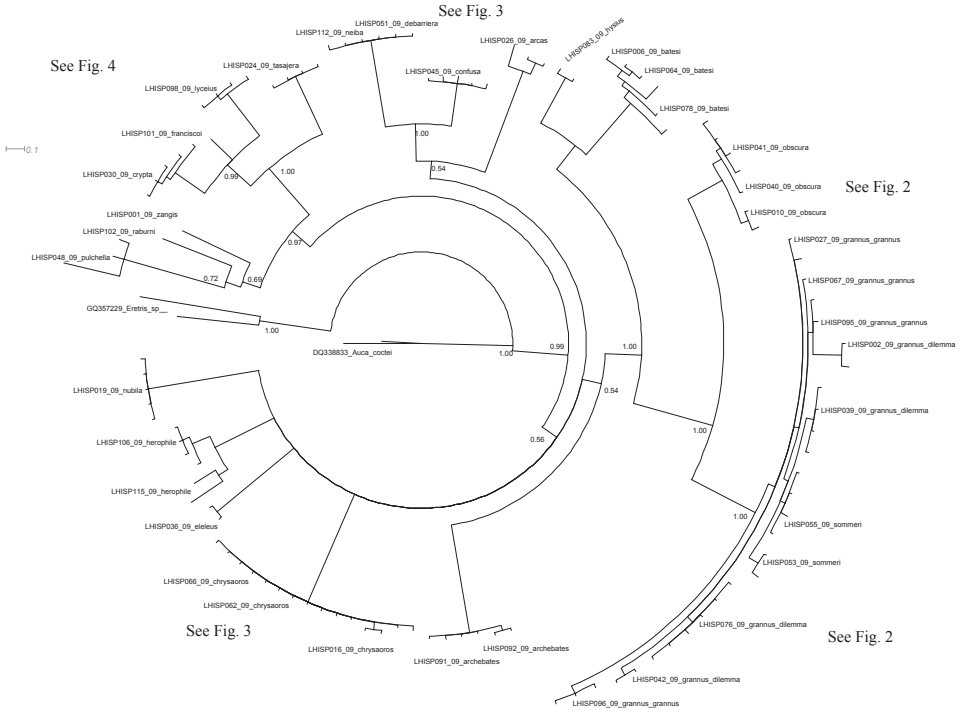

Figure 1. Bayesian inference phylogeny based on 655bp of COI for 111 specimens of the genus *Calisto* (representing ca. 20 species belonging to 26 named taxa), with outgroups of *Eretris* and *Auca* (Nymphalidae: Satyrinae: Prinophilini). The numbers at the nodes indicate posterior probability.

which is found locally throughout the island at higher elevations (Fig. 2, Clade B). Though the latter clade has *C. batesi*/*C. hysius* species complex as its sister clade (Fig. 2, Clade A), the divergence between *C. obscura* and *C. batesi* is substantial at approximately 9%.

2. The allopatric morphologically similar sister species *Calisto batesi*/*C. hysius* (Fig. 2, Clade A), whose species status was questionable based on adult morphology, and whose immature stages are also quite similar (Sourakov 1996), showed a high degree of divergence of ca. 6%, which is twice the rate seen in some sister species in Palearctic Satyrinae (Lukhtanov et al. 2009). For comparison, the divergence within *C. batesi* among well isolated populations throughout Cordillera Central, though still significant, is equal to or less than 1%.

3. *Calisto confusa* and *C. debarriera* appeared as two well-separated clusters (Fig. 3, Clade A). *Calisto debarriera* was originally treated as subspecies of *C. confusa* (Munroe 1951), and later regarded as color variant of *C. confusa* because of its frequent sympatry with the latter (Sourakov per. obs.), and because rearing did not indicate additional morphological characters (Sourakov 1996, 1997). Individuals of both taxa used in our analysis came from the same localities throughout the island, and while they showed interspecific divergence of over 6%, showed divergence of less than ca. 0.2% intraspe-

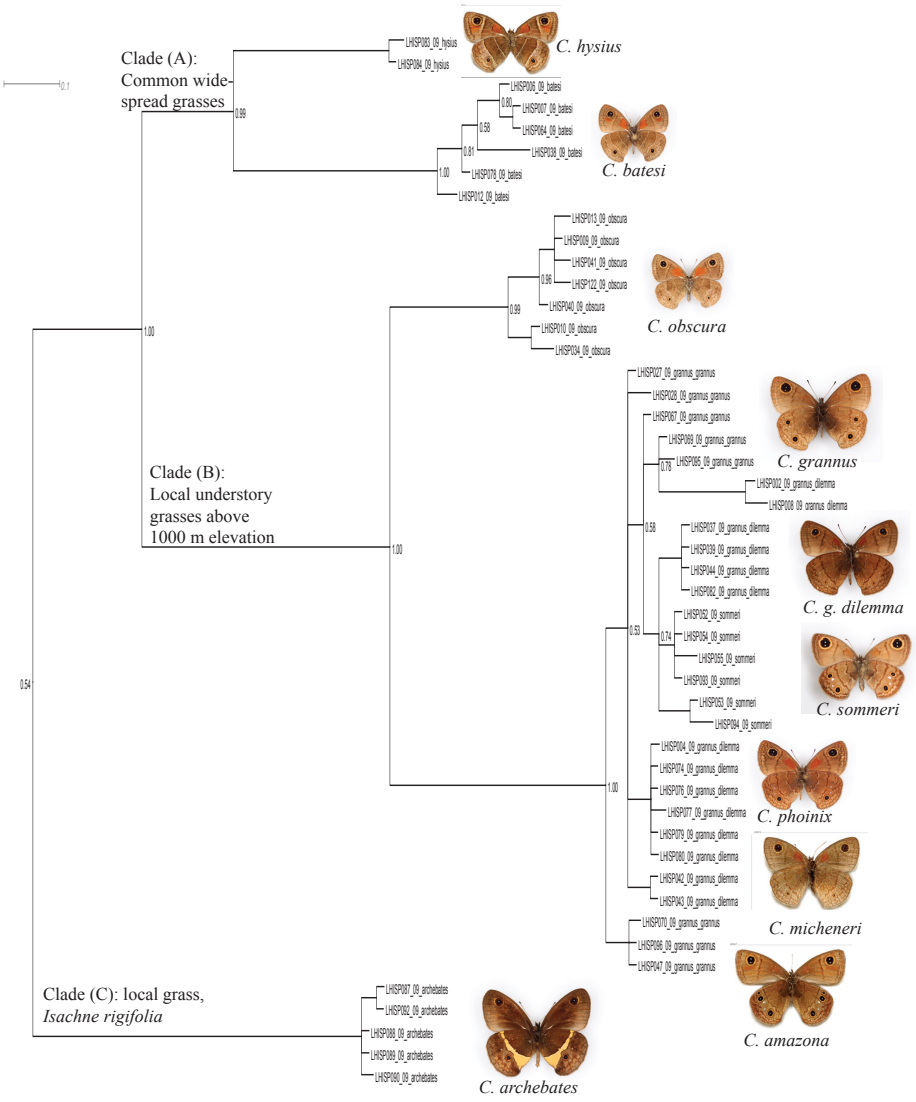

Figure 2. Fragment of the BI tree in Figure 1 with additional information about clades **Clade A:** *Calisto hysius* and *C. batesi* are found allopatrically on two Hispaniolan paleoislands **Clade B:** *Calisto obscura* is a widespread Hispaniolan species. The *Calisto grannus* complex is represented by a number of named populations, mostly but not exclusively found in Cordillera Central, the status of which are revised to subspecies in the present study **Clade C:** *C. archebates* is a local endemic of the southern paleoisland's Sierra de Bahoruco.

cifically. A single specimen with the phenotype of *Calisto neiba* (from Sierra de Neiba) was not divergent from the rest of *C. debarriera*, suggesting that the former is a synonym of the latter.

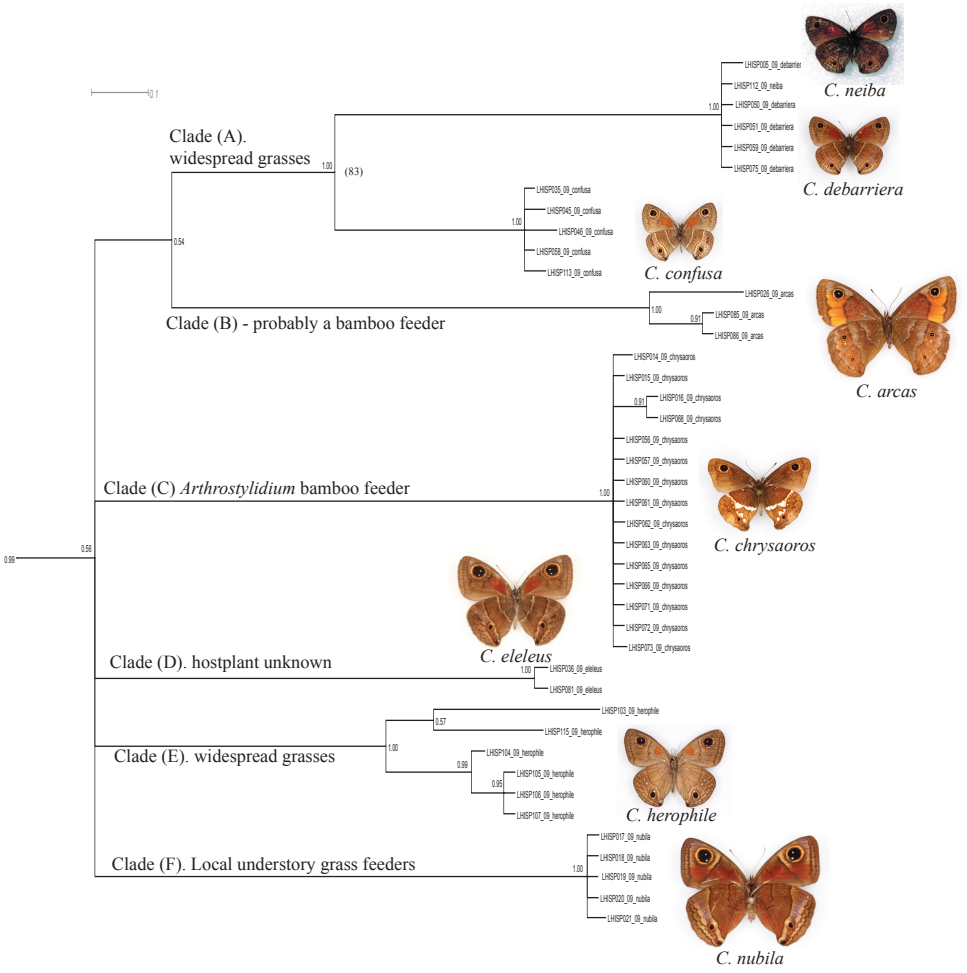

Figure 3. Fragment of the BI tree in Figure 1 with additional information about clades. **Clade A:** *Calisto confusa* and *C. debarriera*/*C. neiba* are morphologically similar and sometimes sympatric, though seemingly occupy different elevations **Clade B:** *Calisto arcas* is an endemic of Cordillera Central's Valle Nuevo area **Clade C:** *Calisto chrysaoros* is found at high elevations on both southern and northern paleoislands in the refugias associated with climbing bamboo grass *Arthrostylidium* **Clade D:** *Calisto eleleus* is now found extremely locally in the Cordillera Central **Clade E:** *Calisto herophile* is distributed on Cuba and Bahamas islands **Clade F:** *C. nubila* is a Puerto Rican endemic.

4. Within the *Calisto grannus* species complex (Fig. 2, clade B), we included at least nine isolated populations from different elevations, which we initially assigned to three taxa: *C. grannus grannus* of high elevations in the Cordillera Central (including a specimen representing the *C. amazona* phenotype), *C. grannus dilemma* (*grannus* individuals with red discal spot on the underside forewing, which includes such taxa as *dilemma*, *micrommata*, *dystacta*, *phoinix*, and *micheneri*) and *C. sommeri*, an isolate from Sierra de Bahoruco. The 28 individuals from these nine populations that are identi-

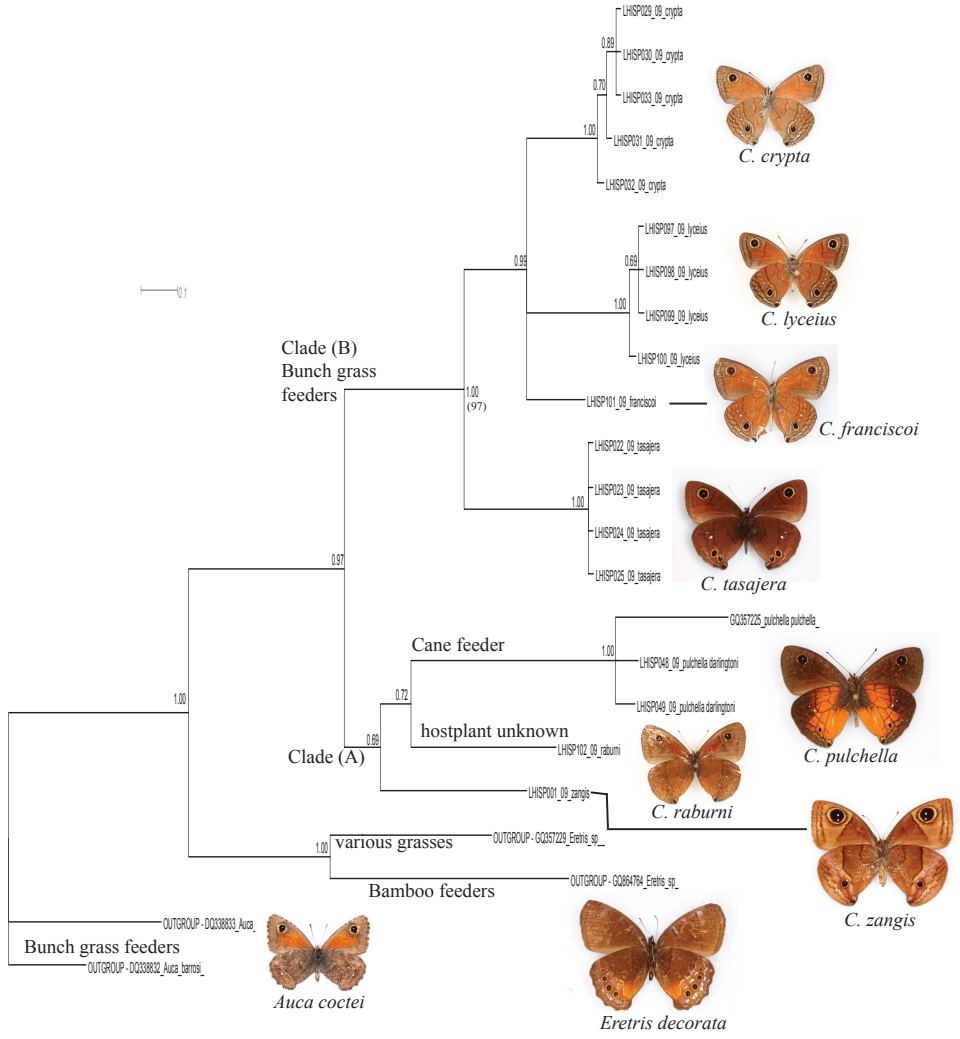

Figure 4. Fragment of the BI tree in Figure 1 with additional information about clades. The outgroups (*Auca* - bunch grass feeder from the southern Andes; *Eretris* - a bamboo-feeding group from Central and South America) and two basal *Calisto* clades **Clade A:** *C. zangis* of Jamaica which is aligned with the Hispaniolan *C. raburni* (a rare highly divergent species with an unknown life history) and *C. pulchella*, a well-known sugar cane pest (the native host plant is unknown) **Clade B:** *C. tasajera* (from the highlands of Cordillera Central) which feeds on *Danthonia domingenisis* bunch grass and *Calisto* of the *lyceius* group feeding on *Uniola virgata* bunch grass in the Hispaniolan lowlands.

fied on the barcode tree as *C. grannus grannus*, *C. grannus dilemma* and *C. sommeri* show geographic, rather than taxonomic, structure. In other words, individuals cluster within populations, separated from other such clusters by 0.5–1.5%, regardless of the taxonomic name applied. For instance, *C. sommeri* of Sierra de Bahoruco appears as

a sister clade to *C. grannus dilemma* from the extreme western portion of Dominican Cordillera Central. The lowland and very common widespread *C. obscura* appears to be a sister taxon to the *C. grannus* species complex, with a divergence of 5–7%.

5. Within the *Calisto lyceius* species complex (Fig. 4, Clade B), lowland desert isolates such as *C. crypta*, *C. franciscoi*, and *C. lyceius*, despite their superficial morphological similarities, proved to be divergent in their barcodes (ca. 4.5%). *Calisto tasajera* González, Schwartz et Wetherbee, 1991 proved to be their immediate relative, found at the high elevations.

6. A widely-distributed pest of sugar cane, *Calisto pulchella* (Fig. 4, Clade A) showed a high degree of divergence (3.5%) between its two described subspecies (*C. pulchella pulchella* from the lowlands and *C. p. darlingtoni* from the Cordillera Central).

7. Three species endemic to islands other than Hispaniola (*Calisto nubila* Lathy,1899, *C. zangis* (Fab., 1775) and *C. herophile* Hübner, 1823) appear to be derived lineages of various Hispaniolan taxa (Fig. 3, Clade D and E; Fig. 4, Clade A). Divergence of these island isolates, though high, does not exceed divergence found within the island of Hispaniola.

8. The maximum divergence within the genus (13.3% between *C. nubila* and *C. grannus*) is almost equivalent to the maximum divergence of *Calisto* from its distant pronophiline relative *Auca* from the southern Andes (14.1%), or from its presumed closest relative *Eretris* (14.4%) (Fig. 4). The average interspecific divergence in *Calisto* was found to be 10%.

Discussion

As a result of the present "DNA barcode" analysis, it is possible to draw a number of taxonomic conclusions (proposed taxonomic changes are summarized in Table 1). *Calisto grannus* represents a recent and incomplete diversification through allopatric isolation, and for now is best considered as a single species, with *C. g. dilemma*, *C. g amazona* stat. n., *C. g micrommata* stat. n., *C. g. dystacta* stat. n., *C. g. phoinix* stat. n., *C. g. sommeri* stat. n., and *C. g. micheneri* stat. n. representing subspecies. Within the *Calisto lyceius* complex, lowland desert isolates such as *C. crypta*, *C. franciscoi*, and *C. lyceius*, despite their superficial morphological similarities, proved to be sufficiently divergent in their barcodes to confirm their species status previously postulated based on male genitalia (Sourakov 2000). The observed divergence within *Calisto pulchella*, which is not only one of the most morphologically divergent species (Sourakov 1996, 1997), but also a widespread and economically important pest of sugar cane (Smyth 1920, Holloway 1933), calls for more research. Interestingly, these results correspond to earlier views (Munroe 1951, Wisor and Schwartz 1985) that there are at least two taxa in *pulchella*, one in the lowlands and another (*C. pulchella darlingtoni*) in the Cordillera Central at 3000–4000 ft elevation. Columbus introduced sugar cane to the island around 500 years ago (Deer 1949), so the current distribution of the species is likely different from its historical distribution. Perhaps, pre-Columbus *C. pulchella* ex-

Table 1. *Calisto* species examined in the present study and resulting nomenclatural changes.

Smith et al. 1994 name	Describer(s)	Status change	Proposed new status
aleucosticha	Correa & Schwartz, 1986	stat. n.	*hysius aleucosticha*
amazona	González, 1987	stat. n.	*grannus amazona*
arcas	M. Bates, 1939		
archebates	(Ménétriés, 1832) (Satyrus)		
batesi	Michener, 1943		
chrysaoros	M. Bates, 1935		
confusa	Lathy, 1899		
confusa debarriera	Clench, 1943	stat. n.	*debarriera*
crypta	Gali, 1985		
dystacta	González, 1987	stat. n.	*grannus dystacta*
eleleus	M. Bates, 1935		
franciscoi	Gali, 1985		
gonzalezi	Schwartz, 1988	syn. n.	*debarriera*
grannus	M. Bates, 1939		
grannus dilemma	González, 1987		
herophile	Hübner, [1823]		
hysius	(Godart, [1824]) (Satyrus)		
lyceius	M. Bates, 1935		
micheneri	Clench, 1944, repl. name	stat. n.	*grannus micheneri*
micrommata	Schwartz & Gali, 1984	stat. n.	*grannus micrommata*
montana	Clench, 1943	syn. n.	*debarriera*
neiba	Schwartz & Gali, 1984	syn. n.	*debarriera*
nubila	Lathy, 1899		
obscura	Michener, 1943		
phoinix	González, 1987	stat. n.	*grannus phoinix*
pulchella	Lathy, 1899		
pulchella darlingtoni	Clench, 1943		
raburni	Gali, 1985		
sommeri	Schwartz & Gali, 1984	stat. n.	*grannus sommeri*
tasajera	González, Schwartz & Wetherbee, 1991		
zangis	(Fabricius, 1775) (Papilio)		

isted as two non-interbreeding allopatric entities, which continued to maintain no or limited gene exchange following sugar cane introduction, but both were able to adapt a new hostplant. We suggest preserving subspecies status for these two entities until further research can be done, which should include multiple specimens from a number of populations, including studying this butterfly in its wild habitat in association with the native hostplant.

Munroe's view that *Calisto confusa* and *C. debarriera* stat. n. are good species is now supported by our DNA data. Munroe found differences only in aedeagus width/length ratio and immediately cast doubt on his finding: "No fresh material was examined, and such a difference might conceivably be the result of distortion of the preparations."

Munroe examined only four *debarriera* specimens, but stated that "in support of this evidence it may be noted that the material of *debarriera* comes from a limited altitude range, which is entirely contained in both the altitudinal and geographic range of the widely distributed *confusa*." In other words, Munroe, though only having available a few old collection specimens, already supposed that he was dealing with two sympatric taxa. Future workers reduced *debarriera* to subspecies (e. g., Smith et al. 1984) and even considered it a synonym of *confusa* after their peripatric/sympatric distribution became more and more evident. However, at the same time, additional representatives of *debarriera* were being described as separate species, such as *C. neiba* syn. n. and *C. gonzalezi* syn. n., based on aberrant isolated populations. Our DNA barcode analysis suggests that *confusa* and *debarriera* are indeed two reproductively isolated species, whose ranges overlap, perhaps as a result of secondary contact following initial speciation through niche partitioning, since *debarriera* is largely a highland species and *confusa* largely a lowland species. A similar confusing situation that existed within the *Calisto hysius* complex, which included *C. hysius*, *C. batesi* (often listed as *C. hysius batesi* (e. g., Munroe 1951)), and *C. aleucosticha* stat. n. is now resolved. *Calisto hysius* mostly occurs on the southern paleoisland (Fig. 5) and shows significant divergence from the mostly northern *C. batesi*, suggesting that these two are distinct species (Fig. 2). *Calisto aleucosticha*, which was described from a few aberrant females of *C. hysius* found on the northern paleoisland by Correa and Schwartz (1986), should be considered a subspecies of *C. hysius*.

Non-Hispaniolan island endemics (*Calisto nubila*, *C. zangis* and *C. herophile*) appear to be derived lineages of various Hispaniolan taxa, indicating several ancient dispersal events from Hispaniola to Puerto Rico, Cuba, and Jamaica. For instance, *Calisto herophile*, which occurs in Cuba and the Bahamas, appears to be a product of dispersal from Hispaniola of the widespread polyphagous *C. confusa* or its immediate ancestor. *Calisto nubila*, endemic to Puerto Rico, which bears morphological similarity to the rare and localized Hispaniolan *C. eleleus* Bates, 1935 (Fig. 3, Clade D), also most likely have originated by dispersal to Puerto Rico from the Hispaniolan clade. Divergence of these island isolates, though great, does not exceed divergence found within the island of Hispaniola, which suggests that they dispersed from Hispaniola when the genus was already undergoing diversification. The low diversity of species on non-Hispaniolan islands as well as the time-frame of *Calisto* evolution, suggests that such taxa arrived there by accidental dispersal, rather than by land bridges or vicariance as hypothesized previously by Miller and Miller (1989).

Calisto zangis, along with *C. pulchella* and *C. raburni* Gali, 1985, are the most morphologically divergent members of the genus in general wing pattern, male and female genitalic structures, and in the immature stages (at least for *pulchella*, for which life history has been studied) (Sourakov 1996, 1997). DNA barcodes also indicate that these three species are strongly separated, suggesting that the origin of Jamaican *C. zangis* is likely an ancient event. The fact that the *C. lyceius*/*C.tasajera* group of bunchgrass-feeding *Calisto* has a close affinity to cane-feeding *C. pulchella* and to the Jamaican *C. zangis*, together forming a clade sister to all other *Calisto*, is of great interest.

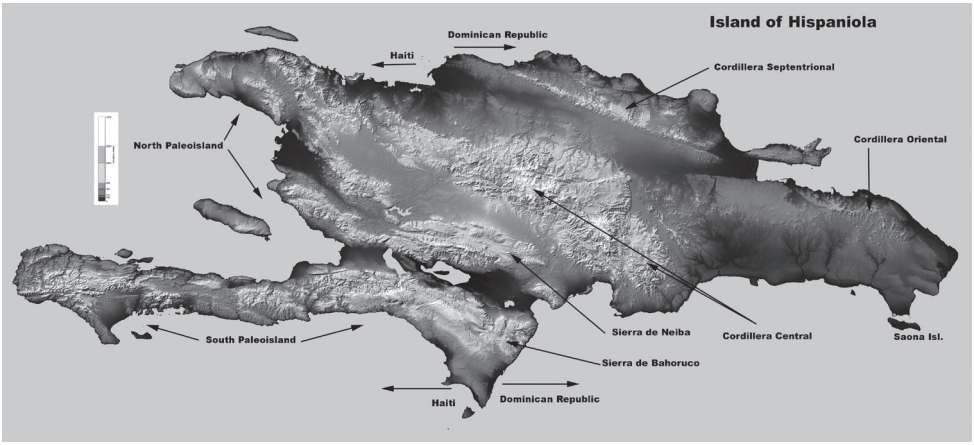

Figure 5. The island of Hispaniola, with some key geological features.

Although the bamboo-feeding *Eretris* were historically regarded as the closest relative to *Calisto* (Miller 1968), our results suggest that the south Andean genus *Auca* may be at least as closely related to *Calisto*, and we suspect that we need to search among lowland bunch-grass feeding satyrines for the closest mainland *Calisto* relative. Feeding on bunch-grasses in low elevation arid habitats may instead be the ancestral state in *Calisto* (e.g., the *C. lyceius* complex) that has been retained in other satyrine genera in Central and South America.

Our results highlight the usefulness of DNA-barcode analysis for routine species-level taxonomic work. DNA-barcoding allowed us to confirm previously observed morphological synapomorphies and test theories based on morphology and ecology alone. For example, the fact that the phenotypically divergent species *C. archebates* (Ménétriés, 1832), which has a yellow stripe traversing the hindwing underside, appeared as sister species to the *C. grannus*/*C. confusa*/*C. batesi* complex was already hypothesized based on immature stage morphology (Sourakov 1996). Further molecular research involving more genes is necessary to establish a robust phylogeny of *Calisto*.

The evolution of Satyrinae has been linked to the evolution and diversity of grasses (Peña and Wahlberg 2008). The DNA barcode divergence found in this study is associated with apparent ecological niche partitioning by species that inhabit a wide variety of habitats and utilize various host plants. We observe evolution of clades that is associated with shifts to new hostplant groups such as bunch grasses, bamboos, canes, etc. These clade-hostplant associations found today are shown in Fig. 2–4. For example, *Calisto arcas* and *C. chrysaoros* are two species whose adults are morphologically highly distinctive but whose life histories are poorly known. Sourakov (1996) described the eggs and first instar larvae of these two species and found that while the life history of *C. arcas* is surprisingly similar to many other *Calisto*, *C. chrysaoros*, which is strongly associated with bamboo, has egg and first instar larva that are highly divergent from the most common *Calisto* phenotype. In the present study, *C. arcas* formed a single clade in the middle of BI tree together with *C. confusa*/*C. neiba* complex (Fig. 3), which sup-

ports previously observed morphological synapomorphies. Yet, the average divergence of *C. arcas* from other *Calisto* (10–12%) is greater than that of *C. chrysaoros* (9–10%).

The butterfly fauna of Hispaniola has evidently been evolving for many millions of years. For instance, an extinct species of an extant neotropical genus of Riodinidae is known from Dominican fossil amber, dating from 15–25 Myr (Hall et al. 2004). Peña et al. (2011) suggested that *Calisto* might be a remnant of the initial colonization of South America by North American Satyrinae, in which case, *Calisto* might be a very old group. Several authors (e. g., Miller and Miller 1989) have suggested that much of the biological diversification found in *Calisto* may be associated with geological events. Indeed, the geological history of the Greater Antilles, the center of distribution for the genus, is complex. The archipelago originated more than 50 million years ago, and since then the component islands have undergone extensive metamorphosis, with Cuba and Hispaniola separating 20–25 million years ago (Pindell 1994). Though it is tempting to assume some role of geological events in speciation of *Calisto*, it has been shown repeatedly that adaptive radiation process is the main driving force behind evolution of species richness in the Caribbean (e.g., Losos et al. 2006). In our opinion, the genus shows a remarkable degree of diversification in comparison with other Caribbean clades, presumably because of low dispersal ability of these butterflies that interacts with topographic isolation within an island of Hispaniola and with exploitation of different habitats with varying rainfall patterns. Inter-island isolation, of course, also contributed to the overall diversity of the genus. However, it is the incredible diversity of habitats, ranging from the hot, dry deserts of the Hispaniolan lowlands to montane forests and grasslands at over 3000 m in elevation, that is responsible for the todays diversity of *Calisto*. These habitats are so variable due primarily to the high central mountain range, which creates strong gradients of temperature and rainfall. In harsher habitats (e. g., deserts, high mountain tops, peripheral localities (Fig. 6 shows two examples)) where numerous unique adaptations are necessary for survival, species may be very local, not spreading to neighboring areas despite the availability of unlimited resources and seeming absence of interspecific competition.

Butterflies, especially grass-feeding butterflies in such a hurricane-prone area, have thus had many chances to colonize every possible habitat and island through dispersal. Even though the genus appears more divergent than most other satyrine genera, it does not seem to be old enough to be influenced too much by geological events related to continental movement. Though recognizing the limited ability of a short DNA strand to give precise time estimates for observed divergence, most models assume that 1.5–3.5% divergence roughly equates to one million years of isolation (e. g., Brower 1994, Kandul et al. 2004, Papadopoulou et al. 2010, Vila et al. 2010). Hence, we can hypothesize based on available data that the genus *Calisto* underwent continuous diversification for some 4–8 Myrs, and thus ancient geological events of continental movement are unlikely to be a factor. Instead, it seems most likely that the diversification of *Calisto* into these numerous different habitats represents traditional Darwinian adaptive radiation, as suspected for other groups of Caribbean insects and vertebrates (e.g., Losos and Schluter 2000; Woods 1989; Liebher 1988).

Figure 6. Examples of habitat diversity on the island of Hispaniola. **A** Valle de Bao (1920 m elevation) at the foothill of Pico Duarte (3098 m elevation), covered with bunch grass, *Danthonia domingenis* - a hostplant of *Calisto tasajera* (top right) **B** Arid south eastern coastal habitat in Boca de Yuma, Altagracia provides an environment for sea oats, *Uniola virgata*, and associated *Calisto lyceius* (top right).

Figure 7. *Calisto montana* holotype (Museum of Comparative Zoology, Harvard, Massachusetts, USA).

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RESEARCH ARTICLE

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Genomic and karyotypic variation in *Drosophila* parasitoids (Hymenoptera, Cynipoidea, Figitidae)

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Abstract

Drosophila melanogaster Meigen, 1830 has served as a model insect for over a century. Sequencing of the 11 additional *Drosophila* Fallen, 1823 species marks substantial progress in comparative genomics of this genus. By comparison, practically nothing is known about the genome size or genome sequences of parasitic wasps of *Drosophila*. Here, we present the first comparative analysis of genome size and karyotype structures of *Drosophila* parasitoids of the *Leptopilina* Förster, 1869 and *Ganaspis* Förster, 1869 species. The gametic genome size of *Ganaspis xanthopoda* (Ashmead, 1896) is larger than those of the three *Leptopilina* species studied. The genome sizes of all parasitic wasps studied here are also larger than those known for all *Drosophila* species. Surprisingly, genome sizes of these *Drosophila* parasitoids exceed the average value known for all previously studied Hymenoptera. The haploid chromosome number of both *Leptopilina heterotoma* (Thomson, 1862) and *L. victoriae* Nordlander, 1980 is ten. A chromosomal fusion appears to have produced a distinct karyotype for *L. boulardi* (Barbotin, Carton et Keiner-Pillault, 1979) (n = 9), whose genome size is smaller than that of wasps of the *L. heterotoma* clade. Like *L. boulardi*, the haploid chromosome number for *G. xanthopoda* is also nine. Our studies reveal a positive, but non linear, correlation between the genome size and total chromosome length in *Drosophila* parasitoids. These *Drosophila* parasitoids differ widely in their host range, and utilize different infection strategies to overcome host defense. Their comparative genomics, in relation to their exceptionally well-characterized hosts, will prove to be valuable for understanding the molecular basis of the host-parasite arms race and how such mechanisms shape the genetic structures of insect communities.

Keywords
Drosophila, Figitidae, parasitoid, genome size, karyotype

Introduction

Each species has a characteristic genome size and chromosome number. This information often serves as a starting point for obtaining whole genome sequence. It is also useful for cytological or PCR-based genotyping and comparative genomics. *Drosophila melanogaster* Meigen, 1830 is by far the best-studied insect. Availability of its annotated sequence data (Flybase 2011) is facilitating rapid progress as details of novel gene functions are uncovered and analysis of gene interaction networks and pathways is becoming possible. Sequencing of the *D. melanogaster* genome also provided the baseline for the analysis of eleven additional *Drosophila* Fallen, 1823 species, spurring detailed investigation of the evolution of biological processes (Crosby et al. 2007).

Many species of *Drosophila* serve as hosts to parasitic wasps (Schlenke et al. 2007). In spite of spectacular progress on the model organism itself, practically nothing is known about the genomics or genetics of the parasitic wasps. *Leptopilina* Förster, 1869 and *Ganaspis* Förster, 1869 species (Figitidae) attack larval stages, emerge as free-living adults from the pupal cases of their hosts (Schilthuizen et al. 1998, Melk and Govind 1999, Allemand et al. 2002). *L. boulardi* (Barbotin, Carton et Keiner-Pillault, 1979) is a specialist parasitoid, while *L. hete*rotoma (Thomson, 1862) is a generalist; these species exhibit distinct strategies to evade or overcome host defense (Schlenke et al. 2007, Kraaijeveld and Godfray 2009, Lee et al. 2009). *Drosophila-Leptopilina* interactions have increasingly become important in understanding innate immunity against natural metazoan parasites and the molecular basis of the arms race between insect host/parasites (Chiu et al. 2006; Kraaijeveld and Godfray 2009, Lee et al. 2009, Paddibhatla et al. 2010).

Karyotypes of only two parasitic wasps attacking *Drosophila* spp., namely, *Leptopilina heterotoma* with n = 10 (Crozier 1975) and *L. clavipes* (Hartig, 1841) with n = 5 (Pannebakker et al. 2004) have been previously reported. These initial results indicate considerable karyotypic diversity within the *Leptopilina* genus, and related taxa. Here we describe the genome sizes and karyotypes of *Leptopilina* species from the *L. heterotoma* and *L. boulardi* clades, as well as that of *Ganaspis xanthopoda* (Ashmead, 1896), and discuss the relationship and significance of these observations.

Material and methods

Wasps were cultured on the *yw* strain of *D. melanogaster* as described in Sorrentino et al. (2004). Origins of the four larval parasitoids of *D. melanogaster*, namely: *Leptopilina boulardi*, *L. heterotoma*, *L. victoriae* Nordlander, 1980 and *Ganaspsis xanthopoda* are given in Table 1.

Table 1. Origins, genome sizes, and gross karyotypic data of *Drosophila* parasitoids. Genome size of wasp species correlates with total chromosomal length deduced from karyotypic analysis. The total length of the haploid *G. xanthopoda* chromosome set differs from both *L. boulardi* and *L. heterotoma* at p<0.001, and from *L. victoriae* at p <0.05; *L. boulardi* differs from both *L. heterotoma* and *L. victoriae* at p<0.001 (T-tests for independent samples).

Genus, species	Locality, strain	Genome size, mean±SE (Mb), no. specimens studied	Chromosome number, (n) 2n/no. (haploid) diploid specimens studied	Total length of haploid set, mean±SE (µm)/no. metaphases studied	Reference/note
Ganaspis xanthopoda	New York	971.5±6.7/4	(9)/(2)	87.7±8.3/3	Melk and Govind 1999
Leptopilina boulardi	G486	370.0±3.2/5	(9)18/(1)1	Not studied	Sorrentino et al. 2002
L. boulardi	17	362.8±1.7/5	(9)18/(7)4	38.6±3.0/7	Schlenke et al. 2007
L. boulardi	France	366.0±2.2/5	Not studied	Not studied	Lanot et al. 2001
L. boulardi	Average	366.3±2.4/15	(9)18/(8)5	38.6±3.0/7	Pooled data
Leptopilina heterotoma	New York	461.9±1.9/6	(10)20/(6)9	58.3±2.1/17	Chiu et al. 2006
L. heterotoma	14	460.0±1.4/5	(10)20/(3)5	Not studied	Schlenke et al. 2007
L. heterotoma	Average	460.9±1.7/11	(10)20/(9)14	58.3±2.1/17	Pooled data
L. victoriae	The Netherlands	520.2±0.8/5	(10)/(3)	63.1±4.5/5	Chiu et al. 2006
Leptopilina (genus)	Average	424.7±11.0/31	N/A/(20)19	54.4±2.3/29	Pooled data

Flow cytometric analysis of genome size, based on nuclei isolated from heads of females of three species of *Leptopilina*, and *Ganaspsis xanthopoda* was carried out as described before (Johnston et al. 2004), except that propidium iodide (PI) was added to each sample to a final concentration of 50 µg/ml (not 5µg/ml). Samples were prepared as follows: (A) Each wasp species alone, (B) *Drosophila* alone, and (C) 4-6 replicates of a wasp head and a *Drosophila* head prepared together, with mean genome size estimates and standard errors of those estimates based on the 4-6 co-preparations. DNA amount was determined as the ratio of the mean fluorescence of the sample 2C divided by the mean fluorescence of the *Drosophila* standard, multiplied by the genome size of the standard (1C *D. melanogaster* = 175 Mb, 1C *D. virilis* Sturtevant, 1916 = 333 Mb).

Chromosomal preparations for karyology were obtained from cerebral ganglia of male and female prepupae of parasitic wasps according to the technique used by Imai et al. (1988) with modifications. For an initial assessment of the main karyotypic features of *Leptopilina* spp., metaphase plates from a few preparations of *L. boulardi* and *L. heterotoma* were stained with Hoechst 33258 (0.2 µg/ml, Molecular Probes) for five minutes. Images were acquired with a Zeiss Laser 510 Scanning Confocal Microscope and formatted with Zeiss LSM5 software. For detailed karyotype analysis, haploid and diploid mitotic divisions were stained with Giemsa and photographed using Zeiss Axioskop 40 FL optic microscope fitted with an AxioCam MRc camera. Metaphase plates with the best chromosomal morphology were used to obtain karyograms. Chromosomes were classified into four groups (metacentrics, submetacentrics, subtelocentrics and acrocentrics) according to Levan et al. (1964). To obtain karyograms, digital

images of metaphase plates were processed with Adobe Photoshop. Measurements of chromosomes were taken using Zeiss AxioVision and then processed with STATISTICA (StatSoft Inc. 1995). Relative lengths of chromosomes (RL) were calculated as percentages of the ratio of a particular chromosome relative to total length of the haploid set. Centromere index (CI) is the percentage of the ratio of length of the short arm relative to total length of the particular chromosome.

Results

Genome sizes

The results of the study of genome sizes of the *Drosophila* parasitoids show almost no intraspecific variation, yet greater than 2.5-fold interspecific variation (Fig. 1; Table 1). The gametic genome size of *Ganaspis xanthopoda* (1 C = 971 Mb) is larger than that of any of the three *Leptopilina* species (370 Mb < 1C < 520 Mb) studied (Fig. 1). In turn, the genome sizes of all parasitic wasps studied in this paper are also larger than those known for all *Drosophila* species, which range from 1C = 136.5 to 331.5 Mb (Gregory and Johnston 2008).

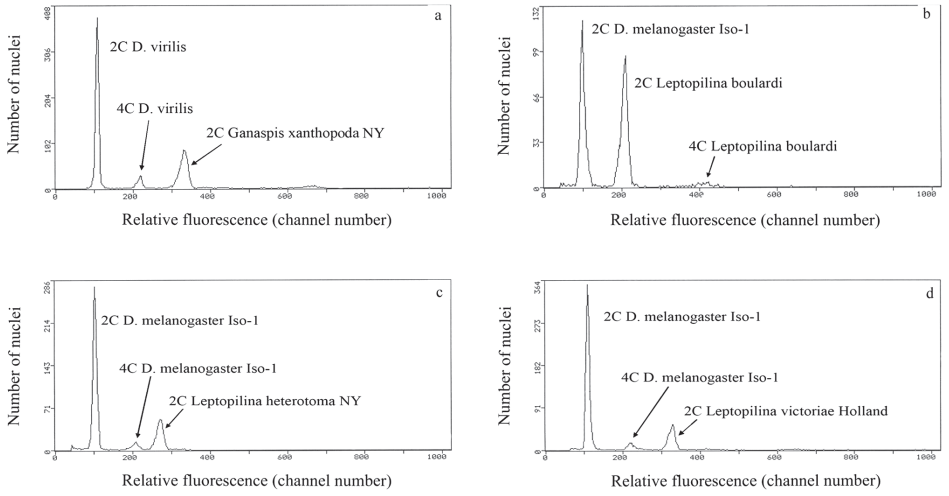

Figure 1. Cytograms showing relative fluorescence and total propidium iodide-stained nuclei of samples and standards to determine genome size. **a** relative fluorescence of PI-stained 2C nuclei from one head of a *Ganaspis xanthopoda* strain NY female co-prepared with 2C and 4C nuclei from one head of a *Drosophila virilis* female standard (1C = 333 Mb) **b–d** relative fluorescence and total PI stained nuclei of co-prepared *Leptopilina* and *D. melanogaster* (1C = 175 Mb) to determine genome size for *L. boulardi* (panel b), *L. heterotoma* (panel c), and *L. victoriae* (panel d). Genome size is calculated as follows: (mean fluorescence channel number of sample 2C peak/mean fluorescence channel number of 2C standard peak) X 1C DNA content of the standard, with the genome size mean and standard error calculated from repeat co-preparations using different individuals of each species.

Our results provide the first information on genome sizes not only of the family Figitidae, but of the superfamiy Cynipoidea as a whole. It is intriguing that the genome sizes of all these parasitoids exceed the average value known for previously studied Hymenoptera, i.e., 360.75 Mb (Tsutsui et al. 2008, Ardila-Garcia et al. 2010), but are fairly close to those of many Chalcidoidea (Tsutsui et al. 2008, Ardila-Garcia et al. 2010), the closest group to cynipoids (see Sharkey 2007).

Karyotypes

Total lengths of haploid chromosome sets of above mentioned species are given in Table 1. The relative lengths and centromere indices of all chromosomes are given in Table 2.

Ganaspis xanthopoda. Nine chromosomes were found in the haploid karyotype of this species (n = 9; Fig. 2a). Chromosomes are long relative to *Leptopilina* spp.

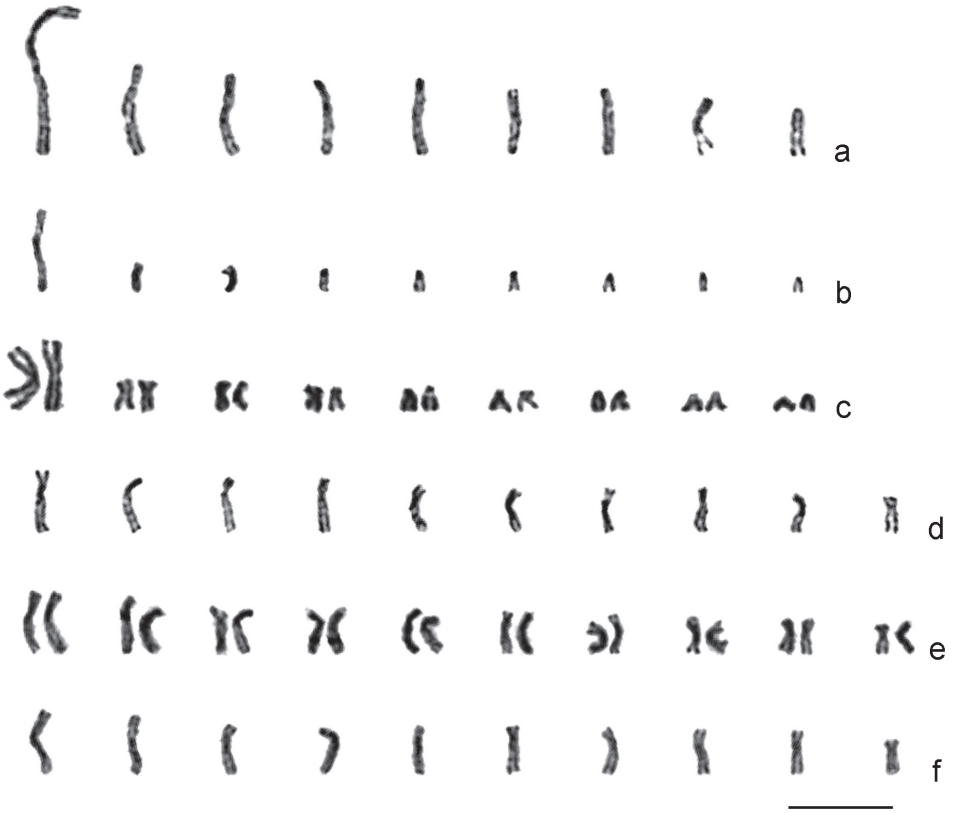

Figure 2. Karyograms of *Drosophila* parasitoids. **a** *Ganaspis xanthopoda*, haploid set **b** *Leptopilina boulardi* (strain 17), haploid set **c** ditto, diploid set **d** *L. heterotoma* (New York strain), haploid set **e** ditto, diploid set **f** *L. victoriae*, haploid set. Scale bar 10 μm.

Table 2. Relative lengths (RL) and centromere indices (CI) of *Drosophila* parasitoids. (mean±SE). Strains and numbers of studied metaphase plates are as in Table 1. Centromere indices are: metacentrics: 37.5-50.0; submetacentrics: 25.0-37.5; subtelocentrics: 12.5-25.0; acrocentrics: 0-12.5, according to Levan et al. (1964).

Species/ chromosome no.	*Ganaspis xanthopoda*		*Leptopilina boulardi*		*L. heterotoma*		*L. victoriae*	
	RL	CI	RL	CI	RL	CI	RL	CI
1	24.17± 0.77	37.50± 5.34	31.13± 0.81	39.97± 2.98	14.21± 0.32	28.26± 1.42	15.49± 0.21	39.27± 4.55
2	12.85± 0.25	20.50± 1.57	13.06± 0.37	35.13± 2.79	11.89± 0.16	30.03± 1.07	11.63± 0.20	30.46± 4.97
3	11.97± 0.11	20.14± 1.28	11.45± 0.27	29.86± 2.56	11.01± 0.11	28.58± 1.44	11.04± 0.17	32.95± 5.05
4	10.59± 0.32	19.80± 3.50	9.19± 0.17	21.03± 2.99	10.51± 0.82	27.72± 1.16	10.48± 0.19	31.88± 4.34
5	9.35± 0.61	22.25± 3.56	8.54± 0.19	18.66± 4.06	10.02± 0.69	28.90± 1.93	9.53± 0.13	33.69± 5.72
6	8.75± 0.43	35.55± 2.49	7.32± 0.19	17.33± 2.79	9.40± 0.11	33.17± 1.89	9.15± 0.13	31.12± 3.84
7	8.39± 0.15	15.86± 0.39	6.95± 0.15	11.86± 3.68	8.92± 0.11	32.12± 1.96	9.02± 0.11	34.72± 4.95
8	7.81± 0.42	43.48± 1.32	6.42± 0.10	13.91± 4.84	8.48± 0.12	30.16± 1.54	8.69± 0.08	41.12± 2.45
9	6.12± 0.10	1.44± 0.73	5.94± 0.13	8.77± 2.60	8.04± 0.11	28.70± 1.49	8.01± 0.26	34.88± 2.57
10	-	-	-	-	7.52± 0.09	31.93± 1.88	6.96± 0.28	36.19± 1.87

(see Table 1 and below); most of them are of similar size. However, the first meta- or submetacentric chromosome is about twice as long as the remaining ones. Most other chromosomes are subtelocentric, except for the sixth submetacentric, eighth metacentric, and last acrocentric ones.

Leptopilina boulardi. As in *G. xanthopoda*, n = 9 (and 2n = 18; Figs 2b and c; Fig. 3a). Moreover, the karyotype of *G. xanthopoda* is superficially similar to that of *L. boulardi* in that the very large first metacentric chromosome is more than twice as long as the second. However, the length of all remaining *L. boulardi* chromosomes is roughly half that of the *G. xanthopoda* chromosomes. Furthermore, chromosomes of the second and third pairs are submetacentric, those of the fourth, fifth, sixth and eighth pairs are subtelocentric, and chromosomes of the seventh and ninth pairs are acrocentric.

L. heterotoma. Consistent with previous observations (Crozier 1975), we found n = 10 and 2n = 20 in this species (Figs 2d and e; Fig. 3b). All chromosomes of the karyotype are submetacentrics that gradually decrease in size.

L. victoriae. This species belongs to the *L. heterotoma* clade (Allemand et al. 2002), and unsurprisingly, its karyotype is similar to that of *L. heterotoma*. The haploid karyotype of *L. victoriae* contains ten submetacentric or metacentric chromosomes (n = 10) of similar size (Fig. 2f). The first chromosome of *L. victoriae* is significantly longer and the fifth and tenth chromosomes are significantly shorter than the corresponding chro-

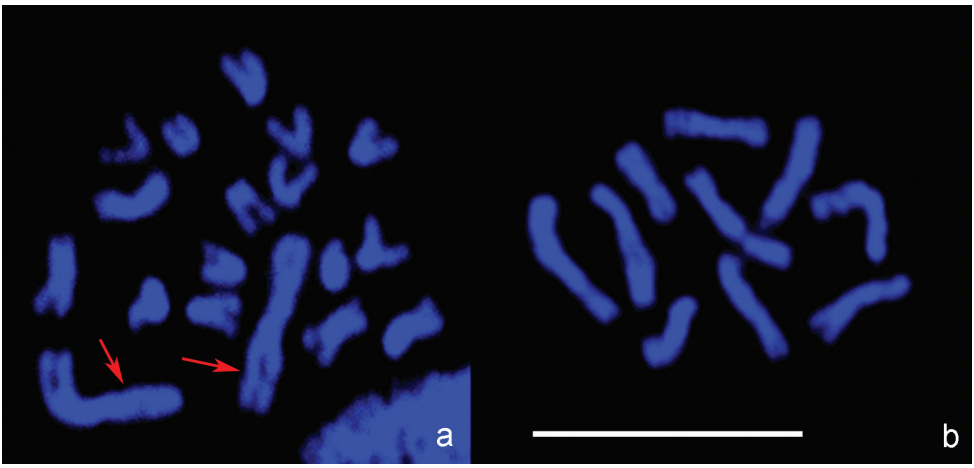

Figure 3. Confocal microscopic images. **a** *L. boulardi*, diploid metaphase plate **b**- *L. heterotoma*, haploid metaphase plate. Arrows point to the pair of large metacentric chromosomes in the karyotype of *L. boulardi* that presumably arose via chromosomal fusion in an ancestral chromosome set with n = 10. Scale bar 10 μm.

mosomes of *L. heterotoma*. In addition, the centromere position in the first and eighth submetacentric chromosomes is significantly different than observed for the apparently metacentric chromosomes of *L. victoriae*.

Discussion

Parasitic wasps make up a significant number of species of all insects (LaSalle and Gauld 1993). However, because of the complete absence of genomic information, the molecular biology and genetics of parasitic wasps of *Drosophila* have lagged behind, even though it is now possible to rapidly sequence genomes of organisms without prior genetic or genomic information.

In a study of genome size of 89 species of bees, wasps, and ants, Ardila-Garcia et al. (2010) hypothesized that genome sizes are constrained by traits associated with parasitism or eusociality. They however found that not all parasitoids have small genomes (Ardila-Garcia et al. 2010). So while it is not altogether surprising that the genomes of the koinobiont parasitoids of *Drosophila* studied here are as large as that of nonparasitic Hymenoptera, it is intriguing that they have such large genomes. Koinobionts keep their host alive; and must develop and emerge before their host is exhausted and dies. Small genomes replicate faster and require fewer resources, which imposes a selection cost on a bloated genome. An antagonist selective force must act on the parasitoid genome. Because of their obligate and intimate relationship with their fly hosts, it is possible that parasitic wasps take up, or share genetic information via transposons. Widespread transfer of genes laterally has recently been documented from *Wolbachia* Hertig, 1936 to insect or nematode genomes (Hotopp et al. 2007). *Wolbachia* has been

associated with many parasitic wasps of *Drosophila* (Vavre et al. 2009). Genomic sequence information will reveal if horizontal transmission of transposons, facilitated by the parasitic life style, may have contributed to the large genome size. In this scenario, different transposon types, with rapid turnover in the genome are expected.

Our karyotypic study provides new insights into the genome structure of *Drosophila* parasitoids. First, the study demonstrates an obvious positive correlation between the genome size and total chromosome length in those parasitic wasps (Table 1; Fig. 4). However, chromosome length in *G. xanthopoda* increases relatively slower than might be expected from its larger genome size (Fig. 4). This observation suggests that a significant portion of the bloated *Ganaspis* genome is repeat sequence that is highly condensed at metaphase. High copy number of satellite DNA is associated with genome size variation in *Drosophila* species (Bosco et al. 2007) and it is possible that a similar discrepancy in transposon or satellite DNA in the *G. xanthopoda* genome accounts for smaller than expected increase in chromosome length (Fig. 4).

Second, our study reveals that genome sizes vary independently of the chromosome number in *Drosophila* parasitoids. This may not be surprising if the large metacentric chromosomes of *L. boulardi* (Fig. 2b; Fig. 3a) and *G. xanthopoda* (Fig. 2a) have an independent origin via chromosomal fusions. Parallel chromosomal fusions are relatively frequent within various lineages of parasitic Hymenoptera (Gokhman 2004, 2009). In addition to *Ganaspis* and *Leptopilina*, chromosome numbers of *Phaenoglyphis villosa* (Hartig, 1841) (n = 10) and *Callaspidia defonscolombei* Dahlbom, 1842 (n = 11) from the same family have been studied (see Gokhman 2009). This information indicates that n = 10 (or

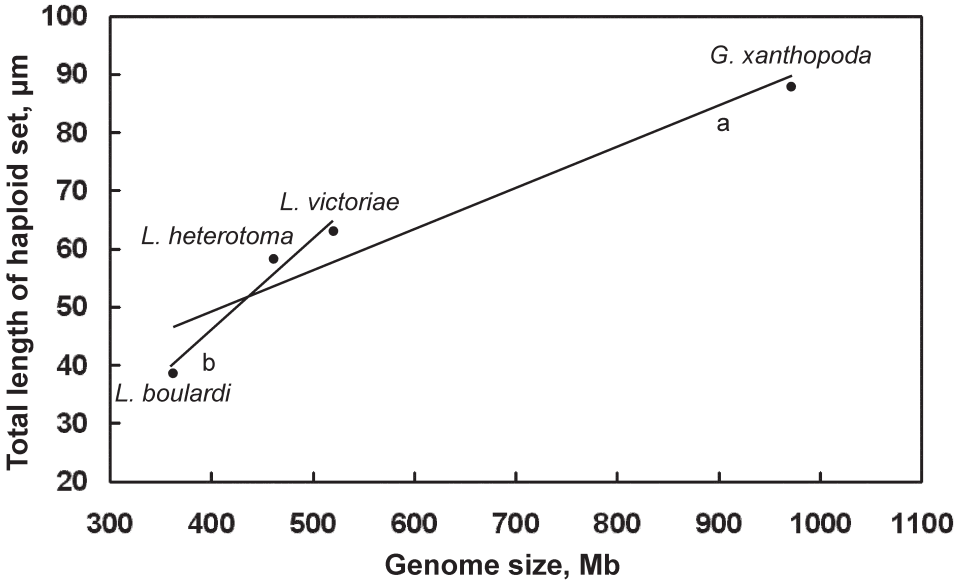

Figure 4. Distribution of genome size/chromosome length of *Drosophila* parasitoids. Mean values are given for each species. Trend lines: **a** for all species combined **b** for *Leptopilina* spp. (i.e. all species excluding *G. xanthopoda*).

a value close to 10) is likely to be an initial chromosome number for species of the *Leptopilina*/*Ganaspis* clade. If this is true, karyotypes with n = 9 found in *G. xanthopoda* and *L. boulardi* as well as that with n = 5 found in *L. clavipes* are likely to have resulted from chromosomal fusions and are therefore derived from a chromosome set that was probably similar to the karyotypes of *L. heterotoma* or *L. victoriae* (see also e.g. Gokhman 2010).

Third, the karyotype provides the scaffold number for future sequencing effort in these insects. When the karyotypic features of the species studied here are superimposed onto their phylogeny (Schilthuizen et al. 1998), clear correspondence is revealed: *L. heterotoma* and *L. victoriae* share very similar karyotypes, and are the most closely related species. In contrast, *L. boulardi* belongs to a distinct clade of the *Leptopilina* genus. Cytogenetic mapping of Expressed Sequence Tags, combined with restriction-site associated DNA (RAD) sequencing (Baird et al. 2008) based on the karyotype would ensure the highest quality genomic assembly, and pave the way for comparative genomics of parasitoid wasps of *Drosophila*. Such comparative genomics will provide insights into the organization of the host and parasitoid genomes and the co-evolution of these insects in nature.

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RESEARCH ARTICLE

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Karyotypic diversity in four species of the genus *Gymnotus* Linnaeus, 1758 (Teleostei, Gymnotiformes, Gymnotidae): physical mapping of ribosomal genes and telomeric sequences

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Abstract

Conventional (Giemsa, C-Banding, Ag-NORs, CMA3) and molecular (5S rDNA, 18S rDNA, telomeric sequences) cytogenetic studies were carried out in specimens of ten distinct fish populations of the genus *Gymnotus* (*G. sylvius* Albert and Fernandes-Matioli, 1999, *G. inaequilabiatus* Valenciennes, 1839, *G. pantherinus* Steindachner, 1908, and *G.* cf. *carapo* Linnaeus, 1758) from different Brazilian hydrographic basins. *G. sylvius* presented a diploid number of 40 chromosomes (22m+12sm+6st), *G. pantherinus* presented 52 chromosomes (32m+18sm+2st), while *G. inaequilabiatus* (42m+10sm+2a) and *G.* cf. *carapo* (38m+12sm+4st) presented 54 chromosomes. The C-banding technique revealed centromeric marks in all chromosomes of all species. Besides that, conspicuous blocks of heterochromatin were found interstitially on the chromosomes of *G. inaequilabiatus*, *G.* cf. *carapo*, and *G. pantherinus*. All four species showed single nucleolus organizing regions confirmed by results obtained through Ag-NORs and FISH experiments using 18S rDNA probes, which showed the NORs localized on the first chromosome pair in *G. inaequilabiatus*, *G.* cf. *carapo*, and *G. pantherinus*, and on pair 2 in *G. sylvius*. CMA_3 staining revealed additional unrelated NORs marks in *G. sylvius* and *G. pantherinus*. The 5S rDNA probes revealed signals on one pair in *G. sylvius* and two pairs in *G. pantherinus*; *G. inaequilabiatus* had about seventeen pairs marked, and *G.* cf. *carapo* had about fifteen pairs marked. It is considered that the high amount of heterochromatin

identified in the chromosomes of *G. inaequilabiatus* and *G.* cf. *carapo* could have facilitated the dispersion of 5S rDNA in these species. Interstitial signals were detected on the first metacentric pair of *G. sylvius* by telomeric probes (TTAGGG)*n* indicating the possible occurrence of chromosomal fusions in this species. The present study reveals valuable cytotaxonomic markers for this group and allows a more precise evaluation of the processes involved in the karyotype differentiation and the interrelationships among different species of the genus *Gymnotus*.

Keywords
FISH, rDNA, cytogenetics, heterochromatin, chromosomal rearrangements

Introduction

Fish species belonging to the order Gymnotiformes, usually known as "tuviras", "electric fish", or "banded knife-fishes", constitute a group endemic to the Neotropical region (Albert and Crampton 2003). This order holds more than 100 species and 27 genera that are grouped in five families: Gymnotidae, Rhamphichthyidae, Hypopomidae, Sternopygidae, and Apteronotidae (Mago-Leccia 1994). Among the Gymnotiformes, the karyotype diversity is better known in *Gymnotus* Linnaeus, 1758 and *Eigenmannia* Jordan and Evermann, 1896 genera (Albert and Crampton 2005).

Gymnotidae is currently composed of the genus *Gymnotus*, with 35 valid species, and *Electrophorus* Gill, 1864 with only one valid species (Froese and Pauly 2011). *Gymnotus* shows the widest geographic distribution in the group, occurring within inland waters of South and Central America, and is found from the Salado River, in the Argentinean "pampas" to San Nicolas River, Mexico, except Chile and Belize (Albert et al. 2005). The genus is more diversified in the Amazon River basin, where 19 species are known, including species not formally described (Crampton et al. 2005, Froese and Pauly 2011).

The available cytogenetics data for *Gymnotus* species evidence a high karyotypic diversity characterized by different diploid numbers observed in some species, as in *G. carapo* Linnaeus, 1758 and *G. inaequilabiatus* Valenciennes, 1839 with 54 chromosomes; *G. sylvius* Albert and Fernandes-Matioli, 1999 which shows 40 chromosomes; *G. pantherinus* Steindachner, 1908 with 52 chromosomes, and *G. capanema* Milhomem et al. in press with 34 chromosomes, the smallest diploid number observed for this genera so far (reviews: Margarido et al. 2007, Milhomem et al. in press). *Gymnotus pantanal* Fernandes-Matioli et al., 2005 presents 40 chromosomes in females and 39 in males, suggesting the occurrence of a multiple sex chromosome system in this species (Silva and Margarido 2005).

The current study was carried out aiming to broaden the cytogenetic data available for *Gymnotus*, mapping the distribution of ribosomal sites and telomeric DNA sequences on the chromosomes of different species of this genus. The data obtained will allow a better understanding of the mechanisms involved in the process of karyotypic differentiation and diversification of this fish group.

Material and methods

Four fish species of *Gymnotus* sampled throughout the different components of the Brazilian hydrographic river basins were cytogenetically analyzed (Figure 1 and Table 1). After analysis, the specimens were deposited in the fish collection of the Laboratório de Biologia e Genética de Peixes (LBP), Universidade Estadual Paulista, at Botucatu, São Paulo, Brazil.

The fishes were euthanized with a lethal dose of benzocaine before the procedures of chromosome preparation. Mitotic chromosome preparations were carried out according to Foresti et al. (1993). The nucleolus organizer regions (NORs) were localized on chromosomes by silver nitrate staining, according to Howell and Black (1980), and C-banding patterns were obtained following the protocol described by Sumner (1972).

Molecular cytogenetic analysis involved the use of GC-specific fluorochrome Chromomycin A_3 (CMA_3) (Schweizer 1976) and probes of specific gene sequences. Fluores-

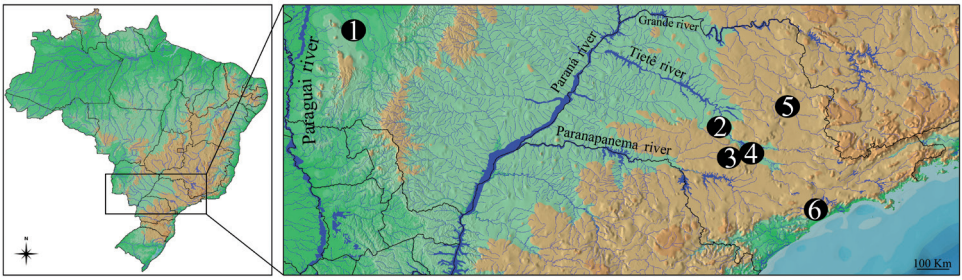

Figure 1. Map of Brazil showing the collection sites of species and populations of *Gymnotus* analyzed. **1** Miranda River, Passo do Lontra – MT, *G.* cf. *carapo* **2** Campo Novo River, Bauru – SP, *G. sylvius* and *G. inaequilabiatus* **3** Água da Madalena River, Botucatu – SP, *G. sylvius* and *G. inaequilabiatus* **4** Araquá River, Botucatu – SP, *G. sylvius* and *G. inaequilabiatus* **5** Mogi-Guaçu River, Pirassununga – SP, *G. sylvius* and *G. inaequilabiatus*; 6. Aguapeú River, Mongaguá – SP, *G. pantherinus*.

Table 1. Specimens of *Gymnotus* analyzed. LBP – deposit voucher number at the fish collection of the Laboratório de Biologia e Genética de Peixes, Instituto de Biociências de Botucatu, UNESP. F – females, M – males.

Species	LBP	Sample Localities	F	M	Coordinates
G. sylvius	11160	Água da Madalena - Botucatu-SP River	20	14	S22°59.25', W48°25.40'
G. sylvius	11155	Araquá – Botucatu-SP River	02	-	S22°47.13', W48°28.89'
G. sylvius	11163	Campo Novo- Bauru-SP River	01	01	S22°23.07', W49°00.55'
G. sylvius	11161	Mogi-Guaçu - Pirassununga-SP River	-	01	S21°55.50', W47°22.29'
G. inaequilabiatus	11154	Água da Madalena - Botucatu-SP River	02	07	S22°59.25', W48°25.40'
G. inaequilabiatus	11158	Araquá – Botucatu-SP River	04	02	S22°47.13', W48°28.89'
G. inaequilabiatus	11152	Campo Novo - Bauru-SP River	06	13	S22°23.07', W49°00.55'
G. inaequilabiatus	11156	Mogi-Guaçu - Pirassununga-SP River	06	17	S21°55.50', W47°22.29'
G. pantherinus	11153	Aguapeú - Mongaguá-SP River	03	02	S24°06.40', W46°43.00'
G. cf. *carapo*	9836	Miranda - Pantanal-MSRiver	03	02	S19°34.34', W57°02.17'

cent *in situ* hybridization was carried out to locate the rDNA genes on chromosomes, according to the procedure established by Pinkel et al. (1986) using stringency of 77%. The 18S rDNA probes were obtained by PCR (Polymerase Chain Reaction) from total DNA of *G.* cf. *carapo* using primers NS1 5'-GTAGTCATATGCTTGTCTC-3' and NS8 5'-TCCGCAGGTTCACCTACGGA-3' (White et al. 1990) and the 5S rDNA probes from total DNA of *Synbranchus marmoratus* Bloch, 1795 using the primers 5SA (5'- TACGCCCGATCTCGTCCGATC-3') and 5SB (5'-GCTGGTATGGC-CGTAGC-3') (Martins and Galetti Jr 1999). The 18S rDNA probe in *G. pantherinus* and 5S rDNA probes in *G. sylvius*, *G.* cf. *carapo* and *G. inaequilabiatus* were labeled with digoxigenin-11-dUTP (Roche Applied Science) by PCR and the detection of hybridization signs was obtained with anti-digoxigenin-rhodamine (Roche Applied Science). The 5S probe in *G. pantherinus* and 18S rDNA probes in *G. sylvius*, *G.* cf. *carapo* and *G. inaequilabiatus* were labeled with biotin-16-dUTP (Roche Applied Science) by PCR and the detection of hybridization signs with avidin-FITC. Telomeric sites were identified with probes for sequences $(TTAGGG)_5$ and $(CCCTAA)_5$ labeled with digoxigenin 11-dUTP (Roche Applied Science) and the hybridization signs were detected with anti-digoxigenin-rhodamine. Chromosome morphology was determined according to arm relationships proposed by Levan et al. (1964), and the chromosomes were arranged in decreasing size order in the karyotypes.

Results

Cytogenetic analysis performed in representatives of four *Gymnotus* fish species evidenced an expressive variation in the diploid number among the species, despite the conservative karyotypic feature among the representatives of the populations. *G. sylvius* presented 40 chromosomes (Fig. 2a); *G. inaequilabiatus* and *G.* cf. *carapo* presented 54 chromosomes (Figs 3a, 5a), and *G. pantherinus* presented 52 chromosomes (Fig. 4a). Data are summarized in Table 2.

The C-banding technique revealed significant differences in the distribution patterns of heterochromatin among the analyzed species. All populations of *G. sylvius* showed small amounts of constitutive heterochromatin restricted to the centromeric areas of all chromosomes, and also blocks coincident with NORs (Fig. 2b). In *G. inaequilabiatus* (Fig. 3b), *G. pantherinus* (Fig. 4b) and *G.* cf. *carapo* (Fig. 5b), besides centromeric and pericentromeric marks, it was possible to observe conspicuous interstitial blocks of heterochromatin in some chromosomes. No numerical or structural polymorphisms related to the presence of supernumerary or sex chromosomes were detected in the samples analyzed.

The impregnation by silver nitrate evidenced that all species and populations of *Gymnotus* analyzed hold a simple pair of chromosomes bearing NORs. The populations of *G. sylvius* showed signals at the interstitial region on the short arms of chromosome pair 2 (highlighted in Fig. 2a). The representatives of the other species showed their ribosomal sites located in the interstitial position on the short arms

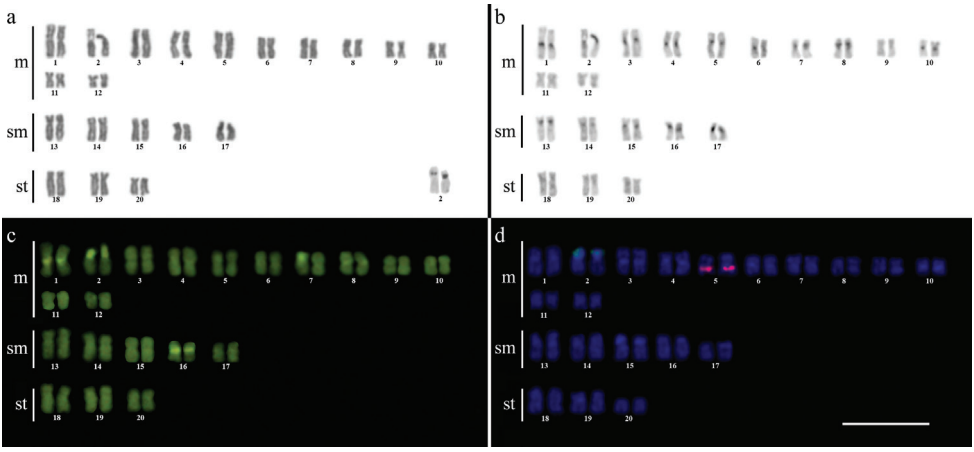

Figure 2a–d. Karyotype of *G. sylvius* after (**a**) conventional Giemsa staining, (**b**) C-banding, (**c**) CMA_3 fluorochrome staining, (**d**) double FISH with 5S rDNA (red) and 18S rDNA (green) probes. Bar = 10μm.

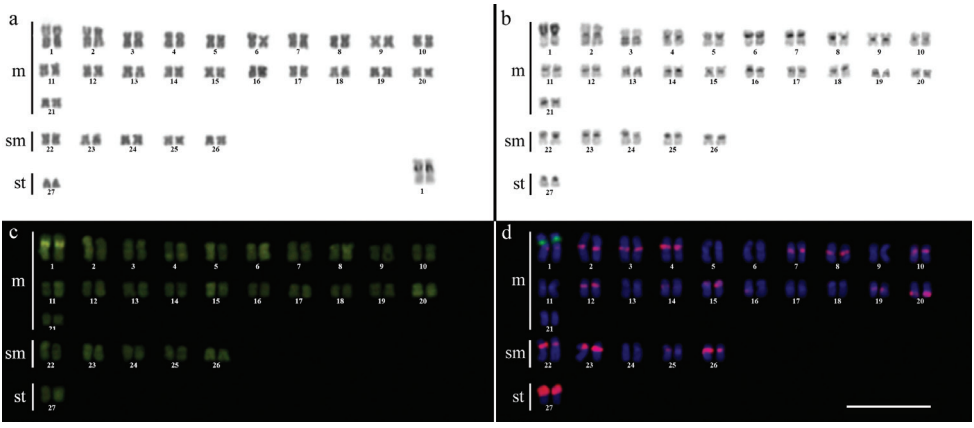

Figure 3a–d. Karyotype of *G. inaequilabiatus* after (**a**) conventional Giemsa staining, (**b**) C-banding, (**c**) CMA_3 fluorochrome staining, (**d**) double FISH with 5S rDNA (red) and 18S rDNA (green) probes. Bar = 10μm.

of chromosome pair number 1 (highlighted in Figs 3a–5a). The use of 18S rDNA probe confirmed the results achieved with silver nitrate staining (Figs 2d–5d), while the hybridization with 5S rDNA probes localized this gene in the pericentromeric position of pair number 4 in the representatives of *G. sylvius* populations (Fig. 2d); in two chromosome pairs (numbers 3 and 5) in *G. pantherinus* (Fig. 4d); in up to 17 chromosomal pairs in the representatives of *G. inaequilabiatus,* and in up to 15 pairs in *G.* cf. *carapo* (Figs 3d, 5d). The coloration with fluorochrome CMA_3 in *G. inaequilabiatus* and *G.* cf. *carapo* marked only the pair bearing the NORs (Figs 3c, 5c), while *G. sylvius* and *G. pantherinus* showed additional marked pairs besides the chromosomes bearing NORs (Figs 2c, 4c).

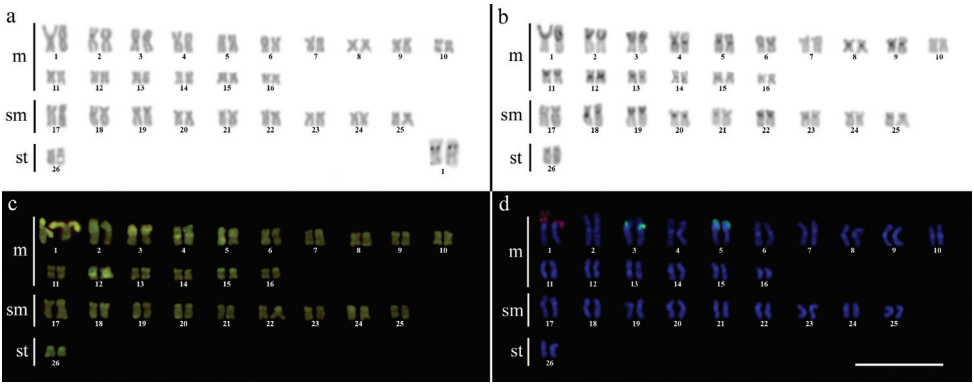

Figure 4a–d. Karyotype of *G. pantherinus* after (**a**) conventional Giemsa staining, (**b**) C-banding, (**c**) CMA_3 fluorochrome staining, (**d**) double FISH with 5S rDNA (green) and 18S rDNA (red) probes. Bar = 10μm.

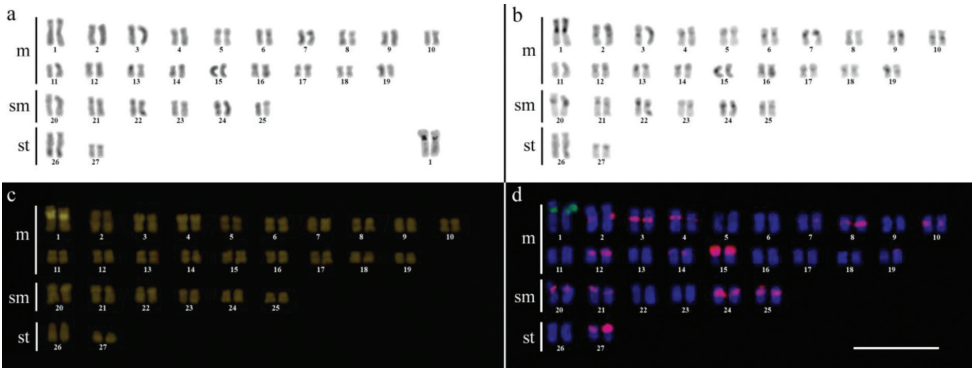

Figure 5a–d. Karyotype of *G.* cf. *carapo* after (**a**) conventional Giemsa staining, (**b**) C-banding, (**c**) CMA_3 fluorochrome staining, (**d**) double FISH with 5S rDNA (red) and 18S rDNA (green) probes. Bar = 10μm.

Table 2. Cytogenetic data on four species of *Gymnotus*. ITS – Interstitial Telomeric Sites; (I) – Interstitial mark.

Species	5S rDNA	18S rDNA	ITS	CMA_3	Karyotypic formulae
G. sylvius	Pair 4	2 (I)	Pair 1	Pairs 1,2 and 16	22m+12sm+6st
G. inaequilabiatus	Up to17 pairs	1 (I)	--	Pair 1	42m+10sm+2a
G. pantherinus	Pairs 3 and 5	1 (I)	--	Pairs 1,3, 4 and 12	32m+18sm+2st
G. cf. *carapo*	Up to 15 pairs	1 (I)	--	Pair 1	38m+12sm+4st

The use of telomeric probes $(TTAGGG)_n$ evidenced signals in the terminal position of all chromosomes in all populations analyzed (Fig. 6). Additionally, conspicuous marks were found along the nucleolar regions in the specimens of *G. inaequilabiatus* (Fig. 6b) and *G.* cf. *carapo* (Fig. 6c). Besides that, interstitial telomeric sites (ITS) were observed in the chromosomes of pair number 1 in *G. sylvius* (Fig. 6a). All data are summarized in Table 2 and represented in an ideogram in Figure 7.

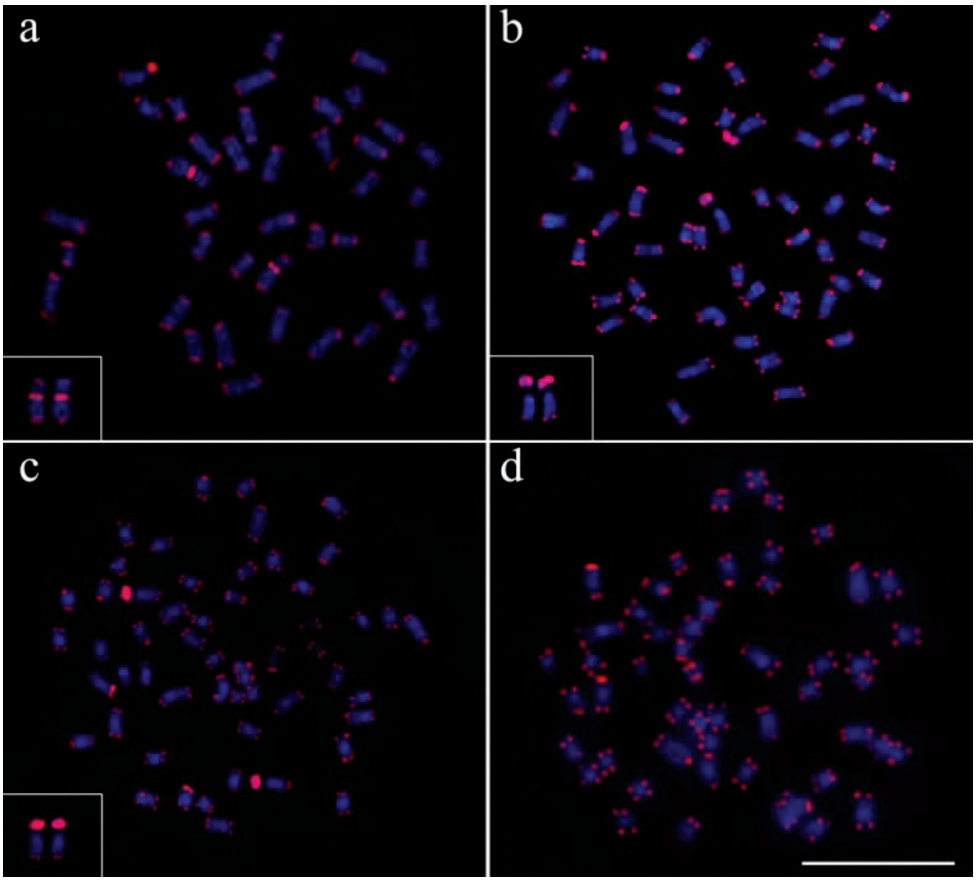

Figure 6a–d. Distribution pattern of telomeric sites in metaphases of the representatives of the four species of *Gymnotus* analyzed. (**a**) *G. sylvius* (featured interstitial telomeric sites – ITS), (**b**) *G. inaequilabiatus*, (**c**) *G.* cf. *carapo* and (**d**) *G. pantherinus*. Bar = 10μm.

Discussion

Available cytogenetic data on the genus *Gymnotus* evidence the occurrence of high karyotypic diversity among the species, notably related to diploid number and karyotypic formulae, ranging from 34 chromosomes in *G. capanema* up to 54 chromosomes in *G. inaequilabiatus* (Fernandes-Matioli et al. 1998, Milhomem et al. in press). In the present work, cytogenetic analysis performed in individuals of different populations of four species of *Gymnotus* confirmed the chromosomal variability, evidencing the occurrence of notable differences among the karyotypes of different species. However, a striking conservation of karyotypic features was observed among the different populations analyzed, mainly among populations of *G. sylvius* and *G. inaequilabiatus*.

The karyotype diversity found in the species may be related to the fact that the representatives of *Gymnotus* are generally endemic organisms living in headwaters, which

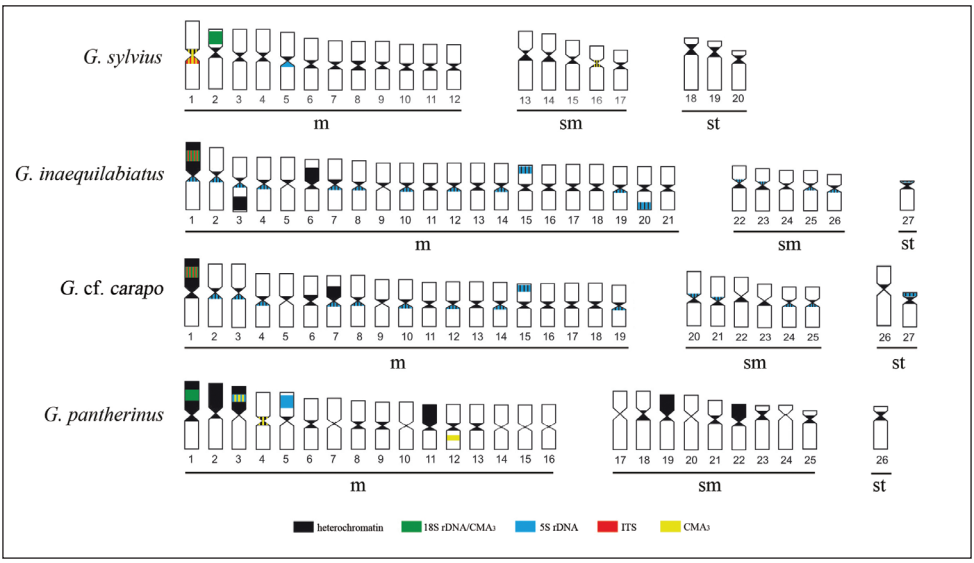

Figure 7a–d. Ideogram showing the hybridization patterns described in this paper. The overlapping signals are represented simultaneously by the respective colors (**a**) *G. sylvius*, (**b**) *G. inaequilabiatus*, (**c**) *G.* cf. *carapo* and (**d**) *G. pantherinus*.

do not migrate long distances. Such characteristic may act to reduce gene flow among different populations, even in the same hydrographic basin, resulting in differences between populations of the same species, as found in samples of *Characidium* Reinhardt, 1867 (Pansonato-Alves et al. 2010, 2011a). However, no significant differences were detected among the karyotypes in the different populations of the species analyzed. Despite the short-distance migratory behavior, the species of the genus *Gymnotus* are widely distributed throughout the Neotropical region and inhabit a wide diversity of environments, ranging from systems of flow rivers to flood plains (Albert et al. 2005, Albert and Crampton 2005). Thus, during the rainy seasons, the representatives of *Gymnotus* inhabiting flood plains could change location by passive dispersal, migrating from one part of the river to another favoring the maintenance of gene flow among populations of different river systems, as may have occurred among populations of *Hoplias malabaricus* Bloch, 1794 (Blanco et al. 2010).

The karyotypic identity observed among populations inside the species of *Gymnotus* reinforces the postulate that cytogenetic characteristics could be considered an important tool for taxonomic diagnostic of species in this fish group (Margarido et al. 2007).

The differences related to the number and morphology of the chromosomes found among the species suggest the occurrence of structural and numerical rearrangements during the process of differentiation. Milhomem et al. (2008) detected possible alterations in karyotype structure in representatives of *G. carapo* with 40 and 42 chromosomes from the Amazon river basin, while Claro and Almeida-Toledo (2010) found this same situation in *G. sylvius* (2n=40) and *G.* cf. *carapo* (2n=54) from the Paraná

river basin. These authors suggested that the differences found in the diploid number of these species might have arisen from chromosomal rearrangements, mainly centric fusions. Notwithstanding these authors' proposition, using whole chromosomes probes in the same sample analyzed by Milhomem et al. (2008), Nagamachi et al. (2010) established that the structural modifications found in the karyotypes could be far more complex than a result of simple fusion or centric fission events. The analysis also revealed that the representatives of the genus *Gymnotus* showed high genomic plasticity, and that the analyzed samples from the Amazon basin, usually denominated cryptic, were, in fact, distinct species.

In the current study, the probes used for telomeric sequence $(TTAGGG)_n$ revealed signals of hybridization on the extremities of all chromosomes in all populations analyzed (Figure 6). However, interstitial telomeric sites (ITSs) were observed in the chromosomes of *G. sylvius*. The presence of these ITSs in some chromosomes could be an indicative of recent centric fusion events, as previously discussed by Claro and Almeida-Toledo (2010) in *G. sylvius* and *G.* cf. *carapo* and Milhomem et al. (2008) in *G. carapo*. These authors proposed that chromosomal fusion events would act as the most important mechanisms of karyotype evolution in this fish group. Further studies by Claro and Almeida-Toledo (2010), using 5-BrdU incorporation in the study of *G. sylvius* and *G.* cf. *carapo* chromosomes, detected homologies among multiple chromosomes in these species, with a complete correspondence of bands, indicating a probable common ancestral origin.

The occurrence of ITS in some chromosomes of *G. sylvius*, as well as its absence in *G. pantherinus,* could be attributed to different factors, such as the occurrence of differences in the type of chromosomal rearrangements, the plasticity of the telomeric sequences or the divergence time of the species, which originated modifications in the sequences and possibly made them undetectable by the FISH technique. In a phylogenetic reorganization of the Gymnotiformes based on molecular and cytogenetic data, Fernandes-Matioli and Almeida-Toledo (2001) suggested that *G. sylvius* constitutes the most derived species amongst the representatives of *Gymnotus*, while *G. pantherinus* apparently differentiated much longer ago. The presence of the ITS in *G. sylvius* and its absence in *G. pantherinus* could be justified by the divergence time between these species. Furthermore, considering that a karyotype presenting 52 chromosomes would characterize the basal genomic group for *Gymnotus* (Fernandes-Matioli and Almeida-Toledo 2001), it could be expected that more chromosomes would present ITS in the karyotype of *G. sylvius*. Thence, the occurrence of only one pair of chromosomes with ITS in this species could be related to later modifications occurred in these sites, making it impossible to be detected by conventional FISH. Such situation is also proposed to occur in *G. capanema* (2n=34), a species with the smallest number of chromosomes within the genus *Gymnotus* with no ITS detected (Milhomem et al. in press). Another possible explanation to the absence of extra ITS in *G. sylvius* would be caused by a loss of the telomere repetition, which could have facilitated events of chromosome fusion (Blasco et al. 1997). This hypothesis also helps to explain why not all fused chromosomes have interstitial telomeric sites.

The identification of nucleolus organizer regions in the four species analyzed through silver nitrate staining and 18S rDNA probes revealed only one chromosome pair containing nucleolar sites and characterizing a simple NORs system, as previously cited (Fernandes-Matioli et al. 1997). The polymorphism in the size of the NOR sites among homologous chromosomes, which is commonly found in fish, was also detected in the species of the genus *Gymnotus* (Foresti et al. 1981, Fernandes-Matioli et al. 1997). These results indicate the conservatism of NORs in this group, not only for its location, generally on the first pair of chromosomes in multiple species studied, but also for its occurrence in only one pair of chromosomes, which characterizes a simple NORs system, a situation also found in other fish groups, such as the cichlids (Feldberg et al. 2003). Withal, analyzing three sympatric species of *Gymnotus*, the motile and dynamic character of these sites was confirmed and permitted identification species-specific Ag-NORs marks, which led the authors to consider this feature as an interesting cytotaxonomic tool (Lacerda and Maistro 2007).

The use of CMA_3 in metaphase chromosomes revealed additional marks to those identified in the ribosomal sites in *G. sylvius* and *G. pantherinus*, indicating the presence of additional GC-rich sequences. Gold and Amemiya (1986) affirmed that the treatment with CMA_3 would mark active and inactive ribosomal sites. Nevertheless, the presence of additional GC-rich sequences in chromosomes without ribosomal cístrons in *G. sylvius* and *G. pantherinus* indicates heterochromatin heterogeneity between these species. The heterochromatin is mainly composed of repetitive DNA sequences, which is thought to evolve in parallel (Dover 1986), resulting in homogenization of sequences within species (Ugarkovic and Plohl 2002). In this way, the distinct heterochromatin differentiation processes in different species of *Gymnotus* could have originated such patterns of CMA_3 staining.

The distribution patterns of 5S rDNA sequences showed a peculiar dispersion of these repetitive sites in the karyotypes of the species in the genus *Gymnotus*. Considering the great blocks of heterochromatin and the variation in the distribution pattern of 5S rDNA sites in *G. inaequilabiatus*, *G.* cf. *carapo,* and even in *G. pantherinus*, it can be considered that this situation might have favored the occurrence of structural rearrangements in the karyotype of these species, since heterochromatic areas are more propitious to breaks, and thus may facilitate the dispersion of this gene sequence. The absence of large heterochromatic blocks and the presence of 5S rDNA in a unique pair of chromosomes in *G. sylvius* could reinforce this hypothesis. Martins and Galletti (2001) related the existence of two classes of 5S rDNA located in different chromosomes of fish belonging to *Leporinus* Spix, 1829 genus. Considering the high degree of dispersion of 5S rDNA in *G. inaequilabiatus* and *G.* cf. *carapo* species, it is possible that different classes of this ribosomal gene might be differentially distributed in the chromosomes of this species. On the other hand, in *G. pantherinus* and *G. sylvius*, the distinct classes of 5S rDNA could be co-located in the same chromosomes.

Recent studies carried out by Cioffi et al. (2010) suggest that transposable elements of *Rex3* retrotransposon type might be associated to the distribution and dispersion of 5S rDNA in the karyotypes of species in the genus *Erythrinus* Bloch and Schneider,

1801. However, the existence of pseudogenes in the genome of *G. inaequilabiatus* and *G.* cf. *carapo* cannot be discarded, since the heterochromatin could have sequences similar to those of 5S rDNA, as suggested to occur in fish belonging to the genus *Characidium* (Pansonato-Alves et al. 2011b) and *Centropyge* Kaup, 1860 (Affonso and Galetti 2005).

Despite the marked karyotypic conservation found inside the populations of *Gymnotus* species, the results achieved in the current work revealed great differences in the chromosome structure in the species of this genus, indicating that all possible evolution ways passed through the differentiation process of chromosomes.

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RESEARCH ARTICLE

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Cytogenetic comparison between two allopatric populations of *Astyanax altiparanae* Garutti et Britski, 2000 (Teleostei, Characidae), with emphasis on the localization of 18S and 5S rDNA

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Abstract

Two populations of *Astyanax altiparanae* (Garutti et Britski, 2000) of the Água dos Patos stream/SP and lake Igapó/PR were analyzed. All individuals showed 2n = 50, however, different karyotypic formulae were observed. The population of the Água dos Patos stream showed 8m +24sm+6st+12a (NF=88) and the population of lake Igapó, 8m+28sm+4st+10a (NF=90). Nucleolus organizing regions (AgNORs) were observed in the terminal position on the short and long arm of different chromosomes of both populations, showing a variation from 3 to 4 chromosomes. Fluorescent *in situ* hybridization (FISH) using 18S rDNA probes revealed only one pair of chromosomes with fluorescent signals in the terminal site on the short arm in the Igapó lake population, while the population of Água dos Patos stream showed 4 fluorescence terminal signals, characterizing a system of simple and multiple NORs, respectively. 5S rDNA fluorescent signals were detected in the interstitial position of a pair of chromosomes in the two studied populations. Some AgNOR sites revealed to be GC-rich when stained with Chromomycin A_3 (CMA_3), however, AT positive regions were not observed. The data obtained show that, despite the conservation of the diploid number and location of 5S DNAr, differences in both the distribution of 18S rDNA and karyotypic formula among the populations were found, thus corroborating the existing data on chromosome variability in *Astyanax altiparanae* that can be significant for cytotaxonomy in this group.

Keywords

Teleostei Characidae, 18S rDNA, 5S rDNA, FISH, karyotypic formula, NORs

Introduction

Astyanax Baird et Girard, 1854, the most common and diversified genus within the family Characidae, has a wide distribution in the Neotropical Region. Due to lack of evidence of monophyly, the genus *Astyanax* is thought to belong to the Incertae sedis group (Lima et al. 2003). Moreover, the presence of several similarities among the species of this genus allows several species to be considered as a compound from a taxonomic viewpoint (Garutti and Britski 2000).

In *Astyanax altiparanae* (Garutti et Britski, 2000) from the upper Parana river basin, previously identified as *Astyanax bimaculatus* (Linnaeus, 1758), all cytogenetic studies accomplished so far reported the occurrence of 2n = 50, with differences in the karyotypic formula among the analyzed populations (Ferreira Neto et al. 2009), which can be explained by the occurrence of chromosome rearrangements, such as pericentric inversions (Domingues et al. 2007).

Besides the differences in karyotypic formula, the nucleolus organizer regions in this species also vary in relation to number and position, as observed by Fernandes and Martins-Santos (2006a), Domingues et al. (2007), Ferreira Neto et al. (2009). However, the same authors found evidence for conservation in relation to the location and number of fluorescent signals of 5S rDNA sites located in the interstitial region of one chromosome pair.

In view of the great chromosome variation observed by other authors in the genus *Astyanax*, the objective of the present work was to characterize the karyotypes of two populations of *Astyanax altiparanae*, with emphasis on the location of 18S and 5S DNAr sites, and compare them with data contained in the literature, in an endeavor toward a better understanding of chromosome evolution within this fish group.

Material and methods

Two populations of *Astyanax altiparanae* were cytogenetically analyzed: twelve specimens (3 males and 9 females) from Água dos Patos stream (22°41'17.7"S; 51° 05'23.9"W), municipality of Iêpe/SP and sixteen specimens (9 males and 7 females) from Igapó lake (23°19'09.38"S; 51°11'44.72"W), municipality of Londrina/PR (Fig. 1). Specimens were deposited in the Museum of Zoology of the Universidade Estadual de Londrina (MZUEL). The samples were collected with the permission of Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA), protocol number 11399-1. This study was approved by the ethics committee of our institution and meets all requirements of the Brazilian environmental laws.

Conventional staining. The specimens were sacrificed after being anesthetized with a solution of benzocaine. Metaphase chromosomes were obtained from kidney cells according to the air drying technique (Bertollo et al. 1978) and stained with 5% Giemsa in phosphate buffer (pH 6.8) The chromosomes were organized as metacentric (m), submetacentric (sm), subtelocentric (st) and acrocentric (a) for the preparation

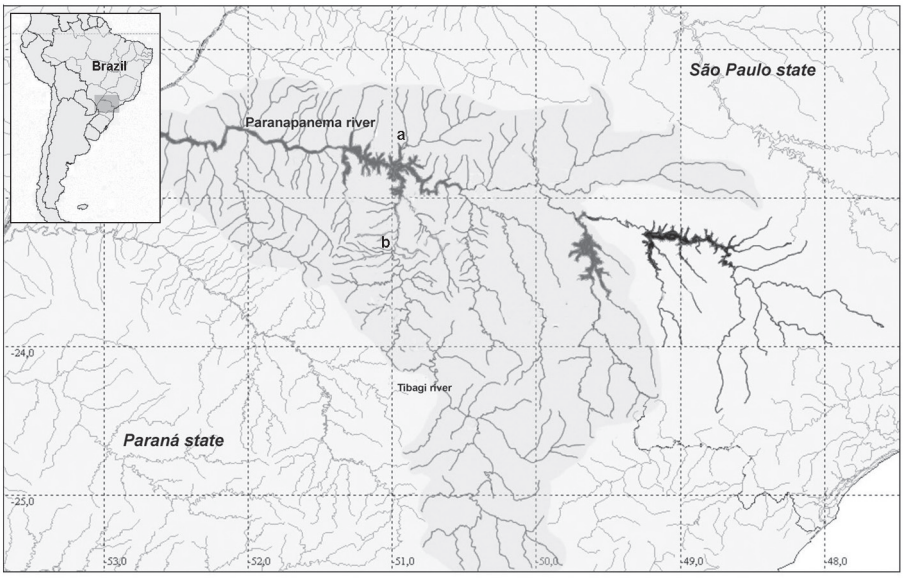

Figure 1. Collection sites of studied specimens. Map of Brazil showing the Paraná and São Paulo states in the selected area (left side). Hydrographic map showing the Paranapanema and Tibagi rivers. In (a) Água dos Patos stream and (b) Igapó lake.

of a karyogram. Metacentric, submetacentric, and subtelocentric chromosomes were considered biarmed and acrocentric uniarmed for determination of the fundamental number (FN) according to Levan et al. (1964).

Fluorescent in situ hybridization (FISH). The *in situ* hybridization procedure was performed according to Swarça et al. (2001). The 18S rDNA probe of *Prochilodus argenteus* Agassiz, 1829 (Hatanaka and Galetti Jr., 2004) and 5S rDNA of *Leporinus elongatus* Valenciennes, 1850 (Martins and Galetti Jr., 1999) were labeled with biotin-14-dATP by nick translation and used as probes. Slides were treated with 30 µl of hybridization mixture (stringency of 70%) containing 100 ng of labeled probe (4 µl), 50% formamide (15 µl), 50% polyethylene glycol (6 µl), 20´ SSC (3 µl), 100 ng of calf thymus DNA (1 µl) and 10% SDS (1 µl). The probe was denatured at 90°C for 10 min, and hybridization was performed overnight at 37°C in a humidified chamber. Post-hybridization washes were carried out in 2´ SSC, 20% formamide in 0.1´ SSC, 0.1´ SSC and 4´ SSC/0.2% Tween 20, all at 42°C. The probe was detected with a solution of 5% BSA and FITC-conjugated avidin (50:0.5, v:v). The post-detection washes were performed in 4´ SSC/0.2% Tween 20 at room temperature. Slides were mounted with 25 µl of a medium composed of 23 ml of DABCO solution (1,4-diaza- bicyclo (2.2.2)-octane (2,3%), 20 mM Tris HCl, pH 8.0, (2%) and glycerol (90%), in distilled water), 1 ml of 2 mg/ml DAPI and 1 ml of 50 mM $MgCl_2$.

Chromosome banding. Active nucleolus organizer regions (NORs) were detected by silver nitrate staining (Howell and Black, 1980). The GC- and AT-rich bands were detected with chromomycin A_3 (CMA_3) and 4'-6-diamino-2-phenylindole (DAPI),

respectively, according to Schweizer (1976). The slides were stained with 0.5 mg/mL CMA_3 for 1 h, washed in distilled water and sequentially stained with 2 µg/ml DAPI for 15 min. Slides were mounted with a medium composed of glycerol/McIlvaine buffer (pH 7.0) 1:1, plus 2.5 mM $MgCl_2$.

All the images were acquired with a Leica DM 4500 B microscope equipped with a DFC 300FX camera and Leica IM50 4.0 software, and optimized for best constrast and brightness with iGrafx Image software.

Results

The two populations of *Astyanax altiparanae* showed 2n = 50, however, different karyotypic formula were evidenced. The population of the Água dos Patos stream showed 8m+24sm+6st+12a (NF=88) (Fig. 2a) and the population of Igapó lake 8m+ 28sm+ 4st+10a (NF=90) (Fig. 2b).

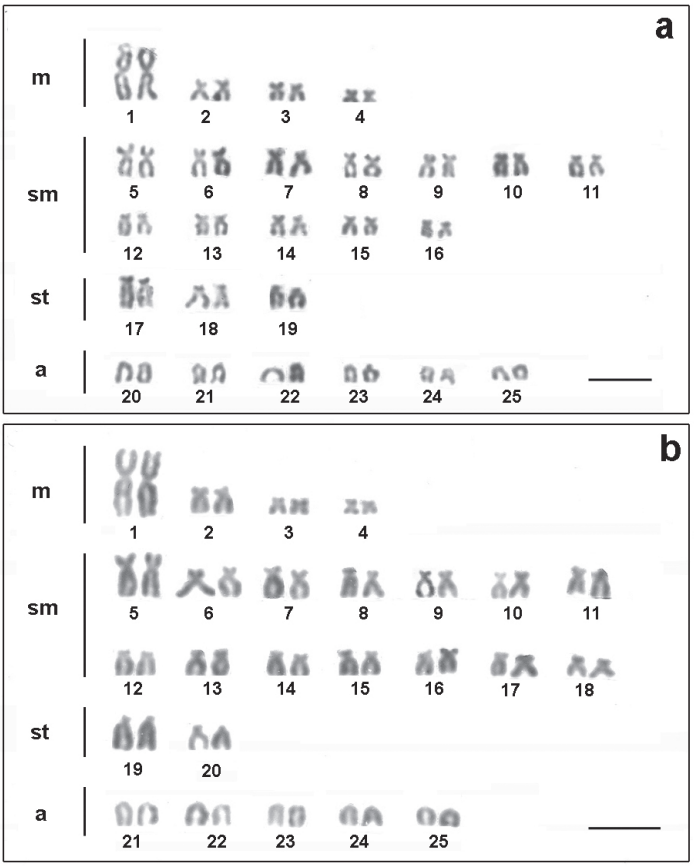

Figure 2. Karyotypes of *Astyanax altiparanae* after conventional Giemsa staining **a** Água dos Patos stream **b** Igapó lake. Bar= 5µm.

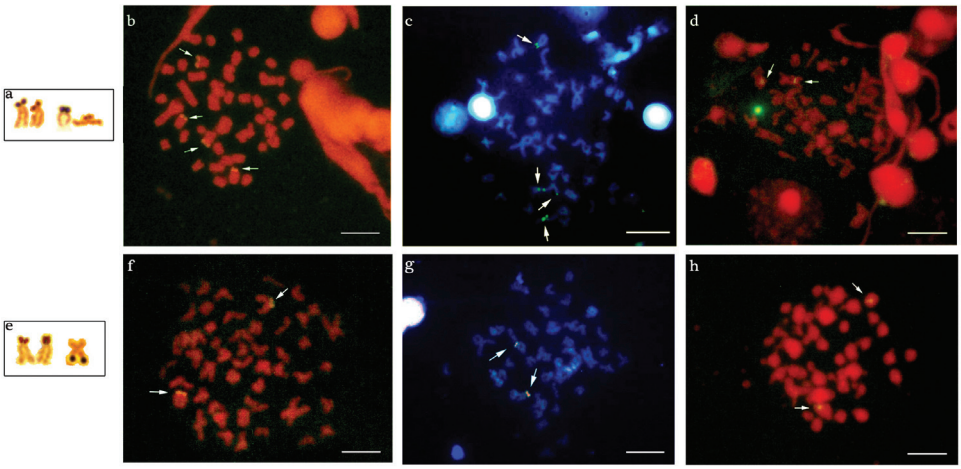

Figure 3. Chromosomes of *Astyanax altiparanae* bearing AgNORs sites: **a** Água dos Patos stream e Igapó lake. Metaphase chromosomes with the arrows showing the 18S rDNA, CMA3/DAPI and 5S rDNA sites in Astyanax altiparanae, respectively: **b, c, d** Água dos Patos stream **f, g, h** Igapó lake. Bar = 5µm.

Multiple NORs sites were detected in the two populations by silver nitrate impregnation, revealing inter- and intra-individual variations. The population of the Água dos Patos stream showes 2 to 4 AgNORs on the short arm, in two equal medium-sized subtelocentric chromosomes, one of which revealed size heteromorphism observed in all metaphases (Fig. 3a).

One to three AgNORs were detected in the population of Igapó lake: a medium-sized submetacentric chromosome with markings on the long arm and also a medium-sized subtelocentric chromosome with signal on the short arm, presenting a small size heteromorphism. The latter was observed in almost all metaphases (Fig. 3e).

The population of the Água dos Patos stream showed 4 markings on the short arm of two medium-sized subtelocentric chromosome pairs after fluorescent in situ hybridization (FISH) with 18S rDNA probe (Fig. 3b). In the population of Igapó lake, only one pair of medium-sized subtelocentric chromosome presenting fluorescent signals on the short arm was detected (Fig. 3f). Both populations exhibited one pair of chromosomes, bearers of the 5S DNAr sites, in the interstitial position (Fig. 3d and h).

In the population of the Água dos Patos stream, 2 to 4 chromosomes with CMA_3^+ terminal blocks were detected: a medium-sized subtelocentric pair with signals of size heteromorphism on the short arm, most frequently visualized in the metaphases; a small-sized acrocentric chromosome with a marking on the short arm; and a medium-sized subtelocentric chromosome with a signal on the long arm (Fig. 3c). In the population of Igapó lake, CMA_3 markings were detected on the short arm of only one pair of medium-sized subtelocentric chromosomes, which disclosed size heteromorphism (Fig. 3g).

The treatment of the chromosomal preparations of the two populations with DAPI showed a homogeneous staining region, and no regions rich in AT base pairs were detected, as can be seen through the superposition of these fluorochrome, as shown in Fig. 3c-g.

Discussion

Cytogenetic studies in *Astyanax altiparanae* from the Água dos Patos stream and Igapó lake revealed a conserved diploid number that has been observed within all the analyzed populations of this species (Table 1) so far. However, differences in the karyotypic formula were found in some of these populations, including the one observed in the present study, probably due to occurrence of chromosomal rearrangements, such as pericentric inversions, thus revealing a variability in the karyotypic macrostructure among the species of this group of fish.

The great variability in the karyotypic macrostructure is also reflected in other chromosome marks of *Astyanax altiparanae*. Variability in AgNOR sites with respect to the number, location and types of chromosomes bearers of such sites is frequently evidenced in this species (Table 1), as corroborated by the present study. Some authors consider that such variations can be ascribed to chromosomal rearrangements and transfer of ribosomic sites (Fernandes and Martins-Santos 2006a, Peres et al. 2008) However, in several cases, transposition events have been held liable for that variability of NORs in the genoma of these animals (Mantovani et al. 2000).

It is worth noting that an AgNORs pair was the most frequently found in the chromosome preparations of the populations analyzed herein. It can be considered a main pair with NOR always active, together with secondary sites, as observed by Pazza et al. (2006) in *Astyanax fasciatus* (Cuvier, 1819).

After FISH with 18S rDNA probe, *Astyanax altiparanae* of Água dos Patos stream showed two chromosome pairs with fluorescent signals on the short arms, coinciding with the sites detected by silver impregnation. The population of Igapó lake, however, showed only one chromosome pair with fluorescent signals on the short arm, coinciding with a pair frequently identified by silver nitrate, thus characterizing a system of simple NORs. The other markings, which had not been identified by FISH, but were observed in this population after the impregnation with silver nitrate, are probably heterochromatic sites with acid proteins that have affinity to silver. Despite the fact that multiple NORs are a common condition among *Astyanax altiparanae* (Table 1), Domingues et al. (2007) and Ferreira Neto et al. (2009) also found a system of simple NORs confirmed by FISH in different populations of this species.

Multiple 18S rDNA sites were also found among other species of the genus *Astyanax*, such as *Astyanax scabripinnis* (Jenyns, 1842) (Souza et al. 2001, Mantovani et al. 2005, Fernandes and Martins-Santos, 2006b) and *Astyanax fasciatus* (Pazza et al. 2006), which are, therefore, a characteristic of this group of fish.

The CMA_3^+ sites of *Astyanax altiparanae* of Igapó lake and Água dos Patos stream were consistent with those marked by silver, however, the AgNORs not detected through CMA_3 in the individuals of Igapó lake may be very small and not detectable by this fluorochrome, or else not all NORs are rich in GC, as suggested by Artoni et al. (1999).

The Ag-NOR heteromorphism observed in the two populations was not seen by FISH, thence, it should be related to the expression of the genes or be ascribable to a

Table 1. Cytogenetic data of different populations of *Astyanax altiparanae.* FN: fundamental number; m: metacentric; sm: submetacentric; st: subtelocentric; a: acrocentric; AgNORs: nucleolar organizer regions; Ref: Reference; PR: Paraná; SP: São Paulo

Locality	2n	FN	Chromosome formulae	AgNORs	CMA_3	18S/28S	5S	Ref.
Mogi Guaçu river/SP	50	88	10m+24sm+4st+12a					1
	50	92	6m+24sm+12st+8a	1 a 5				2
Paranapanema river/SP	50	88	10m+22sm+6st+12a					3
Tibagi river/Sertanópolis/PR	50	90	10m+22sm+8st+10a	2 a 5	5			4
Tibagi river/Limoeiro/PR	50	86	6m+22sm+8st+14a	2 a 5	5			4
Tibagi river/Limoeiro/PR	50	88	10m+22sm+6st+12a	2 a 5	5			4
Couro de Boi river/PR	50	88	8m+20sm+10st+12a	1 a 4	6			4
Três Bocas stream/PR	50	92	10m+28sm+4st+8a	1 a 6	8			4
Claro river/PR	50	90	10m+26sm+4st+10a	1 a 4	6			5
		88	10m+24sm+4st+12a	1 a 4	6			5
		86	10m+22sm+4st+14a	1 a 4	6			5
Paraná river/PR	50	82	32m/sm+18st/a	3		4	2	6
Índios river/PR	50	90	6m+30sm+4st+10a	10	7			7
Paraná river/PR	50	88	6m+26sm+6st+12a	2	5	4	2	7,9
Tibagi river upper/PR	50	92	6m+28sm+8st+8a	4	7	7	2	8
Iguaçu river upper/PR	50	94	6m+30sm+8st+6a	2	4	2	2	8
Keçaba brook/PR	50	88	6m+26sm+6st+12a	3		7	2	9
Tatupeba brook/PR	50	88	6m+26sm+6st+12a	3		4	2	9
Maringá stream/PR	50	88	6m+26sm+6st+12a	1		4	2	9
	50	88	10m+22sm+6st+12a	2 a 3				10
Iguaçu river/PR	50	92	10m+26sm+6st+8a	2 a 5				10
Monjolinho river/SP	50	90	8m+20sm+12st+10a	2		2	2	11
Água dos Patos stream/SP	50	88	8m+24sm+6st+12a	2 a 4	2 a 4	4	2	12
Igapó lake/PR	50	90	8m+28sm+4st+10a	1 a 3	2	2	2	12

References: **1** Morelli et al. 1983 **2** Paganelli 1990 **3** Daniel-Silva and Almeida-Toledo 2001 **4** Pacheco 2001 **5** Pacheco et al. 2001 **6** Almeida-Toledo et al. 2002 **7** Fernandes and Martins-Santos 2004 **8** Domingues et al. 2007 **9** Fernandes and Martins-Santos 2006a **10** Abelini 2007 **11** Peres et al. 2008 **12** Present study.

larger amount of heterochromatin that insert in 18S DNAr cistrons, once it was detected by CMA_3 and by the impregnation with silver nitrate.

After staining with DAPI, markings were not observed in the chromosomes of the two populations of *A. altiparanae,* which, therefore, did not possess any region rich in AT bases. Rosa et al. (2009) used CMA_3 and DAPI fluorochromes in chromosome preparations of *Astyanax laticeps* (Cope, 1894) and observed the occurrence of NORs rich in GC and poor in AT bases, respectively.

In all the populations of *Astyanax altiparanae,* the 5S DNAr sites were located interstitially in one chromosome pair, demonstrating a high stability of those sites. This corroborates the data on other populations of *Astyanax altiparanae* (Table 1) and

of other species of the genus *Astyanax* (Almeida-Toledo et al. 2002, Fernandes and Martins-Santos 2006b, Pazza et al. 2006). The conservation of this pattern can be attributed to the interstitial location of those sites in the chromosomes, whereby the 5S DNAr is protected from the dispersion events that may occur with 45S DNAr, as proposed by Martins and Galetti Jr. (2001).

According to Ferreira Neto et al. (2009), the apparent karyotypic similarity among the populations of *Astyanax altiparanae* strongly suggests an intimate relationship among them. However, the small karyotypic variations detected indicate some evolutionary divergence, probably due to restrictions on gene flow. The data obtained confirm the occurrence of similarity in relation to diploid number and 5S rDNA location, however, differences in the karyotypic macrostructure and in the distribution of 18S rDNA sites are found among the populations. Thence, from the results obtained in this work we corroborate the existing data and, once again, confirm the great chromosome variability of *Astyanax altiparanae* that can be significant for cytotaxonomy in this group.

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RESEARCH ARTICLE

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Comparative chromosome mapping of the rRNA genes and telomeric repeats in three Italian pine voles of the *Microtus savii s.l.* complex (Rodentia, Cricetidae)

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Abstract

The *Microtus (Terricola) savii s. l.* complex is a group of five species/subspecies of the Italian pine voles, which diverged at different times either with or without chromosomal differentiation. The evidence of chromosomal diversification has so far concerned the shape of the sex chromosomes, especially the X chromosome. Three taxa of the group, *M. savii savii*, *M. s. nebrodensis*, and *M. s. tolfetanus* have identical karyotypes with metacentric X chromosomes. The X chromosomes of *M. brachycercus* and *M. b. niethammericus* are, respectively, subtelocentric and acrocentric in shape. The *M. savii* complex has been long an object of conventional karyological studies, but comparative molecular cytogenetic data were completely missing. Therefore, we conducted a comparative chromosomal mapping of rRNA genes (rDNA) and telomeric repeats in three of the five taxa of the group: *Microtus s. savii*, *M. s. nebrodensis,* and *M. b. niethammericus,* each of which belongs to a distinct mitochondrial clade. The survey revealed that differentiation of the clades was accompanied by remarkable changes with regard to the number and locations of the rDNA sites. Thus, *M. s. savii* and *M. s. nebrodensis* have especially high numbers of rDNA sites, which are located in the centromeric regions of, correspondingly, 18 and 13 chromosome pairs, whereas *M. b. niethammericus* shows variable (8–10) and heteromorphic rDNA sites on both centromeric and telomeric regions. Interstitial telomeric sites (ITS), which are believed to indicate possible breakpoints of recurring chromosomal rearrangements, are present on the largest biarmed chromosomes and on the metacentric X chromosomes in *M. s. savii* and *M. s. nebrodensis*. These preliminary results are discussed in the context of recent advances in phylogeny of the group, as well as the rDNA genomic organization and X chromosome rearrangements in the genus *Microtus*.

Keywords
Arvicolinae; chromosomal evolution; sex chromosomes; interstitial telomeric sequences (ITS); rDNA; NORs

Introduction

The Italian endemic pine voles are distributed throughout the Apennine peninsula from the Alps to Sicily (Contoli et al. 2008). The chromosomal and morphological polytypism of this group led to the identification of five forms: "*savii*", "*brachycercus*", "*nebrodensis*", "*niethammericus*", and "*tolfetanus*", ascribed to the *Microtus* (*Terricola*) *savii* complex de Sélys-Longchamps, 1838 (Galleni et al. 1994, 1998, Contoli 2003). The complex genetic structure found in the group (Castiglia et al. 2008) has not been found in the other species of the subgenus *Terricola* (Jaarola et al. 2004). The systematic ranks and relationships of these taxa have been recently reconsidered (Contoli 2008, Contoli and Nappi 2008) in the light of new insight into the phylogeny of the group (Castiglia et al. 2008) and chromosomal morphology (Table 1).

Karyological studies in the *M. savii sensu lato* complex revealed the same diploid number (2n=54) and invariable set of autosomes (NF_a=58) in all these taxa, but only three of the five taxa showed sex chromosomes similar in size and shape. The sex chromosomes distinctiveness and the evidence of male sterility of hybrids between "*brachycercus*" and "*savii*" supported a specific rank of "*brachycercus*", which was first proposed by Galleni et al. (1994) and later accepted by Musser and Carleton (2005). The following analysis of phylogenetic relationships based on mitochondrial cytochrome *b* gene sequence variation revealed a strong similarity of haplotypes of "*brachycercus*" and *"niethammericus"* which indicated a possible co-specificity of the two taxa. Accordingly, Contoli and Nappi (2008) tentatively ascribed "*niethammericus*" to a subspecies of *M. brachycercus*. The difference in the shape of the X chromosomes of these two taxa was considered a polymorphism due to the paracentromeric heterochromatin accumulation (Castiglia et al. 2008), which is a common trend in many species of *Microtus* (see Marchal et al. 2004, Mitsainas et al. 2009). Finally, albeit the karyological similarity with *M. s. savii*, the Sicilian form presently ascribed to *M. s. nebrodensis* showed unexpectedly high genetic divergence, which suggested its possible specific status (Castiglia et al. 2008).

We further investigated the intra- and interspecific chromosomal variation in the Italian pine voles by analysing chromosomal distribution of rDNA and telomeric sequences. At present, we focused on three of the five taxa, i.e. most widespread and abundant *M. s. savii*, the Sicilian *M. s. nebrodensis,* and *M. b. niethammericus*. Each of these taxa belongs to one of the three mitochondrial DNA clades identified in the group (Castiglia et al. 2008). The two taxa of the group, *M. s. tolfetanus* and *M. b. brachycercus*, are missing from the present study. So far, comparative molecular cytogenetic data were not available in this interesting group of arvicoline rodents, which can possibly serve as a model to study chromosomal evolution.

Table 1. The taxonomy and general karyological traits of Microtus savii s. l. complex

Old taxon[1]	New taxon[2]	2n, NFa	Sex chromosomes
M. s. savii	***M. s. savii***	**54, 58**	**X (m), Y (a)**
M. s. nebrodensis	***M. s. nebrodensis****	**- " -**	**- " -**
M. s. tolfetanus	*M. s. tolfetanus*	- " -	- " -
M. brachycercus	*M. brachycercus*	- " -	X (sm), Y (a)
M. s. niethammericus	***M. b. niethammericus***	**- " -**	**X (a), Y (a)**

m – metacentric, sm – submetacentric, a – small acrocentric, A – large acrocentric; * – assignment of species status is possible. [1] Musser and Carleton (2005), Contoli (2003); [2] Contoli (2008).

Materials and methods

Specimens of *Microtus s. savii* (two males and a female) were collected at three sites: Pizzone (Isernia, Molise), Parco dell'Appia and Passo Corese (Roma, Lazio). The specimens of *M. s. nebrodensis* (one male and one female) were trapped on the Nebrodi Mountains (Messina, Sicily). The individuals of *M. b. niethammericus* (two males and one female) were trapped at Farindola (Pescara, Abruzzi). The animals were handled according to the European Code of Practice for the housing and care of animals used in scientific procedures (Council of Europe 1986). As a routine, metaphases were obtained from bone marrow using standard air-drying technique. 1 mg/ml Vinblastin sulfate (Velbe, Lilly) was used as a mitostatic agent. The karyotypes were analysed after standard Giemsa staining. C-banding (Sumner 1972) was performed mainly to discriminate between the acrocentric X chromosomes and the autosomes in *M. niethammericus*. In all specimens, two probes were used for FISH (Fluorescence In Situ Hybridization): 45S rDNA clone of *Xaenopus laevis*, biotin-labelled by random priming (Invitrogen, Life technologies), and a telomeric probe made of two complementary oligonucleotides $(GGGTTA)_7/(TAACCC)_7$ 3'-end-labelled with biotin (M–Medical, Genenco). Standard procedures for hybridization of repetitive sequences were carried out (Lichter et al. 1992). Hybridization was followed by low-stringency (2xSSC/50% Formamide 1x3min; 2xSSC 3x5 min, RT) or high-stringency (1xSSC/50% Formamide at 40°C for 3 min; 2xSSC/50% Formamide at 40°C, 3x3 min; 2xSSC at 40°C, 3x3 min) post-hybridization washes; blocking with 3% BSA in 4xSSC, and three-round signal detection and amplification by Avidin-FITC/biotinylated anti-Avidin (Vector). Slides were customarily stained with propidium iodide and embedded in Vectashield medium (Vector). DAPI (4', 6-diamidino-2-phenylindole) counterstaining facilitated identification of homologues after FISH. Digital images were acquired and elaborated by IPLab software (Photometrics) and then processed in Photoshop CS (Adobe Systems Inc., U.S.).

Results

Specimens of *Microtus s. savii* and *M. s. nebrodensis* showed matching 2n=54 karyotypes with medium-size metacentric X and acrocentric Y chromosomes (Fig. 1a). Specimens of *M. b. niethammericus* had a similar karyotype, but an acrocentric X chromosome of the same size as in the two other taxa. The characteristic C- and DAPI-banding patterns of this X chromosome (Fig. 1b, c) distinguished it from acrocentric autosomes.

Both the number and locations of the rDNA-FISH signals differed remarkably among the specimens (Fig. 2). The number of signal-bearing chromosomes in metaphase plates of *M. s. savii* was as large as 36 (18 chromosome pairs) (Fig. 2a), while 28 signals were distributed on 13 chromosome pairs in *M. s. nebrodensis* (Fig. 2b). The FISH signals were located at centromeres of acrocentric chromosomes in both Savi's pine voles and only one pair of medium-sized acrocentric chromosomes of the Sicilian specimens was marked at both chromosome termini (Fig. 2b). The biarmed autosomes and the sex chromosomes lacked rDNA in both taxa (not illustrated). FISH revealed much lower number of rDNA sites per metaphase plate in *M. b. niethammericus* (Fig. 2c). The overall rDNA-FISH pattern remained constant in different metaphase cells of each individual of *M. b. niethammericus*, but differed slightly among presently studied individuals (8, 9 and 10 signals per cell). In this species, hybridization signals were constantly present on two distinct pairs of homologues, a pair of medium-sized acrocentric chromosomes and the smallest pair of metacentric chromosomes, whereas the remaining 4–6 FISH signals were detected on a small set of apparently non-homologous chromosomes. This pattern persisted under various hybridization conditions.

FISH with the telomeric probe followed by high-stringency post-hybridization washes (PHW) showed an ordinary, all-telomeric, pattern in all the specimens studied. Nonetheless, by decreasing the stringency of PHW we revealed telomeric-like sequences on some chromosomes. Thus, prominent ITS signals were present in the centromeric regions of the metacentric X and the largest biarmed (submetacentric) chromosomes of *M. s. savii* and *M. s. nebrodensis* (Fig. 3a). Other chromosomes including two of the three pars of biarmed autosomes (submetacentric and tiny metacentric) and the Y chromosome did not show telomeric signals even under low-stringency conditions. In *M. b. niethammericus,* FISH detected the same ITS pattern on the largest biarmed chromosomes, but the acrocentric X chromosome lacked any interstitial signal (Fig. 3b).

Discussion

The study revealed a considerable variation in the number and chromosomal distribution of rDNA sites at both intraspecific (between *M. s. savii* and *M. s. nebrodensis*) and interspecific (between the two Savi's pine voles and *M. b. niethammericus*) levels.

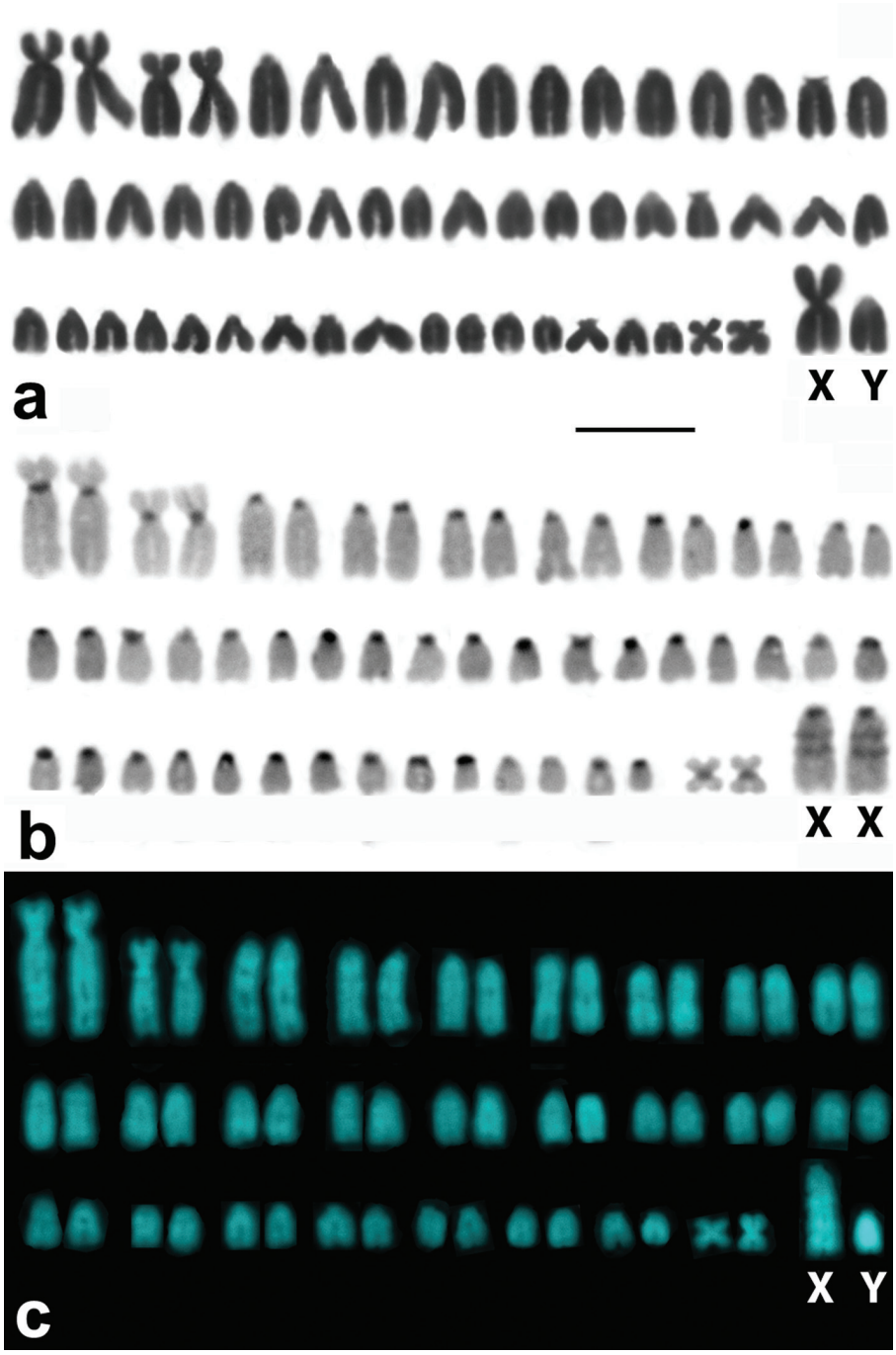

Figure 1. Representative karyotypes of the Italian pine voles. A conventional Giemsa stained male karyotype of a Savi's pine vole exemplified by *M. savii nebrodensis* (**A**) with a large metacentric X chromosome and a small acrocentric Y. C-banded (**B**) and DAPI stained (**C**) chromosome complements of *M. brachycercus niethammericus,* which differ from (**A**) in morphology of the sex chromosomes. The large acrocentric X chromosomes of *M. b. niethammericus* show distinctive prominent bands. *Bar* = 10 μm.

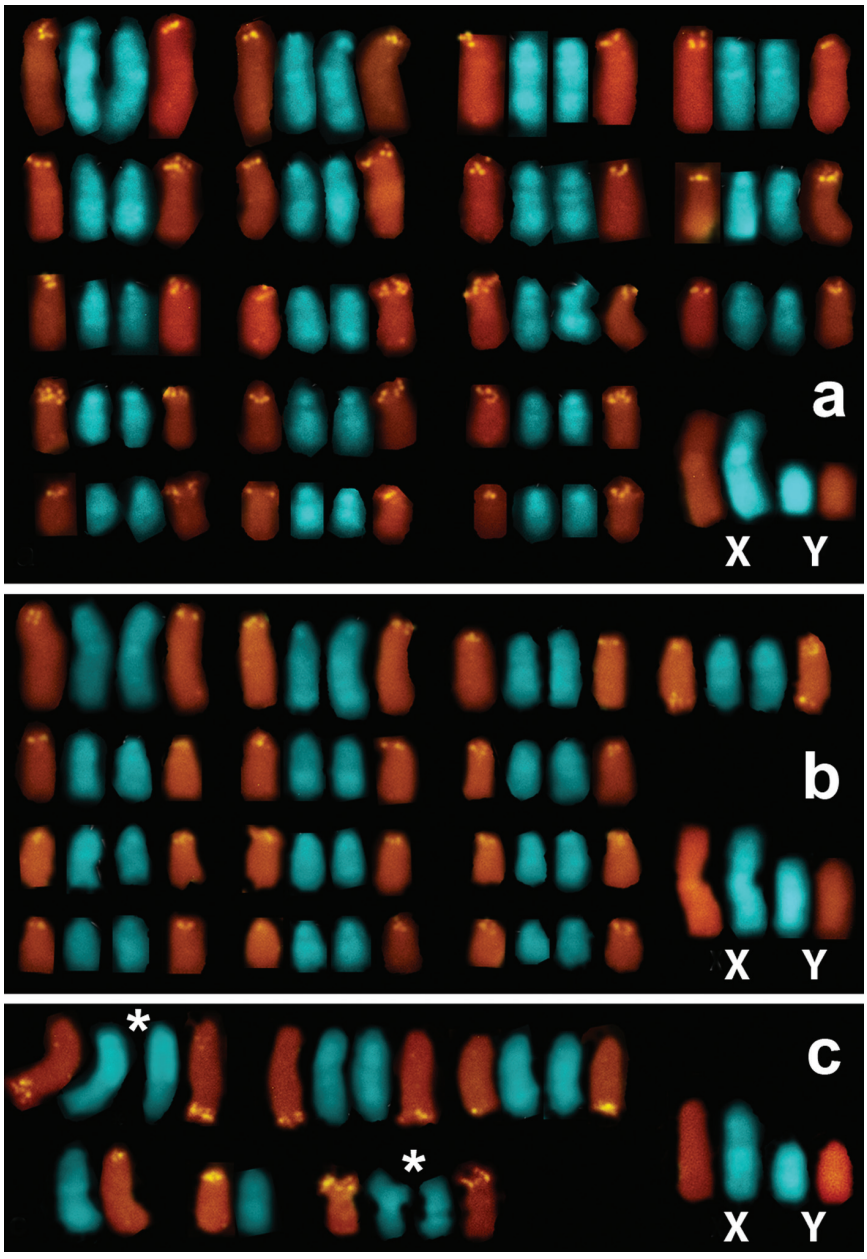

Figure 2. Partial karyotypes composed of rDNA-bearing chromosomes and the sex chromosomes of *Microtus savii savii* (**A**), *M. s. nebrodensis* (**B**), and *M. brachycercus niethammericus* (**C**). The hybridization signals mark centromeric regions of all NOR-bearing chromosomes in *M. savii* subspecies (**A, B**) and, additionally, a telomeric region of a single chromosome in (**B**) (*upper row*). In (**C**), the largest among three individuals of *M. b. niethammericus* set of rDNA-bearing chromosomes composed of two constantly marked chromosome pairs (signed by *asterisks*), one of which represent the smallest biarmed chromosomes, as well as chromosomes with variable rDNA sites, of which two chromosomes are in an apparently heterozygous state. The sex chromosomes lack rDNA-FISH signals in either subspecies.

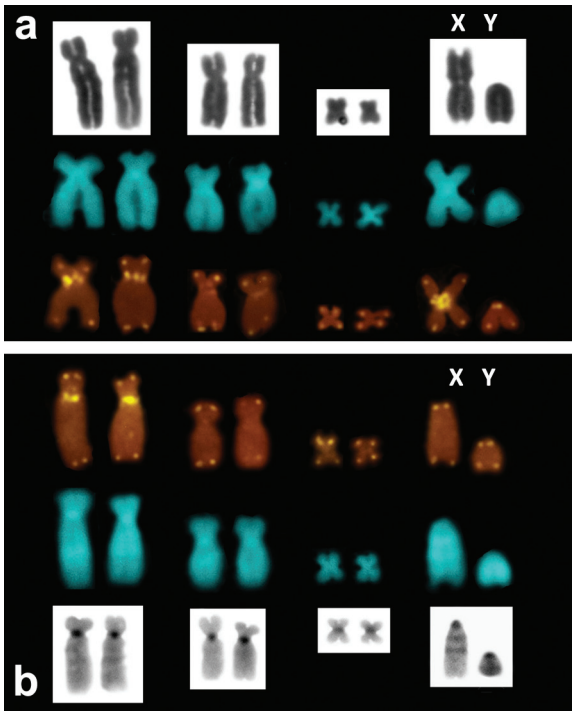

Figure 3. Telomeric FISH signals on the biarmed and the sex chromosomes of *M. savii,* exemplified by *M. s. savii* (**A**) and *M. brachycercus niethammericus* (**B**). Respective chromosome pairs are shown after DAPI counterstaining (*central row* in **A, B**), Giemsa staining (*upper row in* **A)** and after C-banding (*lower row* in **B**). ITS are present on the largest biarmed chromosomes and on the metacentric X chromosome.

The two Savi's pine voles, *M. s. savii* and *M. s. nebrodensis,* separated in middle-early Pleistocene (0.6–1.0 MYA) (Castiglia et al. 2008) have almost identical karyotypes and share the same general rDNA pattern, i.e. an abundant number of rDNA sites, their chiefly centromeric locations, and absence on the sex chromosomes. The intraspecific differences between the two Savi's pine voles concern the exact number of rDNA-bearing chromosome pairs, 13 in *M. s. nebrodensis* and 18 in *M. s. savii*, and the presence of additional telomeric signals on one chromosome pair of *M. s. nebrodensis*.

The rDNA pattern of *M. b. niethammericus* differs peculiarly from the ones of the congeneric species. The mean number of rDNA sites is markedly lower. The sites appear variable in number and size and are located in both centromeric and telomeric regions of a small set of chromosomes. This evidence is in accord with the genetic divergence of "*brachycercus"* clade separated from *M. s. savii* in middle Pleistocene (0.3–0.5 MYA) (Castiglia et al. 2008).

The number and chromosomal locations of NORs (nucleolar organizer regions, rDNA sites) have been comprehensively studied in various species of the genus *Microtus* by conventional silver staining technique (Zagorodnyuk 1992, Mazurok et al. 1996, Mazurok et al. 2001, Mekada et al. 2001, Martínková et al. 2004). Even if this

method reveals not all rDNA sites, but the NORs, which were transcriptionally active in the previous interphase (Ag-NORs), the records reflect a remarkable interspecific variation of NORs in the genus. The putative ancestral karyotype of the genus *Microtus* is considered to be 2n=54 (Zima and Král 1984, Martínková et al. 2004, Lemskaya et al. 2010). Analysing the Ag-NORs data, we noted that the species of *Microtus* with derived karyotypes commonly show minor numbers of NORs (Gornung et al. 2011). In contrast, the species with primitive karyotypes, such as *M. rossiaemeridionalis* (2n=54, NFa=54) (Mazurok et al. 1996) or *M. transcaspicus* (2n=52, NFa=52) (Mazurok et al. 2001), have numerous NORs (up to 16 NOR-bearing chromosome pairs in *M. rossiaemeridionalis*) predominantly located in the centromeric regions of chromosomes.

The increase in the number of NORs in the evolution of different groups of species, so-called rDNA dispersion, is well documented. Reciprocal translocations at the level of C bands are supposed to be the basic underlying mechanism of this event (Hirai et al. 1996). Accordingly, the location of NORs in the C-positive centromeric regions facilitates the dispersion. Moreover, as recently proposed for the genus *Mus*, the accumulation of a large number of rDNA repeats in the centromeric region may represent an important first step of chromosome re-patterning, which may be triggered by modifications of the epigenetic state of DNA (Cazaux et al. 2011). Regardless of a possible mechanism of remodelling of NORs patterns, such as aforementioned translocation events, unequal crossing over or transposition with subsequent amplification of rDNA (Eickbush and Eickbush 2007), the present data imply the evolutionary genomic plasticity of the *Microtus savii* group.

Like in all species of *Microtus* thus studied, except *M. kirgisorum* (Mazurok et al. 2001), NORs have not been detected on the sex chromosomes in the three presently studied taxa. The sex chromosomes of several species of *Microtus* show complex and heterogeneous heterochromatin, which is indicative of a rapid turnover of repetitive sequences in the genus (Modi 1987, Burgos et al. 1988, Marchal et al. 2004). In the *Microtus savii* complex, only satellite DNA Msat-160 has been described (Acosta et al. 2010). According to these data, despite a similar autosomal distribution, the amount of Msat-160 in the pericentromeric regions of chromosomes, including those of the X chromosome, in *M. b. niethammericus* is clearly lower than in *M. s. savii.* Moreover, Msat-160 is present on the Y chromosome of *M. b. niethammericus*, but absent on the Y of *M. savii*. In addition, the report of Galleni et al. (1992) described particular *Alu*I bands on the X chromosomes of *M. brachycercus*.

Presently, we show that while interstitial telomeric-like sequences are marking the largest pair of biarmed chromosomes in either species, they are also present in the centromeric region of the metacentric X chromosome of *M. s. savii* and *M. s. nebrodensis*, whereas absent in the heterochromatic regions of the acrocentric X chromosome of *M. b. niethammericus*. We hypothesize that according to the basal position of *M. s. nebrodensis* in the phylogenetic reconstruction (Castiglia et al. 2008) the metacentric X chromosome should be primitive in the group. It follows that the other forms of the X chromosome found in *M. b. niethammericus* and *M. b. brachycercus* may have originated by pericentric inversion of the ancestral metacentric X with subsequent amplification of

pericentromeric heterochromatin in *M. b. brachycercus*. In view of the fact that clusters of different repetitive DNA including subtelomeric and interstitial telomeric repeats characterize the breakpoints of recurrent chromosomal rearrangements (Azzalin et al. 2001, Nergadze et al. 2004), the presence of interstitial telomeric-like DNA sequences in the presumably primitive metacentric X chromosome is dispuwle. Indeed, according to the evolutionary relationships in a group of voles of another subgenus, *Microtus*, the metacentric X chromosome was supposed to be "derived" respect to the acrocentric and submetacentric morphology (Mazurok et al. 2001, Nesterova et al. 1998). On the other hand, the X chromosomes might have undergone sequential inversions in the chromosomal evolution of the genus. Several different breakpoints were indeed identified on the X chromosomes of some *Microtus* species (Rubtsov et al. 2002).

To date, the evidence of chromosomal diversification in the *M. savii* s.l. complex concerned only the shape of the sex chromosomes, particularly the X chromosome. Presently, we can add several details to this evidence and conclude that significant changes of rDNA genomic organization accompanied the genetic differentiation of the Italian pine voles.

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