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Triploidy in Chinese parthenogenetic *Helophorus orientalis* Motschulsky, 1860, further data on parthenogenetic *H. brevipalpis* Bedel, 1881 and a brief discussion of parthenogenesis in Hydrophiloidea (Coleoptera)

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Abstract

The chromosomes of triploid parthenogenetic *Helophorus orientalis* Motschulsky, 1860 are described from material from two localities in Heilongjiang, China. 3n = 33. All the chromosomes have clear centromeric C-bands, and in the longest chromosome one replicate appears to be consistently longer than the other two. The chromosomes of additional triploid parthenogenetic *H. brevipalpis* Bedel, 1881, from Spain and Italy, are described. In one Italian population one of the autosomes is represented by only two replicates and another appears more evenly metacentric than in material from Spain and the other Italian locality. Parthenogenetic and bisexual specimens of *H. orientalis* are illustrated, along with Pleistocene fossil material. Parthenogenetic *H. brevipalpis* is also illustrated. Parthenogenesis in Hydrophiloidea is discussed. It appears to be rare and, in all cases has been detected by chromosomal analysis of populations in which males are unexpectedly scarce. Parthenogenesis is suspected in *Helophorus aquila* Angus et al., 2014, from northern Qinghai (China), which should be verified in further studies.

Keywords

China, Coleoptera, *Helophorus orientalis*, *H. brevipalpis*, Hydrophiloidea, karyotypes, parthenogenesis, triploidy

Introduction

The family Helophoridae is one of the basal clades of the superfamily Hydrophiloidea (Fikáček et al. 2012). The family comprises about 189 species classified in nine subgenera of which *Rhopalohelophorus* Kuwert, 1886, with about 146 species, is the largest and includes both *Helophorus brevipalpis* and *H. orientalis* (Angus 2015, Smetana 1985). The basic diploid chromosome number for *Rhopalohelophorus*, known in 31 species, is 2n = 22 (Angus 1989, 1996, 1998, 2019, Angus et al. 2005, Angus and Aouad 2009), a number shared with the family Hydrochidae, another of the basal Hydrophiloid lineages (Shaarawi and Angus 1992). The other diploid chromosome number occurring in Helophoridae is 2n = 18, found in the subgenera *Helophorus* s. str. Fabricius, 1775, *Gephelophorus* Sharp, 1915 and *Eutrichelophorus* Sharp, 1915 and occurring in many of the aquatic Hydrophilidae (Angus, 1989).

Parthenogenesis appears to be rare in the Hydrophiloidea and to date has been recorded only in *H. orientalis* Motschulsky, 1860 (Angus 1970) and *H. brevipalpis* Bedel, 1881 in the Helophoridae (Angus 1992), and *Anacaena lutescens* Stephens, 1829 in the Hydrophilidae (van Berge Henegouwen 1986, Shaarawi and Angus 1991). Angus (1992) has shown that in *H. brevipalpis* Bedel, 1881 both diploid and triploid females may coexist in one population.

The aim of the present study was to study the karyotypes of two *Helophorus* species originating from China (*H. orientalis*) and Mediterranean region (*H. brevipalpis*) and to determine the mode of reproduction of the species, bisexual or parthenogenetic, in these unstudied populations.

Material and methods

The material used for chromosome analysis is listed in Table 1. The number of specimens refers to the number from which successful preparations were obtained. The material was collected with a water net in small pools and ditches. The *H. orientalis* was collected by Angus and Jia, the *H. brevipalpis* by Angus.

Following the protocol described by Angus (2006), chromosome preparations were obtained from mid-gut of adult beetles. Beetles were injected with 0.1% colchicine solution in insect saline (0.75% NaCl in distilled water buffered to pH 6.8 with Sörensen's phosphate buffer) and left for 12.5 min. They were then transferred to a 0.48% ($^1/_2$-isotonic) solution of KCl at pH 6.8 in individual solid watch glasses, their abdomens detached, and the midguts removed and left in the solution. The rest of the beetle was removed, killed by immersion in boiling water, and mounted on a card as a voucher. After 12.5 min, the guts were transferred to fixative (3 parts of absolute ethanol and 1part of glacial acetic acid), again in solid watch glasses. The fixative was changed twice, and the guts were then left to stay in fixative for 1 hour, with the watch glasses covered to prevent water being absorbed from the air. For chromosome preparations small pieces of tissue were taken with fine forceps and placed on clean dry slides,

Table 1. The species, location of populations and the number of specimens studied.

Species	Locality	No. examined
Helophorus orientalis Motschulsky, 1860	China, Heilongjiang: Mishan, Dading Shan Forestry Study Centre. 45.3635N, 131.9175E	2♀♀
	China, Heilongjiang: Qitaihe, Shillongshan National Forest. 45.6409N, 131.264E	3♀♀
Helophorus brevipalpis Bedel, 1881	Spain, Leon: Algadefe. 42.215N, 5.590W	2♂♂, 10♀♀ (Angus 1992)
	Italy, Parma: Ponte Scipione. 44.8315N, 9.956E	1♀
	Italy, Reggio Emilia: Near Sologno. 44.375N, 10.402E	5♀♀

cells were disaggregated in a small drop of 45% acetic acid, with the tissue torn apart with fine pins as necessary. Next, a drop of fixative was pipetted on to the cell suspension. This causes the drop to spread over the slide as a thin film. The spreading film can be guided by tilting the slide. Sides were dried horizontally. After at least 1 hour, they were stained with 0.5% Giemsa solution at pH 6.8.

Chromosomes were photographed under oil-immersion (X100 objective) on to high-contrast microfilm. Photographs were printed at X 3000, then scanned into a computer and further processed using Adobe Photoshop.

For C-banding the immersion oil used for photographing the preparations was removed by washing in xylene (2 changes) and absolute ethanol. The slides were then dried vertically. C-banding was done by immersing the slides in saturated barium hydroxide at room temperature for 4 minutes, followed by 1 hour in 2X SSC (Salt-Sodium Citrate: 0.3 M NaCl + 0.03 M trisodium citrate) at 60^{0}C. The C-banding protocol could be repeated if initial results were not satisfactory.

Results

H. orientalis Motschulsky, 1860

The chromosome number of *H. orientalis* was found to be 3n = 33. Mitotic chromosomes, arranged as karyotypes, are shown in Fig. 1a–d. As no data on males are available, the X chromosome cannot be identified. All the chromosomes have distinct centromeric C-bands. There is a gradual decrease in length from chromosome 1 down to chromosome 7, which is about two thirds the length of chromosome 1. Chromosomes 8–11 are slightly smaller, about half the length of chromosome 1. Chromosomes 1–6 are metacentric, 9 is submetacentric and 7, 8 10 and 11 are subacrocentric. A consistent feature of the karyotype is that one replicate of chromosome 1 is consistently larger than the other two. C-banding suggests that the difference between the longer and shorter replicates may be associated with a weakly C-banding region towards the distal end of the long arm. In the more extended preparations (Fig. 1d) there is a second C-band at the distal end of the long arm (obscured by chromosome overlap in one replicate), but in the more contracted preparations (Fig. 1b) the terminal band appears smaller in the long replicate and does not show at all in the other two replicates.

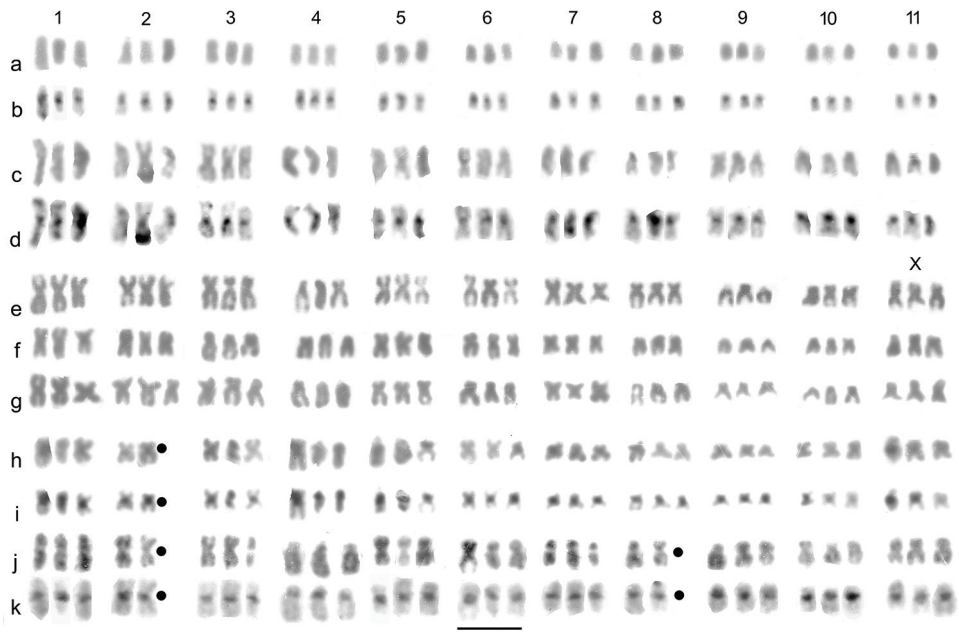

Figure 1. Karyotypes of *Helophorus* spp., females, preparations from mid gut. **a–d** *H. orientalis* **a, b** mishan **a** Giemsa-stained **b** the same nucleus C-banded **c, d** Qitaihe **c** Giemsa-stained **d** the same nucleus C-banded **e–k** *H. brevipalpis* **e, f** Algadefe, Giemsa-stained **e** from Angus (1992) **f** from a different female **g** from Ponte Scipione, Giemsa-stained **h–k** from Sologno **h** Giemsa-stained **i** the same nucleus C-banded **j, k** a preparation from a different female **j** Giemsa-stained **k** the same nucleus C-banded. All preparations from this locality lack one replicate of autosome 2, but the loss of an autosome 8 from **j** and **k** is experimental artefact. Scale bar: 5 μm.

H. brevipalpis Bedel, 1881

The chromosome number of *H. brevipalpis* was found to be 3n = 33. Angus (1992) gave a detailed account of the chromosomes of both sexually reproducing diploid and parthenogenetic triploid *H. brevipalpis*, the triploid material coming from Algadefe (Spain, Provincia de León). Fig. 1e shows the specimen figured by Angus (1992) and Fig. 1f shows a karyogram from a different female. Both appear to show one replicate of autosome 1 shorter than the other two.

It is now possible to add data on Italian material. A specimen from the Provincia di Parma, analysed in 2008 (Angus and Foster 2009), has a karyotype closely resembling that of Spanish material, though the short replicate of autosome 1 is less obvious. Specimens from the Provincia di Reggio Emilia, analysed in the present study appear rather different. Data were obtained from five triploid females in the present study, and the highest chromosome number found was 32 in all cases. The resulting karyograms (Fig. 1h–k) show only two replicates of autosome 2. They also show autosome 3 to be more metacentric than in the Spanish and Parma material (Fig. 1e–g). The karyograms shown in Fig. 1j, k also lack one replicate of autosome 8. This is a

preparation artefact as other preparations from this female have 32 chromosomes. This preparation is illustrated because it shows the form and C-banding of the chromosomes more clearly than the others.

Discussion

As noted in the Introduction, *Helophorus orientalis* was the first *Helophorus* species shown to be parthenogenetic, following laboratory rearing by Angus of females sent to him in 1967 by Prof. C. H. Fernando from Waterloo, Ontario, Canada (Angus 1970). No males were present in this material. Smetana (1985) records male *H. orientalis* from the central Rocky Mountains of America, and there are males among material sent from Logan, Utah to the Natural History Museum in London. Smetana notes that in some populations from Wyoming males account for about 30% of the specimens. Apart from this American material, there are males in the collection of the late G. Lafer of Vladivostok, Russia. A sample from Novitskoye, 12 km south of Partizansk (about 80 km east of Vladivostok) comprised three males and seven females. A further sample from this area, from the village of Prudovoye in the Partizansk region, comprised 10 females, but no males. Lafer's collection contains 38 females from other parts of the Russian Far East (Primorye), but no males. *H. orientalis* is abundant in East Siberia, but only as females. The picture emerging is of the species parthenogenetic over most of its wide distribution range, with bisexual populations in limited, separate areas. *H. orientalis* has a characteristic pronotum, with the internal intervals shining and with very reduced granulation. Fig. 2a shows this feature in a Chinese female, while Fig. 2b shows a Logan male and Fig. 2c shows a female from Waterloo. This pronotum is matched by an English Pleistocene fossil from Brandon, Warwickshire, with radiocarbon dates suggesting an age of about 30,000 years B.P. (Fig. 2d) (Coope 1968, Shotton 1968). *H. orientalis* is now known as a fossil from a number of English sites dating from the Last Glaciation, as well as from the classic Woolly Rhinoceros site at Starunia in the Western Ukraine, where more or less intact beetles may be found (Angus 1973) The Starunia rhinoceros has now been radiocarbon dated at about 33500–40000 years B.P. (Kuc et al. 2012). A fossil female from Starunia is shown in Fig. 3.

As already noted, the longest triplet of chromosomes includes one replicate which is distinctly longer than the other two, possibly associated with differing amounts of weakly C-banding material at the distal end of the long arm. This could suggest that these triploids have a hybrid origin. We know of no other species closely resembling *H. orientalis*, but with such a vast range and long fossil record indicating changes in its distribution, it is possible that different bisexual populations could be, or have been, sufficiently different genetically to cause some chromosomal mismatching if they hybridised. Experimental hybrids between *Helophorus lapponicus* Thomson, 1853 and *H. paraminutus* Angus, 1986 may be relevant here. Angus (1986), working at Karasuk, West Siberia, found that the karyotypes of these two species appeared indistinguishable, so he obtained experimental hybrids between them, with a view to having the

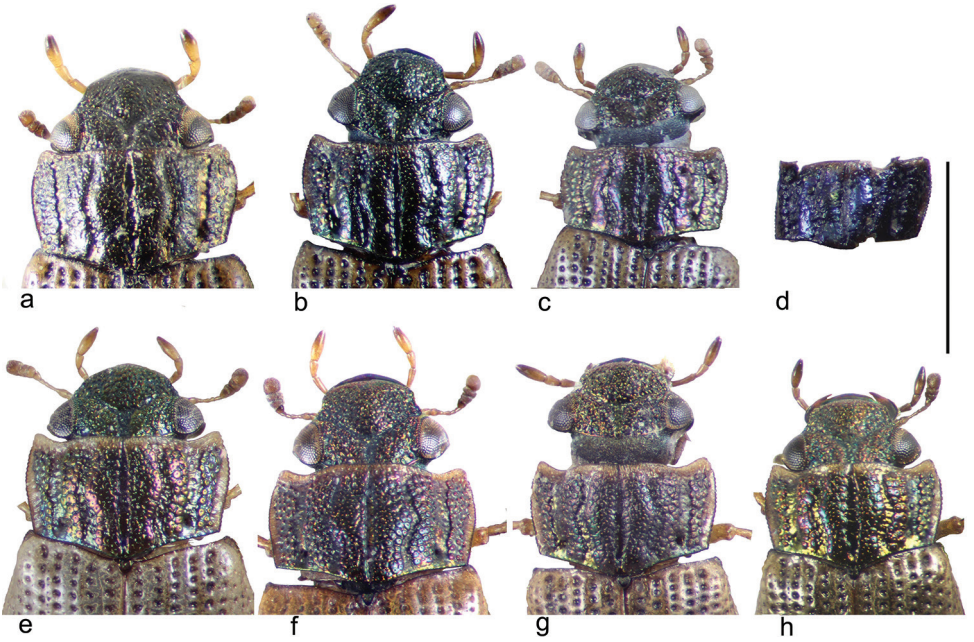

Figure 2. Heads and pronota of *Helophorus* species **a–d** *H. orientalis* **a** triploid female from Qitaihe **b** male from Logan **c** female from Waterloo, Ontario **d** fossil pronotum from Brandon Terrace, Warwickshire **e–h** *H. brevipalpis*, parthenogenetic females **e** from Logan Canyon, Utah **f** triploid from Algadefe **g** triploid from Ponte Scipione **h** triploid from near Sologno. Scale bar: 1 mm.

chromosomes of the two species in exact synchrony in their condensation through mitotic prophase. The result, however, was some irregularities in their condensation, which Angus speculated might be the result of difficulty in uptake of non-histone protein by the condensing chromosomes. At the same time, crosses between Spanish and Swedish *H. lapponicus* resulted in no such irregularities.

The Spanish triploid *H. brevipalpis* also show chromosomal mismatching in the longest triplet, in this case involving one replicate being noticeably shorter than the other two, also shown by the Italian specimen from Parma province, Ponte Scipione. As with *H. orientalis*, *H. brevipalpis* is a distinctive species, but in this case also variable. Angus (1988) undertook detailed analysis of local populations of *H. brevipalpis*. This led to the recognition of a distinct subspecies from eastern Turkey, Syria and Iran. Discriminant functions analysis showed that this subspecies was more distinct from other populations than their variation among themselves, but also showed that these populations grouped into sections with slightly larger or smaller aedeagi. Spanish material comes in the slightly larger aedeagus group and Italian in the slightly smaller group. In terms of frequency of males, most Spanish material is clearly bisexual, and the males and diploid females found by Angus probably reflect the interface between the ranges of bisexual and parthenogenetic populations. It is also worth noting that, surprisingly, the province of Leon is at the edge of the range of *H. brevipalpis*, which is unknown in Spanish Galicia. What seems

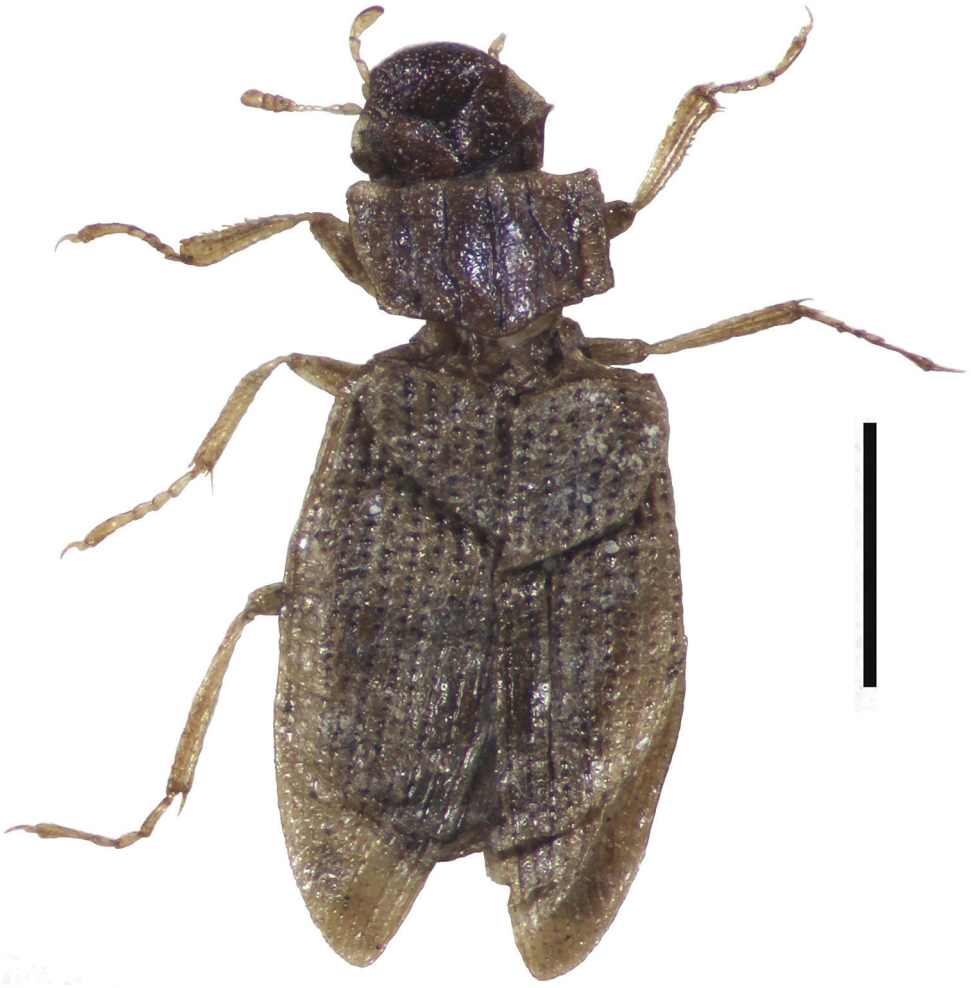

Figure 3. Fossil *H. orientalis* from Starunia. Scale bar: 1 mm.

important here is that the regional variation found in *H. brevipalpis* allows the possibility that the triploids may have resulted from crossing between genetically differing stocks.

The Italian triploids from Sologno differ from the others encountered in having the autosomes of triplet 3 more evenly metacentric than in the other triploids, and in having only two replicates of autosome 2. The difference in triplet 3 must reflect origin from a different bisexual stock, while the loss of one replicate of autosome 2 presumably results from an "accident". In the absence of knowledge of the oogenesis of this species it is not possible to say whether the parthenogenesis has always been apomictic (without any meiosis) or whether there might be automictic parthenogenesis (with meiosis followed by fusion of haploid oocytes) in some diploid females. Apomictic parthenogenesis would result in offspring that are clones of the parent, while automictic parthenogenesis would allow limited variation, and, perhaps, account for the loss of a chromosome.

Parthenogenesis in *Anacaena lutescens* has taken a different course. Shaarawi and Angus (1991) analysed the chromosomes and found that, unlike the situation in *Helophorus*, most parthenogenetic material was diploid, and carried a heterozygous deletion of a small apical section of autosome 8, distal to a secondary constriction. Two triploid populations were found, one from Cumbria (England) and one from the Netherlands. However, these populations differed in the arrangement of autosome 8, indicating that their triploidy evolved independently, after the onset of parthenogenesis.

In all these cases, parthenogenesis was initially suspected on the basis of underrepresentation of males in sampled material. At the moment, we know of one further *Helophorus* species in which this might also be the case. *Helophorus aquila* Angus et al., 2014 was described from the northern part of Qinghai (China). Only 3 males were taken among more than 80 specimens. This area, near the great lake of Qinghai Hu, is readily accessible so this should be verified in further studies.

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Robert Angus thanks Mario Toledo for drawing his attention to probable parthenogenesis in Italian *Helophorus brevipalpis*, and for organising two field trips in which material was collected. He also thanks the Natural History Museum, London for the use of its facilities associated with his continuing position as a Scientific Associate in the Department of Life Sciences, and the State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-sen University, for hosting his visit to China in June 2019. We also thank the reviewers of this manuscript for their helpful suggestions for its improvement.

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RESEARCH ARTICLE

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Cytogenetic and pollen identification of genus *Gagnepainia* (Zingiberaceae) in Thailand

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Abstract

Gagnepainia godefroyi K. Schumann, 1904 and *G. harmandii* K. Schumann, 1904 belong to the genus *Gagnepainia* K. Schumann, 1904 of the Ginger family. They have the potential to be developed as medicinal and attractive ornamental plants. To date, the knowledge on the cytological and reproductive aspects of *Gagnepainia* have not been publicly available. Therefore, the aims of this research are to investigate the cytogenetic and pollen characters of *Gagnepainia* species using light, fluorescence, and scanning electron microscopes. The regular meiotic figures of 15 bivalents are found in both species and presented for the first time. These evidences indicate that *Gagnepainia* is diploid and contains $2n = 2x = 30$ with basic number of $x = 15$. The mean nuclear DNA contents range from 1.986 pg in *Gagnepainia* sp., 2.090 pg in *G. godefroyi* to 2.195 pg in *G. harmandii*. Pollens of all species are monad, inaperturate, prolate with bilateral symmetry, and thick wall with fossulate exine sculpturing. The pollen size of *G. harmandii* (74.506 ± 5.075 μm, 56.082 ± 6.459 μm) is significantly larger than that of *G. godefroyi* (59.968 ± 3.484 μm, 45.439 ± 2.870 μm). Both 2C DNA content and pollen size are the effective characteristics for species discrimination. The reproductive evidence of high meiotic stability and normal pollen production indicate that both *Gagnepainia* species have high fertility and seed productivity, which are in accordance with the broad distribution. The present study provides good cytogenetic and pollen characters not only for plant identification, but also plant fertility assessment through plant genetic resource management and improvement of *Gagnepainia*.

Keywords

Chromosome number, Globbeae, *Hemiorchis*, meiotic figure, nuclear DNA content, palynology

Introduction

Gagnepainia K. Schumann, 1904 is a small tropical ginger genus belonging to tribe Globbeae of Zingiberaceae. It was taxonomically classified into the genus *Hemiorchis* Kurz, 1873, another member within the same tribe. According to taxonomic revision of Schumann (1904), it was separated from *Hemiorchis* and formally placed in a new genus, which was named in honour of François Gagnepain, a French botanist (1866–1952). Despite the fact that *Gagnepainia* is able to phylogenetically form a monophyletic clade with *Hemiorchis*, they are sister genera to each other (Williams et al. 2004, Pospíšilová et al. 2016).

Gagnepainia is a small, deciduous, perennial ginger which has a strong dormancy during the dry period. It has distinctive swollen rhizomes jointed with the base of well-developed pseudostems. The inflorescences consist of the numerous tiny butterfly-like flowers, usually emerging directly from the ground before the emergence of the leafy shoots. Remarkably, the trilobed labellum with a peg-shaped central lobe is a unique characteristic differentiating *Gagnepainia* from closely related genera, especially *Globba* Linnaeus, 1771 and *Hemiorchis* (Leong-Škorničková and Newman 2015). Currently, *Gagnepainia* comprises only two species, which can be characterized by lateral staminode shape and flower colour. *Gagnepainia godefroyi* K. Schumann, 1904 has creamy-white to pale orange flowers with broadly elliptic to obovate lateral staminodes. On the other hand, *G. harmandii* (Baill.) K. Schumann, 1904 has bright green flowers with oblanceolate lateral staminodes (Newman and Simon 2017). Geographically speaking, this genus is widely distributed across the Indo-Chinese and Indo-Burmese areas and Thailand, except the peninsular region (Larsen and Larsen 2006, Leong-Škorničková and Newman 2015). The species of *Gagnepainia* have the ethnomedical potential use for wound healing, inflammation treatment, and hemostasis. Because of their gorgeous flowers, both two species are also cultivated as ornamental plants (Chuakul and Boonpleng 2004, Prathanturarug et al. 2007).

Even though extensive studies on species belonging to the ginger family have been cytogenetically conducted, only chromosome numbers of *Globba* were reported for Globbeae. The species in genus *Globba* contain the diverse chromosome numbers of $2n$ = 20, 22, 24, 28, 32, 34, 48, 64, and 96. However, x = 8 is considered as the primary basic chromosome number (Mahanty 1970, Lim 1972, Takano 2001, Jatoi et al. 2007, Pospíšilová et al. 2016). In the sister genus *Hemiorchis*, only the chromosome number of *H. burmanica* Kurz has been reported as $2n$ = 30 (Pospíšilová et al. 2016).

It is evident that, to date, knowledge on the cytological and reproductive aspects have not publicly been available for the genus *Gagnepainia*. Therefore, the aims of this research are to intensively investigate the cytogenetic characters, including chromosome numbers, meiotic figures, and genome sizes (2C-value), of the genus *Gagnepainia* in Thailand. Pollen morphological study, using light (LM) and scanning electron (SEM) microscopes, were also conducted for the fertility assessment through further genetic resource conservation management and utilization of this genus.

Materials and methods

Sample collection and plant identification

We compiled a total of 19 accessions of *G. godefroyi* and *G. harmandii* in the present study. The accession number of each sample was assigned as PMNN (P. Moonkaew and N. Nopporncharoenkul) and followed by the reference number which referred to the population in district range. The majority of sample collections are from natural habitats in various parts of Thailand, whilst others (PMNN024, 025, 027, 028, and 030) were collected from the Queen Sirikit Botanic Gardens (QSBG), Chiang Mai, Thailand. The list of plant materials with their geographic localities are shown in Table 1. All samples were identified based on the floral characters which were described by Leong-Škorničková and Newman (2015). Representative flowers of each species are shown in Figure 1. Only one accession, PMNN021 from Khong Chiam, Ubon Ratchathani, has not flowered in either natural habitat or cultivation, so we assigned this unknown taxon as *Gagnepainia* sp. Plant samples have been meticulously preserved at Department of Plant Science, Faculty of Science, Mahidol University, Bangkok and QSBG, Chiang Mai, Thailand. The voucher specimens of all accessions are kept, in 70% ethanol, at the BKF and QBG herbaria.

Meiotic chromosome analysis

Meiotic configuration was determined using the aceto-orcein smear and DAPI staining techniques with minor modifications, according to the protocols of Nopporncharoenkul et al. (2017) and Mandáková and Lysak (2016). The young inflorescences, with the majority of closed flower buds of each accession, were harvested in the fields or cultivation at their early emerging stage. To stop all cellular activities and reactions, samples were immediately fixed in Carnoy's fixative (6: 3: 1 v/v of ethanol: chloroform: glacial acetic acid) at room temperature for 24 hours. The fixed flower buds were then transferred and preserved in 70% ethanol at 4 °C until used. Afterwards, selected inflorescences were washed with distilled water twice for 10 min. Each anther, containing the two thecae, was separated from unwanted parts of the closed flower under a stereomicroscope. Finally, a bilocular anther was gently washed with 45% acetic acid at room temperature for 5 min. For individual anther, each theca was used for preparation of meiotic chromosome via either conventional aceto-orcein or fluorescence DAPI (4', 6-diamidino-2-phenylindole) staining.

To further analyze samples, the conventional technique was performed. Anther theca was stained with 1% (w/v) aceto-orcein, and microscopic slide was rapidly moved above the flame of an alcohol burner for three to five times. Warm anther suspension was gently smeared using dissecting needles, and the remaining tissue debris was discarded. A fine cell suspension was covered with a coverslip and tapped vertically with dissecting needles to squash the cells flat. The chromosomes were investigated under an Olympus CX21 light microscope.

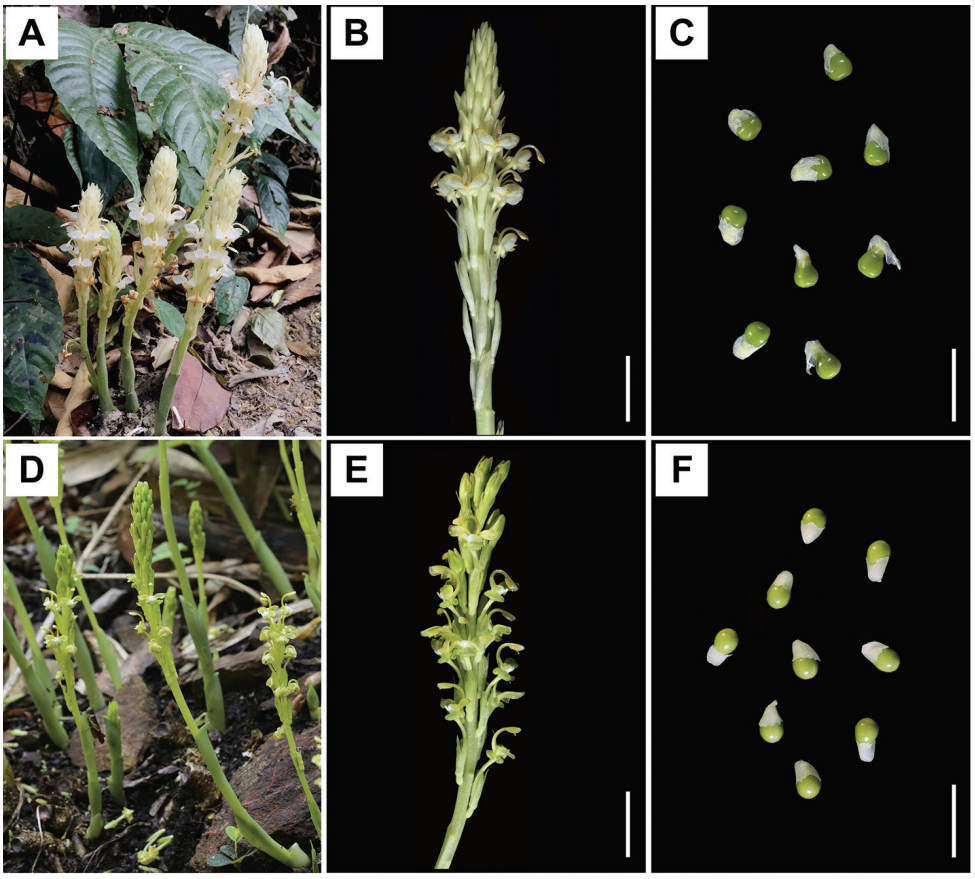

Figure 1. *Gagnepainia* spp. in Thailand. **A–C** *G. godefroyi* **A** inflorescences in habitat (PMNN022) **B** detail of inflorescences (PMNN011) **C** seeds (PMNN011) **D–F** *G. harmandii* **D** inflorescences in habitat (PMNN006) **E** detail of inflorescences (PMNN006) **F** seeds (PMNN006). Scale bars: 2 cm (**B, E**); 1 cm (**C, F**). Photo by N. Nopporncharoenkul.

If the theca staining with aceto-orcein provided the meiotic chromosomes at the right stage, another half theca would be investigated using fluorescence DAPI staining. The theca was placed on an acid-cleaned microscope slide and treated with 10 µl of 45% (v/v) acetic acid. The anther theca was gently tapped with sterile dissecting needles, and the remaining tissue debris was discarded. A fine cell suspension was covered with 18 × 18 mm coverslip, and tapped vertically with dissecting needles to squash the cells flat. The slide was dipped into liquid nitrogen for 5 seconds, and a coverslip was immediately flicked off with a razor blade. The cells on an air-dried slide were stained with 16 µl of fluorochrome DAPI and covered with 22 × 22 mm coverslip. The chromosomes were investigated under an Olympus BX50 epifluorescent microscope connected to a UV source.

Meiotic figures were determined from the pattern of chromosome pairing during late prophase I to anaphase I at 1000× magnification under an Olympus BX50 epifluorescent microscope. The spread chromosomes were captured with an Olympus DP73 digital camera. Cytogenetic characters of each accession were analyzed from at least 20 cells per plant and three plants per accession.

DNA content estimation

The nuclear DNA content (2C-value) was estimated using propidium iodide flow cytometry according to the two-step protocol described by Doležel et al. (2007) with minor modifications. *Musa serpentina* Swangpol & Somana, 2011 clone SS&JS 246 with 2C-value = 1.36 pg was used as the internal reference standard (Rotchanapreeda et al. 2016, Nopporncharoenkul et al. 2017). The young leaves of *Gagnepainia* sample and *M. serpentina* were chopped together using a new sharp razor blade in 1 ml of fresh ice-cold nuclei isolation Otto's buffer I (0.1 M citric acid and 0.5 % Tween 20). The nuclear suspension was filtered through a 42-µm nylon mesh and then centrifuged at 3,500 rpm for 5 min. The supernatant was immediately removed, and the nuclear pellet was then resuspended in 200 µl of ice-cold Otto I solution. After that, 400 µl of Otto II solution (0.4 M $Na_2HPO_4 \cdot 12H_20$ supplemented with 50 µg/ml of propidium iodide (PI), 50 µg/ml of RNase A and 2 µl/ml of β-mercaptoethanol) was applied into the same vial with the nuclear suspension in Otto's buffer I. The nuclear suspension was incubated for about 30 min at room temperature and then analyzed by BD FACSCallibur Flow Cytometer (BD Biosciences, United States). All histograms were analyzed and gated using the BD FACSDiva version 6.1.1 software (BD Biosciences, United States). Each individual plant was re-analyzed for three times on different days and the final nuclear DNA content of each accession was estimated from three individual plants. The genome size (2C-value) of each *Gagnepainia* accession was calculated by sample G0/G1 mean peak divided by reference standard G0/G1 mean peak and multiplied with reference standard 2C-value (1.36 pg).

Pollen morphological study

The fresh pollen grains of 13 accessions were directly collected from anthers of the flowers at anthesis stage, and then preserved in 70% ethanol. Six accessions, including PMNN021, 025, 027, 028, 029, and 030, were excluded from this analysis since we could not collect the pollen samples when they were flowering. The hundred grains from individual plant and three plants of each accession were randomly selected for pollen morphological investigation. Pollen unit, shape, size (polar and equatorial axes), aperture, wall thickness and sculpturing were observed and measured using LM and SEM. For SEM investigation, the samples were dehydrated using an ethanol series

70%, 80%, 95%, and 100%, each step for 5 min. The dehydrated pollens were dried in the air at room temperature for overnight and mounted on an aluminium panel affixed to stubs with carbon tape. Consequently, the stubs were sputter-coated with platinum-palladium in a Hitachi E102 ion sputter for 10 min. The pollen morphology was examined and photographed using a Hitachi SU8010 scanning electron microscope at 5 kV. Pollen terminology, according to an illustrated handbook, was used to describe the pollen features (Hesse et al. 2009).

Statistical analysis

The datasets of pollen sizes (polar and equatorial axes) and nuclear DNA content were initially tested the normal distribution. Analysis of variance (one-way ANOVA) was also conducted using IBM SPSS Statistics version 21.0 software (IBM, United States).

Results

Cytogenetic analyses: chromosomes and genome sizes

We successfully obtained meiotic figures from six and two accessions of *Gagnepainia godefroyi* and *G. harmandii* respectively, whereas other accessions did not contain the cells at the right stage for meiotic chromosome study. Only some accessions of *G. godefroyi* provided a fair quality with low contrast of stained chromosomes when using the conventional aceto-orcein staining method. On the other hand, this technique cannot effectively differentiate the chromosomes from cytoplasm in all cases of *G. harmandii* accessions. Therefore, we need to apply the chromosome-specific DAPI fluorochrome staining for clear demarcation of chromosomes in *Gagnepainia*.

Results from the meiotic analyses of *Gagnepainia* are shown in Figure 2 and summarized in Table 1. The regular meiotic configuration with the 15 pairs of homologous chromosomes or 15 bivalents (15II) is precisely determined during late prophase I to metaphase I in both *G. godefroyi* and *G. harmandii* (Fig. 2). During the anaphase I, the obvious fifteen homologs are completely separated from equatorial plate to each polar, strongly indicates that the species of *Gagnepainia* are diploid and contain the chromosome number of $2n = 2x = 30$ (Figs 2G, H). The chromosome number of the species of *Gagnepainia*, both mitotic and meiotic, is revealed and reported here for the first time.

The genome sizes of the species of *Gagnepainia* were estimated in nuclear DNA content or 2C-value via flow cytometry, compared with the internal standard reference *M. serpentina* clone SS&JS 246 (2C-value = 1.36 pg, Rotchanapreeda et al. 2016). We calculated the nuclear DNA content for the experiments which produced CV < 5%. The mean 2C-values with standard deviation of each species, including *G. godefroyi*, *G. harmandii*, and *Gagnepainia* sp., are 2.090 ± 0.028 (ranges 2.059–2.134), 2.195 ± 0.025 (ranges 2.177–2.212), and 1.986 ± 0.035 pg, respectively (Fig. 3; Table 1).

Table 1. Cytogenetic and palynological characters of *Gagnepainia* accessions analyzed in the present study.

Species	Locality	Accession no.	2*n*	*n*	2C value (pg) ± S.E.	Mean 2C value (pg) ± S.E.	Ploidy level	Pollen size (μm)			
								Polar axis ± S.E.	Mean polar axis ± S.E.	Equatorial axis ± S.E.	Mean equatorial axis ± S.E.
Gagnepainia godefroyi (Baill.) K. Schum.	Chiang Dao, Chiang Mai	PMNN030			2.133 ± 0.021	2.090 ± 0.028	2×		59.968 ± 3.484		45.439 ± 2.870
	Dan Sai, Loei	PMNN027			2.134 ± 0.004		2×				
	Erawan, Loei	PMNN029			2.067 ± 0.016		2×				
	Mae Ramat, Tak	PMNN020			2.059 ± 0.025		2×	61.875 ± 3.551		43.274 ± 1.936	
	Mae Rim, Chiang Mai	PMNN011	30	15II	2.090 ± 0.017		2×	59.427 ± 2.953		45.681 ± 2.185	
	Mae Sot, Tak	PMNN026	30[a]	15II	2.090 ± 0.006		2×	60.740 ± 3.915		45.142 ± 2.764	
	Pha Lat, Mueang, Chiang Mai	PMNN016	30[a]	15II	2.077 ± 0.007		2×	61.255 ± 1.900		46.719 ± 1.956	
	Doi Suthep, Mueang, Chiang Mai	PMNN017	30[a]	15II	2.093 ± 0.006		2×	60.978 ± 2.144		46.679 ± 2.234	
	Nam Nao-Lom Sak, Phetchabun	PMNN025			2.077 ± 0.009		2×				
	Phan, Chiang Rai	PMNN022	30[a]	15II	2.127 ± 0.018		2×	59.166 ± 3.382		41.793 ± 1.901	
	Rong Kwang, Phrae	PMNN028			2.087 ± 0.005		2×				
	Song, Phrae	PMNN005						59.660 ± 3.750		46.385 ± 2.781	
	Thong Pha Phum, Kanchanaburi	PMNN008	30[a]	15II	2.065 ± 0.019		2×	56.651 ± 3.065		45.806 ± 2.181	
	Wiang Pa Pao, Chiang Rai	PMNN004			2.097 ± 0.033		2×	59.957 ± 3.378		47.471 ± 2.657	
G. harmandii (Baill.) K. Schum.	Ban Na, Nakhon Nayok	PMNN024			2.201 ± 0.002	2.195 ± 0.025	2×	69.713 ± 3.807	74.506 ± 5.075	49.621 ± 2.783	56.082 ± 6.459
	Bo Thong, Chonburi	PMNN006	30	15II	2.212 ± 0.031		2×	80.575 ± 4.122		65.625 ± 3.253	
	Khao Chamao, Rayong	PMNN015			2.177 ± 0.014		2×	73.447 ± 2.387		54.881 ± 2.248	
	Mueang Saraburi, Saraburi	PMNN010	30[a]	15II	2.186 ± 0.021		2×	74.289 ± 2.238		54.201 ± 2.355	
Gagnepainia sp.	Khong Chiam, Ubon Ratchathani	PMNN021			1.986 ± 0.035	1.986 ± 0.035	2×				

II bivalent, pg picogram, S.E. = standard error, PMNN = P. Moonkaew and N. Nopporncharoenkul

[a] somatic chromosome number (2*n*) referred from meiotic figure

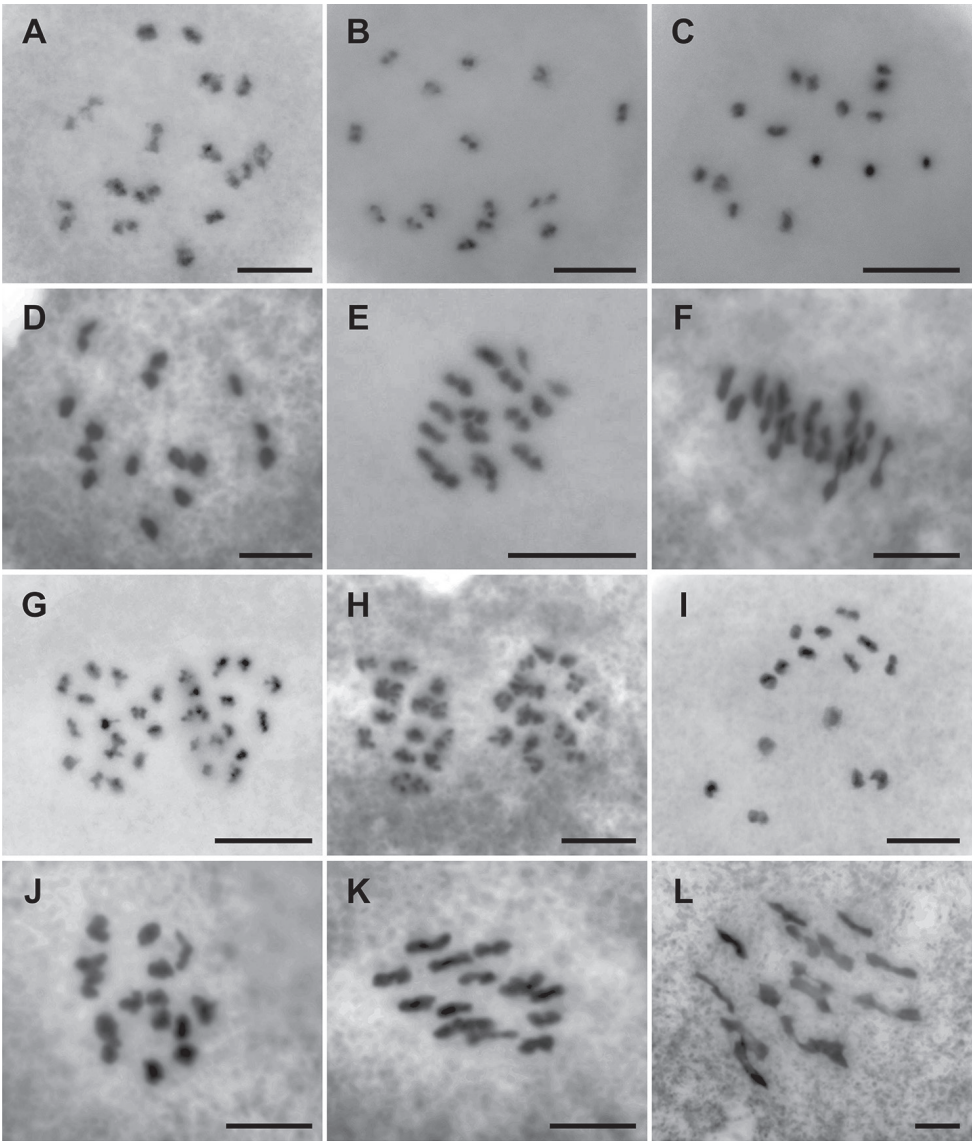

Figure 2. Meiotic chromosomes of *G. godefroyi* (**A–H**) and *G. harmandii* (**I–L**). **A–D** diakinesis **A** PMNN022 **B, C** PMNN017 **D** PMNN008 **E, F** metaphase I **E** PMNN022 **F** PMNN008 **G, H** metaphase I **G** PMNN022 **H** PMNN008 **I, J** diakinesis **I** PMNN006 **J** PMNN010 **K, L** metaphase I, PMNN010. Scale bars: 10 μm.

Pollen morphology

The pollen characters of *Gagnepainia godefroyi* and *G. harmandii* represent the same pattern in both LM and SEM analyses (Fig. 4; Table 1). The pollen grains are monad, inaperturate, prolate with bilateral symmetry. Pollen sizes of *G. godefroyi* and

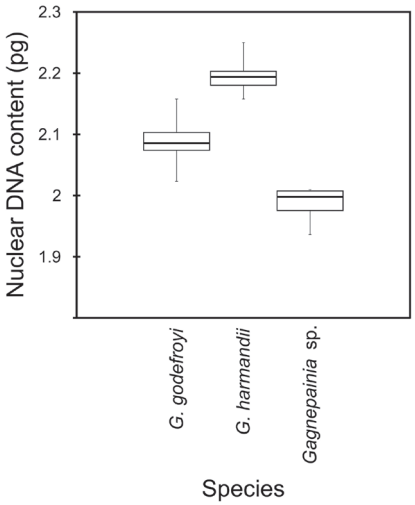

Figure 3. Histograms of the nuclear DNA content of *G. godefroyi* and *G. harmandii*. *Musa serpentina* clone SS&JS 246 was used as the internal reference standard (2C-value = 1.36 pg; Rotchanapreeda et al. 2016).

G. harmandii range from 56.651–61.875 and 69.713–80.575 μm in polar axis and 41.793–47.471 and 49.621–65.625 μm in equatorial axis, respectively. The mean pollen size of *G. harmandii* (74.506 ± 5.075, 56.082 ± 6.459) is significantly larger than that of *G. godefroyi* (59.968 ± 3.484, 45.439 ± 2.870). Pollen wall thickness varies from 1.99 to 3.95 to 3.16 to 5.25 μm in *G. godefroyi* and *G. harmandii*, respectively. The pollen exine of the species of *Gagnepainia* has fossulate sculpturing (Fig. 4).

Discussion

In this study, meiotic chromosomes of the species belonging to *Gagnepainia* are intensively investigated from the young inflorescences with the majority of closed flower buds. Each theca from the same anther is separated and examined cytogenetically using either the conventional aceto-orcein smear, or fluorescence DAPI staining techniques. Unfortunately, the conventional technique with aceto-orcein staining provides the undesirable results of an ambiguous contrast between chromosomes and cytoplasm in both *G. godefroyi* and *G. harmandii*. Because of these results, we precisely examined cytogenetically from another theca using the chromosome-specific DAPI fluorochrome application whenever the nuclei in the cells from the first half theca could not be distinguished using the prior conventional method.

During the microsporogenesis, the regular meiosis with 15 bivalents (15 II) clearly occurs at diakinesis of late prophase I. Moreover, the obvious 30 individual chromosomes during anaphase I are completely separated into 2 sets of 15 chromosomes and moved to each pole in both *Gagnepainia* species analyzed. This meiotic and other evidence, especially the numerous viable seeds found in both natural habitats and in cultivation, strong-

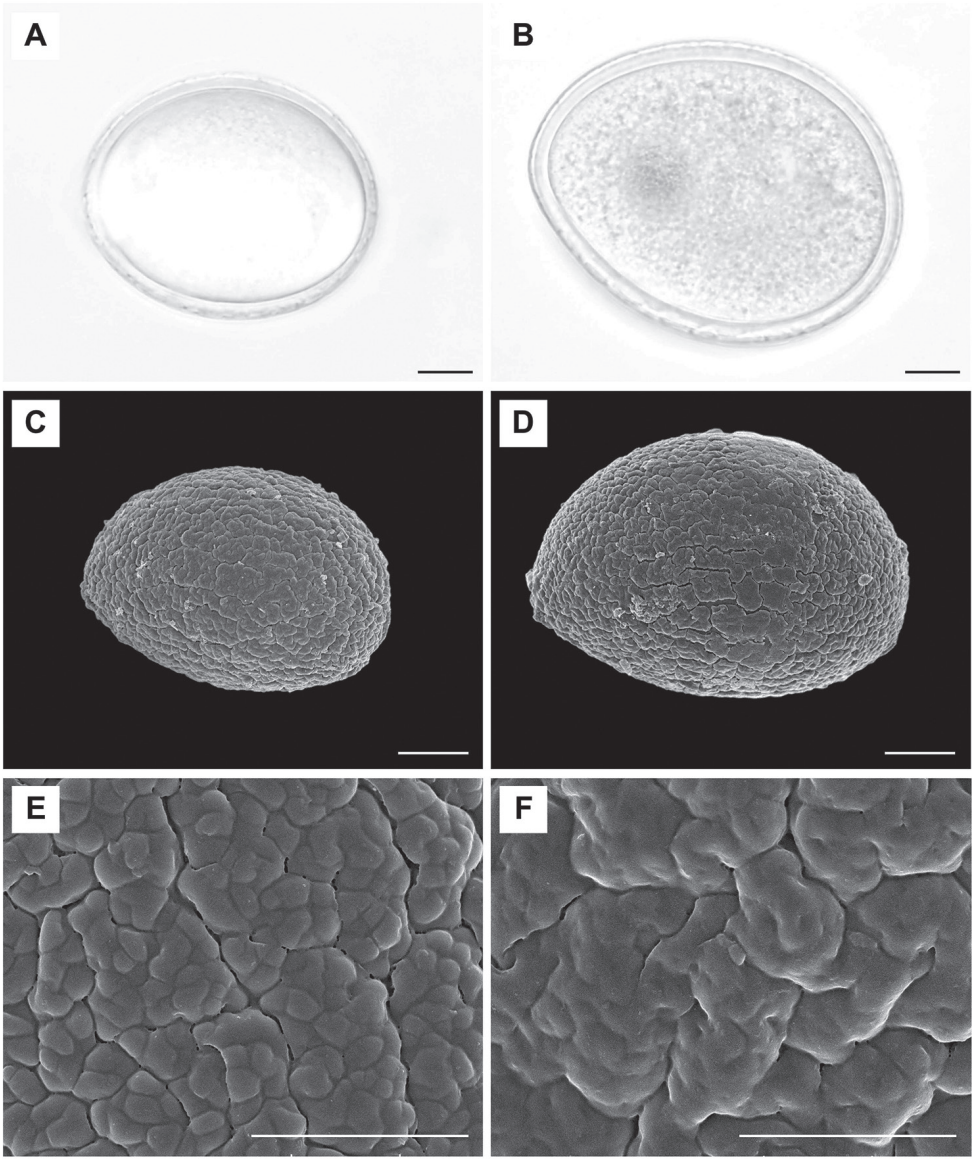

Figure 4. Pollens of *G. godefroyi*, *G. harmandii*, and *Gagnepainia* sp. **A, B** Pollen grains under LM **A** *G. godefroyi* PMNN008 **B** *G. harmandii* PMNN006 **C, D** Pollen grains under SEM **C** *G. godefroyi* PMNN008 **D** *G. harmandii* PMNN006 **E, F** Exine sculpturing under SEM **E** *G. godefroyi* PMNN008 **F** *G. harmandii* PMNN006. Scale bar: 10 μm (**A–D**) and 5 μm (**E, F**).

ly indicate that the species of *Gagnepainia* are diploid and have the chromosome number of $2n = 2x = 30$ (Fig. 2; Table 1). As a result, the basic chromosome number of the genus *Gagnepainia* is $x = 15$. When comparing with the genome size of *Musa serpentina* clone SS&JS 246 (Rotchanapreeda et al. 2016), the range of the nuclear DNA content is found

ranging from 1.986 pg in *Gagnepainia* sp. accession PMNN021 to 2.212 pg in *G. harmandii* accession PMNN006 (Fig. 3; Table 1). Although both *Gagnepainia* species contain the same chromosome number of $2n = 30$, they display the different 2C/2*n* values or average chromosome sizes. To conclude, these characteristics, genome sizes, and 2C/2*n* values are slightly different and able to distinguish between two *Gagnepainia* species.

The monoploid genomes (1C*x*-value) of the genera, belonging to the Ginger family, were classified as very small genome which are less than 3.5 pg (Soltis et al. 2003, Leong-Škorničková et al. 2007, Chandrmai et al. 2012, Sadhu et al. 2016, Basak et al. 2018), such as *Alpinia* Roxburgh, 1810 (0.965–1.108 pg), *Curcuma* Linnaeus, 1753 (0.265–0.473 pg), *Globba* (0.750–0.908 pg), *Hedychium* J. Koenig, 1779 (0.678–1.070 pg), *Kaempferia* Linnaeus, 1753 (1.180–1.863 pg), and *Zingiber* Miller, 1754 (1.800–1.945 pg). However, the 1C*x*-value of *Gagnepainia* (0.993–1.106 pg) is closely related to *Globba*, another genus within the same tribe Globbeae.

According to the previous zingiberaceous chromosome reports, the chromosome number of $2n = 30$ is uniquely found only in tribe Globbeae, especially genus *Hemiorchis* (Pospíšilová et al. 2016). The base chromosome numbers of the family Zingiberaceae have been reported ranging from $x = 6$ to $x = 25$ (Ramachandran 1969, Jatoi et al. 2007). Interestingly, the basic chromosome number $x = 15$ of *Gagnepainia* is a new number in the Ginger family. The chromosome and genome size evidences of the present study fully support the classical taxonomic and phylogenetic classification of Globbeae that *Gagnepainia* is more closely related to *Hemiorchis* than *Globba* (Williams et al. 2004, Pospíšilová et al. 2016). However, *Gagnepainia* and *Hemiorchis* are recognized as distinct genera by use of morphological characters, such as filament length, specific labellum shape, rhizome and tuberous root form (Williams et al. 2004, Picheansoonthon and Tiyaworanant 2010, Pospíšilová et al. 2016).

A monad, inaperturate, prolate with bilateral symmetry, and thick wall with fossulate exine sculptured pollen is recognized as the species of *Gagnepainia* (Fig. 4). The pollen shape and aperture of *Gagnepainia* are similar to those in some genera in Zingiberaceae, especially *Curcuma* and *Hedychium* (Sakhanokho and Rajasekaran 2010, Chen and Xia 2011, Saensouk et al. 2015). However, the nearly smooth pollen exine ornamentation of *Gagnepainia* is obviously different from the echinate exine of *Globba* (Syamsuardi et al. 2010). Amongst the quantitative pollen results, sizes of pollen grains are significant difference between two *Gagnepainia* species, *G. godefroyi* (59.968 ± 3.484 μm, 45.439 ± 2.870 μm) *vs G. harmandii* (74.506 ± 5.075 μm, 56.082 ± 6.459 μm). Therefore, the pollen size is considered as the effective character state that has the potential for species discrimination of *Gagnepainia*.

The high genetic stability with regular meiosis, normal pollen production through producing of numerous viable seeds in natural habitats and cultivation obviously indicates that species of *Gagnepainia* have high fertility and productivity. Theoretically speaking, both *Gagnepainia* species should be broadly distributed in large populations. According to our field studies, these reproductive evidences are in full accordance with previous study that the species of *Gagnepainia* are widely distributed across Indo-China and Thailand, except only the peninsular region which unusually has a high mon-

soon rainfall (Chuakul and Boonpleng 2004, Techaprasan et al. 2010, Promtep et al. 2011, Leong-Škorničková and Newman 2015, Saensouk et al. 2016). Nevertheless, *G. harmandii* has a more restricted distribution around Central and Eastern Thailand than *G. godefroyi*. This might be caused by the habitat destruction and fragmentation through the expansion of human population and plantation. According to fertility and distribution information, the least concern (LC) has recently been assigned as the IUCN conservation status for both species of *Gagnepainia* (Newman and Simon 2017). However, the field observations of these plants are extremely difficult because they have short flowering period, flowers and leaves usually occur at the different time, and plants mostly grow up in the deep forest coexisting with many zingiberaceous plants. Likewise, in several plants of the Zingiberaceae, the lack of the reproductive parts, especially flowers at anthesis, contributes to easily taxonomic misidentification (Nopporncharoenkul et al. 2017).

On the other hand, *Gagnepainia* sp. accession PMNN021 collected from Khong Chiam, Ubon Ratchathani has distinctive swollen rhizomes jointed with the base of the well-developed pseudostem, which is a unique character, only occurring in Gagnepainia. Since the sample collection, this plant has not produced the inflorescences and flowers. After the genome size has been estimated, this accession contains a significantly different genome size from *G. godefroyi and G. harmandii*. Consequently, this accession may be either a variation of two recognized species, new record, or new species, not identifiable until its flower is intensively observed.

Conclusion

We have provided beneficial information on the cytological and reproductive aspects of the species belonging to *Gagnepainia*. First of all, the chromosome number $2n = 30$ with the base number $x = 15$ of the genus *Gagnepainia* is revealed here for the first time and recognized as the new number for the Ginger family. Secondly, the genome and pollen sizes in the present study can be used as the effective characteristics for species discrimination between *G. godefroyi* and *G. harmandii*. This is especially useful as both species of *Gagnepainia* have herbal properties used for treatment of wounds and inflammations, and also have the numerous attractive butterfly-like flowers. Thirdly, they have the high potential to be developed as the commercial medicinal and ornamental pot plants through breeding and genetic improvement programs, such as polyploid induction. Fourthly, the present cytogenetic study has provided not only informative characteristic for species discrimination, but also very useful assessment for plant fertility through *in situ* and *ex situ* conservation strategies, plant genetic resource management, and plant improvement programs. Last but not least, karyotyping of mitotic chromosomes and application of fluorescence in situ hybridization (FISH) should be investigated in future research in order to precisely understand chromosomal evolution between the genera *Gagnepainia* and *Hemiorchis*.

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RESEARCH ARTICLE

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Interspecific cytogenetic relationships in three *Acestrohynchus* species (Acestrohynchinae, Characiformes) reveal the existence of possible cryptic species

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Abstract

The karyotypes and chromosomal characteristics of three *Acestrorhynchus* Eigenmann et Kennedy, 1903 species were examined using conventional and molecular protocols. These species had invariably a diploid chromosome number 2n = 50. *Acestrorhynchus falcatus* (Block, 1794) and *Acestrorhynchus falcirostris* (Cuvier, 1819) had the karyotype composed of 16 metacentric (m) + 28 submetacentric (sm) + 6 subtelocentric (st) chromosomes while *Acestrorhynchus microlepis* (Schomburgk, 1841) had the karyotype composed of 14m+30sm+6st elements. In this species, differences of the conventional and molecular markers between the populations of Catalão Lake (AM) and of Apeu Stream (PA) were found. Thus the individuals of Pará (Apeu) were named *Acestrorhynchus* prope *microlepis*. The distribution of the constitutive heterochromatin blocks was species-specific, with C-positive bands in the centromeric and telomeric regions of a number of different chromosomes, as well as in interstitial sites and completely heterochromatic arms. The phenotypes of nucleolus organizer region (NOR) were simple, i. e. in a terminal position on the *p* arm of pair No. 23 except in *A. microlepis*, in which it was located on the *q* arm. Fluorescence *in situ* hybridization (FISH) revealed 18S rDNA sites on one chromosome pair in karyotype of *A. falcirostris* and *A.* prope *microlepis* (pair No. 23) and three pairs (Nos. 12, 23, 24) in *A. falcatus* and (Nos. 8, 23, 24) in *A. microlepis*; 5S rDNA sites were detected in one chromosome pair in all three species. The mapping

of the telomeric sequences revealed terminal sequences in all the chromosomes, as well as the presence of interstitial telomeric sequences (ITSs) in a number of chromosome pairs. The cytogenetic data recorded in the present study indicate that *A.* prope *microlepis* may be an unnamed species.

Keywords
Repetitive DNA, fish cytotaxonomy, FISH, ITS

Introduction

The family Acestrorhynchidae is a group of South American fishes, for which contradictory views on its phylogenetic position within Characiformes are debated. Based on the analysis of morphological data, for example, Buckup (1998) proposed that the Acestrorhynchidae was part of a monophyletic group, the superfamily Erythrinoidea, which included the families Ctenoluciidae, Hepsetidae, Lebiasinidae, and Erythrinidae. At the same time, also based on morphological data, Lucena and Menezes (1998) suggested that the Acestrorhynchidae (*Acestrorhynchus* Eigenmann & Kennedy, 1903) was a sister group of the family Cynodontidae, consisting of the subfamilies Roestinae (*Roestes* Günther, 1864 and *Gilbertolus* Eigenmann & Ogle, 1907) and Cynodontinae (*Cynodon* Spix & Agassiz, 1829, *Hydrolycus* Müller & Troschel, 1844, and *Rhaphiodon* Spix & Agassiz, 1829), and proposed five synapomorphic characters for *Acestrorhynchus*, supporting thus the monophyly of this genus, as proposed by Menezes (1969b) and Menezes and Géry (1983). Nelson et al. (2016) offered a new classification, based on the most recent data adopted for the determination and classification of characiform taxa, in which the Roestinae was placed as a subfamily of the Acestrorhynchidae, with two genera and six species. Two additional acestrorhynchid subfamilies were also identified, the Heterocharacinae, with four genera and six species, and Acestrorhynchinae, with one genus and 14 species.

The genus *Acestrorhynchus* includes piscivorous fishes with an elongated body and snout, conical teeth and robust canines with a characteristic arrangement in the maxilla, together with a number of other diagnostic traits (Menezes 1969a; Menezes and Géry 1983; Toledo-Piza 2007). The species of the genus *Acestrorhynchus* are widespread in South America, where most of its diversity is concentrated in the Amazon and Orinoco river basins, and the rivers of the Guyanas (Nelson et al. 2016).

The *Acestrorhynchus* species can be distinguished on the basis of their coloration patterns and can be allocated to three groups: (i) *Acestrorhynchus lacustris* group (Menezes 1992), characterized by a well-defined dark spot in the humeral region, (ii) *Acestrorhynchus nasutus* group, defined by the presence of two dark, narrow longitudinal stripes, one which extends from the tip of the snout to the base of the caudal fin, and the other, from the posterior margin of the maxilla to the ventral margin of the caudal peduncle (Toledo-Piza 2007), and (iii) the *Acestrorhynchus microlepis* group, diagnosed by the presence of a small dark spot on the anterior region of the body, immediately posterior to the posterior margin of the operculum, at the origin of the lateral line (Toledo-Piza and Menezes 1996; Toledo-Piza 2007). Pretti et al. (2009) analyzed mitochondrial and nuclear sequences from genomes of 11 of the 14 *Acestrorhynchus*

recognized species and concluded that the genus should be divided into three groups, although the phylogenetic relationships among these three clades did not correspond with the morphological phylogenies.

The cytogenetic data available for *Acestrorhynchus* indicate a conserved 2n = 50 in all species (Falcão and Bertollo 1985; Martinez et al. 2004; Pastori et al. 2009), although the karyotypes varied both within and among species. For example, in *Acestrorhynchus lacustris* (Lütken, 1875) the karyoytpe is composed of 12m+32sm+4st+2a (Falcão and Bertollo 1985) or 8m+34sm+6st+2a in Martinez et al. (2004). These species have karyotypes with a single pair of NOR-bearing chromosomes, except that of *Acestrohhynchus altus* Menezes, 1969, which has two such pairs (Falcão and Bertollo 1985).

The present study examined the karyotypes and chromosomal characteristics of three Amazonian *Acestrorhynchus* species using both conventional and molecular cytogenetic protocols. Our results were compared with the existing data attempting to better understand the chromosomal differentiation of the genus and the rearrangements involved in this process.

Material and methods

The present study analyzed the cytogenetic characteristics of *Acestrorhynchus falcatus* (Block, 1794), *Acestrorhynchus falcirostris* (Cuvier, 1819), and *Acestrorhynchus microlepis*, (Schomburgk, 1841) where the latter species had variation in the chromosome complement of representatives from different collecting localities (Fig. 1, Table 1). The present study followed the ethical standards for zoological research determined by the National Institute of Amazonian Research (INPA) Ethics Committee for the Use of Animals in Research and authorized by protocol number 021/2017. The collection of individuals was authorized by the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA), through SISBIO license number 28095-1. All the specimens were deposited as vouchers in the INPA Fish Collection (Table 1).

Conventional chromosome banding

The chromosomal preparations were obtained following the protocols of Oliveira et al. (1988) and Gold et al. (1990). The active NORs were detected by silver nitrate impregnation (Ag-NORs), following Howell and Black (1980), while constitutive heterochromatin was detected following Sumner (1972).

Molecular cytogenetic protocols

The 5S and 18S ribosomal DNA probes were obtained from the genomic DNA of *A. falcirostris*, which was extracted using the Wizard Genomic DNA Purification kit. The rDNA probes were amplified by polymerase chain reaction (PCR), using the primers

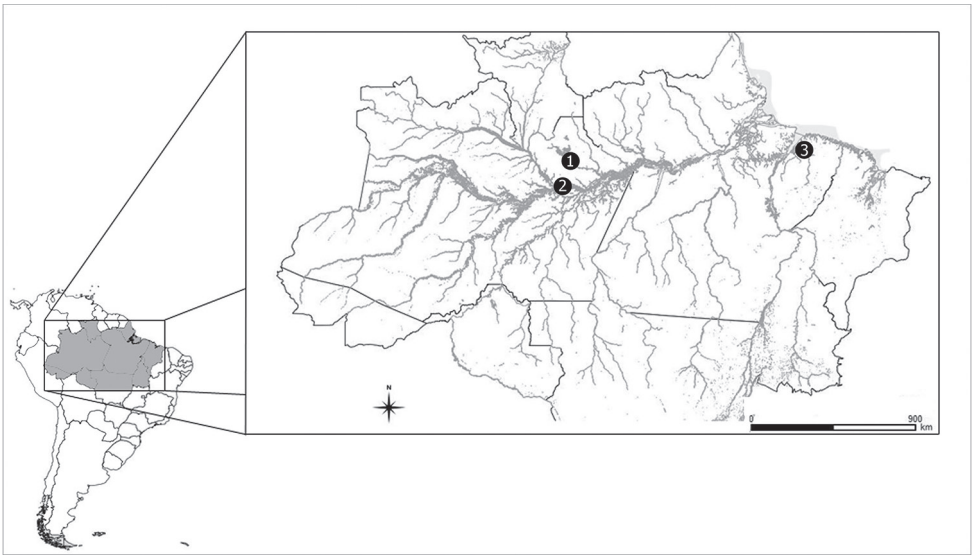

Figure 1. Map of the Brazilian Amazonia region, showing the individual collection localities. **1** *Acestrorhynchus falcirostris* – Balbina reservoir on the Uatumã River, Amazonas state **2** *Acestrorhynchus falcirostris* and *Acestrorhynchus microlepis* – Catalão Lake, at the confluence of the Negro and Solimões rivers, Amazonas state **3** *Acestrorhynchus falcatus*, *Acestrorhycnhus falcirostris*, and *Acestrorhynchus* prope *microlepis* – Apeu Stream, basin of the Guamá River, Pará.

Table 1. The *Acestrorhynchus* species included in the present study, collecting localities, and the number of individuals analyzed. ♂ = male; ♀ = female.

Species	Sampling locations	Hydrographic Basin	Coordinates	Number of analized animals	Vouchers
A. falcatus	Apeu Stream, Pará, Brazil	Guamá River	1°23'20.4"S, 47°59'07.5"W	8♂ 2♀	INPA 57803
A. falcirostris	Catalão Lake, Amazonas, Brazil	Solimões River	3°09'20.4"S, 59°54'47.1"W	1♂ 7♀	INPA 57166
	Balbina UHE, Amazonas, Brazil	Uatumã River	1°55'07.6"S, 59°29'19.7"W	1♂ 2♀	INPA 57167
	Apeu Stream, Pará, Brazil	Guamá River	1°23'20.4"S, 47°59'07.5"W	3♂ 1♀	INPA 57168
A. microlepis	Catalão Lake, Amazonas, Brazil	Solimões River	3°09'20.4"S, 59°54'47.1"W	1♂ 2♀	INPA 57599
A. cf. *microlepis*	Apeu Stream, Pará, Brazil	Guamá River	1°23'20.4"S, 47°59'07.5"W	4♂ 2♀	INPA 57802

18Sf (50-CCG CTG TGG TGA CTC TTG AT-30), and 18Sr (50 - 31 CCG AG-GACC TCA CTA AAC CA- 30) (Gross et al. 2010), 5Sa (50-TAC GCC CGA TCT CGT CCG ATC-3') and 5Sb (5'- CAGGCT GGT ATC GCC GTA AGC-3') (Martins and Galetti 1999). Telomeric segments were generated using non-templated PCR with primers (TTAGGG)5 and (CCCTAA)5 (Ijdo et al. 1991).

The PCR products were verified in 1.5% agarose gel, and quantified in NanoVue Plus (GE Healthcare). The 18S rDNA gene was marked with digoxigenin-11-dUTP (Dig Nick Translation mix, Roche), while the 5S rDNA gene and telomeric sequences were marked with biotin-14-dATP (Biotin Nick Translation mix, Roche), following the manufacturer´s instructions. The hybridization signals were detected using anti digoxigenin-rhodamine (Roche Applied Science) for the 18S rDNA probe, and streptavidin (Sigma-Aldrich) for the 5S rDNA probes and telomeric sequences. Fluorescence

in situ hybridization (FISH) was based on the protocol of Pinkel et al. (1986), with a stringency of 77%. The chromosomes were counter-stained with (2 mg/mL) DAPI in a Vectashield (Vector) mounting medium.

Image analysis and processing

The chromosomes of about 30 metaphases per individual were analyzed and the images were captured using an Olympus BX51 epifluorescence microscope, and processed using Image Pro Plus 4.1 software (Media Cybernetics, Silver Spring, MD, USA). The chromosomes were classified according to Levan et al. (1964).

Results

All *Acestrorhynchus falcatus*, *A. falcirostris*, *A. microlepis*, and *A.* prope *microlepis* individuals possessed invariably 2n = 50 and a fundamental number (FN) 100. Their karyotypes were very similar to each other and composed of 16m+28sm+6st in *A. falcirostris* and *A. falcatus*, while 14m+30sm+6st in *A. microlepis* and *A.* prope *microlepis* (Fig. 2a, d, g, j).

The NORs were located in a distal position on the *p* arms of pair No. 23 in all the species, except for *A. microlepis*, in which the NORs were located on the *q* arms of pair No. 23 (Fig. 2c, f, i, l).

The positive 18S rDNA sites corresponded to the NOR signals in *A. falcirostris* and *A.* prope *microlepis*, at pair No. 23 (Fig. 3a, c, e, g), whereas in *A. falcatus* and *A. microlepis*, the 18S rDNA sites were observed at two chromosome pairs in addition to the single NOR-bearing pair. In *A. falcatus*, these additional 18S rDNA loci resided on the *p* arms of pairs Nos. 12 and 24 (Fig. 3c), while in *A. microlepis* they mapped to the *q* arms of pairs Nos. 8 and 24 (Fig. 3g).

The blocks of constitutive heterochromatin were distributed in centromeric and telomeric regions in karyotypes of all species, though with unique features found in each species, as follows:

A. falcatus: heterochromatin in centromeric and telomeric blocks in pairs Nos. 2, 4, 6, 8, 10, 15, 16, 20, and 22, and in centromeric blocks only in pairs Nos. 1, 3, 5, 7, 9, 11, 13, 14, 17, 19, and 21, while pairs Nos. 12, 23, 24, and 25 have entirely heterochromatic p arms, and pair No. 18 had no clear heterochromatic signal (Fig. 2e).

A. falcirostris: heterochromatin in centromeric and telomeric blocks in pairs No. 1, 3, 4, 5, 7, 8, 9, 10, 12, 14, 15, and 18, in telomeric blocks only in pairs Nos. 2, 6, 17, 19, 20, 21, and 22 and in pericentromeric blocks only in pairs Nos. 11, 16, 23, and 24. Pairs Nos. 13 and 25 have centromeric blocks and terminal blocks on the q arms. In pair No. 23, a differential accumulation of heterochromatin was observed in the *p* arms, with blocks adjacent to the NOR (Fig. 2b).

A. prope *microlepis*: heterochromatin in centromeric and pericentromeric regions. Pair No. 4 also had telomeric signals, while in pairs Nos. 2, 8, 17, 18, 20, 23, 24, and

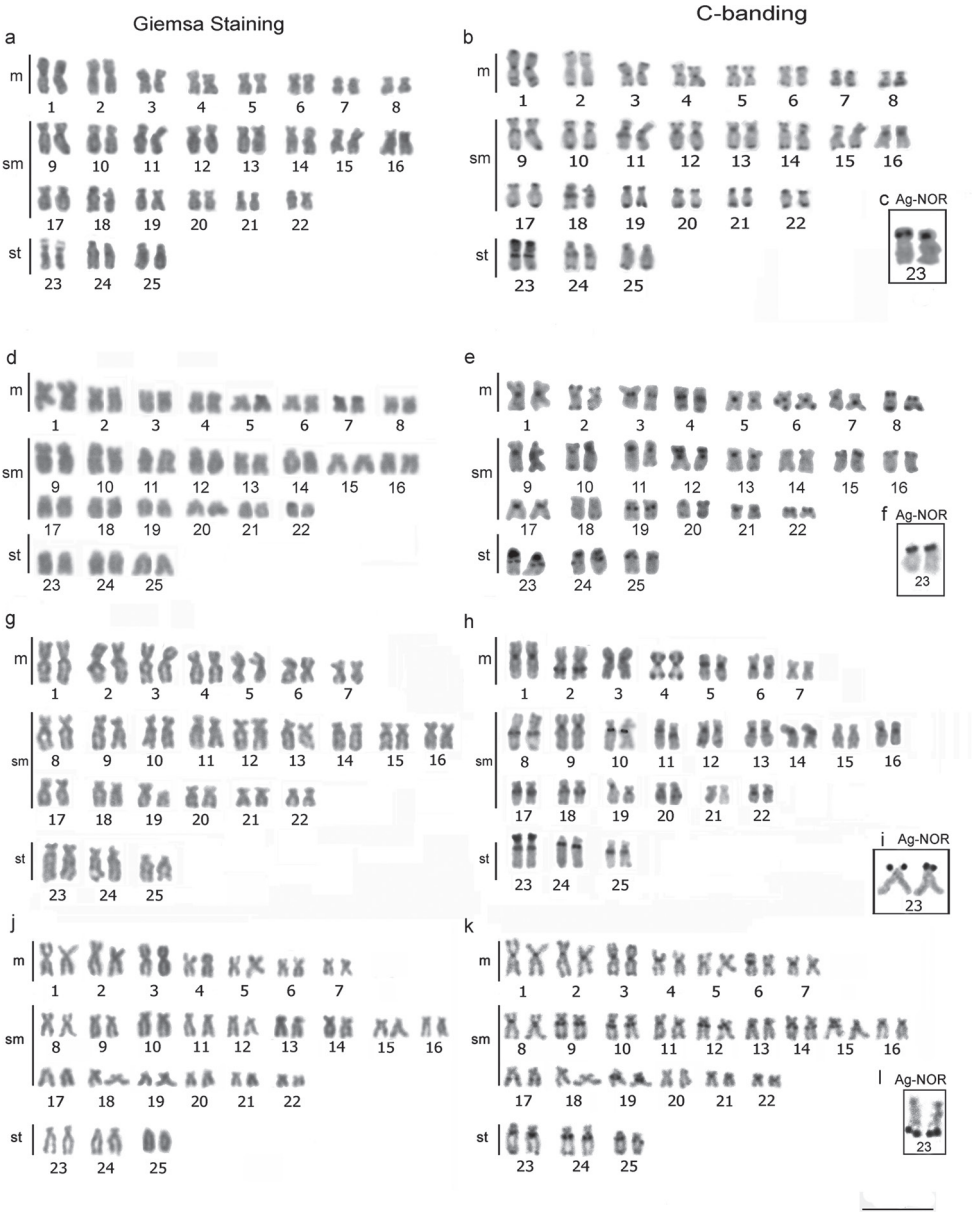

Figure 2. Karyotypes of the species under study arranged from chromsomes stained conventionally with Giemsa, C-banded, and after Ag-NOR impregnation: **a–c** *A. falcirostris* **d–f** *A. falcatus* **g–i** *A.* prope *microlepis* **j–l** *A. microlepis*. Scale bar: 10 μm.

25, there is a block in a more interstitial position. Pair No. 19 displayed size heteromorphism of a heterochromatin block, observed after both Giemsa staining and C-banding (Fig. 2h).

A. microlepis: heterochromatin found primarily in centromeric regions, with some proximal signals, but in a pattern distinct from that observed in *A.* prope *microlepis*,

Figure 3. Karyotypes of the species under study, arranged from chromosomes showing "double" FISH with 18S rDNA (red) and 5S (green) probes **a, c, g** and FISH with (TTAAGG)n probe, in green **b, d, f, h** *A. falcirostris* (**a, b**), *A. falcatus* (**c, d**), *A.* prope *microlepis* (**e, f**), *A. microlepis* (**g, h**). Scale bar: 10 μm.

in terms of the location and position of the heterochromatin on some chromosome pairs (Fig. 2k).

The mapping of the 5S rDNA gene revealed a pericentromeric signal in only one pair in each species (pair No. 17 in *A. falcirostris*, pair No. 3 in *A. falcatus*, *A. microlepis* and A. prope *microlepis*) (Fig. 3a, c, e, g).

Telomeric sequences were detected in the terminal regions of all chromosomes, as expected, but also with additional interstitial telomeric sequences (ITSs) in a number of chromosome pairs in all species under study, displaying species-specific patterns in terms of their localization (Fig. 3b, d, f, h). In *A. falcirostris*, the ITSs were located in 10

Figure 4. Chromosomal pairs with ITSs in comparison with C-positive (C-banding) heterochromatin **a** *A. falcirostris* **b** *A. falcatus* **c** *A.* prope *microlepis* **d** *A. microlepis*. The pairs are arranged irrespective of the type of chromosome. Scale bar: 10 μm.

chromosome pairs, with an accumulation of these sequences in pairs Nos. 12, 13, 23, and 24 (Fig. 4a). In *A. falcatus*, the ITSs were found in six chromosome pairs (Fig. 4b), while in *A.* prope *microlepis*, they were present in 18 pairs, displaying varied signal intensities; and in pair No. 19, the ITSs varied in size between the homologs (Fig. 4c). In *A. microlepis*, ITSs were present in 19 pairs (Fig. 4d).

Discussion

All species analyzed in the present study have invariably 2n = 50 chromosomes, with no morphologically distinguishable sex chromosomes. There is a considerable variation, however, in the karyotype structures and the FN values (Falcão and Bertollo 1985; Martinez et al. 2004; Pastori et al. 2009; present study). One of the main differences between the present study and the formerly published data is the absence of acrocentric chromosomes in the karyotypes of species analyzed here (Table 2). However, these analyzed species encompass all three morphological groups (based on coloration patterns) defined by Menezes (1969a), Menezes and Géry (1983), and Toledo-Piza (2007), i.e., the *Acestrorhynchus lacustris* group (*A. falcatus*), *A. nasutus* group (*A. falcirostris*), and *A. microlepis* group (*A. microlepis* and *A.* prope *microlepis*). Considering these morphological groups for the genus *Acestrorhynchus*, no group-level cytogenetic marker was found (Table 2).

Based on the analysis of morphological characters, Lucena and Menezes (1998), Toledo-Piza (2007) and Mirande (2010) reached the same conclusion that the family Acestrorhynchidae is a sister group of the Cynodontidae, which has a known 2n = 54 (Arai 2011). This would suggest that the ancestral karyotype of *Acestrorhynchus* would have had 54 biarmed chromosomes, which evolved likely through fusions, reducing thus the 2n; and inversions, or reciprocal/nonreciprocal translocations, or centromere

Table 2. Cytogenetic data available for the representatives of the genus *Acestrorynchus*. (2n = diploid chromosome number, FN = Fundamental Number, NOR = Nucleolus Organizer Region, ITS = Interstitial Telomeric Sequence, m = metacentric, sm = submetacentric, st = subtelocentric, a = acrocentric chromosomes p = short arm, q = long arm).

Groups	Species	2n	FN	NOR	Karyotype formulae	N° and location 18S rDNA	N° and location 5S rDNA	N° of pairs ITS	References
lacustris	*A. altus*	50	94	2 pairs	8m+22sm+14st+6a	-	-	-	Falcão and Bertollo [1985]
	A. falcatus	50	100	1 pair	16m+28sm+6st	3 pairs; (p)	1 pair (3); pericentromeric	6 pairs	Present study
	A. lacustris	50	98	1 pair	12m+32sm+4st+2a	-	-	-	Falcão and Bertollo [1985]
	A. lacustris	50	98	-	8m+34sm+6st+2a	-	-	-	Martinez et al. [2004]
	A. pantaneiro	50	86	1pair	36 m-sm+14st-a	-	-	-	Pastori et al. [2009]
microlepis	*A.* cf. *microlepis*	50	100	1pair	14m+30sm+6st	1 pair; (p)	1 pair (3); pericentromeric	18 pairs	Present study
	A. microlepis	50	100	1pair	14m+30sm+6st	2 pairs; (q) and 1 pair bitelomeric	1 pair (3); pericentromeric	19 pairs	Present study
nasutus	*A. falcirostris*	50	100	1 pair	16m+28sm+6st	1 pair; (p)	1 pair (17); pericentromeric	10 pairs	Present study

repositioning, or heterochromatin loss/addition resulting in the maintenance of the complement of biarmed chromosomes, but with distinctly different karyotypes.

A similar scenario is found in the Erythrinoidea (Ctenoluciidae, Hepsetidae, Lebiasinidae and Erythrinidae), a fish groups that are also closely-related to the Acestrorhynchidae (Ortí and Meyer 1997; Buckup 1998). Except for the Hepsetidae, which has 2n = 58 (Carvalho et al. 2017), there has been a reduction in the 2n. In the Erythrinidae, for example, the 2n ranges from 40 to 50 (Oliveira et al. 2015), while most representatives of Lebiasinidae possess 2n = 40 (Moraes et al. 2017), and those of Ctenoluciidae have 2n = 36 (Sousa e Souza et al. 2017).

The comparison of the different markers provides valuable insights into the chromosomal differentiation of *Acestrorhynchus*. In karyotypes of all species, the blocks of constitutive heterochromatin are located primarily in centromeric or telomeric regions, although large heterochromatic blocks are associated with the NORs, as seen as in most species of teleost fish of different families of different orders such as Anguilliformes, Siluriformes, Characiformes, among others (Gornung 2013; Blanco et al. 2014; Salvadori et al. 2018). The NOR phenotype was simple, i.e. one pair of NOR-bearing chromosomes, as observed also in Ctenoluciidae (Sousa e Souza et al. 2017), Cynodontidae (Pastori et al. 2009), and some Erythrinidae species (Bertollo 2007), although multiple NORs are also found in the Lebiasinidae (Moraes et al. 2017). Two species, *A. falcatus* and *A. microlepis*, have multiple 18S rDNA signals, but the Ag-NOR was simple. On the other hand, Falcão and Bertollo (1985) observed multiple Ag-NORs (two pairs) in karyotype of *A. altus* from the Miranda River, Mato Grosso do Sul. As terminal chromosomes regions may be more vulnerable to the transfer of genetic material due to their proximity in the nucleus, (Schweizer and Loidl 1987),

the dispersal of the 18S rDNA sequences in *A. falcatus* and *A. microlepis* may have been facilitated by their proximity to the telomere or by ectopic recombination in meiosis (Pedrosa-Harand et al. 2006; Cazaux et al. 2011; Evtushenko et al. 2016).

The telomeric sequence was a particularly valuable cytogenetic marker, with a species-specific configuration in the four studied taxa, due to the large number of ITSs distributed in different pairs. In fishes, as in other vertebrates, the pericentromeric ITSs are found within or adjacent to the constitutive heterochromatin (Milhomem et al. 2008; Cioffi et al. 2010; Scacchetti et al. 2011; Rosa et al. 2012; Ocalewicz 2013). The ITSs can be classified in six types: heterochromatic (het-ITSs), short (s-ITSs), large ITSs in restricted euchromatic regions (Restricted eu-ITSs), long subtelomeric, fusion, and pericentromeric ones (Lin and Yan 2008; Ruiz-Herrera et al. 2008; Schmid and Steinlein 2016).

In *Acestrorhynchus* species all six ITS types have been observed. Larger sequences were observed in association with the blocks of constitutive heterochromatin in some chromosome pairs as revealed by the C-banding, although a number of the observed ITSs were not associated in any way with the heterochromatin (Fig. 4). It is possible that the het-ITS arose as short sequences through processes such as repair mechanisms (Nergadze et al. 2004, 2007), fusion (Slijepcevic 1998; Bolzán and Bianchi 2006), transposition (Bouffler et al. 1993; Nergadze et al. 2007) or in association with satellite DNA as seen in a species of the family Sparidae (Perciformes) (Garrido-Ramos et al. 1998). These sequences would have increased in length through duplication, in specific independent events in each species, which would then have become integrated with the heterochromatin and become detectable by FISH (Nergadze et al. 2004, 2007; Bolzán and Bianchi 2006).

Other types of ITS, not associated with the heterochromatin would have arisen through terminal translocations, the insertion of telomeric repetitions during the repair of breaks in double-strand DNA, or by the duplication or transposition of genes (Lin and Yan 2008; Ruiz-Herrera et al. 2008; Bolzán 2012). Ruiz-Herrera et al. (2008) concluded that the occurrence of het-ITS is related to the expression of the genes of a specific cellular lineage through epigenetic modifications. No specific function is known in the case of the ITSs that are unrelated to the heterochromatin, although this does not impede their inclusion in the analysis of the evolutionary history of closely-related species. As the chromosomal evolution of *Acestrorhynchus* appears to have been based on a reduction of the number of chromosomes, some of the ITSs may actually be remains of specific rearrangements, although a definitive understanding of this process will require more detailed data from a larger number of species.

In the specific case of *A. microlepis*, remarkable differences were found between the individuals collected at the two localities (Catalão Lake and Apeu Stream, respectively), both in the location of the NORs and the number and location of the 18S rDNA sites. Thus the individuals of Pará (Apeu) were provisionally named *A.* prope *microlepis*. These chromosomal differences may reflect the presence of an unnamed species, that is, a past speciation event, which would have been caused by the geographic distance between the two populations. This distance would have minimized gene flow, isolating the populations, and permitting the fixation of specific rearrangements. A probable rearrangement

was a pericentric inversion involving the NOR carrier pair, since NOR in three species was on the short arm and *A. microlepis* was on the same pair, but located on the long arm.

Another possible type of arrangement is the translocation of major ribosomal 18S sites, which were present in four other sites, in addition to the NORs. This movement may have been facilitated by transposable elements (TEs) associated with the heterochromatin, which has great potential to cause chromosomal rearrangements, as well as through ectopic recombination that can generate intrachromosomal recombination between copies of the same family of transposable elements, arranged in opposite positions (Kidwell 2002; Grewal and Jia 2007; Skipper 2007; Raskina et al. 2008; Delprat et al. 2009; Cioffi et al. 2010; Evtushenko et al. 2016). The genomes of *A. microlepis* and *A.* prope *microlepis* differed also in terms of their ITSs, given not only that the ITSs were present in 19 chromosome pairs in one species, and in 18 pairs in the other one, but also the fact that these chromosomes were different, as well as the polymorphism between the homologs of pair 19 in *A.* prope *microlepis*. In this case, there was a larger ITS in one of the homologs, indicating the translocation of a telomeric sequence to this chromosome (Ruiz-Herrera et al. 2008; Bolzán 2017) and its duplication. An ITS may indicate the presence of chromosomal rearrangements during the evolutionary process, leading to the differentiation of the karyotypes of different species, as observed in several fish families (Meyne et al. 1989; Mota-Velasco et al. 2010; Cioffi and Bertollo 2012; Ocalewicz 2013; Sousa e Souza et al. 2017).

López-Fernández and Winemiller (2003) found subtle differences in the pigmentation and body shape of individuals identified as *A. microlepis*, but concluded that this variability was not sufficient to differentiate species. Furthermore, these authors concluded that *A. apurensis* Toledo-Pizza & Menezes, 1996, described from the Orinoco River in Venezuela was in fact a junior synonym of *A. microlepis*, which occurs in the Negro and Branco rivers in northern Brazil, and in the river basins of the Guyanas. However, the results of the present study indicates that the *A. microlepis* and *A.* prope *microlepis* individuals, while morphologically very similar, have karyotypes with significantly different locations of their NORs and 18S rDNA sites, C-banding patterns, and the pattern of ITSs, including the polymorphism of the homologs of pair No. 19 in *A.* prope *microlepis*.

Cioffi et al. (2018) highlighted the importance of cytogenetic markers for the identification of morphologically similar and/or identical fish groups, as in the case of the *Hoplias malabaricus* (Bloch, 1794), which had seven distinct karyomorphs, including some found in sympatry, supporting the need for a taxonomic review of this group. Cytotaxonomic markers are also useful for the differentiation of species that are often misidentified due to the morphological similarities, as observed in the pike-characins *Boulengerella lucius* (Cuvier, 1816) and *B. maculata* (Valenciennes, 1850), karyotypes of which have distinct patterns of differentiation of the 5S rDNA sequences (Sousa e Souza et al. 2017). Overall, then, certain specific features of the karyotype microstructures of the species analyzed here were found to be diagnostic characters for the diagnosing the diversity of the genus *Acestrorhynchus*.

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RESEARCH ARTICLE

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Cytogenetic data for sixteen ant species from North-eastern Amazonia with phylogenetic insights into three subfamilies

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Abstract

Ants play essential roles in most terrestrial ecosystems and may be considered pests for agriculture and agroforestry. Recent morphological and molecular data have challenged conventional ant phylogeny and the interpretation of karyotypic variations. Existing Neotropical ant cytogenetic data focus on Atlantic rainforest species, and provide evolutionary and taxonomic insight. However, there are data for only 18 Amazonian species. In this study, we describe the karyotypes of 16 ant species belonging to 12 genera and three subfamilies, collected in the Brazilian state of Amapá, and in French Guiana. The karyotypes of six species are described for the first time, including that of the South American genus *Allomerus* Mayr, 1878. The karyotype of *Crematogaster* Lund, 1831 is also described for the first time for the New World. For other species, extant data for geographically distinct populations was compared with our own data, e.g. for the leafcutter ants *Acromyrmex balzani* (Emery, 1890) and *Atta sexdens* (Linnaeus, 1758). The information obtained for the karyotype of *Dolichoderus imitator* Emery, 1894 differs from extant data from the Atlantic forest, thereby highlighting the importance of population cytogenetic approaches. This study also emphasizes the need for good chromosome preparations for studying karyotype structure.

Keywords
Formicidae, karyotype, Neotropical ants, biodiversity

Introduction

Ants are a diverse group of insects comprising more than 16,000 described species and about 6,000 species yet to be described (Ward 2013), and can represent up to 20% of terrestrial animal biomass in tropical regions (Schultz 2000). Considered good indicators of ecosystem diversity or disturbance (reviewed in Andersen 2018), some ant species play important roles in ecosystems (e.g., seed dispersal, plant protection, predation) whereas other species are considered agricultural pests (Hölldobler and Wilson 1990). However, many ant species belong to cryptic species complexes, making accurate description and the understanding of their biogeographical distribution difficult.

Usually, species identification relies on external morphological traits, but this approach is ineffective in cases where two or more species cannot be morphologically differentiated (Bickford et al. 2007). Complementary biological information can be used in these instances (Schlick-Steiner et al. 2010). Considering recent revisions of higher taxa (Ward et al. 2015, 2016, Sosa-Calvo et al. 2017, 2019), cytogenetics could be used to solve taxonomic issues related to the family Formicidae. Cytogenetics is particularly useful in understanding species evolution and population dynamics because chromosome modifications play a direct role in speciation events and generate heritable variation (King 1993, Aguiar et al. 2017).

More than 800 species of Formicidae have been cytogenetically studied to date (reviewed in Lorite and Palomeque 2010, Mariano et al. 2015, 2019). Cytogenetic research of Neotropical ants has focused on species found in the Atlantic forest biome in Brazil, with few data for other regions and countries. Population studies in ant cytogenetics remain scarce, e.g. *Typhlomyrmex rogenhoferi* Mayr, 1862 (Mariano et al. 2006), *Dinoponera lucida* Emery, 1901 (Mariano et al. 2008), *Pachycondyla* spp. (Mariano et al. 2012), and *Camponotus rufipes* (Fabricius, 1775) (Aguiar et al. 2017). However, cytogenetic data can be used to identify cryptic species, which are common in Formicidae (Seifert 2009). Cytogenetic data have advanced our understanding of biology, reproduction, phylogeny, taxonomy, and evolution, and facilitated investigation of cryptic and threatened species (Lorite and Palomeque 2010).

Cytogenetic data are only available for 18 ant species from the Amazon region, mostly (13 species) from French Guiana (Mariano et al. 2006, 2011, 2012, Santos et al. 2010), with four species from the state of Pará, Brazil (Sposito et al. 2006, Mariano et al. 2006, Santos et al. 2012, Mariano et al. 2015), and one species from Amapá, Brazil (Aguiar et al. 2017). Until now, only data for *T. rogenhoferi*, is available for two locations: Pará, Brazil and French Guiana (Mariano et al. 2006). This species shows an interesting cline variation, which highlights the importance of population assays. In the present study, new data for 16 ant species from the Eastern Amazon are presented using cytogenetic analysis (chromosome number and morphology), with phylogenetic insights into three subfamilies.

Material and methods

Ant colonies were collected in French Guiana at three locations: *Montagne des Singes*, Kourou (5.07225N, 52.69407W), *Campus Agronomique*, Kourou (5.17312N, 52.65480W), and Sinnamary (5.28482N, 52.91403W). Colonies were collected in Brazil at Oiapoque, state of Amapá (3.84151N, 51.84112W) (Table 1). Sampling permission was given by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) to Luísa Antônia Campos Barros (SISBIO accession number 32459). Specimens were identified by Jacques Hubert Charles Delabie and deposited in the reference collection at the Laboratório de Mirmecologia, Centro de Pesquisas do Cacau (CPDC/Brazil), as items #5802 and #5803.

Metaphases were obtained from the cerebral ganglia of the larvae after meconium elimination, according to Imai et al. (1988). Chromosome number and morphology of metaphases were analyzed using conventional 4% Giemsa staining. Chromosome morphology was defined according to Levan et al. (1964) using the ratio of chromosome arms (long arm/short arm). Metaphases and chromosomes were karyotyped using Adobe Photoshop CC and measured using Image Pro Plus.

Results and discussion

Sixteen ant species belonging to 12 genera and three subfamilies have been cytogenetically analyzed (Table 1). The karyotypes of six species are described for the first time, including karyotypic information for the genus *Allomerus* Mayr, 1878. Another genus, *Crematogaster* Lund, 1831, is cytogenetically analyzed for the first time in the Neotropical region. Karyotypes of ten species, including the leafcutter ants *Acromyrmex* Mayr, 1865 and *Atta* Fabricius, 1804, previously described in other localities, were compared with our own data.

Ponerinae: Ponerini: *Anochetus* and *Odontomachus*

Anochetus Mayr, 1861 is a monophyletic genus and a sister genus of *Odontomachus* Latreille, 1804 (Larabee et al. 2016, Fernandes 2017). Morphologically, they belong to the subtribe Odontomachiti of trap-jaw ants (Brown-Jr 1976).

Anochetus targionii has 2n = 30 chromosomes (Fig. 1a), which is considered as a modal number according to Santos et al. (2010). *Anochetus* chromosome numbers range from 2n = 24–46, which represents higher karyotype diversity than that found in *Odontomachus* (2n = 32–42) (reviewed in Mariano et al. 2019). However, only 12 morphospecies out of 113 valid species of *Anochetus* have been cytogenetically analyzed: nine from the Indo-Malayan and three from the Neotropics, *A. altisquamis* Mayr, 1887 (2n = 30), *A. horridus* Kempf, 1964 (2n = 46), and *A. emarginatus* (Fabricius, 1804) (2n = 28) (Santos et al. 2010, Mariano et al. 2015).

Table 1. Ant species cytogenetically studied from North-eastern Amazonia. Diploid (2n) and haploid (n) chromosome numbers, karyotypic formulae, sample sizes (numbers of colonies/individuals) and localities are given.

Species	2n(n)	Karyotypic formula	Col/ Ind	Locality
Subfamily Ponerinae				
Anochetus targionii Emery, 1894*	30	2n = 16m+2sm+2st+10a	1/5	Campus Agronomique, Kourou, FG
Odontomachus haematodus Linnaeus, 1758	44	2n = 8sm+18st+18a	3/8	Campus Agronomique, Kourou, FG
Pseudoponera stigma Fabricius, 1804	14	2n = 14m	1/4	Oiapoque, BR
Pseudoponera gilberti (Kempf, 1960)	12	2n = 10m+2sm	1/6	Sinnamary, FG
Subfamily Myrmicinae				
Atta sexdens Linnaeus, 1758	22	2n = 18m+2sm+2st	2/12	Campus Agronomique, Kourou, FG; Oiapoque, BR
Acromyrmex balzani Emery, 1890	38	2n = 12m+10sm+14st+2a	1/10	Campus Agronomique, Kourou, FG
Cyphomyrmex transversus Emery, 1894	24(12)	2n = 14m+6sm+4a (n = 7m+3sm+2a)	2/8	Campus Agronomique, Kourou, FG
Myrmicocrypta sp.	30	2n = 22m+2sm+6a	1/6	Sinnamary, FG
Allomerus decemarticulatus Mayr, 1878*; *Hirtella physophora* Martius et Zuccarini, 1832 †	28	2n = 18m+6sm+2a	4/9	La Montagne des Singes, Kourou, FG
Allomerus octoarticulatus var. demerarae Mayr, 1878*; *Cordia nodosa* Lamarck, 1792 †	44	2n = 4sm+40a	5/12	La Montagne des Singes, Kourou, FG
Allomerus octoarticulatus Mayr, 1878*; *Hirtella physophora* †	44	2n = 4sm+40a	5/11	La Montagne des Singes, Kourou FG
Crematogaster longispina Emery, 1890*	24	2n = 20m+4sm	1/4	Sinnamary, FG
Strumigenys diabola Bolton, 2000*	40	2n = 18sm+12st+10a	1/3	Sinnamary, FG
Wasmannia auropunctata Roger, 1863	32	2n = 16m+13sm+5st	1/6	Campus Agronomique, Kourou, FG
Solenopsis geminata Fabricius, 1804	32 (16)	2n = 14m+12sm+6st (n = 7m+6sm+3st)	1/5	Sinnamary, FG
Subfamily Dolichoderinae				
Dolichoderus imitator Emery, 1894	46	2n = 6m+28sm+12a	1/5	Sinnamary, FG

* first cytogenetic report. † host plant. FG: French Guiana, BR: Brazil

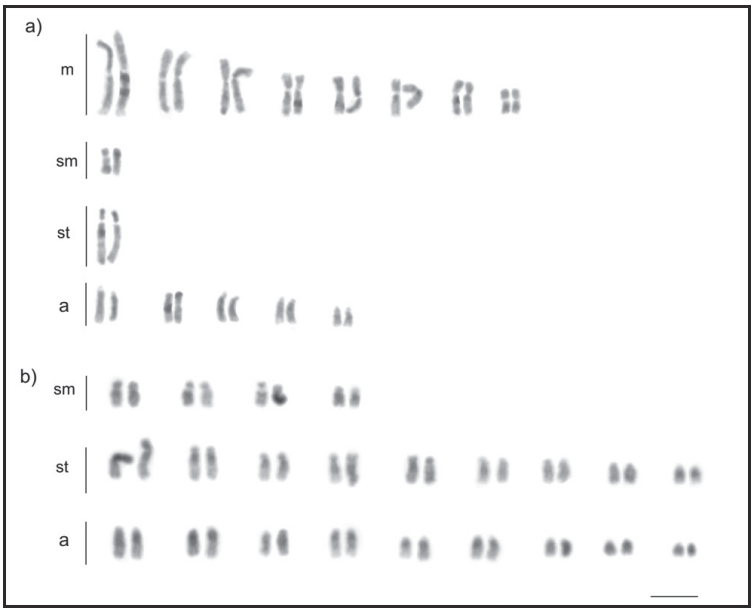

Figure 1. Karyotypes of the tribe Odontomachiti (Ponerinae): **a** *Anochetus targionii* (2n = 30) **b** *Odontomachus haematodus* (2n = 44). Scale bar: 5μm.

Since *Anochetus* diversified earlier than *Odontomachus* (Larabee et al. 2016, Fernandes 2017), higher karyotypic variation in *Anochetus* would be expected (Santos et al. 2010). *Anochetus targionii* has the same chromosome number as *A. altisquamis*, *A. modicus* Brown, 1978, and *A. graeffei* Mayr, 1870. It seems that 2n = 30 chromosomes is the plesiomorphic condition since it is found throughout the genus *Anochetus* and is present in *A. altisquamis*, which is considered a phylogenetically "basal" clade (Larabee et al. 2016, Fernandes 2017). *Anochetus* species also share a constant number of acrocentric chromosomes. Within the lineage of *A. horridus*, chromosome fission seems to have played an important role in recent karyotype evolution, increasing the number of chromosomes in the karyotype. According to Larabee et al. (2016), *A. horridus* diversified around 25 million years ago (MYA), whereas *A. targionii* diversified less than 10 MYA.

Odontomachus haematodus has 2n = 44 chromosomes, of which 18 are acrocentric (Fig. 1b), confirming information provided by Santos et al. (2007, unpublished data) and Aguiar et al. (2012, unpublished data) (reviewed in Mariano et al. 2019). Similar to *Anochetus*, *Odontomachus* species seem to have characteristic karyotypes that are slightly different between *Odontomachus* phylogenetic clades.

The Indo-Pacific species, *O. rixosus* Smith, 1857 and *O. latidens* Mayr, 1867, have 2n = 30 chromosomes but no further information about their karyotypes is available (reviewed in Lorite and Palomeque 2010). The other known karyotype of *Odontomachus* species belongs to the *haematodus* group according to molecular phylogeny (Larabee et al. 2016). All known karyotypes from the *haematodus* group (reviewed in Santos et al. 2010) have 44 chromosomes, including *O. haematodus*, whose karyotype is described

in this study. *Odontomachus chelifer* (Latreille, 1802) has plesiomorphic traits and the highest number of acrocentric chromosomes (40) among Indo-Pacific species (reviewed in Santos et al. 2010). The species *O. meinerti* Forel, 1905 and *O. bauri* Emery, 1892 have 34 and 14 acrocentric chromosomes out of 44 respectively (Teixeira 2018).

This suggests that heterochromatin growth at telomeric regions of shorter arms of acrocentric chromosomes may be significant in *Odontomachus* karyotype evolution. This is in accordance with the Minimum Interaction Theory proposed by Imai et al. (1994), which proposes that reduced interactions between different chromosomes inside the nucleus increases the fitness of the individual.

Ponerinae: Ponerini: *Pseudoponera*

The genus *Pseudoponera* Emery, 1900 has six valid species (Bolton 2019). Two species *Pseudoponera gilberti* and *P. stigma* are near-identical morphologically. Conflicting cytogenetic analyses (Mariano et al. 2012) due to misidentification have recently been resolved and the two species distinguished in samples from the Atlantic forest (Correia et al. 2016). The chromosome number for *P. gilberti* is 2n = 12 (10m + 2sm) and for *P. stigma* was 2n = 14, all of them metacentrics. In spite of minor differences in chromosome morphology of *P. stigma* between Atlantic forest (Correia et al. 2016) and Amazonia, both karyotypes share the same chromosome number.

Myrmicinae: Attini: Attina: *Atta* and *Acromyrmex*

The fungus-growing ants from the genus *Acromyrmex* form a sister group of the genus *Atta* and together are believed to be monophyletic. There are 33 valid species of *Acromyrmex* and 18 species of *Atta* (Bolton 2019), with wide distributions throughout the Neotropics (Delabie et al. 2011). The relationship between *Atta* and *Acromyrmex* became clearer under a combined approach using morphological, molecular, and cytogenetic tools (Cristiano et al. 2013). Cytogenetic data are available for five *Atta* species (Barros et al. 2011, 2014, 2015) from three of the four monophyletic groups according to the molecular phylogeny proposed by Bacci et al. (2009) and 13 species of *Acromyrmex* (reviewed in Barros et al. 2011, 2016, Teixeira et al. 2017).

The leaf-cutter ant *Atta sexdens* has 2n = 22 (Fig. 3a), and chromosome morphology is the same (18m + 2sm + 2st) to that of other *Atta* species from the Brazilian savannah and Atlantic Forest (Barros et al. 2014, 2015). The Amazonian population of *Acromyrmex balzani* analyzed in this study has 2n = 38 chromosomes and the same karyotype (Fig. 3b) as that of the Brazilian savannah and Atlantic forest populations (Barros et al. 2016). The largest metacentric pair of *A. balzani* is large, about twice the length of the largest subtelocentric chromosome previously identified in other Brazilian populations of this species (Barros et al. 2016). In all other *Acromyrmex* species studied so far, the former pair of chromosomes is of similar length. Based on the recent checklist of

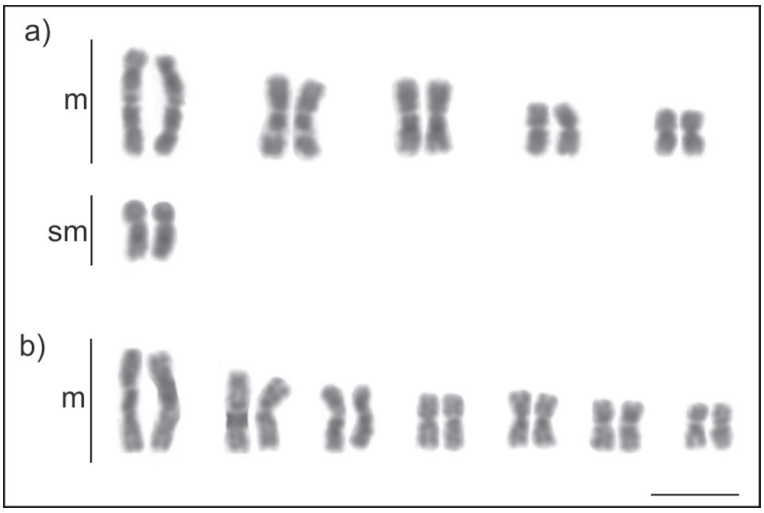

Figure 2. Karyotypes of the genus *Pseudoponera* (Ponerinae): **a** *P. gilberti* (2n = 12) **b** *P. stigma* (2n = 14). Scale bar: 5μm.

the ants of French Guiana (Franco et al. 2019) this is the first record for *A. balzani* in French Guiana and also the first cytogenetic analysis of this species in the region.

So far, all karyotype analyses showed that *Atta* spp. have 2n = 22 chromosomes and *Acromyrmex* spp. have 2n = 38 chromosomes (Barros et al. 2016, Teixeira et al. 2017). *Acromyrmex striatus* (Roger, 1863) is an exception, with 2n = 22 chromosomes, the same as *Atta* spp. and is considered the sister group of leaf-cutter ants (Cristiano et al. 2013). There are variations between the morphological features of certain chromosomes in *Acromyrmex* due to heterochromatin growth (Barros et al. 2016). Interpopulation cytogenetic studies for ants are scarce (e.g., Mariano et al., 2006, 2012, Aguiar et al. 2017) and none are available for leaf-cutter ants.

Myrmicinae: Attini: Attina: *Cyphomyrmex*

The fungus-growing attine *Cyphomyrmex transversus* has 2n = 24 and n = 12 with mostly metacentric and submetacentric chromosomes (Fig. 3c, d) which differs from 2n = 42, all of them acrocentric, observed by Mariano et al. (2019), highlighting the importance of detailed cytogenetic studies in this species. It has a range from northern Brazil to central Argentina including the northeastern regions of Brazil (Kempf 1965). *Cyphomyrmex transversus* and the three other *Cyphomyrmex* species which have been karyotyped (see Sosa-Calvo et al. 2017 for recent taxonomic changes) have chromosome numbers ranging between 2n = 20 and 2n = 42 (reviewed in Mariano et al. 2019). It seems that the high proportion of metacentric chromosomes is characteristic of this genus. In spite of morphological affinity of *C. transversus* (present study) to *C. rimosus* (Spinola, 1851), observed by Kempf (1965), the karyotype divergence

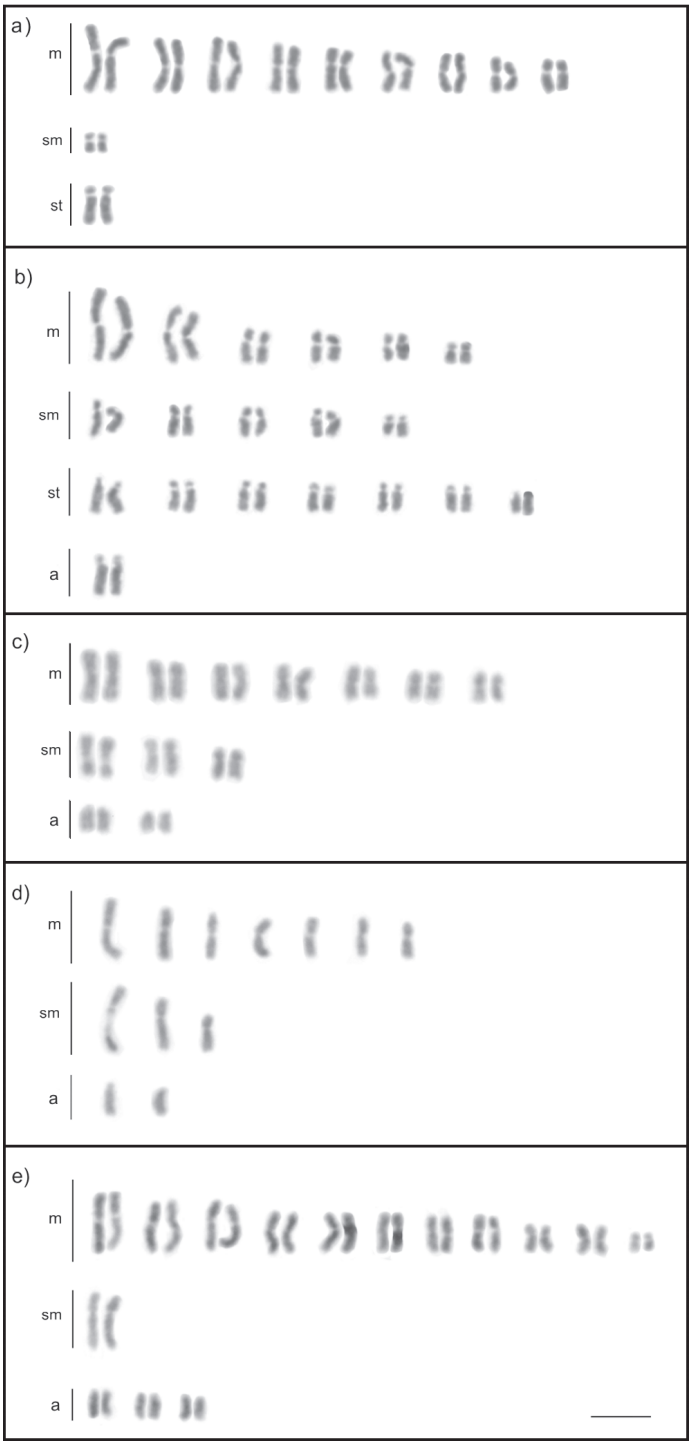

Figure 3. Karyotypes of fungus-growing ants (Myrmicinae, Attini: Attina): **a** *Atta sexdens* (2n = 22) **b** *Acromyrmex balzani* (2n = 38) **c** *Cyphomyrmex transversus* (2n = 24) **d** *C. transversus* (n = 12, male karyotype) **e** *Myrmicocrypta* sp. (2n = 30). Scale bar: 5μm.

between them (2n = 24 and 2n = 32, respectively; Murakami et al. 1998) is puzzling because of the numerical difference of their karyotypes coupled with similar morphology of chromosomes. These findings merit further study using advanced chromosome banding and molecular phylogenetic techniques.

Myrmicinae: Attini: Attina: *Myrmicocrypta*

The fungus-growing species *Myrmicocrypta* sp. had 2n = 30 chromosomes, 18 of them metacentric (Fig. 3e). The studied colony was collected from the cavities of a rotten log. *Myrmicocrypta* Smith, 1860 is the sister genus of *Mycocepurus* Forel, 1893, and both are members of the clade Palleoattina (Sosa-Calvo et al. 2017). *Myrmicocrypta* is widely distributed in the Neotropics, from Mexico to Argentina and includes 27 valid species (Bolton 2019). A recent study by Sosa-Calvo et al. (2019) suggests that only two species can nest in rotten logs: *M. spinosa* Weber, 1937 and the undescribed species, *M*. JSC001. This is a derived characteristic for *Myrmicocrypta* and therefore this clade is apparently monophyletic. The only extant cytogenetic data available for this genus are from the *Montagne des Singes* area, French Guiana (Mariano et al. 2011), about 60 km from where the samples from the present study were collected.

Since the studied sample was identified as an undescribed species, it is possible that the present species is *M*. JSC001. *Myrmicocrypta spinosa* has not been recorded in French Guiana: the samples studied by Sosa-Calvo et al. (2019) included only *M*. JSC001. The species studied by Mariano et al. (2011) had a slightly different karyotype, probably as a result of variation in the chromosome condensation. These results highlight the importance of good chromosome preparations for studying karyotype configuration.

Myrmicinae: Attini: *Allomerus*

This study represents the first cytogenetic analysis for the genus *Allomerus*. *A. decemarticulatus* and the *A. octoarticulatus* species complex had 2n = 28 and 2n = 44, respectively (Fig. 4). The *Allomerus* species are specialist ants inhabiting diverse obligate myrmecophytic plants in South America. *Allomerus decemarticulatus* and *A. octoarticulatus*, have been intensively studied in French Guiana from an ecological perspective: they build galleries using the fungus *Trimmatostroma cordae* Sharma & Singh, 1976 (see Dejean et al. 2005, Ruiz-González et al. 2011). The molecular phylogeny of the genus showed that *A. octoarticulatus* is a complex of two species that cannot be separated morphologically (Orivel et al. 2017). However, these two species are associated with different plants: *A. octoarticulatus* var. *demerarae* inhabits only *Cordia nodosa*, while *A. octoarticulatus* can be associated with several myrmecophytic plant species throughout its distribution range.

The number of acrocentric chromosomes is highly different between these two species, even though meta/submetacentric and acrocentric chromosomes predominate in *A. decemarticulatus* and *A. octoarticulatus*, respectively. According to the Minimum Interaction Theory, centric fissions may have played an important role in the chro-

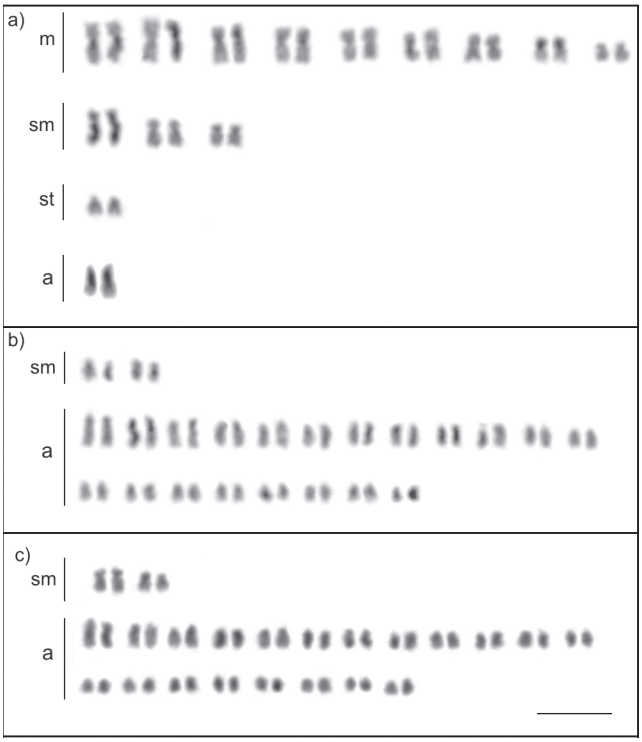

Figure 4. Karyotypes of the genus *Allomerus* (Myrmicinae): **a** *A. decemarticulatus* (2n = 28) **b** *A. octoarticulatus* var. *demerarae* (2n = 44) associated with *Cordia nodosa* **c** *A. octoarticulatus* (2n = 44) associated with *Hirtella* sp. Scale bar: 5μm.

mosome evolution of *Allomerus*; however, the karyotypes of additional species should be investigated to support this conclusion. A comparison between the two species of *A. octoarticulatus*, which nest in different plant species, was also made (Fig. 4b, c). However, basic cytogenetic techniques (chromosome number and morphology) could not differentiate between the two. Additional banding techniques with molecular probes may further illuminate this question in the future.

Myrmicinae: Attini: *Strumigenys*

Strumigenys diabola has 2n = 40 (Fig. 5a) with many chromosomes having short arms (submeta/subtelocentrics). The genus *Strumigenys* Smith, 1860 harbors small cryptic species specialized in preying on collembolans. There are currently more than 800 valid species of *Strumigenys* (Bolton 2019) of which 190 are from the Neotropics. *Strumigenys diabola* are reported in northern and northeastern Brazil and in French Guiana (Janicki et al. 2016). For the Neotropics, cytogenetic data was previously only available for *Strumigenys louisianae* Roger, 1863, which has 2n = 4 (Alves-Silva et al. 2014) and for eight species from Asia and Oceania (Lorite and Palomeque 2010). This is the second cytoge-

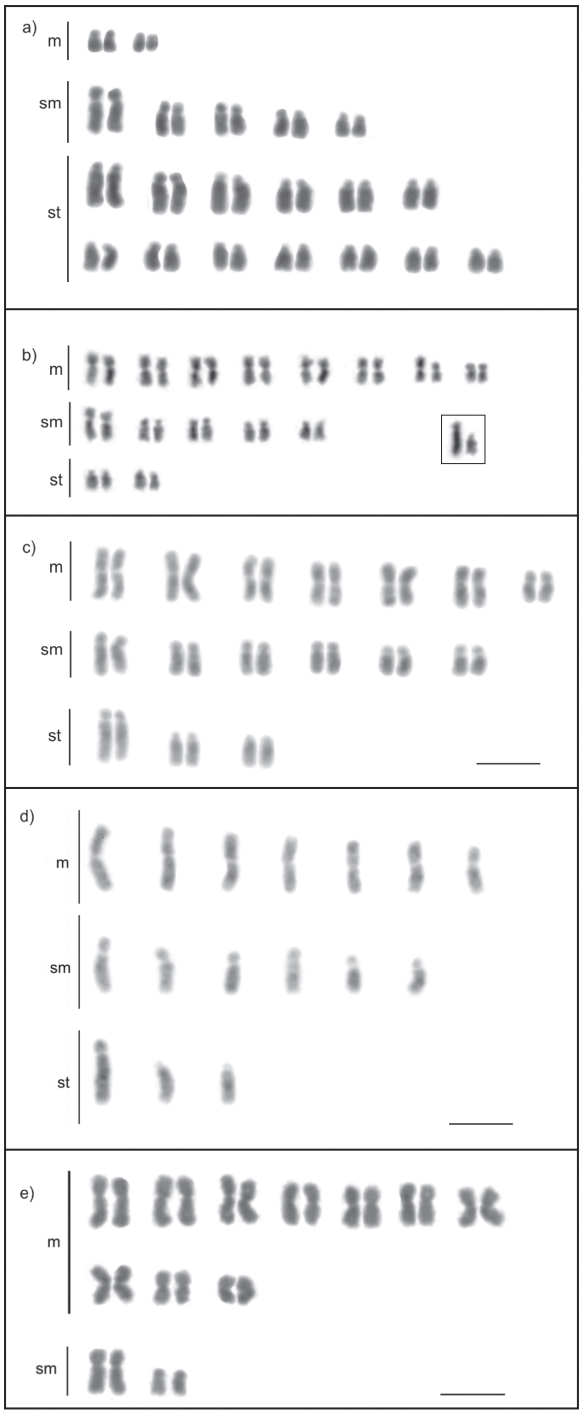

Figure 5. Karyotypes of four genera of Myrmicinae: **a** *Strumigenys diabola* (2n = 40) **b** *Wasmannia auropunctata* (2n = 32) **c** *Solenopsis geminata* (2n = 32) **d** *S. geminata* (n = 16, male karyotype) **e** *Crematogaster longispina* (2n = 24). Box shows the chromosome pair with size heteromorphism. Scale bar: 5μm.

netic record in Neotropics and the absence of data in the *Strumigenys mandibularis* group sensu Bolton (2000) make comparisons with other species impossible. Further studies of this genus will help understanding chromosome evolution and phylogeny of the group.

Myrmicinae: Attini: *Wasmannia*

In this study, workers of *W. auropunctata* had 2n = 32 (Fig. 5b), with one chromosome pair showing considerable size heteromorphism in all individuals analyzed. The genus *Wasmannia* Forel, 1893 includes 11 species and is endemic to the Neotropics. The "little fire ant" *W. auropunctata* is notable because of its reproductive mechanism (Fournier et al. 2005). It has three different genetic systems: haplodiploidy, male clonality, and thelytoky (Foucaud et al. 2006). In this study, the same chromosome number and a similar karyotype from colonies from Ilhéus and Una, southeast Bahia, Brazil were observed (Souza et al. 2011), although there are differences in chromosome classification. The heteromorphic pattern was not described for the Atlantic forest population and therefore needs to be investigated further.

Myrmicinae: Solenopsidini: *Solenopsis*

Our analysis found 2n = 32 in female *Solenopsis geminata* and n = 16 in males with most chromosomes (26) being metacentric or submetacentric (Fig. 5c, d). The genus *Solenopsis* Westwood, 1840 is difficult to identify at the species level, although these species form obvious natural groups (Pacheco and Mackay 2013). The chromosome number for this species in our analysis is the same as that observed in five previously described fire ant species including *S. geminata*, (reviewed in Lorite and Palomeque 2010) which belong to the subgenus *Solenopsis* (Pacheco and Mackay 2013).

We compared our data with those from colonies of *S. geminata* from the USA (Crozier 1970) and India (Imai et al. 1984). The karyotype from French Guiana is similar to that from India, despite certain differences in chromosome classification. Differences in karyotypic formula among various localities and colonies were reported by Imai et al. (1984) based on their observation of the presence/absence of the short arm in some chromosomes as a result of C-band polymorphisms. Those patterns demonstrate the importance of understanding heterochromatin dynamics at the population level for analyzing karyotype evolution of ants.

Myrmicinae: Crematogastrini: *Crematogaster*

The ant genus *Crematogaster* is a global, widespread, and species-rich clade. It currently comprises 498 valid species and is divided into two subgenera, C*rematogaster* sensu stricto and C*rematogaster* (*Orthocrema*) Santschi, 1918 (Blaimer 2012a, b). The subgenus *Orthocrema* is more complex, and numerous clades exist within this group.

Crematogaster longispina, which belongs to the subgenus *Orthocrema*, has 2n = 24, and all chromosomes are meta/submetacentrics (Fig. 5e). This is the first New World *Crematogaster* karyotype ever described, which makes reasonable comparisons difficult. Karyotype data is available for 17 morphospecies of *Crematogaster* from Malaysia, Indonesia, India, Japan, and Australia (reviewed in Lorite and Palomeque 2010).

Within *Crematogaster* spp., the chromosome number ranges from 2n = 24–58, with 10 morphospecies having 2n = 24 or 26. Increasing the number of studied species in the Neotropics may help to understand the chromosome evolution of the group.

Dolichoderinae: *Dolichoderus*

Dolichoderus Lund, 1831 is the most speciose ant genus in the subfamily *Dolichoderinae*, with 130 valid species (Bolton 2019). Chromosomal data is available for seven species collected from the Atlantic forest (Santos et al. 2016) and four species from the Indo-Malayan region (reviewed in Lorite and Palomeque 2010). The genus demonstrates high chromosome variation, 2n = 10–52, and is the most cytogenetically diverse genus within Dolichoderinae. According to the molecular phylogeny produced by Santos et al. (2016), this species occupies a less derived position, which agrees with previous conclusions that suggest that this species belongs to a separate species group (Mackay 1993). The chromosome number already known for this species is 2n = 38, and it also has many meta/submetacentric chromosomes (Santos et al. 2016). However, in this study, additional acrocentric chromosomes were observed in *D. imitator* (2n = 46). Chromosomal intraspecific variation in *Dolichoderus* has not previously been reported. This again emphasizes the importance of karyotypic studies at the level of certain populations, which may represent either geographic clines or a species complex. Enhancing population studies for this species may have important implications for our understanding of both taxonomy and chromosome evolution of Formicidae.

Figure 6. Karyotype of *Dolichoderus imitator* (2n = 46) (Dolichoderinae). Scale bar: 5μm.

Conclusion

Our study increased the number of karyotyped Amazonian ant species from 18 to 34. The karyotype of 16 species were analyzed, six of them for the first time, which permitted comparisons with previously studied species, including population studies of leaf-cutting ants (*Atta sexdens* and *Acromyrmex balzani*). Although cytogenetic analysis of more than 800 ant species is available, there are no data for many genera, including many Neotropical ones. This paper includes the first description of the karyotype of a *Crematogaster* species ever reported for the New World.

Conventional cytogenetics constitutes a powerful tool in characterizing cryptic biodiversity (Cioffi et al. 2018). For example, our study of *D. imitator* showed substantial differences between chromosome numbers of the previously studied Atlantic forest karyotype and that of our study, strongly suggesting the presence of different species. Future studies on molecular cytogenetics will have important implications for understanding the chromosome evolution of ants, focusing especially on the genus *Allomerus* and fungus-growing ants.

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Comparative cytogenetics of the ground frogs *Eupsophus emiliopugini* Formas, 1989 and *E. vertebralis* Grandison, 1961 (Alsodidae) with comments on their inter- and intraspecific chromosome differentiation

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Abstract

South American frogs of the genus *Eupsophus* Fitzinger, 1843 comprise 10 species. Two of them, *Eupsophus vertebralis* Grandison, 1961 and *E. emiliopugini* Formas, 1989 belong to the *Eupsophus vertebralis* group, exhibiting 2n = 28. Fundamental number differences between these species have been described using conventional chromosome staining of few specimens from only two localities. Here, classical techniques (Giemsa, C-banding, CMA_3/DAPI banding, and Ag-NOR staining), and fluorescence *in situ* hybridization (FISH, with telomeric and 28S ribosomal probes), were applied on individuals of both species collected from 15 localities. We corroborate differences in fundamental numbers (FN) between *E. vertebralis* and *E. emiliopugini* through Giemsa staining and C-banding (FN = 54 and 56, respectively). No interstitial fluorescent signals, but clearly stained telomeric regions were detected by FISH using telomeric probe over spreads from both species. FISH with 28S rDNA probes and Ag-NOR staining confirmed the active nucleolus organizer regions signal on pair 5 for both species. Nevertheless, one *E. emiliopugini* individual from the Puyehue locality exhibited 28S ribosomal signals on pairs 4 and 5. Interestingly, only one chromosome of each pair showed Ag-NOR positive signals, showing a nucleolar dominance pattern. Chromosomal rearrangements, rRNA gene dosage control, mobile NORs elements, and/or species hybridization process could be involved in this interpopulation chromosomal variation.

Keywords
Karyotype variations, FISH, Patagonian frogs, ribosomal probe, NOR polymorphism

Introduction

Eupsophus Fitzinger, 1843 is a South American genus of frogs that currently comprises 10 species (Frost 2019, Suárez-Villota et al. 2018a), endemic from the temperate *Nothofagus* forests from Chile and Argentina (Formas 1978, Ibarra-Vidal et al. 2004). Based on ethologic (advertisement calls; Formas and Brieva 1994), morphometrics (Nuñez 2003), molecular (allozymes and DNA sequences; Formas et al. 1992, Blotto et al. 2013), and cytogenetic (Formas 1991, Veloso et al. 2005) analyses, this genus is divided into the *Eupsophus roseus* and the *Eupsophus vertebralis* groups.

The *E. roseus* group is composed of eight species: *E. calcaratus* (Günther, 1881), *E. contulmoensis* Ortiz, Ibarra-Vidal & Formas, 1989, *E. septentrionalis* Ibarra-Vidal, Ortiz, & Torres-Pérez, 2004, *E. nahuelbutensis* Ortiz & Ibarra-Vidal, 1992, *E. insularis* (Philippi, 1902), *E. migueli* Formas, 1978, *E. roseus* (Duméril & Bibron, 1841), and *E. altor* Nuñez, Rabanal & Formas, 2012 (Suárez-Villota et al. 2018a) exhibiting the same diploid number 2n = 30 with some species specific characteristics (*e.g.* fundamental number, sex chromosomes, secondary constriction location; Iturra and Veloso 1986, Veloso et al. 2005, Nuñez et al. 2012). On the other hand, the *E. vertebralis* group, composed of *E. vertebralis* Grandison, 1961 and *E. emiliopugini* Formas, 1989, exhibit 2n = 28, do not have sex chromosomes, and present a secondary constriction in pair 5 (Formas 1991). Moreover, the pair 13 is metacentric in *E. emiliopugini* and telocentric in *E. vertebralis*, differing in their fundamental number (FN = 56 and FN = 54, respectively).

Having in mind the hypothetical ancestrality of telocentric chromosomes in amphibians (Morescalchi 1980), Formas (1991) proposed two alternative hypotheses to explain the origin of the differences on the pair 13 in the *E. vertebralis* group. The first one is a pericentric inversion in a telocentric pair of *E. vertebralis*, which shifted the centromere to the metacentric position in *E. emiliopugini*. The second hypothesis is the addition of heterochromatic segments in the centromeric region of the telocentric pair in *E. vertebralis*, which leads to a metacentric pair in *E. emiliopugini*. Formas (1991) considered the first alternative as a reasonable hypothesis because telocentric and metacentric pairs 13 are the same size.

Although the hypothesis of Formas (1991) is well argued from the data, it should be considered with caution since the conclusions are obtained using only conventional stain and specimens from only two locations, preventing the findings from being extrapolated, and increasing the chance of assuming as true a false premise. Here we combined classical and molecular cytogenetic techniques to characterize the karyotypes of these species using samples from several localities. Thus, we analyzed at population level the nucleolus organizer regions (NORs) position using Ag-NOR banding and

fluorescent *in situ* hybridization (FISH) with 28S rDNA probe. Using FISH with telomeric probe and CMA_3/DAPI banding, we sought interstitial signals, which could suggest chromosomal rearrangements in both species. Our comparative cytogenetic analyses provide a detailed description of the *E. vertebralis* group karyotypes and their inter- and intraspecific chromosome differentiation.

Methods

Sample collection and cytological preparations

Cytological preparations were obtained from 14 and nine individuals of *Eupsophus vertebralis* and *E. emiliopugini*, respectively (See Suppl. material 1: Table S1). These individuals were collected according to permit of Servicio Agrícola y Ganadero (No. 9244/2015) from 15 locations in Southern Chile (Fig. 1). Mitotic plates were obtained from intestine cell suspension. For this purpose, we injected 30 µl/g of 0.1% colchicine (Sigma-Aldrich) into the abdominal cavity of each individual. After 12 hours, the individuals were euthanized with oversaturated benzocaine, according to the recommendations of the Bioethics and Biosecurity Committee of the Universidad Austral de Chile (UACh, resolution No. 236/2015 and 61/15). Immediately after euthanasia, the gut cells were extracted and prepared according to Schmid et al. (1978) protocol. Then, the specimens were included in the herpetological collection of Instituto de Ciencias Marinas y Limnológicas, UACh (voucher numbers in Suppl. material 1: Table S1).

Classical cytogenetic techniques

Mitotic plates were stained with 10% Giemsa for karyotype determination. Active NORs were detected using silver nitrate staining (Ag-NOR) according to (Howell and Black 1980). This chromosomal material was analyzed in Siedentopf trinocular microscope (AmScope T340B-DK-LED) and photographed with AmScope camera using IS capture software. Karyotypes were arranged according to Formas (1991).

To identify constitutive heterochromatic regions, we carried out a C-banding protocol using formamide for DNA denaturation, according to Fernández et al. (2002) and staining with DAPI (1 µg/ml). CG-rich and AT-rich regions were detected using CMA_3/DAPI stains, respectively follow to Schweizer (1976). In this technique, we used pretreated metaphases with formamide according to Pieczarka et al. (2006) as well as FISH pretreated plates (Suárez et al. 2013). For both C-banding and CMA3/DAPI stains, mitotic plates were mounted with Vectashield antifade. Subsequently, metaphases were visualized through a BX61 Olympus microscope, and captured with adequate filter using a DP70 Olympus digital camera with PRO MC Image software. All images were overlaid and contrast enhanced using Adobe Photoshop CS6.

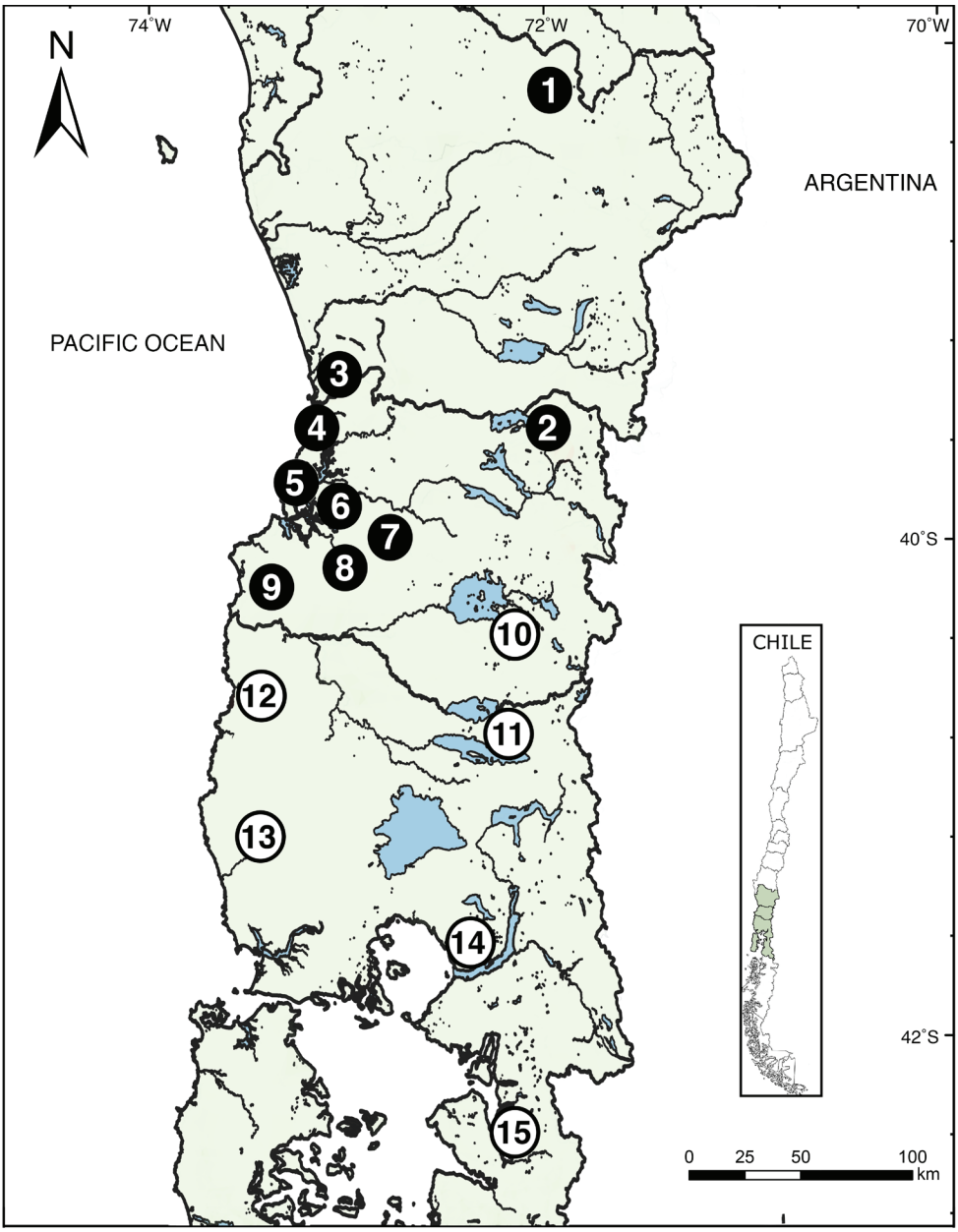

Figure 1. Map depicting 15 collection localities of the *Eupsophus vertebralis* group specimens in Southern Chile. *E. vertebralis* locations are represented by black circles, and *E. emiliopugini* locations are shown with white circles. The numbers inside the circles corresponds with the follow localities: **1**) Tolhuaca, **2**) Lago Pellaifa, **3**) Colegual Alto, **4**) Chanchan, **5**) Oncol, **6**) Llancahue, **7**) Reumén, **8**) Chamil, **9**) Cordillera Pelada, **10**) Los Mañios, **11**) Puyehue, **12**) Pucatrihue, **13**) Cordillera del Sarao, **14**) Parque Alerce Andino, and **15**) Huinay.

Molecular cytogenetic techniques

The physical map of the rDNA genes was detected by FISH on mitotic plates from *E. vertebralis* (from Colegual Alto and Reumén), and *E. emiliopugini* (from Puyehue, Cordillera del Sarao, and Parque Alerce Andino) specimens. For this purpose, 28S rDNA fragment from *E. vertebralis* DNA was amplified using 28SV (5′-AAGGTAGCCAAATGCCTCGTCATC-3′) and 28SJJ (5′-AGTAGGGTAAAACTAACCT-3′) primers (Hillis and Dixon 1991). PCR was carried out according to the manufacturer's instructions for *Taq* Platinum DNA Polymerase (Cat. No. 10966, Invitrogen), at 55 °C of annealing temperature. The 28S probe was PCR-labeled with 11-digoxigenin dUTP (Cat. No. 11093088910, Sigma-Aldrich), hybridized according to Ferreira et al. (2011), and detected with Anti-Digoxigenin-Rhodamine, Fab fragments (Cat. No. 11207750910, Roche).

Telomere detection by FISH was carried out on metaphase chromosomes from *E. vertebralis* (from Tolhuaca, Reumén, and Colegual Alto), and *E. emiliopugini* (from Puyehue, Parque Alerce Andino, and Cordillera del Sarao) specimens. Universal telomeric probes $(TTAGGG)_n$ were PCR-generated and labeled with fluorescein-12-dUTP (Cat. No 11373242910, Roche) (Ijdo et al. 1991). Fluorescent *in situ* hybridization followed to Ferreira et al. (2011) without final immunodetection protocol.

Slide mounting and image capture for both 28S rDNA and telomeric FISH assays were carried out as described previously for C-banding protocol.

Results

Classical cytogenetic techniques

We analyzed 88 mitotic plates showing 2n = 28 for each species, without evidence of sexual chromosomes (Fig. 2). All the *E. emiliopugini* plates showed only chromosomes bi-armed with a FN = 56. The pairs 1, 3, 8–14 were metacentric, pair 7 was submetacentric, and pairs 2, 4–6 were subtelocentric (Fig. 2, top) following the descriptions by Formas (1991).

Mitotic plates of *E. vertebralis* exhibited a telocentric pair 13 presenting FN = 54, while the other karyotypic features were similar to *E. emiliopugini* (Fig. 2, middle). Although it is not clear for all plates, secondary constriction was observed in the short arms of pair 5 from both species (Fig. 2, top and middle, black arrows). In one *E. emiliopugini* specimen collected at Puyehue (hereafter, the Puyehue specimen) was difficult to establish morphological homology among chromosomes of pairs 5 and 4 (Fig. 2, bottom, black arrows).

C-banding and DAPI staining detected predominantly centromeric regions in chromosomes of *E. emiliopugini* and *E. vertebralis* (Fig. 3a, top and middle, respectively). Interstitial heterochromatic signals were detected on the long arms of chromosomes of pair 5 (Fig. 3a, white arrows). When applying C-banding over mitotic plates from Puyehue specimen, secondary constrictions were detected in one chromosome of the pair 4, and in one chromosome of the pair 5 (Fig. 3a, bottom, red arrows). This final arrangement

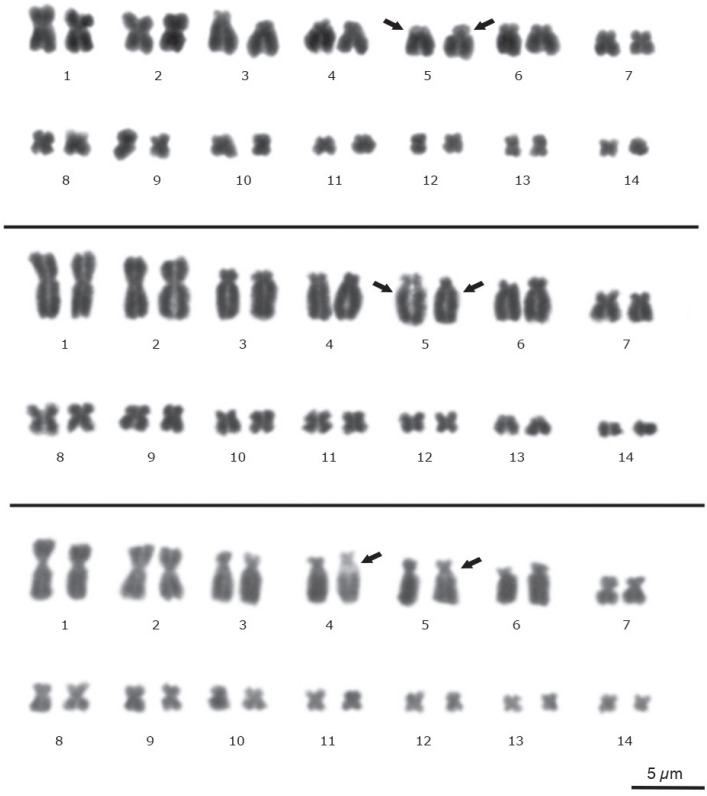

Figure 2. Conventional Giemsa banding on the *Eupsophus vertebralis* group mitotic plates. The *E. emiliopugini*, *E. vertebralis* and *E. emiliopugini* from Puyehue locality karyotypes are shown (top, middle, and bottom, respectively). Note metacentric (top and bottom) or telocentric (middle) pair 13. Secondary constrictions are indicated with black arrows on pairs 4 or 5 (see text for details).

among chromosomes of pairs 4 and 5 was based on Ag-NOR technique as described below. CMA_3 positive signals were detected on pair 5 of both karyotypes (Fig. 3b, top and middle, white arrows), but in that of the Puyehue specimen, these signals were detected in both chromosomes on pairs 4 and 5 (Fig. 3b, bottom, white arrows).

Ag-NOR staining detected active NORs on short arms of chromosomes of pair 5 in both *E. emiliopugini* and *E. vertebralis* karyotypes (Fig. 4a, top and middle, respectively). This technique detected active NORs, corresponding to secondary constriction, on long arm from one chromosome of the pair 4, and on short arm from one chromosome of pair 5 (Fig. 4a, bottom) in the Puyehue specimen.

Molecular cytogenetic techniques

Coincident with Ag-NOR staining results, signals on short arms of chromosomes of pair 5 in both *E. emiliopugini* and *E. vertebralis*, were detected through FISH using 28S rDNA

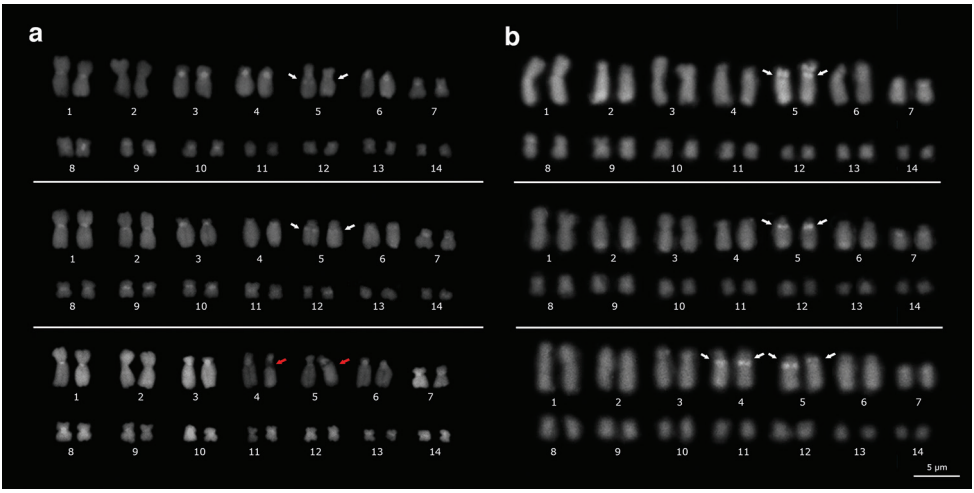

Figure 3. DAPI staining (**a**) and CMA_3 (**b**) on the *Eupsophus vertebralis* group mitotic plates. The *E. emiliopugini*, *E. vertebralis* and *E. emiliopugini* from Puyehue locality karyotypes are shown (top, middle, and bottom, respectively). White arrows indicated heterochromatic interstitial bands in **(a)** and CMA_3 positive signals in (**b**). Red arrows indicated secondary constriction in *E. emiliopugini* Puyehue specimen.

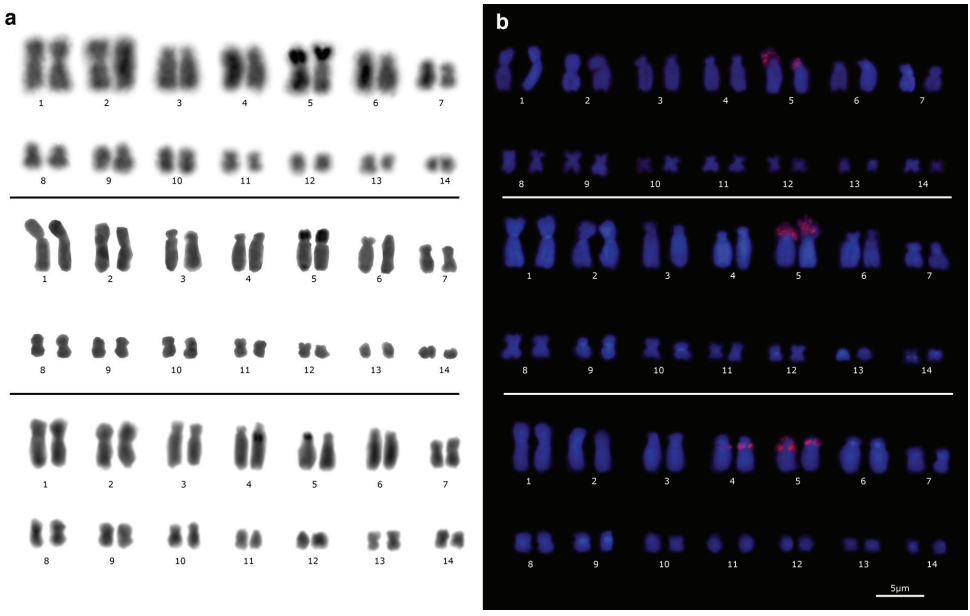

Figure 4. Ag-NOR staining (**a**), and FISH using 28S rDNA probe (**b**) on the *Eupsophus vertebralis* group mitotic plates. The *E. emiliopugini*, *E. vertebralis* and *E. emiliopugini* from Puyehue locality karyotypes are shown (top, middle, and bottom, respectively). Note colocalization of AgNOR and FISH signals on pair 5 (top and middle). FISH signals on four chromosomes, two of them AgNOR stained are observed in *E. emiliopugini* from Puyehue (bottom, see text for details).

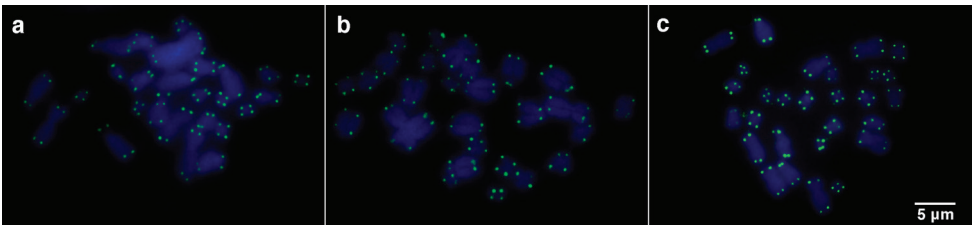

Figure 5. Fluorescent *in situ* hybridization over mitotic plates from the *Eupsophus vertebralis* group, using the telomeric probe. *Eupsophus emiliopugini* (**a**), *E. vertebralis* (**b**), and *E. emiliopugini* from Puyehue locality (**c**) mitotic plates are shown. Note the absence of interstitial signals in all chromosomes.

probe (Fig. 4b, top and middle, respectively). In the Puyehue specimen, this probe detected a long arm region of chromosomes in pair 4 and short arm regions of chromosomes in pair 5 (Fig. 4b, bottom).

Telomeric, but no centromeric or interstitial signals were detected on all chromosomes in both species through FISH using universal telomeric probe (Fig. 5a, b, respectively). This pattern was also observed on mitotic plates from the Puyehue specimen (Fig. 5c).

Discussion

Karyotypic patterns of *E. emiliopugini* and *E. vertebralis*

We present the first comparative cytogenetic study using classical and molecular cytogenetic techniques among specimens from different localities of *E. emiliopugini* and *E. vertebralis*. According with previous works (Formas 1989, 1991), *E. emiliopugini* and *E. vertebralis* exhibit 2n = 28, and FN = 56 and 54, respectively, derived of polymorphisms in pair 13 (Fig. 2). We did not detect sex chromosomes in the *E. vertebralis* group as it was observed by Formas (1991) (Fig. 2). Since, the lineage that gave origin to *E. vertebralis* and *E. emiliopugini* diverged early in the evolutionary history of *Eupsophus* (Suárez-Villota et al. 2018a), and sex chromosomes are detected in some species of the *E. roseus* group (*E. roseus*, *E. migueli*, *E. insularis*, and *E. septentrionalis*; Iturra and Veloso 1986, Cuevas and Formas 1996, Veloso et al. 2005), we agree with the notion that sex chromosomes correspond to an apomorphic condition in *Eupsophus* (Iturra and Veloso 1986, King 1991, Cuevas and Formas 1996, Veloso et al. 2005).

C-banding has been largely used in amphibians to compare karyotypes and to distinguish species with the same diploid number (Bogart 1970, Cuevas and Formas 2003, Nogueira et al. 2015, Sangpakdee et al. 2017, Targueta et al. 2018). Moreover, homogeneous C-banding patterns among related species has been associated with low genetic differentiation (Pellegrino et al. 1997, Lourenço et al. 1998, Bruschi et al. 2012) and enrichment of repetitive elements, characteristic of amphibian chromosomes (Schmid et al. 1978, Bruschi et al. 2012, Zlotina et al. 2017). Therefore, the absence of interspecific variations in heterochromatin banding reported in this

study (Fig. 3), could be associated with the recent and low differentiation between *E. vertebralis* and *E. emiliopugini* as has been reported in divergence times estimates and mitogenomic analyses (Suárez-Villota et al. 2018a, b).

Nucleolus organizer regions (NORs)

Ag-NOR banding combined with FISH using rDNA probes allow us to characterize the NORs in *E. emiliopugini* and *E. vertebralis* (Fig. 4). NORs locus correspond to rDNA coding for 18S rRNA, 5.8S rRNA and, 28S rRNA (Preuss and Pikaard 2007, McStay 2016). Thus, while Ag-NOR staining detects active NORs, FISH checks the total number of loci rDNA (Zaleśna et al. 2017). For both species of the *E. vertebralis* group, excluding the Puyehue specimen, we detected Ag-NOR signals on the short arms of pair 5 (Fig. 4a, top and middle), colocalized with the secondary constriction, and with 28S rDNA FISH signal (Fig. 4b, top and middle, red signal). Therefore, rDNA locus was transcriptionally active in both homologues of pair 5 for *E. emiliopugini* and *E. vertebralis*. Thus, it was not possible to determine a species-specific pattern relative to numbers and locations of NORs between both species. Consequently, NORs polymorphism is not a well indicative of species differentiation in this group as occur in some species of *Alsodes* Bell, 1843 [*A. pehuenche* Cei, 1976, *A. vanzolinii* (Donoso-Barros, 1974) and *A. verrucosus* (Philippi, 1902); Cuevas and Formas 2003]. However, different situation occurs in some species of the *E. roseus* group. For example, *E. contulmoensis* and *E. migueli* show specific Ag-NOR banding patterns (Veloso et al. 2005).

Intraspecific polymorphism in NORs was detected in the Puyehue specimen (Fig. 4a, b, bottom). We observed CMA_3 positive banding and 28S FISH signals on pairs 4 and 5 (four NOR loci, Figs 3b, 4b, bottom), of which only one chromosome of each pair showed secondary constriction (Fig. 2, bottom, black arrows) and Ag-NOR positive signal (Fig. 4a, bottom). The absence of secondary constriction in one chromosome from one pair is a cytologic phenomenon known as differential amphiplasty (Navashin 1928, Pikaard 2000). This phenomenon could be a manifestation of rRNA gene dosage control, regulating the number of active rRNA genes according to the cellular demand, or an epigenetic phenomenon from interspecific hybrids where the expression of rRNA genes inherited from one progenitor are silenced (Pikaard 2000, Tucker et al. 2010). Thus, the four rRNA loci with nucleolar dominance detected in Puyehue specimen could be related with chromosomal rearrangements (Schweizer and Loidl 1987), mobiles NORs (Schmid et al. 2017) or hybrid origin (Pereyra et al. 2009), as it has been also associated to polymorphic NORs in other species.

Hypothesis about the evolution of pair 13

C-banding and CMA_3/DAPI stains results did not show a heterochromatic region in the short arms of metacentric pair 13 of *E. emiliopugini* (Fig. 3a, b, top). Moreover, telomeric

probe hybridized over *E. emiliopugini* and *E. vertebralis* mitotic plates detected telomeric/subtelomeric signals but not interstitial signals (Fig. 5). Therefore, our data did not support the addition of heterochromatic segments in the telocentric pair of *E. vertebralis* and not show insights of inversions in the pair 13 of the *E. vertebralis* group. Since, these phenomena could be expected under hypothesis to explain the differentiation of pair 13 in this group (Formas 1991), we cannot refuse the proposed explanations. In this regard, telomeric sequences at telomeric/subtelomeric region are conserved in vertebrates (Meyne et al. 1989) whereas interstitial telomeric sequences could result from chromosomal rearrangements in animals (Ruiz-Herrera et al. 2002, Vitturi et al. 2002, Castiglia et al. 2006). Therefore, the pericentric inversion proposed by Formas (1991) to explain the differences in pair 13 between *E. emiliopugini* and *E. vertebralis* could be unlikely or it did not include the telomeric regions. Additionally, interstitial telomeric sequences could also be lost, as it has been reported in mammals (Rogatcheva et al. 2002, Castiglia et al. 2006). Thus, we cannot falsify the inversion hypothesis in pair 13 of the *E. vertebralis* group.

In conclusion, our analyses corroborate species-specific cytogenetic pattern differences between *E. emiliopugini* and *E. vertebralis* by detecting metacentric or telocentric pair 13 in populations of these species, respectively. Although, our results do not allow rejecting hypotheses of chromosomal rearrangements or heterochromatin addition in the origin of chromosomes of pair 13, a euchromatic pattern without interstitial telomeric sequences characterized these chromosomes. We reported an intraspecific polymorphism related to number, location, and activation of NORs for one specimen of *E. emiliopugini* from Puyehue locality. Chromosome rearrangements, hybridization event and transposition could be involved in the origin of this polymorphism. Future studies using probes from chromosome 13, more samples of *E. emiliopugini* from Puyehue locality, and molecular sequences analyses will allow a better understanding of the chromosomal evolution in the *E. vertebralis* group.

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Supplementary material 1

Table S1. *Eupsophus* specimens analyzed in the present study

Authors: Camila A. Quercia, Elkin Y. Suárez-Villota, Fausto Foresti, José J. Nuñez

Data type: speciemens data

Explanation note: Map number (Fig. 1), species, localities, coordinates, numbers of samples, and vouchers from herpetological collection of Instituto de Ciencias Marinas y Limnológicas (UACh), are shown.

Link: https://doi.org/10.3897/CompCytogen.v14i1.46852.suppl1

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RESEARCH ARTICLE

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Relationship between meiotic behaviour and fertility in backcross-1 derivatives of the [(*Gossypium hirsutum* × *G. thurberi*)^2 × *G. longicalyx*] trispecies hybrid

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Abstract

Wild cotton species are an important source of desirable genes for genetic improvement of cultivated cotton *Gossypium hirsutum* Linnaeus, 1763. For the success of such an improvement, chromosome pairings and recombinations in hybrids are fundamental. The wild African species *G. longicalyx* Hutchinson & Lee, 1958 could be used as donor of the desirable trait of fiber fineness. Twelve BC1 plants obtained from the backcrossing of [(*G. hirsutum* × *G. thurberi* Todaro, 1877)^2 × *G. longicalyx*] ($A_hD_hD_1F_1$, 2n = 4x = 52) trispecies hybrid (HTL) by *G. hirsutum* (cv. C2) ($A_hA_hD_hD_h$, 2n = 4x = 52) were investigated for meiotic behaviour and plant fertility. Their chromosome associations varied as follows: (2.5 to 11.5) I + (17 to 22) II + (0.31 to 1.93) III + (0.09 to 1.93) IV + (0 to 0.07) V + (0 to 0.14) VI. Their pollen fertility ranged from 4.67 to 32.10 %. Only four BC1 plants produced a few seeds through self-pollination. The remaining BC1 were totally self-sterile and usually presented the highest number of univalents. All BC1 materials produced BC2 seeds (0.44 to 6.50 seeds per backcross) with the number of seeds negatively correlated with the number of univalents ($R^2 = 0.45$, $P < 0.05$). Most BC1 plants gave significantly finer fiber compared to the cultivated *G. hirsutum*. SSR markers showed a segregation of wild alleles among the backcross derivatives and Genomic *in situ* hybridization (GISH) revealed presence of entire chromosomes of *G. longicalyx* as well as recombinant chromosomes in the backcross derivatives. The significance and details of these results are presented and the prospects of successfully exploiting these plant materials are discussed.

Keywords

chromosome, cytogenetics, fiber fineness, *Gossypium* spp, hybrid, *in situ* hybridization, meiosis, plant breeding

Introduction

Cotton is the most important fiber crop in the world. It belongs to the genus *Gossypium* which comprises about 53 species (Wendel and Grover 2015, Wu et al. 2018). Among them, 46 species have been assigned to eight cytologically and geographically defined diploid genome groups (A, B, C, D, E, F, G, and K) with 2n = 2x = 26 chromosomes, and 7 species have been attributed to a tetraploid genome group (AD) with 2n = 4x = 52 chromosomes (Wendel and Grover 2015; Chen et al. 2016; Wu et al. 2018). The genome sizes ranging from largest to smallest in the following order A > F > B > E > C > G > K > D (Zhang et al. 2008) and the affinity between these genomes to the A genome, based on chromosome pairings, follows slightly the same order.

Only four cotton species are cultivated, of which *G. herbaceum* Linnaeus, 1753 (A_1 genome) and *G. arboreum* Linnaeus, 1753 (A_2 genome) are diploid, while *G. hirsutum* Linnaeus, 1763 ($(AD)_1$ genome) and *G. barbadense* Linnaeus, 1753 ($(AD)_2$ genome) are tetraploid (Wendel et al. 2009, Chen et al. 2016, Ulloa et al. 2017). *G. hirsutum* is the main cultivated cotton with more than 90 % of the world production of lint (International Cotton Advisory Committee -ICAC- 2019). Except for these four cultivated types, all the other species of the genus are wild.

In cotton breeding, wild species are an important source of several desirable genes for genetic improvement of *G. hirsutum* such as fiber quality, resistance to diseases and insect pests, or tolerance to abiotic stress. The wild species *G. longicalyx* Hutchinson & Lee, 1958 (F_1 genome) could be used as donor of the desirable traits of fiber fineness, length and strength, which are very important to textile industry (Demol et al. 1978, Ndungo et al. 1988, Nacoulima et al. 2016). *G. longicalyx* appears to be a mixed genome related to all the other cotton genomes (except D genome) and phylogenetic analysis suggests a close relationship between its F_1 genome and the A genome (Cronn et al. 2002). In 2007, Konan et al. have created the HTL trispecies hybrid by crossing the [*G. hirsutum* ($(AD)_1$ genome) × *G. thurberi* Todaro, 1877 (D_1 genome)]^2 hexaploid to *G. longicalyx* (F_1 genome). This hybrid was totally self-sterile and its interspecific status was confirmed using SSR markers and cytogenetic analysis (Konan et al. 2007), but no data have been published so far concerning the meiotic behaviour and the fertility of its progeny.

In interspecific breeding programs, carrying out continuous cytological analysis is very important for plant selection because it provides information concerning the degree of meiotic irregularities, viability of gametes, chromosome pairing and genetic recombination (Lavinscky et al. 2017). For introgression of the desirable characters from the donor into the recipient, homoeologous recombinations are essential and the occurrence of bivalents and multivalents is important because they are indicative of chromosome recombination. It is important that genetic compatibility exists between the involved species to allow chromosome pairing, and it is especially fundamental that this pairing leads to real exchanges of chromatin between the donor chromosomes and the recipient ones; the success of hybridization depends on the recombination efficiency.

Another responsible factor for the success of breeding is the selection of genotypes with a high percentage of viable gametes (Techio et al. 2006). Pollen viability is considered a valuable tool for assessing the fertility of the male gamete and can be determined by using different methods: staining techniques, *in vitro* and *in vivo* germination tests, or analyzing final seed set (Abdelgadir et al. 2012). For staining techniques, different dyes such as acetic-carmine, Alexander's solution, fluorescein diacetate, and Lugol reagent can be used. These chemical dyes react with cellular components of the mature pollen grain, indicating whether the pollen is viable or unviable (Lavinscky et al. 2017).

The observations of both meiotic behaviour and plant fertility can thus help reducing the time needed for producing new hybrid cultivars, since plants with meiotic irregularities and/or unviable pollen grains can be rejected for selection of more stable genotypes (Lavinscky et al. 2017).

The objective of this study was to develop backcross progenies from the HTL hybrid, and to analyse their meiotic behaviour and their fertility with the long-term objective of introgressing the improved fiber fineness trait from *G. longicalyx* into *G. hirsutum.*

Material and methods

Plant material

[(*Gossypium hirsutum* cv. C2 × *G. thurberi* G27)^2 × *G. longicalyx* G17] ($A_hD_hD_1F_1$, 2n = 4x = 52) trispecies hybrid created by Konan et al. (2007) was backcrossed to *G. hirsutum* (cv. C2) to produce BC1 progenies. Crosses were achieved as follows: flowers of HTL trispecies hybrid were emasculated in the afternoon before anthesis and the stigma was covered by a small plastic sachet; pollen was applied to stigmas between 08:00 and 11:00 h the following morning. To avoid capsule shedding, a small piece of cotton wool containing a drop of the growth regulator solution (100 mg l^{-1} naphtoxyacetic acid + 50 mg l^{-1} gibberellic acid) recommended by Altman (1988) was applied on the ovary just after pollination.

Twenty seven BC1 seeds were hulled and cultivated *in vitro* on Murashige and Skoog medium (Murashige and Skoog 1962) because of their lack of germinative vigour. After a week of *in vitro* culture, seedlings were acclimated in a growth chamber (12 h of light, 55%–60% relative humidity and 28–26 °C day-night air temperatures). Twelve surviving BC1 adult plants were multiplied by grafting on *G. hirsutum* vigorous seedlings and cultivated in greenhouses for morphological observations, SSR marker analyses, pollen quality evaluation, meiosis analyses, selfings, backcrossings and, fiber fineness analyses. Plants were grown, in greenhouse, in 5 liter pots filled with a 3:2:1 (v:v:v) sterile mixture of compost, sand and peat. A dozen BC2 seeds belonging to the progeny of a randomly chosen BC1 plant were germinated on moist filter paper in a petri dish at 30 °C, for fast-growing root tips production in order to carry out genomic *in situ* hybridization.

Analysis of meiosis

Cytological analyses on the plant material produced were performed on their pollen mother cells (PMC) at meiosis. Suitable flower buds of each plant were collected between 09:00 and 11:00, according to the weather conditions, and fixed in fresh Carnoy's II fluid (glacial acetic acid 1: chloroform 3: and ethanol 6) for 72 hours at 4 °C. They were then stored at 4 °C in 70 % ethanol until their evaluation. To obtain meiotic plates, a few anthers were squashed in a drop of 1.5 % acetic-carmine solution on a microscope slide, debris were removed with fine forceps, and the slide was covered with a coverslip and heated a few seconds over a flame to improve chromosome staining. With pressure on the coverslip, pollen mother cells were flattened to spread out chromosomes. Observations were made with a Nikon Eclipse E800 photomicroscope (Nikon, Tokyo, Japan) under oil immersion. We concentrated our observations on metaphase I stage where chromosome arrangements (univalents, bivalents, and multivalents) were counted. But meiotic abnormalities such as laggard chromosomes at Anaphase I, Telophase I, Metaphase II, Anaphase II and final abnormal products of meiosis were considered as well.

Evaluation of the plant fertility

To have an indication of pollen quality, about 300 pollen grains per plant were analyzed. Flowers were collected in the morning on the day of anthesis. Pollen grains were dipped in a drop of 1.5 % acetic-carmine solution on a slide for 30 minutes and were analysed under a stereomicroscope Nikon Eclipse E800 (Nikon, Tokyo, Japan). Only fully stained and large pollen grains were scored as viable and non-aborted. The quantity of viable pollen was estimated as the percentage of stained pollen.

The self-fertility of the BC1 plants was assessed by determining the average number of seeds obtained from 30 self-pollinated flowers of each BC1 genotype. The cross-fertility of the BC1 plants was assessed by counting the average number of BC2 seeds obtained per backcross.

Fiber fineness analysis

For fiber fineness analysis, the fibers were combed and a tuft of parallel fibers was cut from the seed. Their free points were also cut and the median region was placed on a slide and covered with a cover glass. We let one or two drops of 18 % NaOH solution penetrate by capillarity into the fibers. The NaOH solution swells the fibers. The diameter of at least 100 fibers was then measured with the software NIS-Elements BR 2.30 using the Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan) equipped with a digital JVC KY-F 58E camera (JVC, Yokohama, Japan). The ribbon width was determined by

dividing the mean of the diameters measured by the 1.3 Summers coefficient (Roehrich 1947; Nacoulima et al. 2016). All the data collected were subjected to the analysis of variance (ANOVA) using the software Statistica 7.1 (Stat Soft France). The least significant difference (LSD) was used to establish differences between means at P = 0.05.

DNA isolation and microsatellites marker analysis:

SSR marker analysis was achieved to check molecular segregation among the BC1 plants. Total genomic DNA was isolated from young fresh leaf tissues following the mixed alkyltrimethylammonium bromide (MATAB) method described by Lacape et al. (2003). SSR markers BNL 836, BNL 2662 and BNL 3279, developed at Brookhaven National Laboratory (BNL) and showing polymorphism between the three parental species were used. Clone sequences used for these primer definitions are available at http://ukcrop.net/perl/ace/search/cottonDB.

Polymerase chain reactions (PCR) were performed in 10 µL volume containing approximately 25 ng of template DNA, 0.6 U of Taq DNA Polymerase, 2.5 mM $MgCl_2$, 1× Polymerase Buffer, 2 µM of each forward and reverse primers, and 0.2 mM of dNTPs mix. A PTC-200 thermal cycler (BioRad, Belgium) was used, with a PCR conditions consisting of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, with a final 72 °C extension for 8 min. Amplification products were separated on 6.0 % denaturing polyacrylamide gel and visualized by silver stain according to the protocol of Benbouza et al. (2006). The microsatellite bands were photo-documented and analyzed.

Genomic *in situ* hybridization

To know whether the meiotic behaviour observed in the HTL trispecies hybrid and its BC1 derivatives allows recombination between *G. hirsutum* and *G. longicalyx* chromosomes, genomic *in situ* hybridizations (GISH) were performed using fast-growing root tips of twelve BC2 seeds belonging to the progeny of a randomly chosen BC1 plant.

DNA probe labelling

Total genomic DNAs were labelled by nick translation method with digoxigenin-11-dUTP (Roche, Switzerland) and biotin-14-dATP (Invitrogen life technologie, Carlsbad, USA) according respectively to the labelling protocol of the manufacturers. To reveal chromosomes or chromatin of *G. longicalyx* in the HLT hybrid and in BC2 derivative, Digoxigenin-11-dUTP was used to label total genomic DNA of *G. longicalyx* and biotin-14-dATP to label total genomic DNA of *G. hirsutum*.

Chromosome preparation

For mitotic metaphase chromosomes preparations, fast-growing root tips were collected in 0.04 % 8-hydroxyquinoline for 4 hours at room temperature (RT) and fixed for 48 h in a fresh fixative fluid (3:1 ethanol: acetic acid) at 4 °C. After washing in distilled water (2 × 10 min), treating in 0.25 N HCl (10 min), rinsing in distilled water (10 min) and treating in a 0,01M citrate buffer (10 min), root tips were digested in an enzyme solution (5 % cellulase Onozuka R-10, 1 % pectolyase Y-23 in citrate buffer) at 37 °C for 1 hour. The enzyme mix was removed by rinsing in distilled water for 10 min, and on a clean glass slide a single root tip was spread in one or two drops of fresh fixative (3:1 ethanol : acetic acid) using a fine-pointed forceps. Slides were stored at -20 °C until needed.

In situ hybridization

In situ hybridization was performed according to the protocol used by D'Hont et al. (1995). Slides were treated with RNAse A (1 µg/ml) at 37 °C for 1 hour, denatured for 2.5 min in 70 % deionized formamide in 2 × SSC (sodium saline citrate) at 70 °C, then dehydrated in an ethanol series of 70 %, 95 %, 100 % for five min at -20 °C, followed by air-drying. The hybridization mixture was 30 µl per slide and it contained 15 µl of 100 % deionized formamide, 3 µl of 20 × SSC, 6 µl of 50 % (w/v) dextran sulfate, l µl of 20 % SDS (sodium dodecyl sulphate) and 250 ng of each of the two DNA probes. The hybridization mixture was denatured for 10 min at 75 °C, chilled on ice for at least 5 min and added to the slide. Hybridization was performed in a humid chamber at 37 °C overnight.

Hybridization signal detection

After hybridization, to dissociate non-specific and imperfect hybrids, posthybridization stringent washes were performed successively in 2 × SSC, 0.5 × SSC, 0.1 × SSC, for 10 min each wash at 42 °C and in 2 × SSC for 10 min at 37 °C. Slides were afterwards incubated with 200 µl (per slide) of 5 % BSA-4SSC/Tween for 10 min at 37 °C. Both the BSA (bovine serum albumin) and the detergent Tween bind to the unoccupied sites, preventing subsequent non-specific binding of the antibodies. Biotin detection with Texas Red, digoxigenin with FITC (fluorescein isothiocyanate) and amplification were achieved as follows: Slides were incubated for 45 min at 37 °C three times; the first time with 50 µl of 5µg/ml Texas Red-avidin in TNB (100 mM Tris HCl (pH 7.5), 150 mM NaCl, 0.5 % Blocking reagent), the second time with 50 µl of a mixture of 12.5 µg/ml Biotinilated anti-avidin + 2 µg/ml anti-Dig FITC in TNB and lastly with 50 µl of a mixture of 5 µg/ml Texas Red-avidine + 5 µg/ml FITC-conjugated rabbit anti sheep in TNB. Each incubation was followed by two washes in TNT (100 mM Tris HCl (pH 7.5), 150 mM NaCl, 0.05 % Tween 20) at 37 °C for 5 min. The slides were wash for 1 min in 2 × SSC at 37 °C and dehydrated

in an ethanol series of 70 %, 95 %, 100 % for 1 min at RT. Chromosome preparations were counterstained with DAPI (4,6-diamidino-2-phenylindole) in Vectashield. Slides were examined with an epifluorescence Nikon Eclipse E800 microscope (Nikon, Tokyo, Japan) using appropriate filters and a JVC KY-F 58E camera (JVC, Yokohama, Japan). Images were captured and processed with the softwares ArcSoft PhotoStudio 2000 4.3 and Adobe Photoshop 7.

Results

BC1 plants and their morphological observation

On 183 backcrosses achieved with the HTL hybrid and *G. hirsutum*, only 27 BC1 seeds (i.e. 0.15 seeds per cross) were obtained with generally one seed per boll. Thirteen of these seeds gave rise to viable plants. Among these plants, only twelve produced flower buds and could be submitted to cytogenetic analysis. An important segregation regarding morphological characters was observed among the BC1 plants. The plant heights ranged from 133 cm (BC1/10) to 241 cm (BC1/2), while the heights of *G. hirsutum* and the HTL were on average 150 and 274 cm respectively. The size of the BC1 plants leaves was variable but all of them were bigger than the leaves of *G. thurberi* and *G. longicalyx*, and close to those of *G. hirsutum*. The colour of the flowers was pale cream, light yellow or yellow. The BC1/10 plant had flowers with red spot at the base of the petals like *G. thurberi*. It was the sole BC1 plant that presented this trait. No pollen grains were noted on the anthers of the BC1/12 genotype. The anthers of this plant remained indehiscent even after the flower opened.

Fiber fineness analysis

Table 1 shows the results of fiber fineness analysis. Among the three parental species, *G. longicalyx* had the finest fiber with 5.94 µm ribbon width against 17.765 and 15.769 µm for *G. hirsutum* and *G. thurberi* respectively. The ribbon width of the HTL trispecies hybrid was 12.649 µm, while it ranged from 13.039 to 16.276 µm for the BC1 plants. Among these BC1 plants, BC1/4, BC1/3, BC1/11, and BC1/6 had the lowest ribbon width (13.039–13.416 µm) while BC1/1 and BC1/2 had the highest values (16.235 and 16.276 µm respectively).

SSR marker analysis

All the SSR markers used revealed polymorphism between the parental species. They also showed segregation of the diploid species alleles among the BC1 plants (Table 2). For each of the three primers, seven BC1 plants (BC1/1, BC1/4, BC1/9, BC1/10, BC1/11

Table 1. Ribbon width of parental species and the BC1 progenies of the HTL trispecies hybrid.

Genotype	Number of fibers analysed	Ribbon widh (µm) ± standard deviation	LSD grouping
G. hirsutum (cv. C2)	107	17.765 ± 0.130	H
G. thurberi	107	15.769 ± 0.130	I
G. longicalyx	113	5.940 ± 0.126	A
HTL hybrid	120	12.649 ± 0.123	B
HTL BC1/1	112	16.235 ± 0.127	J
HTL BC1/2	111	16.276 ± 0.128	J
HTL BC1/3	122	13.414 ± 0.122	D
HTL BC1/4	110	13.039 ± 0.128	C
HTL BC1/5	108	15.347 ± 0.129	H
HTL BC1/6	124	13.336 ± 0.121	CD
HTL BC1/7	110	14.822 ± 0.128	G
HTL BC1/8	115	14.457 ± 0.125	F
HTL BC1/9	111	14.358 ± 0.128	EF
HTL BC1/10	111	14.081 ± 0.128	E
HTL BC1/11	114	13.41 ± 0.126	D
HTL BC1/12	111	14.327 ± 0.128	EF

Table 2. Distribution of homozygous (hh) and heterozygous (hl, ht or hlt) for SSR marker BNL832, BNL3279 and BNL2662 in the BC1 progeny (h: allele of *G. hirsutum*; l: allele of *G. longicalyx*; t: allele of *G. thurberi*).

Genotype	BNL 836	BNL3279	BNL2662
HTL BC1/1	hh	hh	hh
HTL BC1/2	hl	hl	hl
HTL BC1/3	hl	hl	hl
HTL BC1/4	hh	hh	hh
HTL BC1/5	hl	hlt	hlt
HTL BC1/6	hl	hl	hl
HTL BC1/7	hh	hl	hl
HTL BC1/8	hh	ht	ht
HTL BC1/9	hh	hh	hh
HTL BC1/10	hh	hh	hh
HTL BC1/11	hh	hh	hh
HTL BC1/12	hh	hh	hh

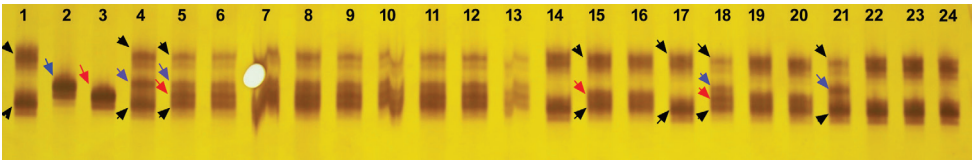

Figure 1. Polyacrylamide gel with BNL3279 SSR marker presenting allele segregations in the parental species, the hexaploid, the HTL tri-species hybrids and the BC1 plants. **1** *G. hirsutum* **2** *G. thurberi* **3** *G. longicalyx* **4** the hexaploid (*G. hirsutum* × *G. thurberi*)^2 **5–13** HTL tri-species hybrid plants (*G. hirsutum* x *G. thurberi*)^2 × *G. longicalyx* **14–24** BC1 plants (HTL x *G.* hirsutum). Black arrow: allele of *G. hirsutum*, Blue arrow: allele of *G. thurberi*, Red arrow: allele of *G. longicalyx*.

and BC1/12) were homozygous exhibiting only *G. hirsutum* alleles, while three BC1 plants (BC1/2, BC1/3 and BC1/6) were heterozygous showing alleles from *G. hirsutum* and *G. longicalyx*. For BNL327 and BNL2662, the BC1/5 plant was heterozygous with alleles from the three parental species and BC1/8 was heterozygous with alleles from *G. hirsutum* and *G. thurberi*. For BNL836, these two plants were respectively heterozygous (with alleles from *G. hirsutum* and *G. longicalyx*) and homozygous (with alleles from *G. hirsutum*). Figure 1 gives an example of the alleles segregation observed with BNL3279.

Plant fertility

HTL BC1 plants presented great variations among them regarding pollen grain shape, size and stainability, unlike the pollen of the HTL parental species (*G. hirsutum*, *G. thurberi* and *G. longicalyx*) which had uniform size and were easily stainable with acetic-carmine (Fig. 2). The mean proportion of stainable pollen grains approached 100 % for the parental species, and ranged from 4.67 to 32.10 % for the BC1 plants (Table 3). The results of the self-pollinations are presented in Table 3. In the parental species, all the self-pollinated flowers gave mature bolls with a large number of well formed seeds. No mature capsule could be obtained by self-pollination of a large part of the HTL BC1 progeny (BC1/2, BC1/4, BC1/6, BC1/8, BC1/9, BC1/11 and BC1/12). A few mature capsules containing 0 to 12 seeds were produced by five BC1 plants (BC1/1, BC1/3, BC1/5, BC1/7 and BC1/10).

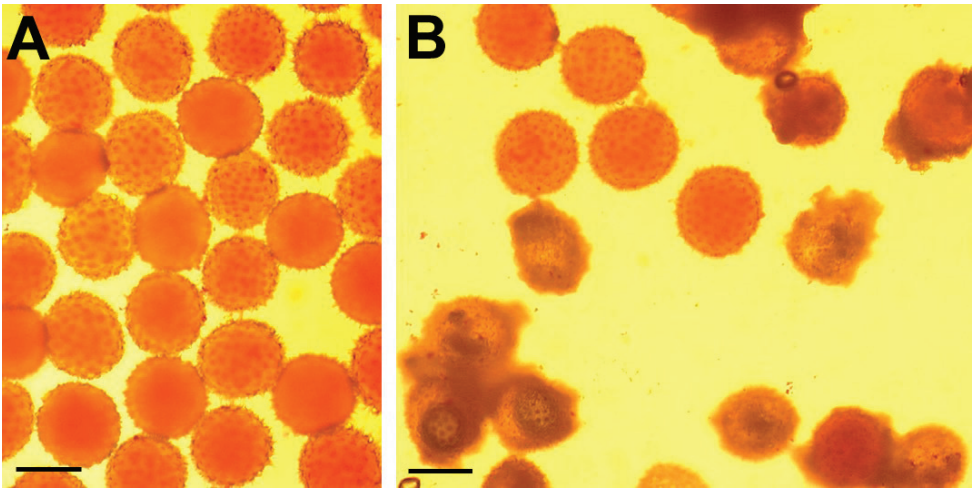

Figure 2. Cotton pollen grain viability revealed by acetic-carmine staining. **A** *G. hirsutum* viable pollen grains with good colour and uniform size **B** mixture of viable and non viable pollen grains of a [(*G. hirsutum* × *G. thurberi*)^2 × *G. longicalyx*] backcross-1 plant. Scale bars: 100 µm.

Table 3. Self-fertility of the first backcrossing (BC1) progeny of [(*G. hirsutum* × *G. thurberi*)^2 × *G. longicalyx*] trispecies hybrid (HTL) and its parental species.

Genotype	Total number of examined pollen grain (% of stainable pollen grain)	No of self-fertilized flowers	No of aborted capsules	No of capsules harvested		No of seeds harvested	Average number of seeds per self-fertilized flower
				No of capsules without seeds	No of capsules containing seeds		
G. hirsutum (cv. C2)	200 (100.00)	30	0	0	30	1020	34
G. thurberi	131 (100.00)	30	0	0	30	450	15
G. longicalyx	200 (100.00)	30	0	0	30	180	6
HTL BC1/1	362 (15.19)	30	26	4	0	0	0
HTL BC1/2	321 (4.67)	30	30	0	0	0	0
HTL BC1/3	348 (14.37)	30	28	1	1	1	0.03
HTL BC1/4	383 (22.45)	30	30	0	0	0	0
HTL BC1/5	416 (11.78)	30	26	1	3	6	0.2
HTL BC1/6	352 (23.39)	30	30	0	0	0	0
HTL BC1/7	405 (32.10)	30	27	0	3	12	0.4
HTL BC1/8	383 (13.84)	30	30	0	0	0	0
HTL BC1/9	267 (9.36)	30	30	0	0	0	0
HTL BC1/10	332 (9.94)	30	28	0	2	9	0.3
HTL BC1/11	335 (10.45)	30	30	0	0	0	0
HTL BC1/12	Indehiscent anthers	30	30	0	0	0	0

Table 4. Cross-fertility of the HTL BC1 plants (as females) with *G. hirsutum*.

Backrcrosses	No of pollinated flowers	No of capsules harvested		No of seeds harvested	Mean number of seeds per backcross
		No of capsules without seeds	No of capsules containing seeds		
HTL BC1/1 × *G. hirsutum*	20	10	10	11	0.55
HTL BC1/2 × *G. hirsutum*	33	0	33	92	2.79
HTL BC1/3 × *G. hirsutum*	17	1	16	36	2.12
HTL BC1/4 × *G. hirsutum*	43	20	23	42	0.98
HTL BC1/5 × *G. hirsutum*	37	0	37	191	5.16
HTL BC1/6 × *G. hirsutum*	23	13	10	22	0.96
HTL BC1/7 × *G. hirsutum*	43	0	43	219	5.09
HTL BC1/8 × *G. hirsutum*	74	8	66	157	2.12
HTL BC1/9 × *G. hirsutum*	75	53	22	33	0.44
HTL BC1/10 × *G. hirsutum*	56	7	49	364	6.5
HTL BC1/11 × *G. hirsutum*	66	39	27	38	0.57
HTL BC1/12 × *G. hirsutum*	21	12	9	10	0.48
Total	**508**	**163**	**345**	**1215**	**2.39**

Table 4 shows the results obtained for the backcrossing to *G. hirsutum* of the different BC1 plants. All the BC1 plants gave seeds by backcrossing. The number of BC2 seeds produced per BC1 plants ranged from 10 to 364 seeds with an average of 0.44 to 6.50 seeds per cross. The total number of BC2 seeds obtained after pollination of 508 flowers was 1215 seeds. BC1/10, BC1/5 and BC1/7 gave the highest mean numbers of seeds per backcross (6.5, 5.16 and 5.09 respectively), while BC1/9, BC1/12, BC1/1, BC1/11, BC1/6 and BC1/4 gave mean numbers of seeds inferior to 1 (0.44, 0.48, 0.55, 0.57, 0.96 and 0.98 respectively).

Meiosis of BC1 plants

Meiosis studies were performed on the HTL BC1 progeny, and also on *G. hirsutum* as control. With the exception of the HTL BC1/12 plant which had 54 chromosomes, all the analyzed HTL BC1 plants had 52 chromosomes. At metaphase I, chromosomes of *G. hirsutum* paired perfectly with 26 bivalents (Fig. 3A). The meiosis of *G. hirsutum* was regular, stable and normal tetrads with four normal cells were observed as final products. Chromosome associations at metaphase I in the HTL BC1 progeny were variable, with usually a mixture of univalents and bivalents (Fig. 3B) and sometimes multivalents. Cytological data concerning these genotypes are summarized in Table 5. In the BC1 plants, the chromosome associations varied as follows: (2.5 to 11.5) I + (17 to 22) II + (0.31 to 1.93) III + (0.09 to 1.93) IV + (0 to 0.07) V + (0 to 0.14) VI. On an average, the number of paired chromosomes observed in BC1 plants varied from 40.50 to 49.50. The plants BC1/10 and BC1/7 gave the lowest mean numbers of univalents (2.5 and 3.75 univalents respectively). The highest number of univalents was found with BC1/11 (11.50 on average).

Unlike observations made in *G. hirsutum*, PMC meiosis in the BC1 progeny was mostly abnormal (Fig. 4) and asynchronous with different meiosis phases in the same flower bud. In these plants, from metaphase I onwards (Fig. 4C) meiosis showed abnormalities. The most common meiotic abnormality found was irregular chromosome segregation, characterized by precocious chromosome ascension and laggards. This irregular chromosome segregation affected all meiotic phases, generating genetically unbalanced microspores. Irrespective of the BC1 plants the meiotic behaviour was mostly the same, with univalents showing precocious ascension at metaphase I (Fig. 4C) or remaining as laggards at anaphase I, telophase I (Fig. 4E), metaphase II (Fig. 4F) and anaphase II (Fig. 4G). Chromosomes did not have the same rhythm in cell division and laggards were engulfed by extra nuclei so that at the end of the second division, meiotic products were mostly represented by polyads (more than 4 cells), generally with microspores of different sizes. In the BC1 plants, the final products of meiosis were, thus, completely abnormal, with a predominance of polyads even if some sporadic normal tetrads were observed (Fig. 4H).

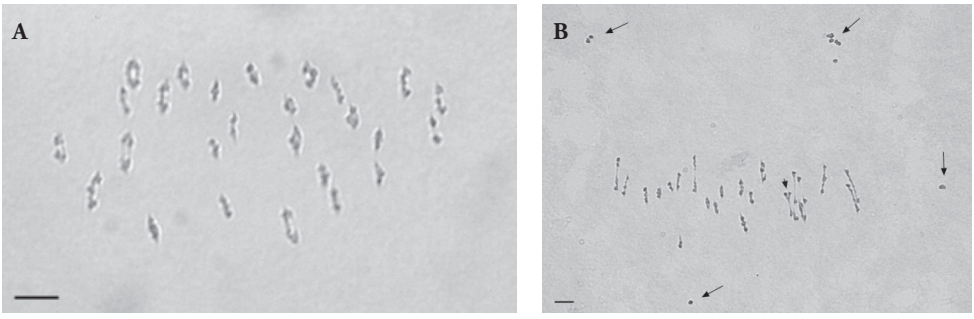

Figure 3. Meiotic metaphase I plates in *G. hirsutum* and in the HTL BC1/4 plant. **A** Meiotic metaphase I cell showing 26 bivalents in control *G. hirsutum* **B** meiotic metaphase I cell showing 8 univalents (long arrows), 20 bivalents and 1 quadrivalent (short arrow) in HTL BC1/4. Scale bars: 5 μm.

Table 5. Meiotic pairing in *G. hirsutum* and in the HTL BC1 progeny.

Genotype	No of analyzed c ells	Chromosome configuration						Chromosome Number	Average No of chromosomes paired
		I	II	III	IV	V	VI		
G. hirsutum	10		26					52	52
HTL BC1/1	30	6.90 (1–12)	21.07 (17–24)	0.53 (0–3)	0.33 (0–2)	0.03 (0–1)		52	45.23
HTL BC1/2	32	9.37 (4–16)	20.66 (18–24)	0.31 (0–2)	0.09 (0–1)			52	46.62
HTL BC1/3	30	5.23 (0–12)	21.93 (16–26)	0.43 (0–2)	0.43 (0–2)			52	46.90
HTL BC1/4	30	7.73 (4–12)	20.67 (16–24)	0.37 (0–2)	0.40 (0–2)	0.03 (0–1)		52	42.20
HTL BC1/5	12	4.75 (2–10)	21 (19–24)	0.75 (0–3)	0.75 (0–2)			52	47.25
HTL BC1/6	33	5.85 (2–10)	21.67 (17–24)	0.45 (0–4)	0.36 (0–2)			52	46.15
HTL BC1/7	30	3.83 (0–8)	22.8 (19–25)	0.37 (0–3)	0.33 (0–2)			52	48.03
HTL BC1/8	37	8.63 (4–14)	18.94 (13–23)	0.51 (0–4)	0.97 (0–4)			52	43.32
HTL BC1/9	21	10.38 (5–15)	18.90 (15–22)	1.14 (0–3)	0.09 (0–2)			52	41.62
HTL BC1/10	14	2.5 (0–5)	22 (0–2)	1.07 (0–2)	0.57 (0–1)			52	49.50
HTL BC1/11	32	11.50 (7–17)	17.81 (10–22)	1 (0–4)	0.47 (0–3)			52	40.50
HTL BC1/12	14	5.28 (0–8)	17 (14–20)	1.93 (0–6)	1.93 (0–4)	0.07 (0–1)	0.14 (0–1)	54	48.71

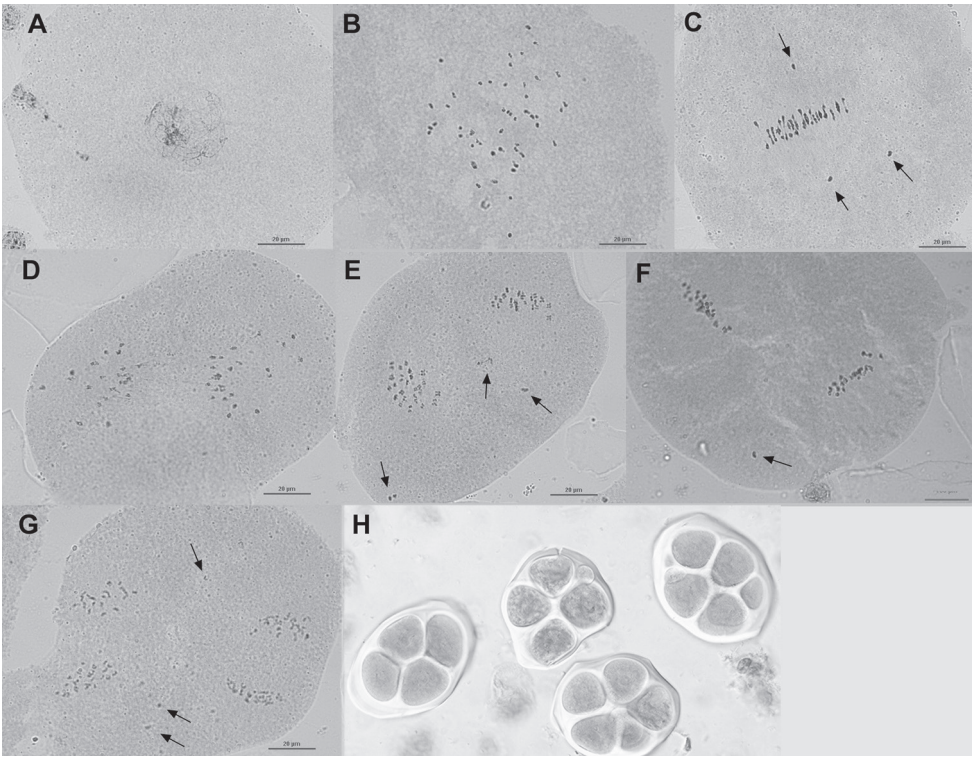

Figure 4. Meiotic aspect with abnormalities in HTL BC1 plants. **A** Leptotene **B** diakinesis **C** metaphase I with univalent chromosomes in early ascension (arrow) **D** anaphase I **E** telophase I with the presence of laggard chromosomes (arrows) **F** metaphase II with laggard chromosomes (arrows) **G** anaphase II with presence of laggard chromosome (arrow) **H** microsporocytes with a mixture of tetrad and polyads with micronuclei. Scale bars: 20 μm.

GISH analysis

Figure 5 shows results of GISH analysis. For the HTL hybrid and the BC2 progeny, 52 chromosomes appeared in blue when they were counterstained with DAPI (Fig. 5A, C). When FITC detection and Texas Red detection were superimposed, three populations of chromosomes are differentiated for the HTL trispecies hybrid (Fig. 5B). Thirteen chromosomes appeared green and were those from *G. longicalyx*; thirteen large chromosomes appeared yellow-orange and were those from *G. hirsutum* A subgenome, twenty six small chromosomes appeared red and were those from *G. hirsutum* D-subgenome and *G. thurberi* D1 genome since the D genome is comprised of the smallest chromosomes (Phillips and Strickland 1966; Konan et al. 2009; Li et al. 2016). For the Backcross-2 progenies, GISH revealed presence of entire chromosomes of *G. longicalyx* as well as recombinant chromosomes (Fig. 5D).

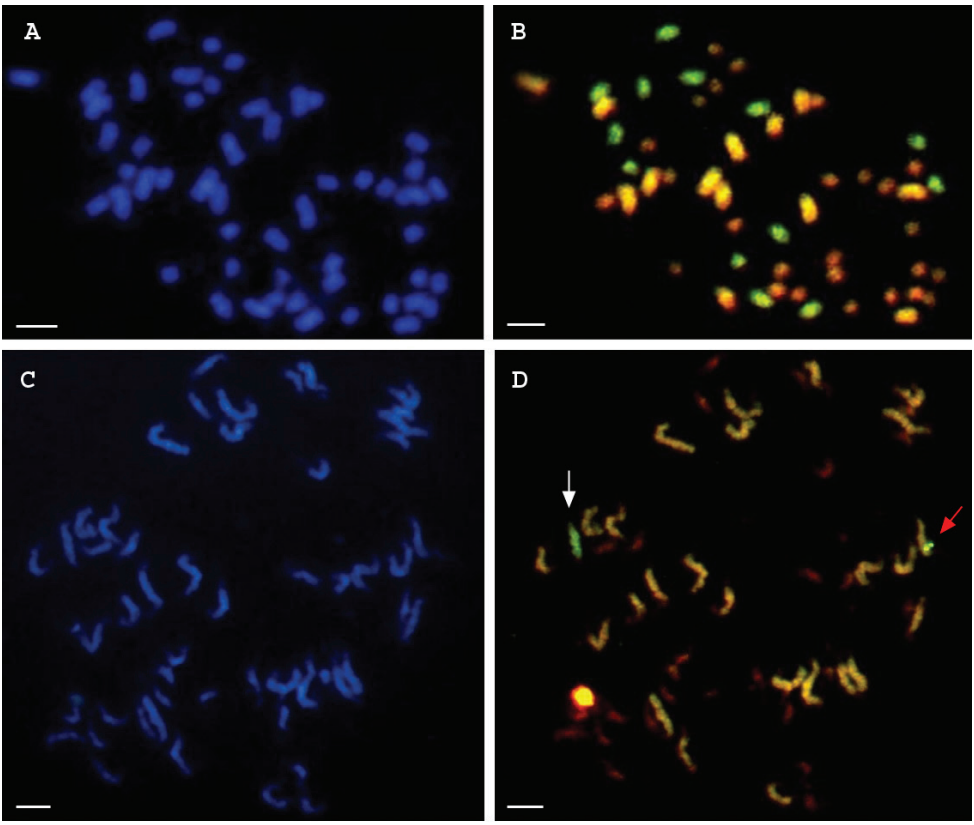

Figure 5. Genomic *in situ* hybridization on mitotic metaphase chromosomes of the HTL trispecies hybrid [(*G. hirsutum* × *G. thurberi*)^2 × *G. longicalyx*)] and a BC2 progeny. **A** Mitotic metaphase showing 52 chromosomes of the HTL hybrid revealed by counterstaining with DAPI **B** mitotic metaphase showing in the HTL hybrid 13 green chromosomes from *G. longicalyx*, 13 yellow-orange chromosomes from the A subgenome of *G. hirsutum* and 26 red chromosomes from *G. thurberi* and the D subgenome of *G. hirsutum* revealed after the superimposition of FITC detection and Texas Red detection **C** mitotic metaphase showing 52 chromosomes in a HTL BC2 revealed by counterstaining with DAPI **D** mitotic metaphase in a HTL BC2 showing an entire chromosome of *G. longicalyx* (white arrow) and an intergenomic recombination (red arrow) showing movement of *G. longicalyx* chromatin into a chromosome of the A subgenome of *G. hirsutum*. Scale bars: 5 μm.

Discussion

Compared with the homologous chromosome pairing in the parental *G. hirsutum* species which showed only bivalents, the meiotic behaviour of HTL BC1 progeny was abnormal. In these plants, a disturbed meiosis was observed and, at metaphase 1, chromosomes paired imperfectly and gave, in addition to bivalents, some univalents and multivalents. The same abnormalities were observed by Konan et al. (2007) with the HTL trispecies hybrid. Moreover, *G. hirsutum* gave normal tetrad and 100% pollen

fertility unlike the BC1 plants. The normal and harmonious course of meiosis in pollen mother cells of *G. hirsutum* with regular bivalent formation and normal tetrad production after cytokinesis ensured 100 % pollen viability; whereas the abnormalities observed in the meiosis of the BC1 plants caused the formation of sterile gametes and low percentages of pollen viability. These results indicate a link between meiotic behaviour and pollen fertility. They suggest that the irregular chromosome pairing observed in the BC1 plants is one of the main reasons of the problem of fertility observed. Usually in plant species with normal genomes, homologous chromosomes tend to have high matching rate unlike the homoeologous chromosomes in hybrid plants derived from interspecific crosses (Kaur and Singhal 2019). Actually, an essential event in meiosis is the chromosome pairing, not only for the occurrence of recombination but also for the correct chromosome segregation. Working on meiotic irregularities and pollen viability in the genus *Passiflora* Linnaeus, 1753, Souza et al. (2003) showed that when an error occurs in the chromosome pairing, the segregation can happen in an unbalanced way and gametes can receive an unbalanced number of chromosomes, leading to decrease of viable gametes. This is in accordance with several other authors (Kammacher 1966, Shuijin and Biling 1993, Zanders et al. 2014, Potapova and Gorbsky 2017, Wang et al. 2017) who found that the irregular chromosome pairing could cause unequal meiotic division and unbalanced chromosome segregation, leading to incomplete or abnormal chromosome sets (i.e. aneuploidy) after cytokinesis and causing sterile gametes.

Konan et al. (2007) observed the same meiotic abnormalities with the HTL trispecies hybrid (14.13 I + 15.10 II + 1.03 III + 0.9 IV + 0.03 V + 0.13 VI). But there was globally, greater chromosome pairing in the BC1 plants than in the trispecies hybrid (40.50 to 49.50 instead of 37.85 paired chromosomes in HTL), supporting their relative higher pollen fertility. In general, progression toward increasing frequencies of plants that form 26 bivalents at metaphase I were observed in the advanced backcross generations of other cotton trispecies hybrids exploited in breeding programs (Vroh et al. 1999, Mergeai 2006). This was also the case for the HTL BC1 progeny. This observation indicates that backcrossing has promoted cytological stability. He et al. (2017) made the same observation in their works on chromosome pairing in backcross progeny of the hybrid *Triticum aestivum* Linnaeus, 1753 and *Elytrigia elongata* (Host ex P. Beauvois, 1812) Nevski, 1933.

Four BC1 genotypes were able to produce a few seeds through self-pollination. These plants which produced also the highest number of BC2 seeds per backcross were characterized by a better meiotic stability. Their relative lower univalent rate and higher paired chromosome rate could explain their higher self- and cross-fertility. Globally, the number of BC2 seeds produced per backcross was negatively correlated with the average number of univalents at Metaphase I ($R = -0.67$, $P < 0.05$) indicating a deleterious role of unpaired chromosomes on the fertility of the hybrid plants. This is in accordance with Bikchurina et al. (2018) who reported that univalents impair pollen fertility and seed production.

However, irregular meiotic behaviour cannot be the sole explanation of the observed sterility problems. Indeed, some of the HTL BC1 plants presenting a few uni-

valents were totally self-sterile, indicating that additional factors may reduce the actual gamete viability of the BC1 plants. The same observation was earlier made by Brown and Menzel (1950) in the [(*G. hirsutum* × *G. arboreum*)^2 × *G. harknessii* Brandegee, 1889] and [(*G. hirsutum* × *G. herbaceum*)^2 × *G. harknessii*] trispecies hybrids and by Kammacher (1956) in the allotetraploid hybrid (*G. arboreum* × *G. thurberi*)^2. These three hybrids characterised by relatively good chromosome pairing rates (respectively 24.6, 22.36 and 22.14 bivalents) were also self-sterile. Actually, each species has a balanced complex of many genes and a structural organization of the chromosomes that regulates pairing and ensures the genetic stability of the species (Mursal and Endrizzi 1976, Jeridi et al. 2011). Hybridization between species can destroy this stability. Homoelogous chromosomes of two close species can differ by several little chromosomal rearrangements appeared during genome differentiation (Jeridi et al. 2011). Even if hybrids between such species do not show irregular chromosome configuration at meiosis owing to their apparent homology, the genetic stability is however disturbed and the hybrids can be sterile (Jeridi et al. 2011). Sterility of plants with regular meiosis has been also associated with disturbances of tapetal development and degeneration (Narkhede et al. 1967, Papini et al. 1999, Wang et al. 2015). Tapetum is the innermost of the four sporophytic layers of the anther wall that plays an important role in the male fertility of pollen grains. It comes into direct contact with the developing male gametophyte and contains all the nutrients for microspore development and maturation, such as callose, sporopollenin and proteins (Wang et al. 2015). Studies have proven that tapetal tissue has a secretory role, providing nutrients required for microspore and pollen grain development, and defects in tapetal tissue can lead to pollen abortion (Chiavarin et al. 2000, Wang et al. 2015). While studying the stage of pollen grain degeneration of male sterile plants in sorghum, wheat, tomato, sugar-beets, maize etc. earlier authors (Narkhede et al. 1967) have already reported that meiosis in these male sterile plants appeared to be normal, but after the pollen grains were partially formed they aborted and the anthers lacked viable pollen prior to dehiscence. According to these authors even if the meiosis was found to be normal in the male sterile plants, the pollen grains deteriorated after their formation because the tapetum in anthers of such plants did not deteriorate and release the food material necessary for the normal development of the pollen grains. It was stated that there was a possible association of the nonviable character of pollen grains with the nutritive role of tapetum in male sterile plants. Recently, there have been a large number of reports that confirmed this statement (Ma et al. 2015, Wang et al. 2015, Li et al. 2017, Zheng et al. 2019).

Although only four BC1 plants produced a few seeds through self-pollination, all the BC1 plants gave seeds by backcrossing when they were used as female. This result indicates that instead of the male sterility presented by most of BC1 plants, the potential for female reproduction remains. Konan et al. (2007) made the same observation with the HTL trispecies hybrid.

Evaluation of fiber fineness showed that *G. longicalyx* had the finest fiber among the parental species, and the HTL trispecies hybrid and some of its BC1 progenies

gave finer fiber than the main cultivated cotton *G. hirsutum*. This result confirms that *G. longicalyx* is a good donor for fiber fineness. The plants BC1/3, BC1/4, BC1/6, and BC1/11 which gave the lowest ribbon width are interesting genetic stocks which can be selected for further improvement of this trait in a breeding program. However, as one of the main success factors of breeding is the selection of genotypes with a high percentage of viable gametes (Lavinscky et al. 2017), the BC1/10 stock which was the most stable plant (with the lowest univalent number, the highest paired chromosome number, the highest cross fertility, and among the best self-fertile plants) and had a fairly good fiber fineness can be selected as well.

SSR marker analysis revealed segregation of diploid alleles among the BC1 plants indicating the differential presence or absence of the diploid species chromosomes and/or chromosome recombinations. This allele segregation supports the segregation observed in BC1 plants regarding fiber fineness trait and the other morphological traits. For the success of an interspecific breeding program, homoeologous recombinations are crucial. Interspecific hybridization finds its justification in the possibility for genetic material exchanges between the genome of the different target species. The degree of homology between the parental genomes is fundamental for the occurrence of intergenomic recombination in successive backcross progenies. In general, chromosomes of closely related genomes tend to pair more often than chromosomes of genomes that are more distantly related (Kimber and Yen 1990, He et al. 2017). The occurrence of chromosome pairing provides a basis for recombination. According to some authors (Brown and Menzel 1952; Endrizzi 1957; Philips and Strickland 1966; Konan et al. 2009) the D subgenome of *G. hirsutum* retains sufficient chromosome homology with the D_1 genome of *G. thurberi*, and the F_1 genome of *G. longicalyx* is more closely related to the *G. hirsutum* A subgenome. Therefore, in the hybrid plants, D_1 chromosomes of *G. thurberi* should mostly pair with the chromosomes of *G. hirsutum* D subgenome, while F_1 chromosomes of the donor species (*G. longicalyx*) should mostly pair with the chromosomes of *G. hirsutum* A subgenome. The formation of a high number of bivalents and some multivalents as it was observed in these hybrids is an indication that genomic exchanges can occur during the meiosis between wild genome chromosomes and the chromosomes of *G. hirsutum* subgenomes. GISH revealed a clear distinction of the *G. hirsutum* and *G. longicalyx* chromosomes in the trispecific hybrid, showing that it is possible to differentiate *G. longicalyx* and *G. hirsutum* chromatin. For the backcross derivative progenies, GISH revealed presence of entire chromosomes of *G. longicalyx* as well as recombinant chromosomes, indicating that segregation of *G. longicalyx* chromosomes and introgression of this species chromatin into *G. hirsutum* occur. These results show that it is possible to introgress chromosome segments of *G. longicalyx* into *G. hirsutum*. Segregation observed in the BC1 plants regarding morphological characters could hence be attributed to the differential presence or absence of the diploid species chromosomes and to homoelogous chromosome recombination as suggested by SSR marker analysis. The current results suggest that the development of balanced upland cotton varieties integrating *G. longicalyx* genetic material and exhibiting good fiber fineness could be feasible.

Conclusion

Cotton fibers sustain one of the world's largest industries, the textile industry, for wearing apparel, home furnishings, and medical supplies. Further improvement of cotton fiber quality is much desired. Most of the BC1 plants studied have presented an increased number of paired chromosomes compared to the parental HTL trispecies hybrid. Moreover, all of them could produce seeds through backcrossing and some were self-fertile. The plant material is gaining stability. Some BC1 plants exhibited interesting fiber fineness and recombination is possible between the donor species chromosomes and *G. hirsutum* chromosomes. These results are promising for the introgression of the improved fiber fineness trait from *G. longicalyx* into upland cotton.

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The occurrence of dot-like micro B chromosomes in Korean field mice *Apodemus peninsulae* from the shore of the Teletskoye Lake (Altai Mountains)

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Abstract

The data on the changes in the cytogenetic structure of the geographic population of Korean field mouse *Apodemus* (*Alsomys*) *peninsulae* Thomas, 1907 at the southern shore of the Teletskoye Lake (Altai Republic) are presented. In 1980 no dot-like microchromosomes were found in 34 mice captured on the southern and northern coasts of the Teletskoye Lake. In 2011, a 1.6-fold (from 2.7 to 4.3) increase in the mean number of B chromosomes compared to the rate estimated there earlier in 1980 was discovered. In 11 of the 15 mice (73%) captured in 2011, the karyotypes contained 1–2 dot-like micro B chromosomes and 1–5 macro B chromosomes. The pollution of the territory by the remains of the rocket fuel components may be an appropriate explanation for the cause of the karyological changes observed in *A. peninsulae* in this region.

Keywords

Apodemus (*Alsomys*) *peninsulae*, B chromosome dynamics, dot-like micro B chromosomes, karyotype

Introduction

The story of studying and describing B chromosomes (Bs) dates back to 1907, when Edmund Wilson (1907), working on hemipteran chromosomes, noticed those that appeared to be additional to the main karyotype and were present only in a fraction of individuals. However, the term 'B chromosome' was only established 11 years later. In 1928, Lowell Fitz Randolph working on variation in maize chromosomes proposed to call stable chromosomes of the standard complement 'A chromosomes', and those coming additional to the standard complement and being variable in number and morphology, 'B chromosomes' (Randolph 1928). B chromosomes were discovered very early in the history of cytogenetics. By 2004, Bs have been found in 55 of 4629 living species of mammals (Vujošević and Blagojević 2004).

Rubtsov and Borisov (2018) suggest several models of B chromosome origin, while the article by Vujošević et al. (2018), provides an updated list of 85 mammalian species with Bs, and gives a detail description of research experiments accomplished on these species. The research article by (Makunin et al. 2018) provides novel data on B chromosome content and evolution in the red fox (the first mammalian species with Bs, whose genome has recently been sequenced and assembled (Kukekova et al. 2018), and in the raccoon dog, the carnivore species, where B specific coding genes were discovered almost 13 years ago. Using new generation sequencing, the authors argue that the origin of B chromosome in these species is independent. Through the analysis of B content in different mammals they conclude a frequent and independent re-use of the same genomic regions in B chromosome formation. They suggest that such a re-use may be connected with gene functions. By definition, these chromosomes are not essential for the life of the species, their presence is not necessary for some (as a rule, for most) individuals of the species, thus, the population can consist of individuals with 0, 1, 2, 3 (etc.) B chromosomes, but limited to the population by their critical number, i.e., with the maximum known number of Bs in the individuals in the population (Vujošević and Blagojević 2004, Vujošević et al. 2018). B chromosomes have been reported in six species of the genus *Apodemus* (*A. peninsulae* Thomas, 1907, *A. agrarius* Pallas, 1771, *A. sylvaticus* Linnaeus, 1758, *A. flavicollis* Melchior, 1834, *A. mystacinus* Danford & Alston, 1877, *A. argenteus* Temminck, 1844). High frequencies of Bs were recorded particularly in *A. peninsulae* and *A. flavicollis* (Zima and Macholán 1995, Vujošević and Blagojević 2004, Borisov and Zhigarev 2018). Through the wide geographical range *A. peninsulae* karyotypes contain from 48 to 78 chromosomes and the vast majority of individuals of this species have supernumerary B chromosomes (Borisov and Zhigarev 2018).

Earlier (Borisov and Zhigarev 2018), we analyzed B chromosome variation in Korean field mouse *Apodemus peninsulae* (Rodentia, Muridae) based on a 40-year study of karyotypes collected from geographically distant populations in East Siberia, North Mongolia, China and the Russian Far East. In *A. peninsulae* up to five morphotypes were revealed. In the East Asian mouse, there are up to 30 B chromosomes differing in number and having a diverse morphology varying from dot-like micro pointed and

small acrocentric B chromosomes to meta- and submetacentric B chromosomes of different size (small, medium and large) (Borisov and Zhigarev 2018). In Siberian mouse populations 1–10 macro B chromosomes, 1–30 dot-like micro B chromosomes and different combinations of macro and micro B chromosomes could be observed. While in mice populations of other regions individuals with no B chromosomes are frequent, all individuals in Siberian populations have B chromosomes of different types which make stable inheritable population systems (Borisov and Zhigarev 2018). Notably, it is customary for the Siberian geographical populations of the species to have a different number of B chromosome morphotypes, which together form stably heritable population systems.

The pattern of evolutionary dynamics of Bs can be distinctly different between geographical populations, and both the parasitic and the heterotic models can be applied to explain the maintenance of Bs in different populations. Further studies are desirable to improve our understanding of the complicated evolutionary dynamics of Bs in the *A. peninsulae* (Borisov 1990, 2008, Rubtzov et al. 2009, Rubtsov and Borisov 2018). The Korean field mouse has thus become a good mammalian model for studies of evolutionary dynamics and effects of Bs on the host genome (Rubtzov et al. 2009, Borisov and Zhigarev 2018, Rubtsov and Borisov 2018). B chromosome frequencies in *A. peninsulae* show temporal variation. Comparison of Bs from the population from Altai Republic, trapped in the 1980 and 2002, showed that a mean number of Bs in this population has almost tripled in 22 years (Rubtzov et al. 2009). The question is how widely this phenomenon occurred along the Teletskoye Lake shore. Earlier B. Kral (1971) and we in 1980 (Borisov 1990, 2008) studied the cytogenetic structure of the population of *A. peninsulae* on the southern shore of the Teletskoye Lake. Therefore, now we have an opportunity to compare these data with the data on the state of the population in 2011, i.e. 30 years later.

Material and methods

We have analyzed new data on the chromosome sets of 15 *A.peninsulae* caught in 2011 in localities on the southern extremity of the Teletskoye Lake (Republic of Altai, Russia) (Fig. 1, localities Nos. 2 and 3): locality in the tourist centre at Karagai (4♀♀ and 7♂♂), locality at the Kyga River mouth in the vicinity of the settlement Chiri (The Altaiskiy State Nature Reserve) (3♀♀ and 1♂).

Chromosome preparations were made from marrow cells after intraperitoneal injection of 0.5 ml of 0.04% colchicine solution (Ford and Hamerton 1956). For defining the number and morphotypes of B chromosomes at least 20 metaphase cells from each animal were examined.

Previously, repeated DNA of Bs in the Korean field mouse has been analyzed by FISH with DNA probes generated by microdissection of A and B chromosomes followed by DOP-PCR (Karamysheva et al. 2002, Rubtsov et al. 2004). It was shown that all B chromosomes were composed of a large amount of repeated DNA sequences.

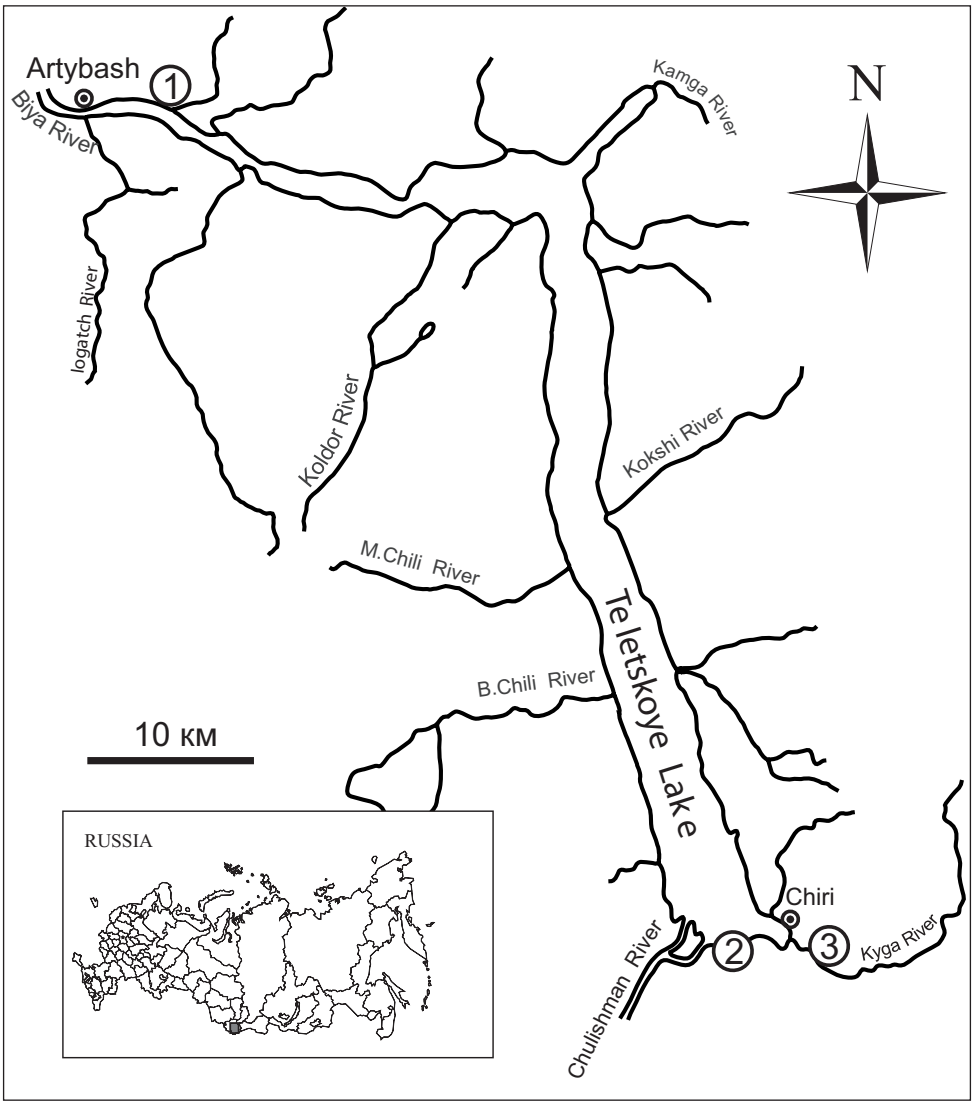

Figure 1. Schematic map of the study area and sampling localities of the mice *Apodemus peninsulae*: **1** locality in the vicinity of Artybash settlement **2** tourist centre at Karagai **3** locality at the Kyga River mouth.

The repeats were classified in terms of their homology and predominant location. But in this work, the routine dyeing was enough for the analysis of variability of B chromosome. B chromosomes were split by morphotype into five classes (Borisov and Zhigarev 2018), four of which are represented by macro B chromosomes more than 0.1 the size of the largest acrocentric chromosome (3.5–4.0 microns). The first class included large two-arm macro B chromosomes, from half the size of the largest autosome to the size comparable to that of the entire chromosome. The second class included smaller macro B chromosomes from 0.5 to 0.3 the size of the largest autosome. The third class

was represented by small two-arm B chromosomes approximately 0.3–0.1 the size of the largest autosome. The fourth class comprised very rarely found and as a rule relatively small acrocentric B chromosomes, equal in sizes to B chromosomes of the third class. The fifth class included micro B chromosomes; these are dot-like B chromosomes with centromeres of unclear position. The frequency distribution of B chromosome classes was calculated from all metaphase plates of the studied individuals. Calculations of chromosome dimensions and registration of B chromosomes of *A. peninsulae* was performed using microscope D 5000 Leika.

Results and discussion

The karyological analysis of 15 specimens of *A. peninsulae* from the southern shore of the Teletskoye Lake (Fig. 2, Table 1) captured in 2011 has shown that apart from the major stable chromosome set comprising 48 acrocentric A chromosomes, the karyotypes of these mice contained 2–6 B chromosomes. The variable part of the karyotype is represented by a combination of 0–1 large two-arm, 0–3 average two-arm, 0–3 small two-arm, 0–1 small acrocentric, and 0–2 dot-like micro B chromosomes. Small acrocentric and dot-like micro B chromosomes have been revealed in 14 (93%) of 15 studied mice from the southern coast of the Teletskoye Lake (Table 1).

This result differs from the result obtained from the same population in 1980 (Borisov 2008). We also observed an increase in the number of macro B chromosomes and occurrence of dot-like microchromosomes in karyotypes of the majority of specimens in *A. peninsulae* from the northern shore of the Teletskoye Lake in the vicinity of Artybash settlement (Fig. 1, locality No. 1) in 2006 as compared to 1980 (Fig. 2, d) (Borisov 1990, 2008). In 2011 the average number of B chromosomes in karyotypes of *A. peninsulae* increased 1.6-fold (from 2.7 to 4.2) ($td \approx 4.1$, $p >> 0.9999$) (Table 1). Moreover, in 1980, in mice from the southern shore of the Teletskoye Lake no small acrocentric and dot-like microchromosomes were observed.

The study of 57 mice in 1971, 1978, 1980, 1986, 1988, and 1990 revealed no small acrocentric and dot-like microchromosomes (Kral 1971, Borisov 1990, 2008). For the first time small B chromosomes were discovered in four specimens of the Artybash population in 2002 (Borisov 2008). The subsequent study of the population in 2006 revealed an increase in the number of mice with small B chromosomes. Among 17 mice captured in 2006 small acrocentric and dot-like micro B chromosomes were revealed in 13 (76%) specimens (Borisov 2008). It has been established, therefore, that at present in populations of *A. peninsulae* inhabiting the shore of the Teletskoye Lake the process of reorganization of the B chromosome system is occurring. It consists in the growth of the average number of B chromosomes, due to, besides other factors, the increase in the number of small B chromosomes (Table 1, Fig. 2b).

There are not sufficient data for establishing the causes of the phenomenon discovered, so only suggestions with a certain degree of probability could be made. First of all, it is noteworthy that the changes in the B chromosome system of *A. peninsulae*

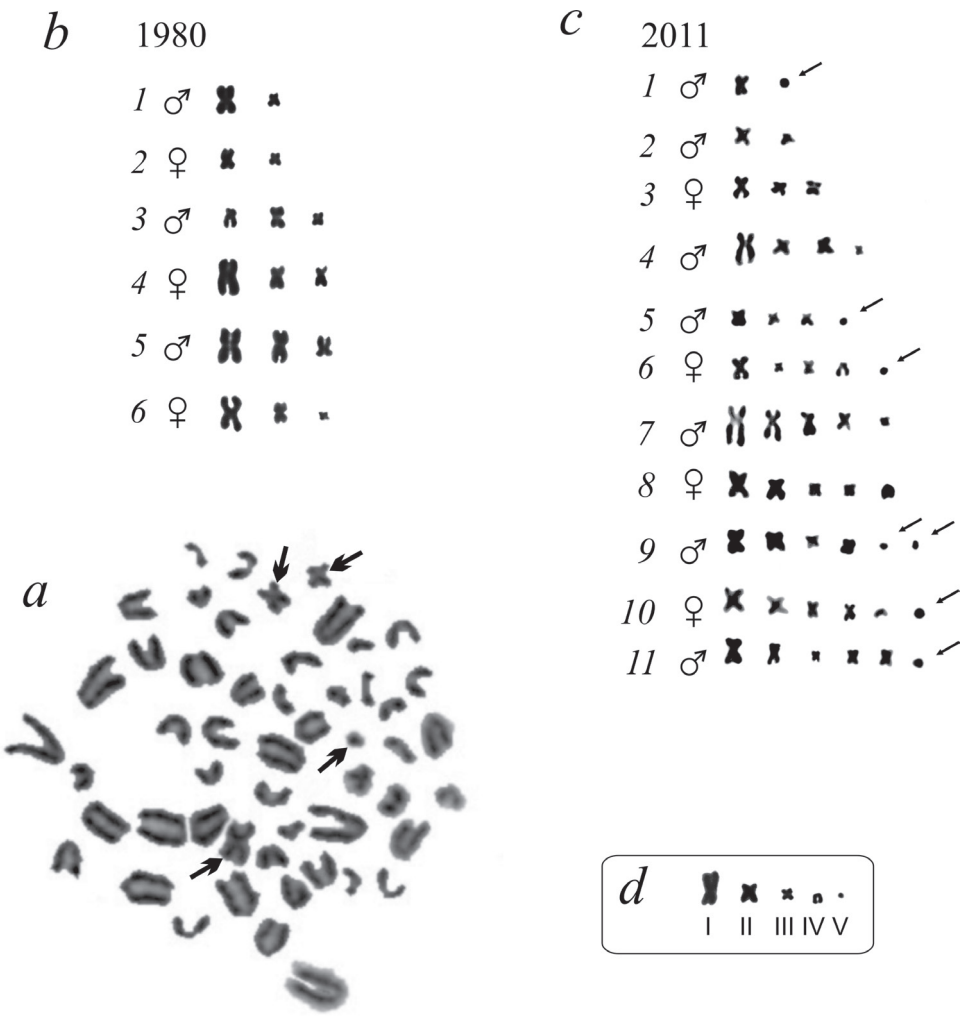

Figure 2. Metaphase plate of *Apodemus peninsulae* caught on the southern shore of the Teletskoye Lake in 2011 with different B chromosomes indicated with thick arrows (**a**) and the individual variants of the B chromosome system in mice from the southern shore of Teletskoye Lake revealed in 1980 (**b**) and 2011 (**c**) (arrows indicate the dot-like micro B chromosomes). Roman numerals denote five classes of identified B chromosomes (**d**).

have occurred throughout the past 30 years; therefore the factor responsible for them probably arose at the same period of time. According to the present concepts, the processes of the rise of dot-like micro B chromosomes of *A. peninsulae* and human small supernumerary marker chromosomes (sSMC) are associated with the increase in chromosome breaks on the boundaries of the pericentromeric heterochromatic regions. The reorganization of primary micro B chromosomes into macro B chromosomes is supposed to occur as a result of segment duplications and inversions of highly repetitive DNA sequences (Rubtzov et al. 2009, Rubtsov and Borisov 2018). The factors

Table 1. Locality, year of capture, and B chromosome system in *Apodemus peninsulae* from the southern shore of Teletskoye Lake.

No.	Sex	Variant of B chromosome system	Totall number of B chromosomes	B chromosome classes				
				I	II	III	IV	V
Karagai, 2011 (locality No. 2)								
1	♂	X •	2	0	1	0	0	1
2	♂	X ^	2	0	1	0	1	0
3	♀	X X x	3	0	2	1	0	0
4	♂	X x x •	3	1	2	0	0	1
5	♂	x x x •	4	0	0	3	0	1
6	♀	X x x ^ •	4	1	0	2	1	1
7	♂	X X x x •	5	1	1	2	0	1
8	♀	X X x x ^	5	1	1	2	1	0
9	♂	X X x x • •	5	0	2	2	0	2
10	♀	X X x x ^ •	6	1	1	2	1	1
11	♂	X X X X x •	6	1	3	1	0	1
$\bar{X}_B$ (Karagai, 2011)			**4.1**	**0.55**	**1.27**	**1.36**	**0.36**	**0.82**
Kyga River, 2011 (locality No. 3)								
12	♀	X x x •	4	0	1	2	0	1
13	♂	X x x ^	4	0	1	2	1	0
14	♀	X X x x •	5	1	1	2	0	1
15	♀	X X X x •	5	1	2	1	0	1
$\bar{X}_B$ (Kyga River, 2011)			**4.5**	**0.50**	**1.25**	**1.75**	**0.25**	**0.75**
$\bar{X}_B$ (totall)			**4.2** ± 0.33	**0.53** ± 0.13	**1.26** ± 0.21	**1.47** ± 0.24	**0.33** ± 0.13	**0.80** ± 0.15

$\bar{X}_B$ – mean number of B chromosomes per individual (with standard error o mean).

underlying the increase of chromosome ruptures resulting in formation of micro B chromosomes are possibly the impact of different mutagenic environmental factors or viral infections. No data on the prevalence of viral infections in *A. peninsulae* in the Altai Mountains are currently available. However, the Altai Mountains and Teletskoye Lake, in particular, are known as a region where space-rocket second stages containing remains of rocket propellant components, including one of the strongest mutagens – heptyl, have been falling for over 30 years (Panin and Perova 2006). Accumulation of heptyl in soil, vegetation cover and foodstuff of mice could be responsible for the karyological changes observed in *A. peninsulae* in that region. An indirect evidence that technogenic pollution destabilizes B chromosome systems of *A. peninsulae* is the fact that in another population of *A. peninsulae* inhabiting the territory extending for

200 km in the flood-lands of the Yenisei River left bank – the region under severe radiation pollution, karyotypes contained only dot-like micro B chromosomes (from 4 to 30) (Bolsunovsky et al. 2007).

The changes in the cytogenetic structure of the geographic mice population in the Altai Mountains (Borisov 2008, and the present report) and the microevolutionary processes occurring there are unique and require further study. The pollution of the territory by the remains of the rocket fuel components may be an appropriate explanation for the cause of the karyological changes observed in *A. peninsulae* in this region. The role of this and other new natural and man-caused factors affecting the nature of this region is yet to be studied. Possibility of an impact of migration on mice with such volatile karyotype is unlikely in our opinion.

All authors declare that there is no conflict of interests exists. All of the authors have contributed substantially to the manuscript and approved the submission.

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RESEARCH ARTICLE

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Cytogenetics of entelegyne spiders (Arachnida, Araneae) from southern Africa

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Abstract

Spiders represent one of the most studied arachnid orders. They are particularly intriguing from a cytogenetic point of view, due to their complex and dynamic sex chromosome determination systems. Despite intensive research on this group, cytogenetic data from African spiders are still mostly lacking. In this study, we describe the karyotypes of 38 species of spiders belonging to 16 entelegyne families from South Africa and Namibia. In the majority of analysed families, the observed chromosome numbers and morphology (mainly acrocentric) did not deviate from the family-level cytogenetic characteristics based on material from other continents: Tetragnathidae (2n♂ = 24), Ctenidae and Oxyopidae (2n♂ = 28), Sparassidae (2n♂ = 42), Gnaphosidae, Trachelidae and Trochanteriidae (2n♂ = 22), and Salticidae (2n♂ = 28). On the other hand, we identified interspecific variability within Hersiliidae (2n♂ = 33 and 35), Oecobiidae (2n♂ = 19 and 25), Selenopidae (2n♂ = 26 and 29) and Theridiidae (2n♂ = 21 and 22). We examined the karyotypes of Ammoxenidae and Gallieniellidae for the first time. Their diploid counts (2n♂ = 22) correspond to the superfamily Gnaphosoidea and support their placement in this lineage. On the other hand, the karyotypes of Prodidominae (2n♂ = 28 and 29) contrast with all other Gnaphosoidea. Similarly, the unusually high diploid number in *Borboropactus* sp. (2n♂ = 28) within the otherwise cytogenetically uniform family Thomisidae (mainly 2n♂ = 21–24) supports molecular data suggesting a basal position of the genus in the family. The implementation of FISH methods for visualisation of rDNA clusters facilitated the detection of complex dynamics of numbers of these loci. We identified up to five loci of the 18S rDNA clusters in our samples. Three different sex chromosome systems (X0, $X_1X_2$0 and $X_1X_2X_3$0) were also detected among the studied taxa.

Keywords
Karyotype, sex chromosomes, meiosis, rDNA FISH, NOR, acrocentric, Gnaphosoidea, Araneoidea, Oecobioidea, RTA clade

Introduction

With nearly 50000 species, spiders represent the second largest order of arachnids (World Spider Catalog 2019). The order consists of three main lineages: Mesothelae, Mygalomorphae and Araneomorphae (e.g. Garrison et al. 2016); the last one, with 96 described families, is one of the most diverse groups of arachnids. Araneomorphae (so-called modern spiders) were traditionally divided into Haplogynae and Entelegynae, based on the differences in complexity of their copulatory organs (e.g. Coddington et al. 2004). However, recent phylogenomic studies have disputed Haplogynae monophyly (Bond et al. 2014, Garrison et al. 2016). The monophyly of Entelegynae is well-supported, and the group comprises approximately 80% of extant spider species. Our knowledge about spider cytogenetics also mirrors the diversification disparity among the spider groups. Entelegynae, being the most speciose spider taxon, also represents the most frequently analysed group (86% of all 843 analysed spiders) (Araujo et al. 2019).

Based on their cytogenetic characteristics, spiders can be assigned into several groups displaying different dynamics of karyotype evolution. Their assignment into these groups correlates with the parcelling of spider diversity into the main evolutionary lineages. The basal groups Mesothelae (2n♂ = 80 or 96) (Suzuki 1954) and Mygalomorphae (2n♂ = 14–128, average 61) (Král et al. 2013) typically show higher numbers of chromosomes, a condition regarded as ancestral for spiders (Suzuki 1954). Concerning Araneomorphae, the haplogyne superfamily Dysderoidea represents the most cytogenetically distinct group. It is characterized by lower diploid numbers (2n♂ = 5–40, average 13) and holokinetic chromosomes. This chromosome type is unique in spiders, and evolved only once in this order (e.g. Král et al. 2006, 2019). The rest of haplogyne spiders typically possess a low number of biarmed chromosomes (2n♂ = 9–33, average 21) (Král et al. 2006), with the exception of the presumably polyploid family Caponiidae (2n♂ = 55–152) (Král et al. 2019).

Compared to haplogyne and mygalomorph spiders, the karyotypes of entelegynes are considerably less diversified. Their respective interfamilial diploid number (2n) ranges are low (2n♂ = 10–52, average 27), and the majority of species possess exclusively acrocentric chromosomes (Araujo et al. 2019). The ancestral condition in entelegynes is hypothesised to be 40 acrocentric autosomes (Král et al. 2006), while a reduction in 2n is likely the leading evolutionary trend (Suzuki 1954). An increasing chromosome number in Entelegynae karyotype evolution is theoretically regarded as problematic, since simple fission of an acrocentric chromosome leads to the formation of an acentric fragment. The fragment is unable to attach to a spindle microtubule and thus cannot be transferred into the daughter cell (e.g. Schubert and Lysak 2011).

A prominent feature of spider karyotypes is the presence of unusual sex chromosomes. The majority of species display the X_1X_20 system (male: X_1X_2, female: $X_1X_1X_2X_2$), which is considered to be an ancestral state in spiders (Suzuki 1954). This system is otherwise rare in animals (White 1976). Interestingly, X_1X_20 evolved into a variety of sex chromosome systems, resulting in an enormous diversity of male heterogamy modes. Namely, systems with up to 13 differentiated X chromosomes in mygalomorphs (Král et al. 2013), X_1X_2Y systems in several Haplogynae families (Král et al. 2006), and various neo-sex chromosome systems, reported from three Entelegynae families: Salticidae (Maddison and Leduc-Robert 2013), Agelenidae (Král 2007) and Sparassidae (Sharp and Rowell 2007). In entelegynes, besides the proposed ancestral X_1X_20 and neo-sex chromosome systems, X0 and $X_1X_2X_30$ systems are often present (Araujo et al. 2012, Kořínková and Král 2013). Rather exceptionally, some members of three Entelegynae families (Corinnidae, Sparassidae and Tetragnathidae) display an $X_1X_2X_3X_40$ system (Data and Chatterjee 1983, 1988, Araujo et al. 2012). To further add to the complexity of systems of differentiated sex chromosomes in spiders, Král et al. (2013) proposed the existence of additive XY pair(s), with a weak level of differentiation. However, such structures are not distinguishable by conventional karyotype examination in entelegynes.

Due to the conservative aspects of karyotype features in entelegynes, our knowledge of chromosomal evolution in this group could be broadened by the implementation of molecular cytogenetic approaches. Because of the limited number of banding techniques available for invertebrates, the fluorescence *in situ* hybridization (FISH) for visualisation of nucleolus organizer regions (NORs) is a convenient choice in terms of methodology. The NORs are composed of clusters of genes coding most of the rRNA, namely major rDNA loci (18S, 5.8S and 28S rRNA genes). The application of FISH in spider chromosome studies is scarce (Vítková et al. 2005, Zhao et al. 2010, Suzuki and Kubota 2011). Major rDNA clusters were successfully identified via FISH in five species of entelegynes (Forman et al. 2013, Rincão et al. 2017) and one mygalomorph (Král et al. 2013).

Southern Africa includes three of the 36 global biodiversity hotspots (Mittermeier et al. 2011, Noss et al. 2015), with many groups displaying a typical Gondwanan distribution (e.g. Beron 2018). Despite the importance of this geographical region and the relatively good knowledge of cytogenetics in other arachnid groups, e.g. scorpions (e.g. Šťáhlavský et al. 2018a), harvestmen (e.g. Svojanovská et al. 2016, Šťáhlavský et al. 2018b) and pseudoscorpions (e.g. Šťáhlavský et al. 2006, 2012), our knowledge about spiders here is limited. A few attempts have been made to elucidate the karyotype diversity of mygalomorphs and haplogynes from this region (Král et al. 2006, 2013, 2019). However, despite the enormous diversity of entelegynes, and our comparatively good knowledge about their cytogenetics worldwide, there is a significant lack of karyotype data from sub-Saharan Africa (Araujo et al. 2019). So far, only the social spider species *Stegodyphus dumicola* Pocock, 1898 (Eresidae) ($2n♂ = 26$) has been subjected to cytogenetic analyses (Avilés et al. 1999).

In this study, we analysed the karyotypes of 38 species representing 16 entelegyne families (Araneoidea, Oecobioidea and RTA clade groups) from South Africa and Namibia, to gain knowledge about entelegyne cytogenetics from this biogeographical

region. Additionally, we analysed major rDNA clusters via FISH in 11 species. Our results also address the status of Prodidominae and the overall composition of Gnaphosoidea, which highlights the utilization of cytogenetic methods as an important tool to bring additional perspectives for the study of entelegyne taxonomy and systematics. We use the molecular phylogenetic framework and classification established in Wheeler et al. (2016), which represents the most complete assessment of spider diversity to date, and also includes large number of genera sampled and karyotyped in our study.

Material and methods

Specimen and locality data of 55 entelegyne samples (38 species) analysed in this study are reported in Table 1. Vouchers were deposited in the National Museum, Bloemfontein, South Africa (NMBA). Our analyses were based exclusively on males, in order to determine the sex chromosome systems based on the analysis of meiosis in the heterogametic sex. Chromosome preparations were obtained by the "plate spreading" method (Traut 1976), adapted for arachnids (Šťáhlavský and Král 2004). During the procedure, gonads are hypotonized in 0.075 M KCl (20 min), fixed in methanol: acetic acid (3:1) solution (20 min), dissociated and spread in a drop of 60% acetic acid on a microscope slide on a hot plate (40–45 °C). The chromosomes were stained in a 5% Giemsa solution in modified Sörensen phosphate buffer (30 min) (Dolejš et al. 2011).

Chromosomes were documented with an ORCA-AG monochromatic camera (Hamamatsu) on an Olympus IX81 microscope operated by Cell^R. Standard karyotype characteristics, such as number, relative size, and morphology of the chromosomes, were analysed from photographs using the LEVAN plugin (Sakamoto and Zacaro 2009) for the IMAGEJ 1.47 program (http://imagej.nih.gov/ij/). The 2n was established by analysing at least ten well-spread nuclei for each species. Classification of chromosome morphology follows Levan et al. (1964). The sex chromosome systems of certain species were identified during meiosis of the heterogametic sex, either by segregation or their behaviour in prophase I (see e.g. Král et al. 2011).

Major rDNA clusters were detected by FISH, with the 18S rDNA probe, as described in Forman et al. (2013). Briefly, biotine-labelled probe was hybridized on the chromosomal preparations. Signal was detected by streptavidine-Cy3, followed by one round of signal amplification using biotinylated antistreptavidine and streptavidine-Cy3. The chromosomes were stained with Fluoroshield with DAPI (4',6-diamidino-2-phenylindole) (Sigma-Aldrich) and observed on an Olympus IX81 microscope with an ORCA-ER camera (Hamamatsu). The photographs were pseudocolored (red for Cy3 and blue for DAPI) and superimposed with Cell^R software (Olympus).

Results and discussion

We obtained cytogenetic data for 38 species of entelegyne spiders belonging to 16 families (Table 1). Except for the cosmopolitan *Oecobius putus* Blackwall, 1859 (Oecobiidae), all

taxonomically determined species were analysed for the first time. We also provide the first cytogenetic information for the spider families Ammoxenidae and Gallieniellidae, and the gnaphosid subfamily Prodidominae, which until recently (Azevedo et al. 2018) was considered as an independent family. The 2n of the examined species ranged from 19 to 42. The observed sex chromosome systems were either X0, X_1X_20 or $X_1X_2X_30$. The X_1X_20 was the most frequently occurring system (detected in 75% of species). The acrocentric morphology of the whole chromosome complement was detected in 92% of species. Chromosomes of the remaining species were most likely acrocentric as well, but the lack of well-spread plates prevented us from determining the morphology of certain chromosomes. We present our results divided into four main groups: Araneoidea, Oecobioidea, RTA clade (except Dionycha), and Dionycha (subdivided into three parts), according to their placement within Entelegynae phylogeny (Wheeler et al. 2016). Results of FISH are presented and discussed in a separate section, as well as general aspects of Entelegynae karyotype evolution.

I. Araneoidea

The superfamily Araneoidea comprises more than 15 families of ecribellate orb-weavers (Wheeler et al. 2016, Dimitrov et al. 2017, Fernández et al. 2018). We analysed members of two families of this highly diversified group, which presents the first insights into the cytogenetics of African araneoids.

Tetragnathidae Menge, 1866

Tetragnathidae is a species-rich family with a cosmopolitan distribution, with 25 species represented in South Africa (Dippenaar-Schoeman et al. 2010, World Spider Catalog 2019). We examined an undetermined species of *Pachygnatha* Sundevall, 1823. We found its karyotype complement displaying 24 chromosomes, gradually decreasing in size (Fig. 1A), with an X_1X_20 sex system and acrocentric morphology in all chromosomes (Fig. 1D). These findings are consistent with karyotypes known from other studied members of the genus (e.g. Gorlov et al. 1995). During the course of prophase I, sex chromosomes exhibited distinctive associations by their centromeres and superspiralization (Fig. 1B, C). Contrary, in metaphase II condensation of the X became weaker (Fig. 1D), as reported in other tetragnathids (Král et al. 2011).

Theridiidae Sundevall, 1833

This cosmopolitan and diverse family includes about 57 South African species (Dippenaar-Schoeman et al. 2010, World Spider Catalog 2019). Our dataset included two distinct species of *Argyrodes* Simon, 1864 and *Theridion* cf. *purcelli* O. P.-Cambridge, 1904 (Table 1). Both *Argyrodes* displayed 21 acrocentric chromosomes (Fig. 1E, H). We detected one X chromosome, which was positively heteropycnotic from preleptotene until pachytene (not shown), rather less condensed in metaphase II (Fig. 1G), and subsequently slightly positively heteropycnotic in anaphase II. The X chromosome was

Table 1. List of examined species, including summary of the cytogenetic data: 2n of male, chromosome morphology (A = completely acrocentric), sex chromosome system, length ratio of sex chromosomes (N = number of measured nuclei) and number of NOR loci. Locality data (EC – Eastern Cape; FS – Free State; MP – Mpumalanga; NAM – Namibia; NL – KwaZulu-Natal; NP – National Park; WC – Western Cape; ZA – South Africa) and sample size (m mature male, sm submature male). * = unidentifiable specimens.

Family/*species*	2n	Chromosome morphology	Sex chromosome system	X ratio (N)	Number of 18S rDNA loci	Locality	GPS (S/E)	Sample size
I. Araneoidea								
Tetragnathidae								
Pachygnatha sp.	24	A	X_1X_20	1:0.96 (8)	–	ZA-MP: God's Window	24.8747, 30.8910	1m
Theridiidae								
Argyrodes cf. *convivans* Lawrence, 1937	21	A	X0	–	4	NL: Tembe	27.0276, 32.4083	1m
Argyrodes sp.	21	A	X0	–	–	NL: Ndumo	26.8749, 32.2109	2m
Theridion cf. *purcelli* O. P.-Cambridge, 1904	22	A	X_1X_20	1:0.80 (6)	–	NL: Pongola Reserve	27.3601, 31.9848	1m
II. Oecobioidea								
Hersiliidae								
Hersilia sericea Pocock, 1898	35	A	$X_1X_2X_30$	1:0.92:0.71 (9)	–	NL: Vernon Crookes	30.2749, 30.6092	1m
Neotama corticola (Lawrence, 1937)	33	A	$X_1X_2X_30$	1:0.89:0.76 (10)	–	ZA-EC: Port St. Johns	31.5977, 29.5346	1m
Oecobiidae								
Oecobius navus Blackwall, 1859	19	A	X0	–	–	ZA-EC: Hogsback	32.5914, 26.9303	2m
Oecobius putus O. P.-Cambridge, 1876	25	A	$X_1X_2X_30$	–	–	ZA-FS: Bloemfontein	29.0949, 26.1621	2m
III. RTA - non-Dionycha								
Ctenidae								
Ctenus cf. *pulchriventris* (Simon, 1896)	28	A	X_1X_20	1:0.86 (14)	–	ZA-MP: Sudwala Caves	25.3713, 30.6965	2m
Oxyopidae								
Peucetia striata Karsch, 1878	28	A	X_1X_20	1:0.84 (4)	–	ZA-FS: Bloemfontein	29.0488, 26.2152	1m
Sparassidae								
Olios sp.	42	A	X_1X_20	1:0.93 (20)	1	NL: Ndumo	26.8749, 32.2109	1sm
Sparassinae sp. cf. *Olios*	42	A	X_1X_20	1:0.93 (20)	4	NAM: south of Etosha	19.6208, 15.8858	1sm
Thomisidae								
Borboropactus sp.	28	A	X_1X_20	1:0.76 (5)	–	NL: Pietermaritzburg	29.6050, 30.3462	1sm
Xysticus sp.	23	A	X0	–	–	NL: Ndumo	26.8749, 32.2109	2sm
IVa. RTA clade Dionycha - "Prodidomidae Simon, 1884, Prodidominae (*sensu* Azevedo et al. 2018)"								
Prodidomus simoni Dalmas, 1919	29	A	X1X2X30	1:0.94:0.91 (7)	–	NL: Ndumo	26.8855, 32.3124	4m
Theuma sp.	28	A	X1X20	1:0.68 (12)	–	ZA-FS: Bloemfontein	29.04876, 6.2152	1sm
IVb. RTA - Dionycha Part A - (Gnaphosoidea *sensu lato*, (Wheeler et al. 2016))								
Ammoxenidae								
Ammoxenus amphalodes Dippenaar & Meyer, 1980	22	A	X_1X_20	?	–	ZA-FS: Bloemfontein	29.0986, 26.1550	1m
Ammoxenus psammodromus Simon, 1910	22	A	X_1X_20	1:0.86 (4)	–	ZA-FS: Bloemfontein	29.0986, 26.1550	1m
Gallieniellidae								
Austrachelas natalensis Lawrence, 1942	22	A?	X_1X_20	1:0.80 (8)	–	NL: Ithala Reserve	27.5426, 31.2824	1m
Gnaphosidae								
Camillina maun Platnick & Murphy, 1987	22	A	X_1X_20	1:0.90 (4)	–	NL: Cornationweg	27.6946, 31.0609	1m
Camillina maun Platnick & Murphy, 1987	22	A	X_1X_20	1:0.92 (11)		NL: Manzengenya	27.2361, 32.7076	2m
Zelotes fuligineus (Purcell, 1907)	22	–	X_1X_20	1:0.94 (7)	–	NL: Cornationweg	27.6946, 31.0609	1m
Zelotes sclateri Tucker, 1923	22	A	X_1X_20	1:0.82 (10)	2	NL: Ithala Reserve	27.5426, 31.2824	2m
Zelotes sclateri Tucker, 1923	22	A	X_1X_20	1:0.88 (10)	–	NL: Ndumo	26.8855, 32.3124	1m
Zelotes sp.	22	A	X_1X_20	1:0.85 (8)	–	NL: Ndumo	26.8854, 32.3124	1sm

Family/*species*	2n	Chromosome morphology	Sex chromosome system	X ratio (N)	Number of 18S rDNA loci	Locality	GPS (S/E)	Sample size
Trachelidae								
Afroceto plana Lyle & Haddad, 2010	22	A	X_1X_20	1:0.85 (23)	1	NL: Ndumo	26.8855, 32.3124	2m
Trochanteriidae								
Platyoides walteri (Karsch, 1887)	22	A	X_1X_20	1:0.93 (10)	3	NL: Royal Natal NP	28.7101, 28.9336	1m, 1sm
IVc. RTA clade - Dionycha Part B								
Cheiracanthiidae								
Cheiramiona kirkspriggsi Lotz, 2015	24	A	X_1X_20	1:0.77 (6)	1	NL: Ithala Reserve	27.5426, 31.2824	1m
Salticidae								
Baryphas ahenus Simon, 1902	28	A	X_1X_20	1:0.98 (7)	–	NL: Tembe	27.0276, 32.4083	1sm
Cyrba lineata Wanless, 1984	28	A	X_1X_20	1:0.95 (16)	–	NL: Ndumo	26.8749, 32.2109	2m
Holcolaetis zuluensis Lawrence, 1937	28	–	X_1X_20	1:0.76 (4)	–	NL: Ndumo	26.8855, 32.3124	1m
Myrmarachne laurentina Bacelar, 1953	28	–	X_1X_20	1:0.84 (6)	–	NL: Ndumo	26.8855, 32.3124	1m
Menemerus minshullae Wesołowska, 1999	28	A	X_1X_20	1:53 (7)	–	NL: Ndumo	26.8749, 32.2109	1m
Nigorella hirsuta Wesołowska, 2009	28	A	X_1X_20	1:0.92 (7)	–	ZA-FS: Bloemfontein	29.0483, 26.2112	1m
Thyene ogdeni Peckham & Peckham, 1903	28	A	X_1X_20	1:0.89 (10)	–	NL: Tembe	27.0276, 32.4083	1m
Thyenula haddadi Wesołowska, Azarkina & Russell-Smith, 2014	28	A	X_1X_20	1:0.90 (16)	2	NL: Royal Natal NP	28.6909, 28.9415	1m
Thyenula leighi (Peckham & Peckham, 1903)	28	A	X_1X_20	1:0.84 (10)	–	NL: Ophathe	28.3742, 31.3898	1m
Selenopidae								
Anyphops sp.*	26	A	X_1X_20	1:0.77 (8)	–	ZA-WC: Mossel Bay	34.1634, 22.1065	1m
Anyphops sp.*	26	A	X_1X_20	1:0.75 (9)	4	NL: Ndumo	26.8749, 32.2109	1sm
Selenops sp. 1*	26	A	X_1X_20	1:0.78 (19)	4	NL: Pongola Reserve	27.3602, 31.9848	1sm
Selenops sp. 1*	26	A	X_1X_20	1:0.76 (34)	–	NL: Ophathe	28.3937, 31.3942	1sm
Selenops sp. 2*	29	A	$X_1X_2X_30$	1:0.94:0.87 (15)	1	NAM: Omuthiya	18.3770, 16.6005	1 m

acrocentric (Fig. 1F, G, I). In contrast, *Theridion* cf. *purcelli* showed 2n♂ = 22, with an X_1X_20 sex chromosome system. Its chromosomes were acrocentric and both X displayed positive heteropycnosis from pachytene to anaphase II (Fig. 1J–L). Other members of both genera have often been reported to possess 22 acrocentric chromosomes (Datta and Chatterjee 1983, Srivastava and Shukla 1986, but see latter for *Argyrodes*), which is frequently the case in other theridiids too (Araujo et al. 2019).

II. Oecobioidea

Alongside the families Uloboridae and Deinopidae, the superfamily Oecobioidea (comprising families Hersiliidae and Oecobiidae) forms the so-called "UDOH grade" (Fernández et al. 2018), which is consistently recovered by molecular data in proximity to the RTA clade (Garrison et al. 2016, Wheeler et al. 2016, Fernández et al. 2018). The superfamily Oecobioidea historically formed part of Eresoidea; however, this grouping was never supported by molecular analyses (Miller et al. 2010, Wheeler et al. 2016, Fernández et al. 2018).

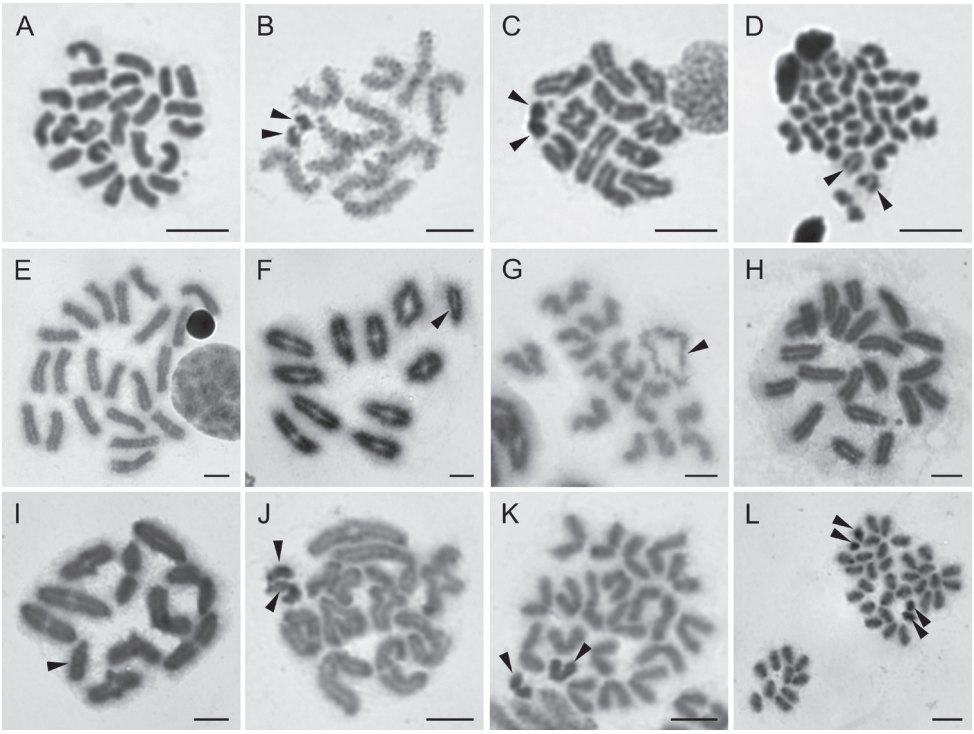

Figure 1. Chromosomes of Tetragnathidae (**A–D**) and Theridiidae (**E–L**). *Pachygnatha* sp. (2n♂ = 24, X_1X_20) **A** mitotic metaphase **B** late pachytene with positively heteropycnotic sex chromosomes **C** diakinesis **D** half of metaphase II, with slightly less condensed X chromosomes. *Argyrodes* cf. *convivans* (2n♂ = 21, X0) **E** mitotic metaphase **F** diakinesis with isopycnotic X univalent **G** half of metaphase II with despiralised acrocentric X chromosome. *Argyrodes* sp. (2n♂ = 21, X0) **H** mitotic metaphase **I** diakinesis with isopycnotic X chromosome. *Theridion* cf. *purcelli* (2n♂ = 22, X_1X_20) **J** diplotene, X_1X_2 associate on the periphery of the plate **K** metaphase II **L** anaphase II. Arrowheads indicate sex chromosomes. Scale bars: 5 μm.

Hersiliidae Thorell, 1870

Hersiliids are a small family distributed in the tropics and subtropics. Twelve species have been reported from South Africa (Dippenaar-Schoeman et al. 2010, World Spider Catalog 2019). We analysed two of them: *Hersilia sericea* Pocock, 1898 and *Neotama corticola* (Lawrence, 1937). Male diploid counts were 35 and 33, respectively (Fig. 2A–F). Complements of both species were fully acrocentric (Fig. 2C, E, F). Both species exhibited $X_1X_2X_30$. Different spiralization of the sex chromosomes, reflected by positive or negative heteropycnosis, was apparent during meiosis (Fig. 2B–F). The karyotype of *H. sericea* differed from its congener *H. savignyi* Lucas, 1836 (Bole-Gowda 1958) by the higher 2n and an additional X chromosome. On the other hand, the same karyotype formula was found in *Hersiliola bayrami* Danişman, Sancak, Erdek & Coşar, 2012 (Kumbıçak et al. 2018). This discrepancy reflects rather higher dynamics

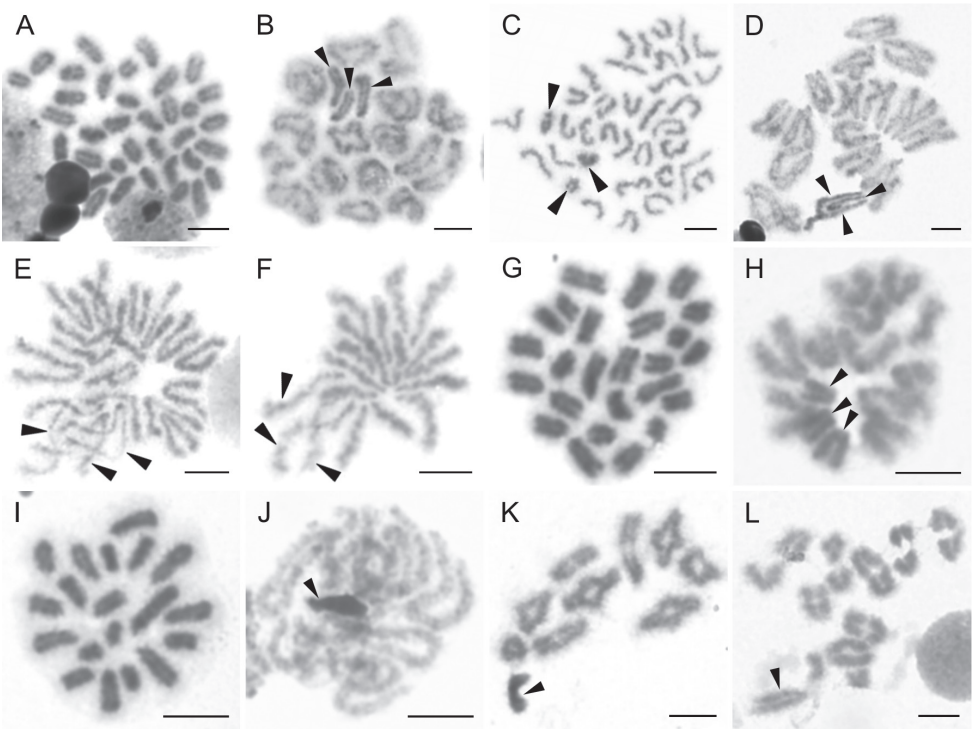

Figure 2. Chromosomes of Hersiliidae (**A–F**) and Oecobiidae (**G–L**). *Hersilia sericea* (2n♂ = 35, $X_1X_2X_3$0) **A** mitotic metaphase **B** early metaphase I, $X_1X_2X_3$ shows slightly positive heteropycnosis **C** metaphase II, note positively heteropycnotic $X_1X_2X_3$. *Neotama corticola* (2n♂ = 33, $X_1X_2X_3$0) **D** early diakinesis, sex chromosomes **E** half of anaphase I with despiralised sex chromosomes **F** quarter of anaphase II with despiralised sex chromosomes. *Oecobius putus* (2n♂ = 25, $X_1X_2X_3$0) **G** mitotic metaphase **H** half of anaphase I with positively heteropycnotic sex chromosomes. *O. navus* (2n♂ = 19, X0) **I** mitotic metaphase **J** pachytene with compact X **K** diakinesis X univalent shows positive heteropycnosis **L** metaphase I with already isopycnotic X. Arrowheads indicate sex chromosomes. Scale bars: 5 μm.

in the karyotypes of hersiliids, as seen in *Neotama* Baehr & Baehr, 1993 (this study), as well as in other members of the family (Forman et al. in prep.), and a tendency for convergent 2n reduction in entelegynes (Kořínková and Král 2013).

Oecobiidae Blackwall, 1862

The cosmopolitan family Oecobiidae consists of about a hundred species, of which five can be found in South Africa (Dippenaar-Schoeman et al. 2010, World Spider Catalog 2019). Two representatives of the genus *Oecobius* Lucas, 1846 (*O. navus* Blackwall, 1859 and *O. putus* O. Pickard-Cambridge, 1876) were subjects of our investigation. This genus contains small cribellate spiders, including a few synanthropic, cosmopolitan species. In agreement with the previous findings of Mittal (1983), we found the *O. putus* male karyotype to contain an acrocentric set of 25 chromosomes and an $X_1X_2X_3$0 sex

chromosome system (Fig. 2G, H). In contrast, we found the chromosomal complement of *O. navus* to be substantially different from the previous species, comprising 19 acrocentric chromosomes (Fig. 2I). The karyotype constitution of *O. navus* was likely derived by a series of chromosomal fusions. Interestingly, these rearrangements also involved the sex chromosome complement, and resulted in an X0 formation. A fusion-based origin of the *O. navus* karyotype is supported by two features: 1) a low ability of acrocentric chromosomes to be subjected to fissions, and 2) by the length of X in *O. navus*, which was the longest chromosome of the karyotype (Fig. 2K, L). The behaviour of both $X_1X_2X_30$ and X0 sex chromosomes in male meiosis included positive heteropycnosis in early prophase (Fig. 2J), which also persisted in the latter phases (Fig. 2K). Karyotype variability of *Oecobius* is unusually high for an entelegyne genus; a male formula of $2n♂ = 22, X_1X_20$ is also known from *O. cellariorum* (Dugès, 1836) (Youju et al. 1993).

RTA clade

The RTA clade comprises lineages united by the presence of the retrolateral tibial apophysis on the male palps (Griswold et al. 2005). The internal relationships of this group, and also the group itself, did not receive sufficient support in analyses based on traditional Sanger-sequenced loci (Wheeler et al. 2016). In recent phylogenomic analyses, albeit with less exhaustive taxon sampling, the RTA clade was recovered as a monophyletic clade with mostly resolved relationships (Garrison et al. 2016, Fernández et al. 2018). These analyses yielded a similar organization the of RTA clade into several main subclades, but their internal relationships often differed (Garrison et al. 2016, Wheeler et al. 2016, Fernández et al. 2018). Following Wheeler's et al. (2016) classification, we obtained results from both Dionycha and non-Dionycha clades.

III. RTA clade non-Dionycha

Here we studied four non-Dionycha lineages, namely three families belonging to the Oval calamistrum clade (Ctenidae, Oxyopidae and Thomisidae) and the family Sparassidae.

Ctenidae Keyserling, 1877

Wandering spiders are distributed worldwide, except for New Zealand (Jocqué and Dippenaar-Schoeman 2006). They are represented by approximately 520 species, of which seven are known from South Africa (Dippenaar-Schoeman et al. 2010, World Spider Catalog 2019). The family as a whole was not recovered as monophyletic (Wheeler et al. 2016); however, both *Anahita* Karsch, 1879 and *Ctenus* Walckenaer, 1805 belong to the monophyletic "core ctenids". Since the first report of a ctenid karyotype, of *Anahita fauna* Karsch, 1879 by Chen (1999), our knowledge of the cytogenetics of this family has increased considerably, now comprising data for 11 species, with male karyotypes of $2n♂ = 22, X_1X_20$, $2n♂ = 28, X_1X_20$, and $2n♂ = 29, X_1X_2X_30$ (Araujo et al. 2014,

Kumar et al. 2017, Rincão et al. 2017). Here we report the karyotype of *Ctenus* cf. *pulchriventris* (Simon, 1897). We observed 28 chromosomes in the male of this species (Fig. 3B). We were able to confirm acrocentric morphology of all autosomes and both X chromosomes (Fig. 9A). The X chromosomes displayed positive heteropycnosis and parallel associations from pachytene (Fig. 3A) to early metaphase I (Fig. 3B), followed by higher condensation (or late decondensation) in anaphase I (not shown). Both 2n = 28 and X_1X_20 represented the most common constitution in males of Ctenidae, and have been reported so far from eight species, including all examined species of the genus *Ctenus* (Araujo et al. 2019).

Oxyopidae Thorell, 1870

Lynx spiders comprise more than 450 species distributed all over the world, of which 41 species have been recorded from South Africa (Dippenaar-Schoeman et al. 2010, World Spider Catalog 2019). In this paper, we analysed the species *Peucetia striata* Karsch, 1878. The male of this species displayed a diploid number of 28 chromosomes (Fig. 3C), with the X_1X_20 sex chromosome system. Sex chromosomes differed slightly in length (the ratio of sex chromosomes - 1:0.86) (Fig. 3D, Table 1). The sex chromosomes showed a different pattern of staining from the autosomes during meiosis I. This positive heteropycnosis was evident from early prophase I (not shown) until metaphase I (Fig. 3D). Later, the sex chromosomes became isopycnotic, and it was not possible to distinguish them from autosomes during metaphase II (Fig. 3E).

Currently, 26 species belonging to five genera have been analysed cytogenetically (Araujo et al. 2019). Our results from *P. striata* from South Africa were similar to the characteristics of Indian (Bole-Gowda 1950, Parida and Sharma 1987, Sharma and Parida 1987) and Turkish (Kumbıçak 2014) representatives of the genus. The main trends in the karyotype evolution of Oxyopidae were the reduction of 2n and change of the sex chromosome system to an X0 type (Stávale et al. 2011). These changes were also observed in two species of *Peucetia* Thorell, 1869 from Brazil (Stávale et al. 2011), and were particularly evident in the genus *Oxyopes* Latreille, 1804 (see Araujo et al. 2019). The centric fusions of the chromosomes in *Oxyopes salticus* Hentz, 1845 resulted in one of the lowest diploid numbers (2n♂ = 11, X0) known among entelegyne spiders (Stávale et al. 2011).

Sparassidae Bertkau, 1872

Huntsman spiders, represented by 56 species in South Africa (Dippenaar-Schoeman et al. 2010), are a diverse family predominantly found between 40°N to 40°S latitude, with the exception of the Palearctic genus *Micrommata* Latreille, 1804 (Jocqué and Dippenaar-Schoeman 2006, World Spider Catalog 2019). The family was placed with low support as sister to the Oval calamistrum clade + Dionycha (Wheeler et al. 2016). However, in recent phylogenomic analyses (Fernández et al. 2018) the family was recovered as sister to a clade that would roughly correspond to the "marronoid clade" in

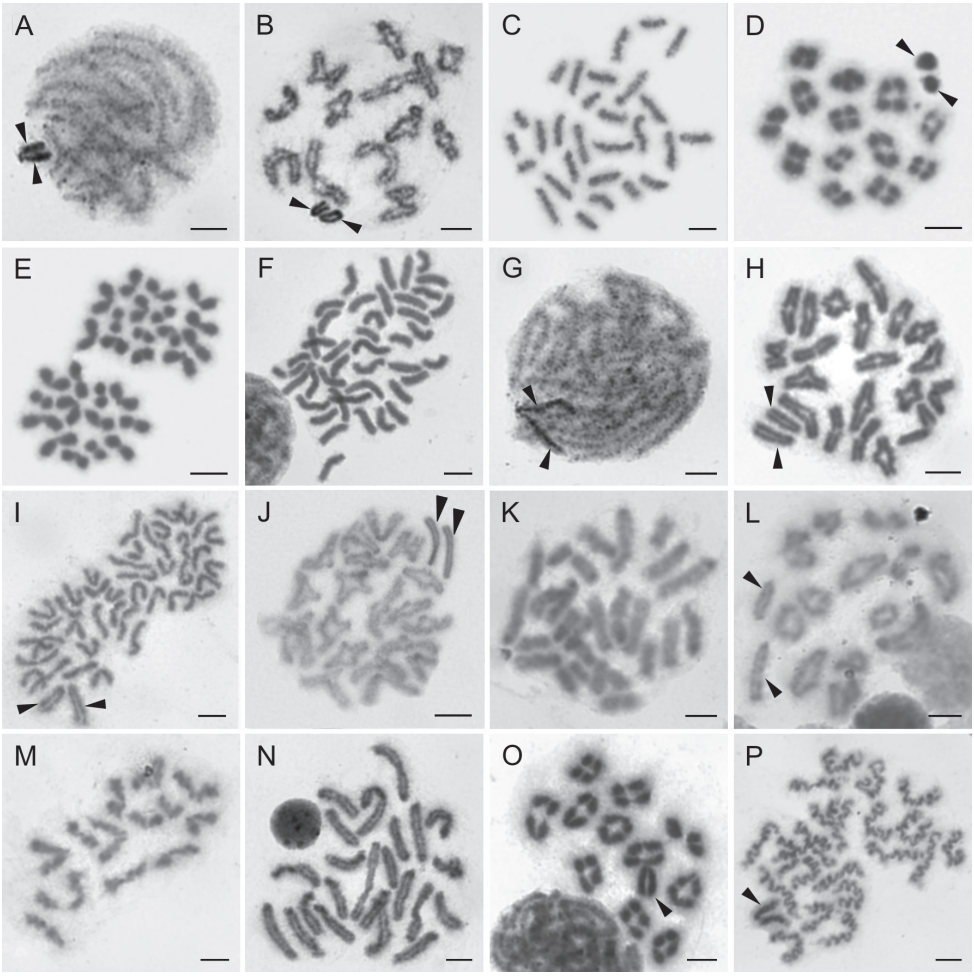

Figure 3. Chromosomes of Ctenidae (**A–B**), Oxyopidae (**C–E**), Sparassidae (**F–J**) and Thomisidae (**K–P**). *Ctenus* cf. *pulchriventris* (2n♂ = 28, X_1X_20) **A** pachytene, X_1X_2 associate on the periphery of plate **B** diakinesis. *Peucetia striata* (2n♂ = 28, X_1X_20) **C** mitotic metaphase **D** metaphase I **E** metaphase II, sex chromosomes isopycnotic. *Olios* sp. (2n♂ = 42, X_1X_20) **F** mitotic metaphase **G** pachytene **H** late diakinesis **I** metaphase II **J** Diakinesis of Sparassinae sp. cf. *Olios* (2n♂ = 42, X_1X_20). *Borboropactus* sp. (2n♂ = 28, X_1X_20) **K** mitotic metaphase **L** metaphase I **M** half of metaphase II without sex chromosomes. *Xysticus* sp. (2n♂ = 23, X0) **N** mitotic metaphase **O** metaphase I, note early segregation of one bivalent **P** prometaphase II. Arrowheads indicate sex chromosomes. Scale bars: 5 μm.

Wheeler et al. (2016). We analysed *Olios* sp. from South Africa (Fig. 3F–I) and one penultimate male of an unidentified genus resembling *Olios* Walckenaer, 1837 from Namibia (Fig. 3J). Both males possessed 2n = 42 (Fig. 3F, J), an X_1X_20 sex chromosome system (Fig. 3H, J) and acrocentric morphology of all chromosomes (Fig. 3F, I). Sex chromosomes differed only slightly in their length (the ratio of sex chromosomes – 1:0.93) (Table 1) and showed positive heteropycnosis only during early prophase

until pachytene (Fig. 3G). During this phase, they were associated by their centromeric regions (Fig. 3G), and were later located together at the periphery of the nucleus (Fig. 3H–J). The karyotypes of both specimens analysed here show the same characteristics as *O. lamarcki* (Latreille, 1806) from India (Bole-Gowda 1952), whereas *Olios* sp. from Australia possesses an additional sex chromosome (Rowell 1985). Both X_1X_20 and $X_1X_2X_30$ systems are common in Sparassidae (see Araujo et al. 2019); exceptions include unique $X_1X_2X_3X_40$ (Datta and Chatterjee 1983) or neo-sex chromosome systems (Sharp and Rowell 2007).

Thomisidae Sundevall, 1833

Crab spiders represent a diverse cosmopolitan family, with more than 130 species known from South Africa (Dippenaar-Schoeman et al. 2010, World Spider Catalog 2019). We analysed two unidentified species belonging to the genera *Borboropactus* Simon, 1884 and *Xysticus* C. L. Koch, 1835. The male of *Borboropactus* sp. displayed 28 acrocentric chromosomes (Fig. 3K) and an X_1X_20 system. The sex chromosomes showed conspicuous difference in length (Fig. 3L, Table 1). *Xysticus* sp., on the other hand, displayed 23 acrocentric chromosomes (Fig. 3N), including a single acrocentric X (Fig. 3O, P), which corresponds to the characteristics of the genus reported in the literature (e.g. Hackman 1948, Gorlov et al. 1995, Kumbıçak et al. 2014) and is also typical for most thomisid genera (see Araujo et al. 2019). Presumably, the 2n decreases in Entelegynae karyotype evolution (Suzuki 1954, Kořínková and Král 2013). The higher number of chromosomes detected in *Borboropactus* would thus indicate an ancestral position within Thomisidae, which was further supported by the results of molecular phylogenetic analyses recovering *Borboropactus* at the base of the Thomisidae clade, albeit with low support (Benjamin et al. 2008, Wheeler et al. 2016). However, Wunderlich (2004) doubted the genus' placement within Thomisidae and established a monogeneric family Borboropactidae (but see Wheeler et al. 2016). The phylogenetic position of the genus, along with the fact that all other thomisids display a lower diploid number, support that $2n♂ = 28$, X_1X_20 represents an ancestral condition in this family. Our results thus suggest that the reduction of 2n, accompanied by X chromosome fusions, could play a role in the karyotype evolution of Thomisidae.

IV. RTA clade Dionycha

Dionycha, the two-clawed spiders, are a diverse group comprising about 17 families, representing a third of known spider species diversity. The group received moderate support in Wheeler et al. (2016), but the internal relationships remain largely unresolved. The exact composition of Dionycha also became a matter of debate recently, due to the conflicting position of Sparassidae (Ramírez 2014, Wheeler et al. 2016, Fernández et al. 2018). The molecular analyses recovered most of the Dionycha diversity placed in three main clades (Wheeler et al. 2016). The first clade comprised Prodi-

domidae, a family that was traditionally placed within Gnaphosoidea (see below); the second clade, "Dionycha part A", comprised most of the Gnaphosoidea and few other families; and the third clade, "Dionycha part B", included corinnids, jumping spiders, miturgids and other families.

Albeit with limited sampling, the phylogenomic analyses recovered the group as monophyletic, with its subdivision into two main clades concordant with the "Dionycha part A" and "Dionycha part B" (Fernández et al. 2018). Based on a morphological analysis of Gnaphosoidea, the family Prodidomidae was transferred to Gnaphosidae, losing its family-level status (Azevedo et al. 2018), whereas the remaining gnaphosoid families remained valid. In this paper, we analysed eight dionychan families from South Africa, including the subfamily Prodidominae (Gnaphosidae). This sampling comprises species representing all of the major Dionycha clades (*sensu* Wheeler et al. 2016), including the first cytogenetic records of Prodidominae, Ammoxenidae and Gallieniellidae.

IVa. "Prodidomidae Simon, 1884, Prodidominae (*sensu* Azevedo et al. 2018)"

The position of prodidomines remains uncertain. In molecular analyses, they were placed as a sister lineage to all remaining Dionycha (Wheeler et al. 2016). However, based on morphological evidence, the family was recently transferred to Gnaphosidae and established as one of its subfamilies (Azevedo et al. 2018). Prodidomines comprise over 300 species with a tropical and subtropical distribution, of which 26 species are known from South Africa (Dippenaar-Schoeman et al. 2010, World Spider Catalog 2019). In this paper, we report the first chromosomal data for the group.

We analysed two species, *Theuma* sp. and *Prodidomus simoni* Dalmas, 1919, representing two formerly recognized prodidomid subfamilies, Theuminae and Prodidominae, respectively (Wheeler et al. 2016). The subadult male of *Theuma* sp. displayed 2n = 28 (Fig. 4A) and an X_1X_20 sex chromosome system. All chromosomes were acrocentric (Fig. 4A, D). The X_1 and X_2 sex chromosomes differed considerably in length (the ratio of sex chromosomes – 1:0.68) (Fig. 4C, D, Table 1) and showed positive heteropycnosis during meiosis I (Fig. 4B, C), as well as during meiosis II (Fig. 4D). They were associated by their centromeric regions during pachytene (Fig. 4B), and later in prophase I they remained in close proximity to each other at the periphery of the nucleus (Fig. 4C, D). The males of *P. simoni* also possessed acrocentric chromosomes (Fig. 4H), but displayed a higher chromosome number of 2n = 29 (Fig. 4E), due to a different sex chromosome system. The species possessed an $X_1X_2X_30$ system, with the sex chromosomes similar in length (the ratio of sex chromosomes – 1:0.94:0.91, respectively). The course of the heteropycnosis was similar to *Theuma* sp.; the chromosomes were positively heteropycnotic and closely located during the whole meiosis I (Fig. 4F–H). Interestingly, the differences in the length of the sex chromosomes between both species indicated either a fusion of two X chromosomes or fission of X_1. Despite the data presented here constituting the only

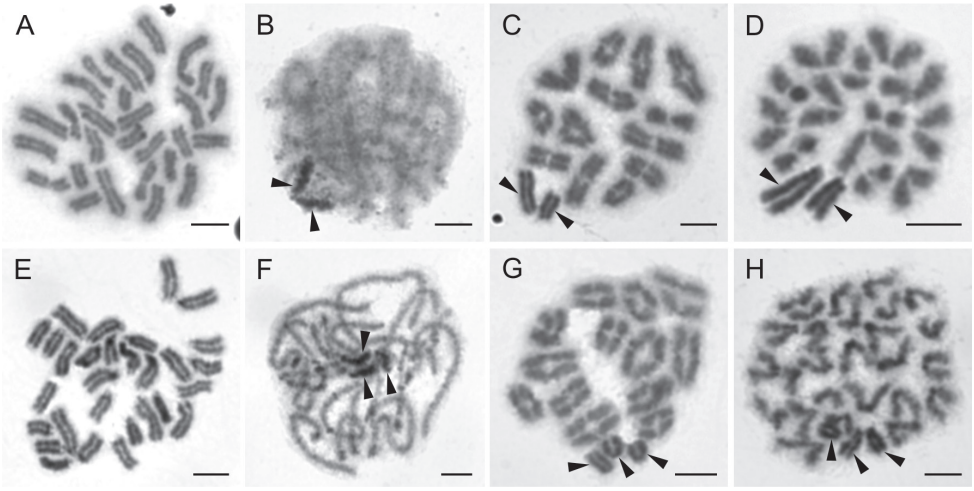

Figure 4. Chromosomes of Prodidominae (Dionycha). *Theuma* sp. (2n♂ = 28, X_1X_20) **A** mitotic metaphase **B** early pachytene with positively heteropycnotic X_1X_2 **C** diakinesis, note a difference in size of sex chromosomes **D** half of late metaphase II with sex chromosomes. *Prodidomus simoni* (2n♂ = 29, $X_1X_2X_30$) **E** mitotic metaphase **F** pachytene note positively heteropycnotic $X_1X_2X_3$ **G** metaphase I **H** early metaphase II, note positively heteropycnotic sex chromosomes. Arrowheads indicate sex chromosomes. Scale bars: 5 μm.

information about the chromosomes of prodidomines, they bring a relevant perspective on the placement of the family within Gnaphosidae (see below).

IVb. RTA clade Dionycha Part A – [Gnaphosoidea *sensu lato* (Wheeler et al. 2016)]

Altogether, we analysed nine species from five families belonging to this clade, and provide the first insights into the karyotypes of Ammoxenidae and Gallieniellidae. Several of the families analysed here, namely Ammoxenidae, Gallieniellidae and Trochanteriidae, were not recovered as monophyletic in previous molecular and morphological phylogenetic analyses, and formed a grade of lineages within the Gnaphosoidea (Wheeler et al. 2016). Therefore, we interpret the obtained results only in the context of Gnaphosoidea.

Ammoxenidae Simon, 1893

Ammoxenidae is a small family of termitophagous spiders, currently comprising four genera and 18 species distributed across southern Africa and Australia (World Spider Catalog 2019). We analysed two species of the genus *Ammoxenus* Simon, 1893 (*A. amphalodes* Dippenaar & Meyer, 1980 and *A. psammodromus* Simon, 1910). Males of *A. psammodromus* had 22 acrocentric chromosomes including an X_1X_20 sex chromosome system (Fig. 5A–D). The X chromosomes of this species paired together during pachytene (Fig. 5B) and showed positive heteropycnosis. Subsequently, they

became isopycnotic in metaphase I and II (Fig. 5C). The X_1 and X_2 differed slightly in length (Table 1). Despite the limited results we obtained for male *A. amphalodes*, we were able to confirm 2n = 22, X_1X_20 (Fig. 5E) and acrocentric chromosomes (Fig. 9B) in this species too.

Gallieniellidae Millot, 1947

Recent molecular analyses cast doubts on the monophyly of this family, splitting the group into two lineages with uncertain placement within "Dionycha part A" clade (Wheeler et al. 2016). The family Gallieniellidae has a typical Gondwanan distribution, spanning across the Afrotropical region, Madagascar, Australia and Argentina (Jocqué and Dippenaar-Schoeman 2006). We analysed one species, *Austrachelas natalensis* Lawrence, 1942, which displayed 2n♂ = 22, including an X_1X_20 sex chromosome system. The orientation of the chromosomes in mitotic anaphase suggests an acrocentric morphology (Fig. 5F). The length of the autosomes decreased gradually, and the X_1 and X_2 differed slightly in length (the ratio of sex chromosomes – 1:0.80) during diakinesis (Fig. 5H). They showed intensive positive heteropycnosis and parallel associations during pachytene (Fig. 5G), and became isopycnotic in diakinesis and metaphase I (Fig. 5H).

Gnaphosidae Pocock, 1898

Gnaphosidae is a diverse family with a cosmopolitan distribution. We analysed four species belonging to the genera *Camillina* Berland, 1919 and *Zelotes* Gistel, 1848, both belonging to the subfamily Zelotinae. The males of all species displayed a diploid number of 22 chromosomes and X_1X_20 sex chromosome system (Fig. 6A–H). The acrocentric morphology of all chromosomes was confirmed in *Camillina maun* Platnick & Murphy, 1987 (Fig. 9C), *Zelotes sclateri* Tucker, 1923 (Fig. 6G), and *Zelotes* sp. (Fig. 9D). During pachytene to late diakinesis, the X_1 and X_2 were displaying positive heteropycnosis and pairing in parallel on the periphery of nuclei (Fig. 6A, C, E, F). Despite the enormous species diversity of the family, gnaphosids show extremely conservative karyotype properties, with 2n♂ = 22 and X_1X_20 representing the most common constitution (Araujo et al. 2019).

Trachelidae Simon, 1897

Trachelids, recently elevated to family level (Ramírez 2014), include more than 230 species distributed worldwide with exception of Australia (World Spider Catalog 2019). We analysed one South African representative, *Afroceto plana* Lyle & Haddad, 2010. The males of this species had 2n = 22, X_1X_20 (Fig. 6I), with all chromosomes being acrocentric (Fig. 6L). The X_1 and X_2 differed slightly in length (the ratio of sex chromosomes – 1:0.85) (Fig. 6J, K, Table 1). Both sex chromosomes displayed intensive positive heteropycnosis and parallel association during pachytene (Fig. 6J). Both

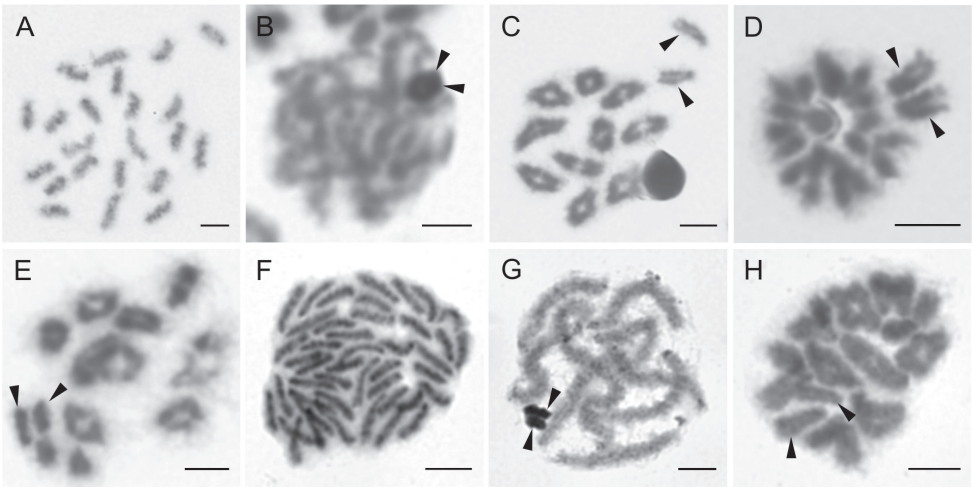

Figure 5. Chromosomes of Ammoxenidae (**A–E**) and Gallieniellidae (**F–H**). *Ammoxenus psammodromus* (2n♂ = 22, $X_1X_2$0) **A** mitotic prometaphase **B** pachytene **C** metaphase I with slightly negatively heteropycnotic X_1X_2 **D** half of anaphase II including sex chromosomes. *A. amphalodes* (2n♂ = 22, $X_1X_2$0) **E** metaphase I. *Austrachelas natalensis* (2n♂ = 22, $X_1X_2$0) **F** mitotic anaphase, orientation of chromatids suggests acrocentric morphology of chromosomes **G** pachytene X_1X_2 shows strong positive heteropycnosis **H** early metaphase I with already isopycnotic sex chromosomes. Arrowheads indicate sex chromosomes. Scale bars: 5 μm.

heteropycnosis and their location on the periphery of the nucleus persisted during diakinesis (Fig. 6K) and metaphase II (Fig. 6L). The karyotype characteristics, i.e. the 2n, chromosome morphology and the sex chromosome system, fully correspond to the karyotype of *Trachelas japonicus* Bösenberg & Strand, 1906 from Japan (Suzuki 1952). *Trachelas* sp. from India, representing the only remaining analysed trachelid, possesses 2n♂ = 24, $X_1X_2$0 (Datta and Chatterjee 1983).

Trochanteriidae Karsch, 1879

Trochanteriidae is another gnaphosoid family with a mainly Gondwanan distribution, but also extending to East Asia. In South Africa, the family is represented by nine species of the genus *Platyoides* O. Pickard-Cambridge, 1891 (Dippenaar-Schoeman et al. 2010, World Spider Catalog 2019), of which we analysed one species. *Platyoides walteri* (Karsch, 1887) displayed 2n♂ = 22, $X_1X_2$0 (Fig. 6M, O), with all of the chromosomes being acrocentric (Fig. 6M, P) and decreasing gradually in length. X_2 was smaller than X_1 and probably represented the smallest chromosome of the complement. Sex chromosomes were positively heteropycnotic from leptotene and became aligned by their centromeres at pachytene (Fig. 6N). Positive heteropycnosis was obvious during metaphase I (Fig. 6O), but it became less intensive during metaphase II (Fig. 6P). These results correspond to the information available for the only trochanteriid analysed to date, *Plator pandeae* Tikader, 1969 from India, which also exhibits 2n♂ = 22, $X_1X_2$0 (Srivastava and Shukla 1986).

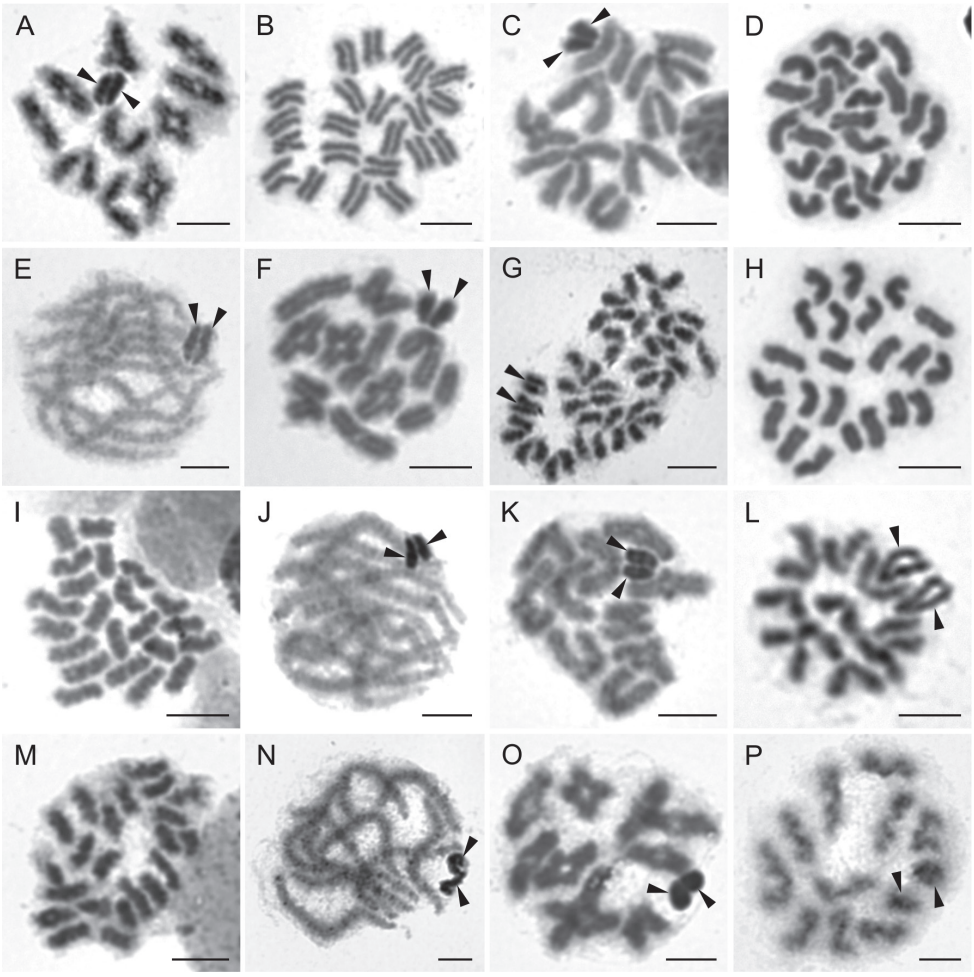

Figure 6. Chromosomes of Gnaphosidae (**A–H**), Trachelidae (**I–L**) and Trochanteriidae (**M–P**) **A** diakinesis of *Camillina maun* (2n♂ = 22, X_1X_20). *Zelotes fuligineus* (2n♂ = 22, X_1X_20) **B** late mitotic metaphase **C** diakinesis, X_1X_2 show positive heteropycnosis. *Z. sclateri* (2n♂ = 22, X_1X_20) **D** mitotic metaphase **E** pachytene, sex chromosomes pair in parallel on the periphery of nucleus **F** diakinesis **G** metaphase II, sex chromosomes are nearly isopycnotic **H** mitotic metaphase of *Zelotes* sp. (2n♂ = 22, X_1X_20). *Afroceto plana* (2n♂ = 22, X_1X_20) **I** mitotic metaphase **J** pachytene, X_1X_2 show parallel association **K** diakinesis **L** half of metaphase II with sex chromosomes. *Platyoides walteri* (2n♂ = 22, X_1X_20) **M** mitotic metaphase **N** pachytene, sex chromosome associate by their centromeric regions **O** diakinesis, with positively heteropycnotic sex chromosomes **P** half of prometaphase II with sex chromosomes. Arrowheads indicate sex chromosomes. Scale bars: 5 μm.

IVc. Dionycha part B

Following the results of Wheeler et al. (2016), this group forms a monophyletic clade comprising eight families with mostly unresolved relationships. In the present study, we analysed three of them.

Cheiracanthiidae Wagner, 1887

The family, restored by Ono and Ogata (2018), currently includes 12 genera and more than 350 species (World Spider Catalog 2019). In *Cheiramiona kirkspriggsi* Lotz, 2015, we identified 2n♂ = 24, X_1X_20 (Fig. 7A, D). All autosomes were acrocentrics and gradually decreased in length. The X_1 and X_2 differed in length substantially (the ratio of sex chromosomes – 1:0.77) during diakinesis (Fig. 7C). They also showed positive heteropycnosis during the whole course of meiosis (we did not observe anaphase II). Gonosomes started associating at zygotene by their (probably distal) ends, and were arranged in parallel during pachytene and diakinesis (Fig. 7B, C). They were localized close together during meiosis II too (Fig. 7D).

The genus *Cheiracanthium* Koch, 1839, closely related to *Cheiramiona* Lotz & Dippenaar-Schoeman, 1999 (Lotz and Dippenaar-Schoeman 1999, Ramírez 2014), representing the most cytogenetically examined cheiracanthiid genus so far (Araujo et al. 2019), commonly displays 2n♂ = 26 and X_1X_20 (see Araujo et al. 2019). The karyotype of *Cheiramiona kirkspriggsi* was probably derived from this state, by tandem fusion or a series of lesser translocations, leading to degeneration of the donor autosome, as the reduction of 2n is presumably the leading trend of karyotype evolution in Entelegynae (Kořínková and Král 2013). The X_1X_20 sex chromosome system is also present in most analysed cheiracanthiid species (see Araujo et al. 2019), with exception of *Cheiracanthium saraswatii* Tikader, 1962, *C. melanostomum* (Thorell, 1895) and *C. murinum* (Thorell, 1895), with $X_1X_2X_30$ (Datta and Chatterjee 1983, Srivastava and Shukla 1986). Interestingly, *C. saraswatii* and *C. melanostomum* also possess a distinctly higher number of chromosomes (2n♂ = 43), while the rest of the species have 2n♂ = 22–28, with 26 being the most frequent.

Salticidae Blackwall, 1841

Jumping spiders are the most diverse spider family, with about more than 6100 species globally and 350 species distributed in South Africa (Wesołowska and Haddad 2018, World Spider Catalog 2019). Paralleling their diversity, with 160 karyotyped species the family is also well-investigated in terms of cytogenetics. Most of the species exhibit 2n♂ = 28, X_1X_20 (see Araujo et al. 2019). However, numerous cases of neo sex chromosome formation have been reported in American representatives (Maddison and Leduc-Robert 2013).

We analysed nine species of South African salticids. Consistent with the majority of published data, we found a 2n♂ = 28, X_1X_20 system in all of the species analysed in this study, namely *Baryphas ahenus* Simon, 1902 (Fig. 7E), *Cyrba lineata* Wanless, 1984 (Fig. 7F, G), *Holcolaetis zuluensis* Lawrence, 1937 (Fig. 7H, I), *Myrmarachne laurentina* Bacelar, 1953 (Fig. 7J, K), *Menemerus minshullae* Wesołowska, 1999 (Fig. 7L, 9E), *Nigorella hirsuta* Wesołowska, 2009 (Fig. 7M), *Thyene ogdeni* Peckham & Peckham, 1903 (Fig. 7N) and two species of *Thyenula* Simon, 1902, *T. haddadi* Wesołowska, Azarkina & Russell-Smith, 2014 (Fig. 7O) and *T. leighi* (Peckham & Peckham, 1903) (Fig. 7P). A completely acrocentric karyotype was detected in *B. ahenus* (Fig. 7E), *C. lineata*

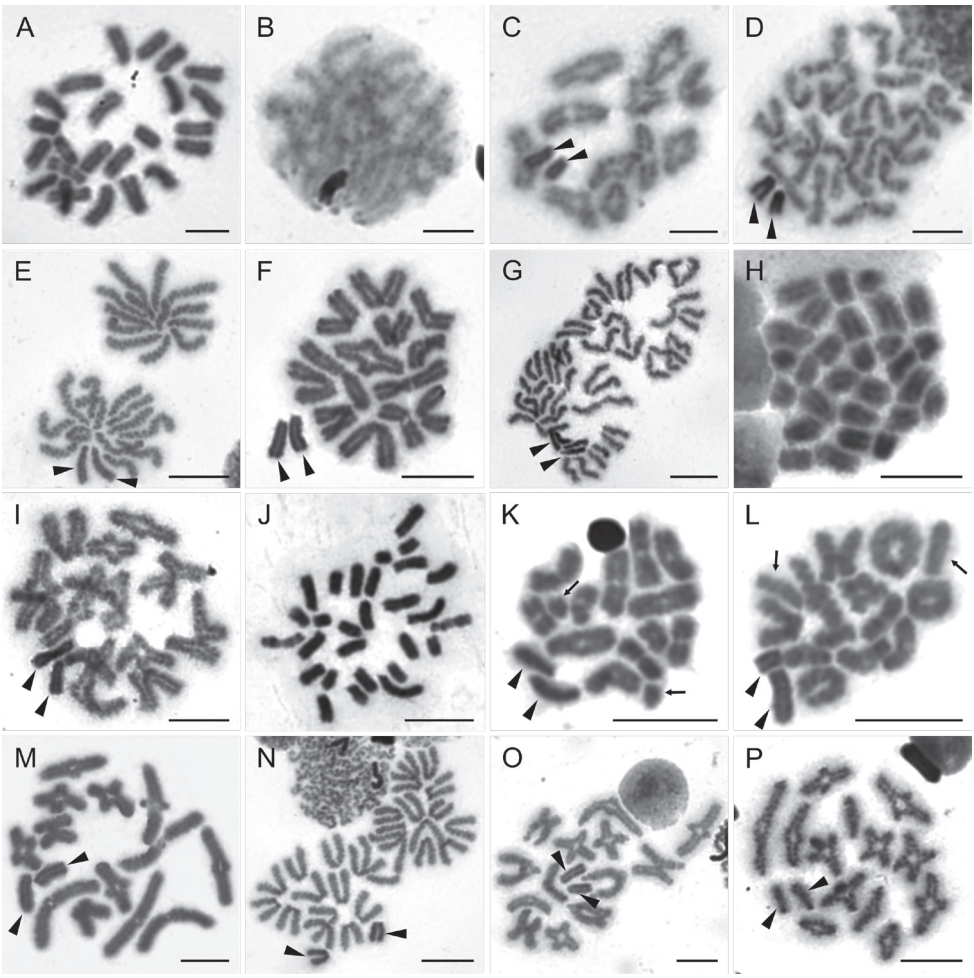

Figure 7. Chromosomes of Cheiracanthiidae (**A–D**) and Salticidae (**E–P**). *Cheiramiona kirkspriggsi* (2n♂ = 24, $X_1X_2$0) **A** mitotic metaphase **B** pachytene, sex chromosomes associate on the periphery of nucleus **C** diakinesis with positively heteropycnotic X_1X_2 **D** metaphase II **E** *Baryphas ahenus*, (2n♂ = 28, $X_1X_2$0) prometaphase II. *Cyrba lineata* (2n♂ = 28, $X_1X_2$0) **F** diakinesis **G** metaphase II. *Holcolaetis zuluensis* (2n♂ = 28, $X_1X_2$0) **H** mitotic metaphase **I** diakinesis, with positively heteropycnotic X_1X_2. *Myrmarachne laurentina* (2n♂ = 28, $X_1X_2$0) **J** mitotic metaphase **K** early metaphase I, note one bivalent with early segregation (arrows) **L** *Menemerus minshullae* (2n♂ = 28, $X_1X_2$0) diakinesis, note one bivalent with early segregation (arrows) **M** Diakinesis of *Nigorella hirsuta* (2n♂ = 28, $X_1X_2$0) **N** Metaphase II of *Thyene ogdeni* (2n♂ = 28, $X_1X_2$0) X_1X_2 are positively heteropycnotic **O** *Thyenula haddadi* (2n♂ = 28, $X_1X_2$0) diakinesis **P** *Thyenula leighi* (2n♂ = 28, $X_1X_2$0) diakinesis. Arrowheads indicate sex chromosomes. Scale bars: 5 μm (**A–D**), 10 μm (**E–P**).

(Fig. 7G), *M. minshullae* (Fig. 9F), *N. hirsuta* (Fig. 9G), *T. ogdeni* (Fig. 7N) and both species of *Thyenula* (Fig. 9H, I). Unfortunately, in the remaining two species the chromosome plates were of insufficient quality to allow the identification of the morphology of the whole chromosome complement. Our dataset thus further supports the conservatism of 2n in this highly diversified group of spiders (Araujo et al. 2019).

Selenopidae Simon, 1897

Selenopids can be considered a smaller family, distributed in the tropics and subtropics (Jocqué and Dippenaar-Schoeman 2006). We analysed two specimens of *Anyphops* Benoit, 1968 and two subadult males of *Selenops* Latreille, 1819 from different localities in South Africa, and one male from Namibia (Table 1). Specimens of both genera from South Africa displayed a similar karyotype of 2n♂ = 26 (Fig. 8A, E), with acrocentric chromosomes gradually decreasing in length (Fig. 8A,E, D, H). The sex chromosome system X_1X_20 was identified in all South African specimens; the X_1 and X_2 differed in length (the ratio of sex chromosomes – 1:0.75–0.78) (Fig. 8C, G). Both sex chromosomes showed intensive positive heteropycnosis and parallel association during pachytene (Fig. 8B, F). Their pairing, location on the periphery of the nucleus and positive heteropycnosis also persisted during diakinesis (Fig. 8C, G) and metaphase II (Fig. 8D, H). On the other hand, the male of *Selenops* sp. 2 from Namibia displayed 2n = 29 (Fig. 8I), with acrocentric morphology of all chromosomes (Fig. 8I, L) and an $X_1X_2X_30$ sex chromosome system (Fig. 8K). In contrast to the species from South Africa, the sex chromosomes of the Namibian representative were of similar length (Table 1), but the characteristics concerning the heteropycnosis and behaviour during meiosis were similar to the other species. The sex chromosomes were associated during pachytene (Fig. 8J) and positively heteropycnotic during the whole meiosis (Fig. 8J–L).

Interestingly, only the data obtained from the Namibian specimen, namely the 2n and the sex chromosome system, were comparable to karyotypes described in three other karyotyped selenopids (Suzuki 1952). The 2n♂ = 29, $X_1X_2X_30$ was reported from species belonging to the genera *Makdiops* Crews & Harvey, 2011 and *Selenops* from India (Sharma et al. 1959, Mittal 1966, Prakash and Prakash 2014). This indicates that the reduction of 2n could have occurred in southern Africa. Interestingly, species with the reduced autosome number also possess an X_1X_20 system, which could indicate that the ancestral sex chromosome constitution was $X_1X_2X_3$, with a subsequent reduction to X_1X_20. However, additional research on this topic will be necessary in order to answer this question.

Distribution of major rDNA loci

We applied 18S rDNA FISH on 11 species from eight different families, including: i) one araneoid (Fig. 10A, B); ii) two species belonging to the non-dionychan RTA clade (family Sparassidae, Fig. 10C, D); iii) three gnaphosoids (Fig. 10E–G); and iv) five non-gnaphosoid Dionycha (Fig. 10H–L), including three members of the family Selenopidae. Numbers of clusters varied from one to five loci (Table 1). We did not observe an 18S rDNA signal on the X chromosome in any of the analysed species. Most of the species possessed a single locus, namely *Olios* sp. from South Africa (Sparassidae) (Fig. 10C), *Afroceto plana* (Trachelidae) (Fig. 10E), *Cheiramiona kirkspriggsi* (Cheiracanthiidae) (Fig. 10H), and *Selenops* sp. 2 from Namibia (Selenopidae) (Fig. 10L). The signals were located in the distal positions on the long arms in all species

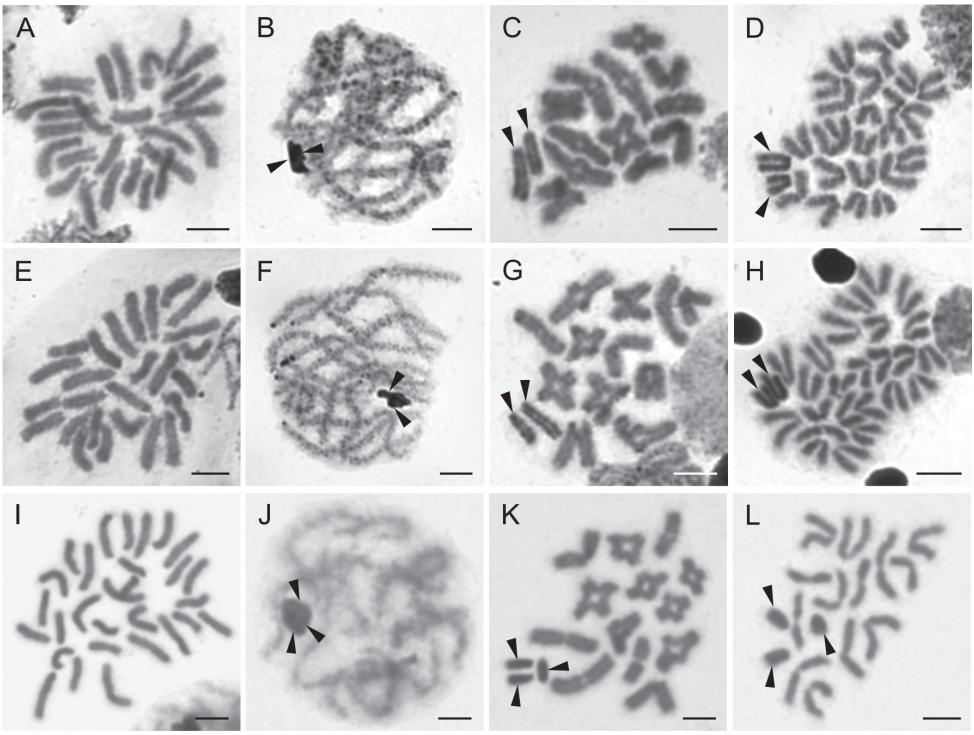

Figure 8. Chromosomes of Selenopidae. *Anyphops* sp. (Mossel Bay) (2n♂ = 26, X_1X_20) **A** mitotic metaphase **B** pachytene, note close association of sex chromosomes **C** diakinesis **D** metaphase II, note positive heteropycnosis of X chromosomes. *Selenops* sp. 1 (Ophathe) (2n♂ = 26, X_1X_20) **E** mitotic metaphase **F** pachytene, note close association of X_1X_2 **G** diakinesis **H** metaphase II. *Selenops* sp. 2 (Namibia) (2n♂ = 29, $X_1X_2X_30$) **I** mitotic metaphase **J** pachytene, note close association of $X_1X_2X_3$ **K** metaphase I **L** half of metaphase II with sex chromosomes. Arrowheads indicate sex chromosomes. Scale bars: 5 μm.

(e.g. Fig. 10C, L). Moreover, in less spiralized chromosomes (e.g. in pachytene), the signal could be confirmed in a terminal position (Fig. 10H). We found two distal loci in *Zelotes sclateri* (Gnaphosidae) (Fig. 10F) and *Thyenula haddadi* (Salticidae) (Fig. 10I). Interestingly, we identified three 18S rDNA loci in *Platyoides walteri* (Fig. 10G), and even four in *Argyrodes* cf. *convivans* (Fig. 10A, B), all of them in distal positions.

In some cases, we found a different number of loci in members of the same family. Namely, two South African representatives of Selenopidae (*Selenops* sp. 1 Ophathe/Pongola and *Anyphops* sp. Ndumo/Mossel Bay) displayed four distal loci (Fig. 10J, K), while *Selenops* sp. 2 from Namibia only had one (Fig. 10L). We observed even higher variation between the two analysed species from the family Sparassidae. One male from South Africa only had a single 18S rDNA locus (Fig. 10C), whereas we found five loci in *Olios* sp. from Namibia (Fig. 10D), all of them likely in a distal position. Both sparassids differed in the number of clusters, despite having the same 2n. This indicates that genome dynamics in entelegynes could be substantial, even though the genome rearrangements do not manifest themselves by changing of 2n and chromosome morphology.

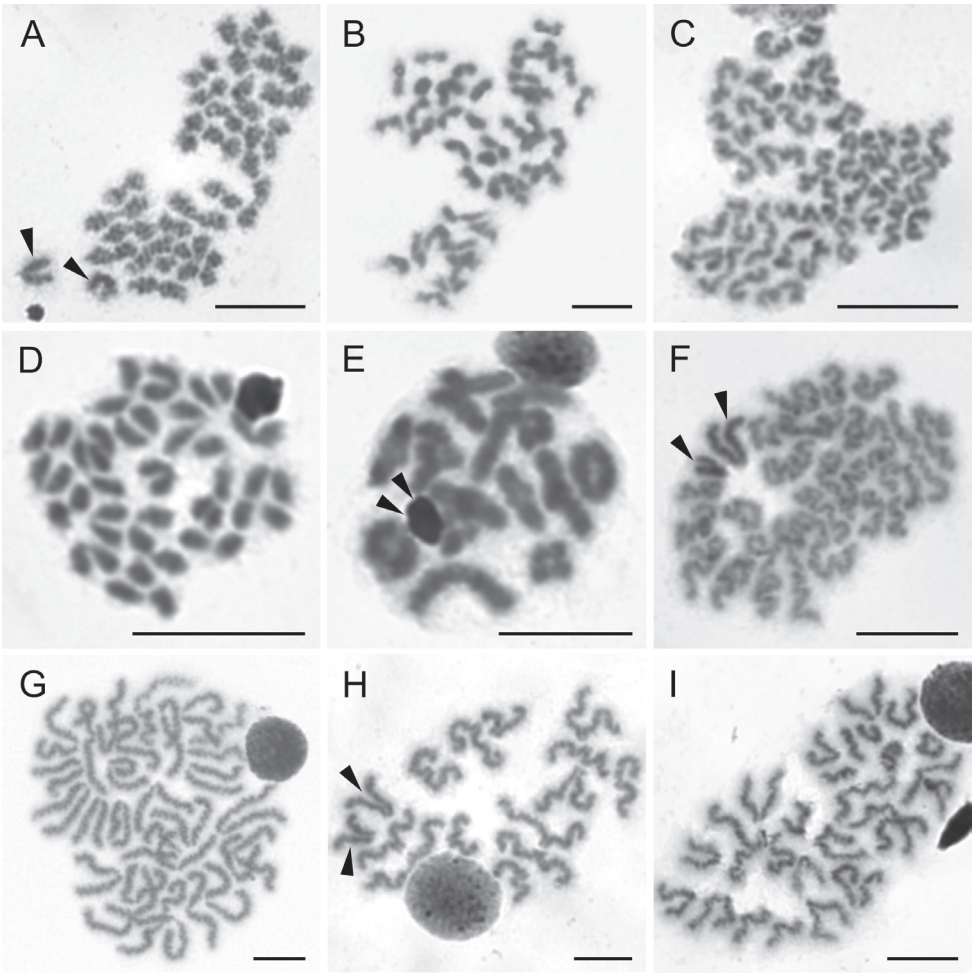

Figure 9. Chromosomes of Ctenidae (**A**), Ammoxenidae (**B**), Gnaphosidae (**C, D**), and Salticidae (**E–I**). *Ctenus* cf. *pulchriventris* (2n♂ = 28, $X_1X_2$0) **A** prometaphase II. *Ammoxenus amphalodes* (2n♂ = 22, $X_1X_2$0) **B** two metaphases II. *Camillina maun* (2n♂ = 22, $X_1X_2$0) **C** metaphase II. *Zelotes* sp. (2n♂ = 22, $X_1X_2$0) **D** metaphase II. *Menemerus minshullae* (2n♂ = 28, $X_1X_2$0) **E** metaphase I **F** metaphase II. *Nigorella hirsuta* (2n♂ = 28, $X_1X_2$0) **G** metaphase II. *Thyenula haddadi* (2n♂ = 28, $X_1X_2$0) **H** metaphase II, one sister cell with sex chromosomes. *Thyenula leighi* (2n♂ = 28, $X_1X_2$0) **I** diakinesis. Arrowheads indicate sex chromosomes. Scale bars: 10 μm.

The position of major rDNA loci has previously been examined in a limited number of entelegynes, namely in one lycosid species (Forman et al. 2013) and four ctenids (Rincão et al. 2017), but we were able to observe broad variation in their number, ranging from one (this study) up to ten (Forman et al. 2013). Notably, the number of loci can differ at family level (this study), or even intraspecifically (Forman et al. 2013). The number of major rDNA loci in entelegynes is very dynamic and more work is necessary to evaluate its usefulness for the cyto-systematics of spiders. Interestingly, the position of the loci on

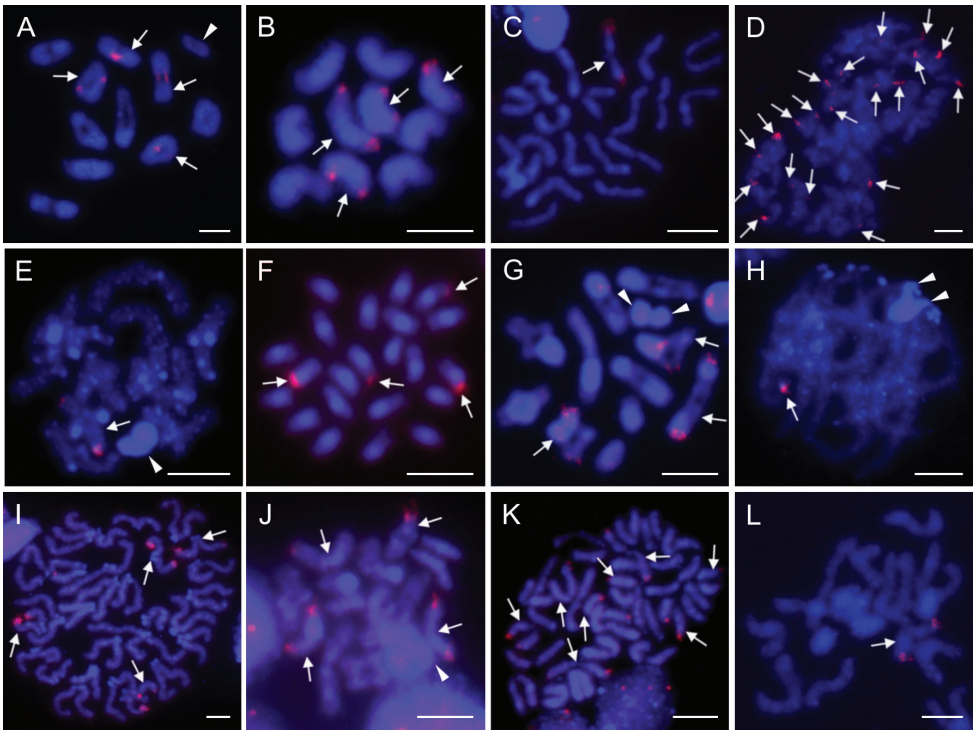

Figure 10. Chromosomes of entelegyne spiders from South Africa after FISH with 18S rDNA probe (red signal). Arrows point on bivalents with signals (**A, E, G, H, J**), certain chromosomes with signals (**B, C, F, I, K, L**), or certain signals (**D**) **A** *Argyrodes* cf. *convivans* (Theridiidae), diplotene **B** *Argyrodes* cf. *convivans*, half of metaphase II (without sex chromosomes), note distal (opposite centromere) positions of loci **C** *Olios* sp. from South Africa (Sparassidae), metaphase II, one sister cell without sex chromosomes, note distal position of locus **D** *Olios* sp. from Namibia (Sparassidae), metaphase II, signals at distal parts of acrocentric chromosomes **E** *Afroceto plana* (Trachelidae), diplotene **F** *Zelotes sclateri* (Gnaphosidae), mitotic metaphase **G** *Platyoides walteri* (Trochanteriidae), diplotene **H** *Cheiramiona kirkspriggsi* (Cheiracanthiidae), pachytene, with distal signal on one bivalent **I** *Thyenula haddadi* (Salticidae), metaphase II, distal signals on two chromosome pairs **J** *Anyphops* sp. (Mossel Bay) (Selenopidae), diplotene **K** *Selenops* sp. 1 (Pongola Reserve) (2n♂ = 26) (Selenopidae), metaphase II, two sister cells **L** *Selenops* sp. 2 (Selenopidae), half of metaphase II. Arrowheads indicate sex chromosomes, where distinguished. Scale bars: 5 μm.

the distal ends of the acrocentric chromosomes seems to be conservative in Entelegynae. The absence of major rDNA loci on the X chromosomes in entelegynes contrasts with our knowledge generated from the basal groups of haplogyne spiders, where the NORs have been found on sex chromosomes via silver impregnation (Král et al. 2006).

General trends of entelegyne karyotype evolution

Our cytogenetic results from southern African entelegynes fit with our knowledge of the general trends in karyotype diversification of the group. Compared to other ma-

jor clades of spiders (Král et al. 2006, 2013, 2019), Entelegynae karyotypes are more homogenous in 2n ranges and very conservative in morphology, which is nearly exclusively acrocentric (Araujo et al. 2019, this study). The X_1X_20 sex determination system is dominant in entelegynes. Alternatively, the presence of X0 and $X_1X_2X_30$ is also common (Araujo et al. 2013, Kořínková and Král 2013, this study). The leading trend of karyotype diversification is a decrease in diploid counts, which convergently occurred among the groups.

In case of Araneoidea, our results from *Pachygnatha* and *Theridion* showed the typical karyotype conservatism in both Tetragnathidae and Theridiidae. On the other hand, *Argyrodes* (Theridiidae) displayed chromosomal rearrangements unusual for entelegynes. Stávale et al. (2010) described inversions of autosomes in *A. elevatus*, accompanied by a Robertsonian fusion of X chromosomes, leading to an X0 system. Because we can confirm acrocentric morphology of the sex chromosomes in both *Argyrodes* species examined here, it is thus likely that the X0 sex system evolved via tandem fusion of an ancestral acrocentric X_1 and X_2. An alternate scenario for the origin of the X0 sex system in South African *Argyrodes* could be explained by centric fusion of X chromosomes and their subsequent pericentric inversion. In both cases, the X0 sex chromosome systems among the *Argyrodes* species evolved independently. This makes *Argyrodes* an interesting model for research of X_1X_20/X0 transition mechanisms.

In comparison to other Entelegynae families, Oecobioidea karyotypes represented a dynamic system. Hersiliidae, despite limited data availability for only four species (Bole-Gowda 1958, Kumbıçak et al. 2018, this study), displayed three different diploid counts. Interestingly, Oecobiidae showed the highest diversity in 2n among Entelegynae families, approaching both upper and lower ranges of Entelegynae diploid number (or fundamental number, respectively), ranging from $2n♂ = 19$, X0 (this study) to $2n♂ = 42$, X_1X_20 (Suzuki 1950). Therefore, the Oecobioidea present an intriguing group that could provide important insights into some fundamental trends of Entelegynae karyotype evolution. Further studies of the African fauna, notably, endemic genera of Hersiliidae (*Tyrotama* Foord & Dippenaar-Schoeman, 2005) and Oecobiidae (*Uroecobius* Kullmann & Zimmermann, 1976 and *Urocteana* Roewer, 1961) could contribute significantly to this topic in the future.

Despite the rare utilization of cytogenetic markers in Entelegynae phylogenetics, the results presented in this paper could have an implication for the group's systematics. The RTA clade represents the most diversified group of entelegynes. The nondionychan members of the RTA clade analysed in this paper showed a broad range of diploid numbers in entelegynes with acrocentric chromosomes. The 2n of examined sparassids ranked among the highest in entelegynes, neighbouring the proposed ancestral state for the group ($2n♂ = 42$, Král et al. 2006). We also described karyotypes of two members of the families Ctenidae and Oxyopidae, traditionally placed within the superfamily Lycosoidea. Both species analysed showed $2n♂ = 28$, X_1X_20 in males, which is a hypothesised ancestral condition for whole Lycosoidea (Dolejš et al. 2011). In both families, a decreasing autosome number has been reported (Stávale et al. 2011, Araujo et al. 2014), with exception of some Ctenidae representatives, where chromosome count increases due to the formation of a $X_1X_2X_30$ system (e.g. Araujo

et al. 2014). Finding the same condition in basal Thomisidae (*Borboropactus*) could mean that 2n♂ = 28 is an ancestral state for the whole family, which also fits with the proposed placement of thomisids within the Lycosoidea (Polotow et al. 2015), which was also supported by phylogenomic analyses (Fernández et al. 2018). However, the karyotype formula 2n♂ = 28, $X_1X_2$0 is common among the Entelegynae families, and it is also proposed as the ancestral condition for many of them (Kořínková and Král 2013). Therefore, the diploid number itself cannot be interpreted as a strong argument for Thomisidae placement within the Lycosoidea.

All representatives of the superfamily Gnaphosoidea analysed in this paper displayed the same karyotype of 22 acrocentric chromosomes and $X_1X_2$0, which confirmed that such a constitution is widespread not only within Gnaphosidae, but also among the other Gnaphosoidea families. Despite the limited data, higher 2n has not been found in any species of Gnaphosidae, Ammoxenidae, Gallieniellidae and Trochanteriidae (see Araujo et al. 2019, this study). These findings suggest that Gnaphosoidea are extremely conservative, and the few species that exhibit a formula other than 22 acrocentrics represent an exception to the rule. For example, Trachelidae, a group closely related to the Gnaphosoidea, comprises species with both 22 and 24 chromosomes (Suzuki 1952, Datta and Chatterjee 1983). The 2n♂ = 22, $X_1X_2$0 can be thus considered an ancestral state for both Gnaphosidae, as well as the majority of Gnaphosoidea families. The karyotypes of subfamily Prodidominae are in contrast with the characteristics of Gnaphosoidea listed above. We detected three more autosome pairs (or two if we will include *Trachelas* sp.) in their karyotypes. An increasing autosomal number is not common in entelegynes (Suzuki 1954), and fissions of acrocentric chromosomes in general are very unlikely. If we consider prodidomines as an internal group of Gnaphosidae, we would have to assume three such independent events. For this reason, we can safely conclude that prodidomines' position within Gnaphosidae family is highly unlikely. Our conclusion is further supported by the results of the molecular analyses (Wheeler et al. 2016) that recovered Prodidomidae as a sister clade to Dionycha.

Our results of the Dionycha part B clade taxa confirm the 2n conservatism within the families Salticidae and Cheiracanthiidae. On the other hand, a substantial variability was observed in Selenopidae. Both *Selenops* and *Anyphops* individuals from South Africa share karyotype features, namely: 2n♂ = 26, $X_1X_2$0 and four 18S rDNA loci, while *Selenops* sp. 2 from Namibia possesses 2n♂ = 29, $X_1X_2X_3$0 and a single 18S rDNA locus.

Conclusions

This study improves our knowledge about entelegyne karyotypes and brings new information about taxa from an understudied biogeographical region. The data proceeding from South Africa and Namibia are consistent with the information available for entelegyne karyotypes from other continents. Here, we confirmed the stability of the karyotype characteristics, namely acrocentric morphology, the prevalence of $X_1X_2$0, and relatively small ranges of 2n in most families. On the other hand, we found variability of 2n

within the families Hersiliidae, Oecobiidae, Gnaphosidae, Selenopidae and Thomisidae. Our cytogenetic data challenge the current placement of Prodidomidae as an internal group of Gnaphosidae, although admittedly further taxa should be analysed to resolve this conundrum, and thus highlight the utility of cytogenetics for taxonomy and systematics. Our study expands our knowledge about major rDNA loci distribution in the Entelegynae, and reveals surprising variability in the number of loci among certain taxa.

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RESEARCH ARTICLE

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New data on karyotype, spermatogenesis and ovarian trophocyte ploidy in three aquatic bug species of the families Naucoridae, Notonectidae, and Belostomatidae (Nepomorpha, Heteroptera)

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Abstract

We report the karyotype, some aspects of spermatogenesis, and ovarian trophocytes ploidy in three aquatic bug species: *Ilyocoris cimicoides* (Linnaeus, 1758), *Notonecta glauca* Linnaeus, 1758, and *Diplonychus rusticus* Fabricius, 1871 from previously unexplored regions – South Europe (Bulgaria) and Southeast Asia (Vietnam). Our results add considerable support for the published karyotype data for these species. In *I. cimicoides*, we observed achiasmate male meiosis – the first report of achiasmy for the family Naucoridae. More comprehensive cytogenetic studies in other species of the Naucoridae are required to elucidate the role of achiasmy as a character in the systematics of the family.

Our observations on the association between phases of spermatogenesis and developmental stages in *I. cimicoides* and *N. glauca* differ from the previously published data. In these species, we assume that the spermatogenesis phases are not strongly associated with certain developmental stages. For further cytogenetic studies (on the Balkan Peninsula), we recommend July as the most appropriate month for collection of *I. cimicoides* and *N. glauca*.

In the ovaries of both species, we studied the level of ploidy in metaphase and interphase trophocytes. In *I. cimicoides*, diploid and tetraploid metaphase trophocytes were found. Heteropycnotic elements, observed in interphase trophocytes of this species, represented the X chromosomes. It allowed us to determine the trophocytes ploidy at interphase (2n was repeated up to 16 times). The situation with *N. glauca* was different. The metaphase trophocytes were diploid and we were not able to determine the ploidy of interphase trophocytes since such conspicuous heteropycnotic elements were not found. The scarce data available suggest a tendency for a low level of trophocyte ploidy in the basal infraorders (Nepomorpha and Gerromorpha) and for a high level in the more advanced Pentatomomorpha. Data about this character in species from other infraorders are needed to confirm that tendency.

Keywords

achiasmate male meiosis (achiasmy), B-chromosomes, karyotype, nurse cells, South Europe and Southeast Asia, *Diplonychus rusticus*, *Ilyocoris cimicoides*, *Notonecta glauca*

Introduction

Ilyocoris cimicoides (Linnaeus, 1758), *Notonecta glauca* Linnaeus, 1758, and *Diplonychus rusticus* Fabricius, 1871 are common predators in freshwater basins. The first two species are broadly distributed across the Palearctic region. *Ilyocoris cimicoides* (Naucoridae) inhabits most of Europe and Asia from Anatolia to Siberia and North China (Fent et al. 2011). *Notonecta glauca* (Notonectidae) occurs in Europe, North Africa, western parts of Central Asia, reaching northwest China (Polhemus 1995, Linnavuori and Hosseini 2000, Kanyukova 2006, Fent et al. 2011). *Diplonychus rusticus* (Belostomatidae) is widespread in the warmer regions of India, Sri Lanka, Southeast Asia, Malaysia, Sumatra, Java, Borneo, Sulawesi, Philippines, New Guinea, China, and Japan (Chen et al. 2005, Polhemus and Polhemus 2013). Although these three species have broad ranges, karyotype data for them have been published only from few regions: *I. cimicoides* – northern of the Danube River (Steopoe 1929); *N. glauca* – England (Browne 1916, Angus et al. 2004), Finland (Halkka 1956), Netherlands (Pantel and Sinety 1906), *D. rusticus* – India (Bawa 1953, Jande 1959). A recently described variation in chromosome number between different populations of two broadly distributed species of Nepomorpha (Angus et al. 2017) have raised the question if there are such cytogenetic differences between populations of other broadly distributed species (*I. cimicoides*, *N. glauca*, and *D. rusticus*).

In many insect species, spermatogenesis completes at the final preimaginal developmental stage, so that the testes of adults contain only spermatids/spermatozoa (Dumser 1980, Gillot 1995). In Heteroptera, adults are traditionally used for cytogenetic studies as at this stage, accurate species identification is easy and spermatogenesis still occurs (e.g. Nokkala and Nokkala 1999, Grozeva and Nokkala 2003, Lanzone and Souza 2006, Castanhole et al. 2008, Golub et al. 2018, Grozeva et al. 2019, for extensive bibliography see Papeschi and Bressa 2006). However, in last (V) instar and adults of nepomorphan species *I. cimicoides* and *N. glauca*, testes were shown to con-

tain only spermatids/spermatozoa. Spermatogenesis has been observed only in earlier developmental stages: instar III and IV nymphs of *I. cimicoides* (in Papáček and Gelbič 1989) and instar IV and V of *N. glauca* (Papáček and Soldán 1992). Spermatogenesis was not the focus of the cited studies; the authors' comments are based only on histological analysis of testes.

A detailed cytogenetic analysis could elucidate the association of certain stages of meiosis with definite instars. Such data would be useful in further cytogenetic studies of these species (e.g. to collect the most appropriate developmental stage with meiotic or mitotic divisions).

In hemipteran species (incl. Heteroptera), the ovaries consist of meroistic telotrophic ovarioles, characterised by a tropharium in the apex and a vitellarium in the basal part (Ma and Ramaswamy 1987). The organisation of the tropharium has been studied in species of each of the infraorders of Heteroptera but Enicocephalomorpha (Eschenberg and Dunlap 1966, Heming-van Battum and Heming 1986, Bilinski et al. 1990, Jawale and Ranade 1990, Simiczyjew et al. 1996, Štys et al. 1998, more references in Štys et al. 1998 and in Simiczyjew et al. 1998). Significant differences in the species of the basal infraorders compared to those of the more advanced infraorders have been found (Simiczyjew et al. 1998). Unlike the organisation of the tropharium, very little attention has been given to the ploidy of trophocytes (nurse cells). The level of trophocyte ploidy in heteropteran ovarioles has been reported only for three species of two infraorders. Choi and Nagl (1977) measured diploid DNA content increase in trophocytes of *Gerris najas* (De Geer, 1773) – 16-fold increase; Cave (1975) – in *Oncopeltus fasciatus* (Dallas, 1852) – 128-fold increase; Dittmann et al. (1984) in *Dysdercus intermedins* Distant, 1902) – 124-fold increase. The first species (*G. najas*) belongs to one of the basal (Gerromorpha), while the last two species – to one of the most advanced (Pentatomomorpha) infraorders. In the ovaries of insects of another hemipteran group – aphids, the level of trophocyte ploidy is suggested to be species specific (Michalik et al. 2013), but for the true bugs there is no confirmation of that.

The aim of the present cytogenetic study was to examine *Ilyocoris cimicoides*, *Notonecta glauca*, and *Diplonychus rusticus* originating from previously unexplored regions – South Europe (Bulgaria) and Southeast Asia (Vietnam), in order to 1) check cytogenetic differences between populations; 2) analyse the relationship between the developmental stages and the phases of spermatogenesis in testes for *I. cimicoides* and *N. glauca*; and 3) determine the ploidy level of trophocytes in ovaries on the example of *I. cimicoides* and *N. glauca*.

Material and methods

Specimens of *Diplonychus rusticus* (Belostomatidae) (4 males) were collected in September 2018 from Vietnam: Ca Mau Province, Tran Van Thoi District, Tran Hoi commune, U Minh Ha National Park, 09.22521N, 104.95898E (Fig. 1). Specimens

Figure 1. *Diplonychus rusticus*, male carrying eggs and typical habitat of the species in U Minh Ha National Park, Ca Mau Province, Vietnam.

of *Ilyocoris cimicoides* (Naucoridae) (16 males, 7 females) and *Notonecta glauca* (Notonectidae) (19 males and 6 females) were collected during the period September 2018-August 2019 from three different localities in Bulgaria: Pernik Province, Choklyovo blato Marsh, 42.40252N, 22.82234E; Sofia Province, artificial pond in a park in the City of Sofia, 42.66355N, 23.30742E; Sofia Province, pools near the Town of Ihtiman, 42.459837N, 23.804966E. For cytogenetic studies, the insects were fixed in the field in 3:1 fixative (96% ethanol: glacial acetic acid). The gonads were dissected out and squashed in a drop of 45% acetic acid. The coverslips were removed using dry ice. Slides were dehydrated in fresh fixative (3:1) and air-dried. The preparations were stained using the Schiff-Giemsa (Grozeva and Nokkala 1996) and C-banding (Grozeva et al. 2004) method. The chromosomal location of 18S rDNA clusters was determined by the well-known FISH protocol (Grozeva et al. 2011, 2015, 2019, Golub et al. 2019).

Giemsa stained preparations were analysed under an Axio Scope A1 – Carl Zeiss Microscope) at 100× magnification and documented with a ProgResMFcool – Jenoptik AG digital camera. FISH preparations were analysed under a Leica DM 6000 B microscope and images were acquired using a Leica DFC 345 FX camera and Leica Application Suite 3.7 software with an Image Overlay module.

The specimens and the chromosome preparations used for this study are stored at the Lab of Cytotaxonomy and Evolution, Institute of Biodiversity and Ecosystem Research, BAS (Sofia, Bulgaria).

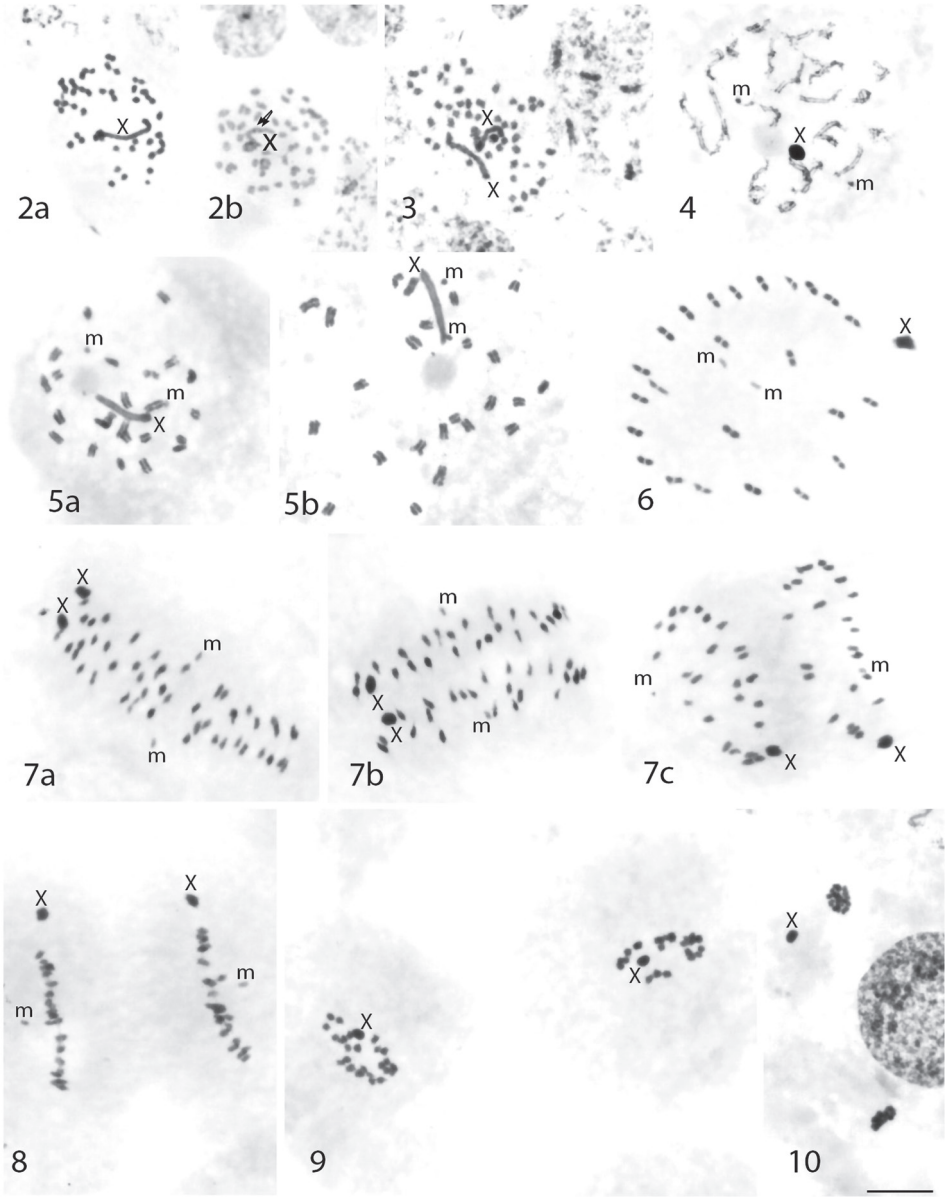
Results and discussion

Description of the karyotype
***Ilyocoris cimicoides*, 2n = 51 (48A + 2m + X) ♂**

The morphology of the testes of the examined males and the ovaries of the females matched the descriptions given by Papáček and Gelbič (1989) and Papáček et al. (1997), respectively.

Like all heteropteran species (Ueshima 1979, Papeschi and Bressa 2006, Kuznetsova et al. 2011), the chromosomes of this species are holokinetic – without localized centromere. In studied male and female nymphs, mitotic metaphases consisted of 48 autosomes (Figs 2 a, b, 3). Except the 48 autosomes, the karyotype included a pair of very small m-chromosomes (see below the description of the meiotic metaphase I), difficult for observation in mitotic cells. The X chromosomes were the largest chromosomes of the complement. At spermatogonial metaphase, the X chromosome displayed interstitial heterochromatin blocks after C-banding (Fig. 2b). In the early condensation stage (late meiotic prophase), we observed 24 bivalents consisting of two side-by-side aligned chromosomes without any sign of chiasmata between them, a pair of m-chromosome univalents, and the heteropycnotic X chromosome, which usually appeared close to the nucleolus (Fig. 4). At the late condensation stage, the X still tended to be close to the nucleolus (Fig. 5a, b). At metaphase I (MI), the autosomal bivalents were similar in size. Most of them formed a ring. The m-chromosome pair and some of the autosomal bivalents laid inside the ring (Fig. 6). The X chromosome was usually seen close to the periphery of the ring. The post-reduction for the sex chromosomes in male meiosis is another specific cytogenetic character of Heteroptera being typical for the majority of the studied species and higher taxa of this group (Ueshima 1979, Kuznetsova et al. 2011). Such was the case in *I. cimicoides*: the X was observed in all the examined anaphase I (AI) (Fig. 7 a–c), telophase I (TI) nuclei (Fig. 8) and in all daughter cells at metaphase II (MII) (Fig. 9). At AI, it was easy to distinguish the m-chromosomes, which were going ahead of the set (Figs 7, 8). At MII, only the large X could be recognized reliably (Fig. 9). At telophase II (TII), the second (equational for the autosomes but reductional for the sex chromosomes) division resulted in two types of daughter cells – with and without X chromosome (Fig. 10).

Cytogenetic data on *I. cimicoides* have been published by Divaz (1915) and Steopoe (1929). Divaz (1915) studied the spermatogenesis of *I. cimicoides* from Serbia, but he did not actually deal with the karyotype: he focused on the presence and behaviour of specific chromatophilic bodies ("corpuscules archoplasmiques"). Our results confirmed the chromosome formula of 2n = 51 (48A + 2m + X), and the chromosomes behaviour for male *I. cimicoides* reported by Steopoe (1929), based on specimens (without information of their developmental stage) collected north of the Danube River. These authors did not mention anything about the formation of chiasmata. In the present study, we provide the first report for achiasmate male



Figures 2–10. *Ilyocoris cimicoides* (testis/ovary): Schiff-Giemsa (**2a, 3–10**), C-banding (**2b**) **2a, b** spermatogonial metaphase (arrows indicate heterochromatin blocks) **3** oogonial metaphase **4–8** primary spermatocytes: **4** early condensation stage **5a, b** late condensation stage **6** metaphase I **7 a–c** anaphase I **8** telophase I **9, 10** secondary spermatocytes: **9** metaphase II **10** two telophases II – one with X, another without X. Scale bar:10 μm.

meiosis in *I. cimicoides* and in the whole family Naucoridae. The achiasmate type of meiosis, called also "achiasmy" (Satomura et al. 2019), as a rule, is restricted to the heterogametic sex (White 1973). Hitherto, achiasmate male meiosis has been found

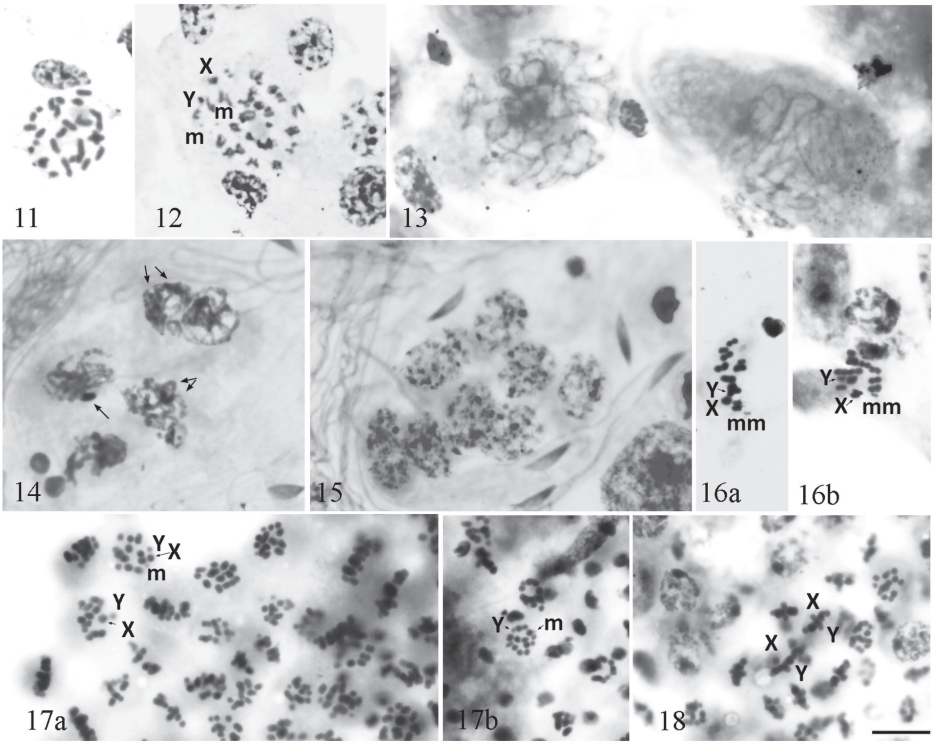
in eight other families of three Heteroptera infraorders, namely Cimicomorpha: Anthocoridae, Microphysidae, Cimicidae, Miridae, and Nabidae; Leptopodomorpha: Saldidae; Nepomorpha: Micronectidae and Corixidae (Stoianova et al. 2015, more references in Kuznetsova et al. 2011). Most of the studied families seem to be homogenous in respect to the presence/absence of chiasmata. Based on the cited data it is suggested that in Heteroptera achiasmy is a stable cytogenetic characteristic at the family level (Grozeva et al. 2008, Kuznetsova et al. 2011). However, heterogeneity in respect to this character has been observed in family Corixidae (Nepomorpha) (Stoianova et al. 2015). Achiasmy is reported for two *Cymatia* species, while the rest examined species of the Corixidae display chiasmata. The achiasmy in *I. cimicoides* (present study) reveals the heterogeneity of the Naucoridae in respect to this character. Hitherto, cytogenetic studies have been published for eight other naucorid species (Papeschi 1992, for more references see Ueshima 1979). Two of them, *Pelocoris lautus* Berg, 1879 and *P. binotulatus* (Stål, 1862), were shown to display chiasmate meiosis in males (Papeschi 1992), while for another six species no information on the presence/absence of chiasmata has been provided (for references see Ueshima 1979). It is noteworthy that in another insect order (Diptera) heterogeneity in the type of male meiosis (achiasmate/chiasmate) has been reported even at genus level (see Satomura et al. 2019). Taking into consideration these new findings, more comprehensive cytogenetic studies in other species of the family Naucoridae are required to elucidate the type of meiosis (chiasmate/achiasmate) as a character in the systematics of the family.

***Diplonychus rusticus*, 2n = 28 (24A + 2m + XY) ♂**

The internal reproductive system of the examined adult males confirmed the morphological descriptions given for *Diplonychus rusticus* by Pendergrast (1957: as *Sphaerodema rusticum* Fabricius, 1871). Every colorless testis consisted of one more or less spherical follicle, decreasing in diameter from the apex to the vas deferens, which expanded to a vesicula seminalis. Such structure of the male reproductive system was described and illustrated well in another Belostomatidae species – *Lethocerus patruelis* (Stål, 1854) (Grozeva et al. 2013).

Chromosome complement in males of *D. rusticus* (as *S. rusticum*) from India was published as 2n = 28 (24A + 2m + XY), together with drawings of the chromosomes at different stages of spermatogenesis (Bawa 1953, Jande 1959). These authors claim a symmetric karyotype and describe in detail all stages of spermatogenesis: spermatogonial mitosis and the behaviour of the chromosomes during both meiotic divisions.

We studied males of this species from Vietnam, collected in U Minh Ha National Park. Spermatogonial metaphases resembled those of *D. rusticus*, *D. annulatus* (Fabricius, 1781) and *D. molestus* (Dufour, 1863) (as *D. subrhombeus* (Mayr, 1871)) studied from India (Bawa 1953, Jande 1959). They consisted of 28 chromosomes (Fig. 11) but it was difficult to identify individual chromosomes in the set. At early spermatogonial



Figures 11–18. *Diplonychus rusticus* (testis) **11** spermatogonial metaphase **12** spermatogonial anaphase **13–16** primary spermatocytes: **13** leptotene **14** pachytene **15** diffuse stage **16a, b** metaphase I **17, 18** secondary spermatocytes: **17 a, b** metaphase II **18** anaphase II. Scale bar:10 μm.

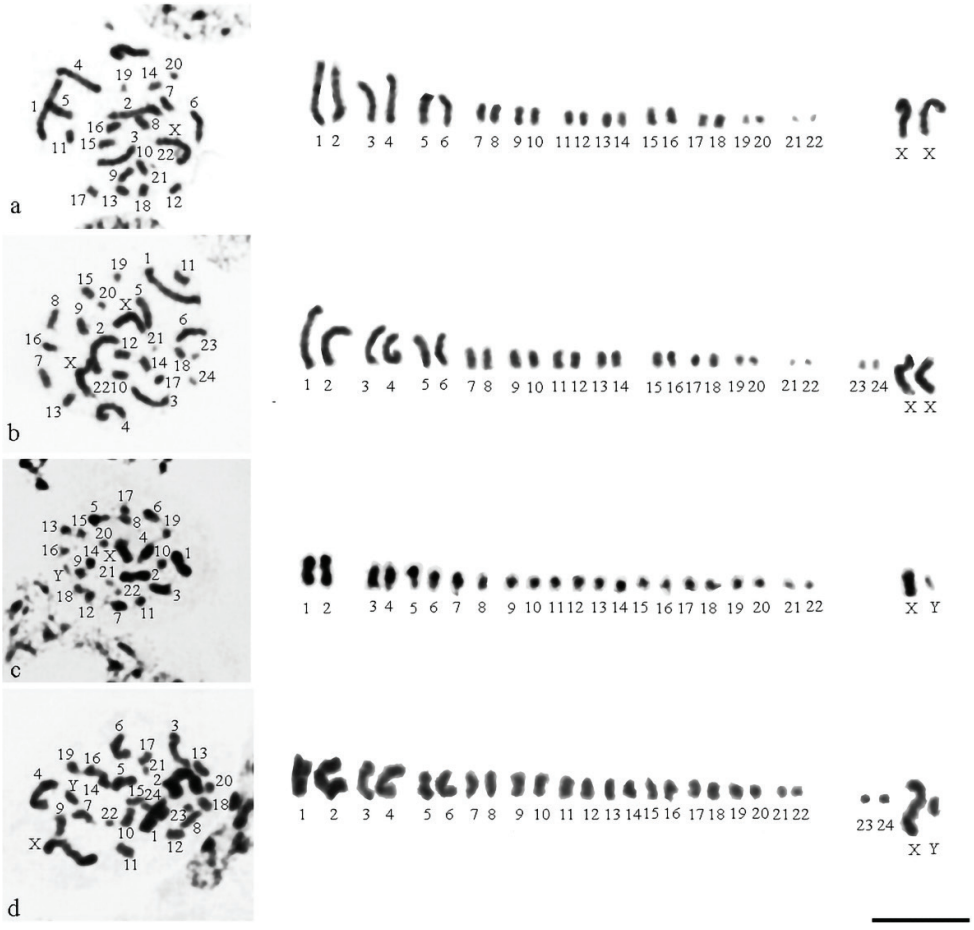
anaphase, the chromosomes were split in chromatids, lying in parallel (Fig. 12), which is the case for holokinetic chromosomes (for instance, Lukhtanov et al. 2019) and one could recognize the X, Y and m-chromosomes. At leptotene (Fig. 13), long and thin chromosomes began to thicken, the sister chromatids were not visible as separate entities. At pachytene (Fig. 14), one or two heteropycnotic bodies of the sex chromosome heterochromatin could be observed. During the diffuse stage, the sex chromosomes were more often associated with each other (Fig. 15). At MI, 12 bivalents, two sex chromosomes as univalents, and a pseudobivalent of the m-chromosomes could be seen (Fig. 16 a, b). At MII, every plate consisted of 12 autosomes, a pseudobivalent of the sex chromosomes, and one m-chromosome (Fig. 17 a, b). The first meiotic division was thus reductional for the autosomes and equational for the sex chromosomes. At AII, the sex chromosomes were going ahead to the poles (Fig. 18). The study of males from Vietnam fully confirmed the observations and description on the spermatogenesis of this species from India (Bawa 1953, Jande 1959). Here, we provide for the first time, photographs of the spermatogenesis stages for *D. rusticus*.

***Notonecta glauca*, 2n = 24 (20A + 2m + XY) ♂**

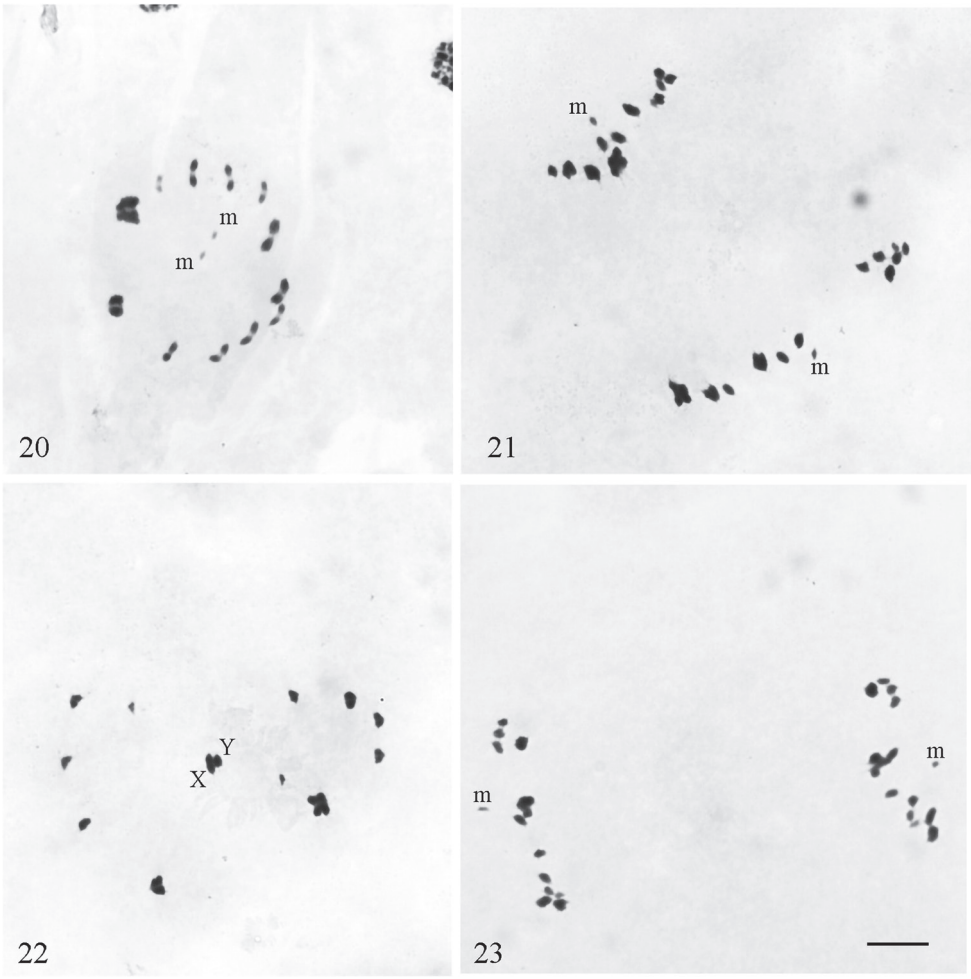
The ovaria of the females and the testes of the males examined matched the morphological descriptions given by Papáček and Soldán (1987, 1992, respectively).

In gonads of females and males, we found mitotic metaphase plates with 24 chromosomes including two sex chromosomes (Fig. 19 a, c). As an exception, only two mitotic metaphase plates with 26 elements were found: one in a ovariole (Fig. 19 b), in which we found mitotic metaphase plates with 24 chromosomes and one in a testis (Fig. 19 d), in which we found mitotic metaphase plates with 24 chromosomes.

In adults collected in July, we found the advanced stages of spermatogenesis, from MI (Fig. 20) to AII (Fig. 23). At MI, 10 autosomal bivalents and the X and Y chromosomes formed a ring with a pseudobivalent of m-chromosomes in it (Fig. 20). It



Figures 19. *Notonecta glauca* (testis/ovary) **a, b** mitotic metaphase in a ovariole: **a** with 24 chromosome elements **b** with 26 chromosome elements **c, d** mitotic metaphase in a testis: **c** with 24 chromosome elements **d** with 26 chromosome elements. Scale bar:10 μm.



Figures 20–23. *Notonecta glauca* (testis/ovary) **20, 21** primary spermatocytes: **20** metaphase I **21** anaphase I **22, 23** secondary spermatocytes: **22** metaphase II with 13 chromosome elements **23** anaphase II. Scale bar:10 μm.

was difficult to distinguish the sex chromosomes in the majority of the meiotic stages. At all the examined AI nuclei, 13 chromosome elements could be counted with the m-chromosomes going ahead of the set (Fig. 21). At MII, a pseudobivalent of the sex chromosomes was placed inside a ring formed by the autosomes; the m-chromosome was indistinguishable (Fig. 22).

Our observations confirm the chromosome formula of 2n = 24 (20A + 2m + XY) and post-reduction of the sex chromosomes reported by Browne (1916) and Halkka (1956) for males of *N. glauca*, collected in England and Finland, respectively. Angus et al. (2004) studied the karyotype of *N. glauca* in midgut cells from both male and female specimens collected in England. He found some specimens with 2n = 26 along with

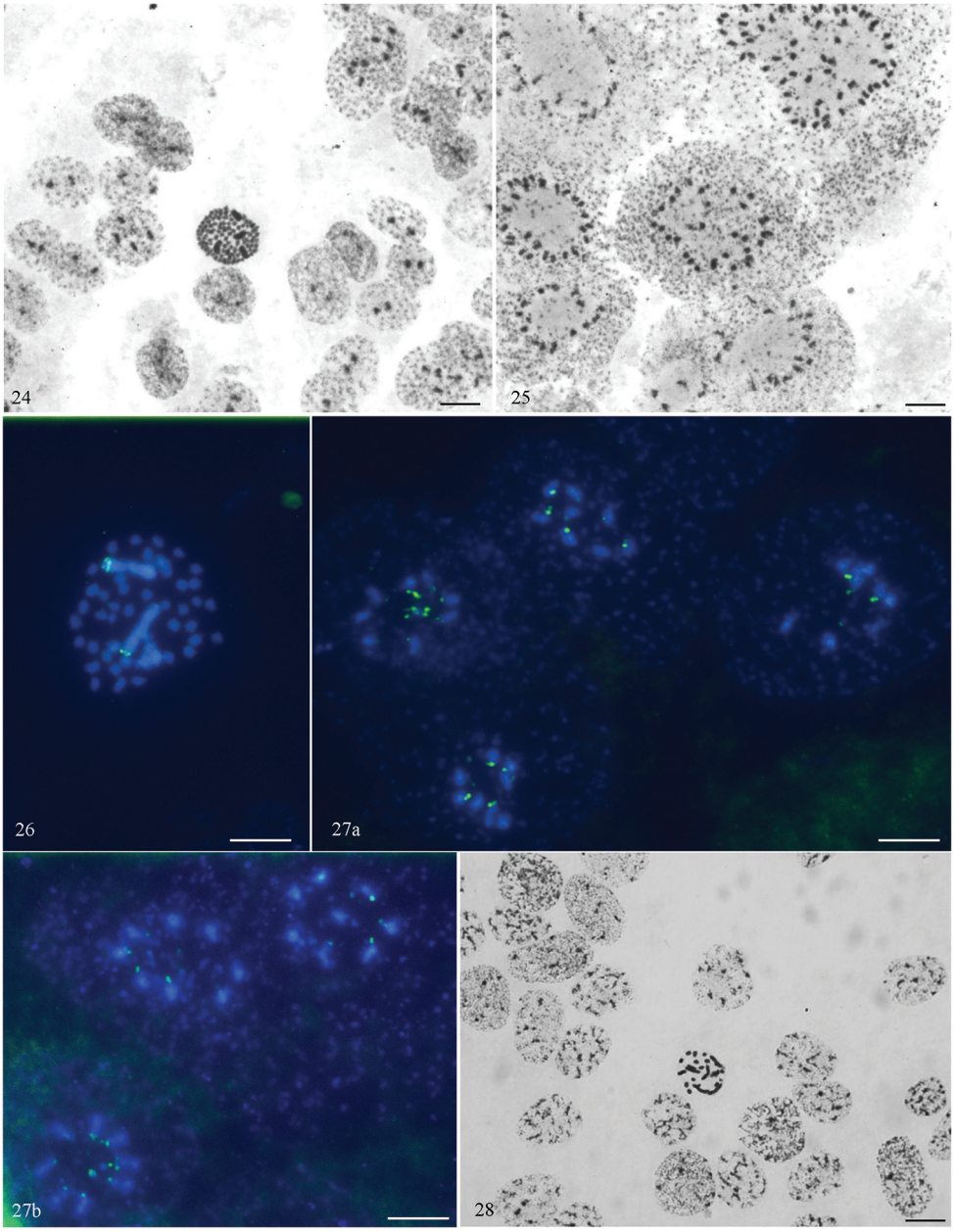
the regular 2n = 24. Our observations of mitotic metaphase plates with 26 chromosomes confirm the occasional occurrence of an extra pair of chromosomes, which was interpreted by Angus et al. (2004) as that of the B-chromosomes. In the present study, C-banding technique was performed but it did not provide additional information.

Association between phases of spermatogenesis and developmental stages in *Ilyocoris cimicoides* and *Notonecta glauca*

Our observations of the association between phases of spermatogenesis and developmental stages in *I. cimicoides* and *N. glauca* differ from those published for these species by Papáček and Gelbič (1989) and Papáček and Soldán (1992), respectively (see Table 1). In *I. cimicoides*, we observed meiotic divisions in instar V, while Papáček and Gelbič (1989) reported the instar V to have spermatids and spermatozoa only. In adults of *N. glauca*, we observed meiotic divisions, while Papáček and Soldán (1992) found only spermatids and spermatozoa. According to our observations, in both *I. cimicoides* and *N. glauca*, males of the same stage of development show different stages of spermatogenesis if they are collected in different seasons (Table 1). For these species, we assume thus that the spermatogenesis phases are not strongly associated with certain developmental stages. It could be speculated that spermatogenesis phases in these species follow the seasonal changes of factors such as temperature and/or photoperiod. There are no publications about the influence of temperature and/or photoperiod on spermatogenesis in heteropteran species. Nevertheless, the influence of these factors on the development and reproduction of Heteroptera has been well documented (Dunbar and Bacon 1972, Ali and Ewiess 1977, Spence et al. 1980, Braman and Pendley 1993, Nagai and Yano 1999, Niva and Takeda 2003, Zerbino et al. 2013, Gusev and Lopatina 2018, Santos et al. 2018). Temperature effect on the development of subspecies *Ily-*

Table 1. Stages of spermatogenesis and spermiogenesis observed in the testes of IV and Vth instars and adult of *I. cimicoides* and *N. glauca*.

Developmental stage	*Ilyocoris cimicoides*		*Notonecta glauca*	
	After Papáček and Gelbič (1989)	**Present data**	**After Papáček and Soldán (1992)**	**Present data**
Instar III	primary spermatocytes and as result of MI – secondary spermatocytes	spermatogonial stages	primary spermatocytes	spermatogonial stages
Instar IV	primary spermatocytes and as result of MI – secondary spermatocytes	spermatogonial stages and meiotic prophase stages	primary spermatocytes and as result of MI – secondary spermatocytes	spermatogonial stages
Instar V	collected in the end of the summer – bundles of spermatozoa	collected in July and August – from PMI to telophase II	disappearance "of the zone of spermatogonia" (the end of the spermatogonial divisions)	spermatogonial stages and meiotic prophase stages
		collected in September – spermatids/spermatozoa		
Adult	mature spermatozoa	spermatids/spermatozoa	No information on the month of collection – spermatids/spermatozoa	collected in July – from MI to AII
				collected in September – spermatids/spermatozoa



Figures 24–28. *Ilyocoris cimicoides* and *Notonecta glauca* (ovarioles): Schiff-Giemsa (**24, 25, 28**) and FISH with 18S rDNA (**26, 27**) **24–27** *Ilyocoris cimicoides*: **24** interphase trophocytes with 2 and 4 heteropycnotic elements, and a metaphase tetraploid trophocyte **25** interphase trophocytes with 16 and about 32 heteropycnotic elements **26** 18S rDNA signals on the sex chromosomes in a oogonial diploid metaphase plate **27 a, b** 18S rDNA signals on the heteropycnotic elements in interphase octoploid trophocytes **28** *Notonecta glauca*: interphase trophocytes and a mitotic metaphase diploid trophocyte. Scale bars: 10 μm.

ocoris cimicoides exclamationis (Scott, 1874) from Japan has also been reported (Kaneda and Yoshiyasu 2007). We have no data on the environmental factors in the collection sites; therefore, the present study cannot contribute to understanding the influence of environmental factors on the spermatogenesis in the studied species. Nevertheless, for practical purposes, we assume that in Bulgaria (in regions at less than 800 m a.s.l.) and probably on the Balkan Peninsula, July is the most appropriate month for collecting *I. cimicoides* and *N. glauca* for cytogenetic studies (Table 1).

Trophocyte ploidy in the ovarioles of *N. glauca* and *I. cimicoides*

In the ovaries of *I. cimicoides* and *N. glauca*, we studied the level of ploidy both in mitotic (metaphases) and in interphase trophocytes (nurse cells). Among the trophocytes of *I. cimicoides*, only diploid and tetraploid metaphases were found (Fig. 24). In *I. cimicoides*, we observed interphase trophocytes with conspicuous heteropycnotic elements, which varied in number from 2 to 32 (Figs 24, 25), almost always an even number. In cells with a higher (8–32) number of elements, the exact counting was often impeded by the clumping of the elements. In ovarian mitosis of *I. cimicoides*, hybridization signals after FISH for 18S rDNA were found in the telomeric region of the sex chromosomes (Fig. 26). The same hybridization signals were likewise found in heteropycnotic elements of trophocytes (Fig. 27 a, b). This suggests that the heteropycnotic elements found in the trophocytes represent the sex chromosomes. Thus, in *I. cimicoides* the interphase trophocytes with high number of heteropycnotic elements, i.e. X chromosomes, were highly polyploid (2n was repeated up to 16 times). The situation with *N. glauca* was different. We did not find interphase trophocytes with such conspicuous heteropycnotic bodies (Fig. 28) and we were able to determine the ploidy (2n) only in mitotic trophocytes (Figs 19, 28).

The level of trophocyte ploidy in the ovarioles of *I. cimicoides* (Nepomorpha) (present study) is the same (16 times increase) as reported for *Gerris najas* (Gerromorpha) (Choi and Nagl 1977). Much higher level of trophocyte ploidy has been reported for the two hitherto studied species of infraorder Pentatomomorpha: *Oncopeltus fasciatus* – 128 times increase (Cave 1975); *Dysdercus intermedins* – 124 times increase (Dittmann et al. 1984). The scarce data presently available suggest a tendency for a low level of trophocyte ploidy in basal infraorders (Nepomorpha and Gerromorpha) and a high such level in the more advanced Pentatomomorpha. Data on additional species of the same and other infraorders are needed to confirm this tendency.

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Patterns of chromosomal variation in Mexican species of *Aeschynomene* (Fabaceae, Papilionoideae) and their evolutionary and taxonomic implications

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Abstract

A cytogenetic analysis of sixteen taxa of the genus *Aeschynomene* Linnaeus, 1753, which includes species belonging to both subgenera *Aeschynomene* (Léonard, 1954) and *Ochopodium* (Vogel, 1838) J. Léonard, 1954, was performed. All studied species had the same chromosome number (2n = 20) but exhibited karyotype diversity originating in different combinations of metacentric, submetacentric and subtelocentric chromosomes, chromosome size and number of SAT chromosomes. The plasticity of the genomes included the observation in a taxon belonging to the subgenus *Aeschynomene* of an isolated spherical structure similar in appearance to the extra chromosomal circular DNA observed in other plant genera. By superimposing the karyotypes in a recent phylogenetic tree, a correspondence between morphology, phylogeny and cytogenetic characteristics of the taxa included in the subgenus *Aeschynomene* is observed. Unlike subgenus *Aeschynomene*, the species of *Ochopodium* exhibit notable karyotype heterogeneity. However the limited cytogenetic information recorded prevents us from supporting the proposal of their taxonomic separation and raise it to the genus category. It is shown that karyotype information is useful in the taxonomic delimitation of *Aeschynomene* and that the diversity in the diploid level preceded the hybridization/polyploidization demonstrated in the genus. The systematic implications of our results and their value can be extended to other Dalbergieae genera as knowledge about the chromosomal structure and its evolution increases.

Keywords
infraspecific taxa, karyotypes, Leguminosae, New World, satellites, secondary constrictions

Introduction

Aeschynomene Linnaeus, 1753 (Fabaceae, tribe Dalbergieae s. l. Cardoso et al. 2013) is a diverse genus of subfamily Papilionoideae (Papilionoid legumes) distributed in the tropics and subtropics of the world (Lavin et al. 2001, Klitgaard and Lavin 2005). The number of new described species has increased rapidly in last decades (Queiroz and Cardoso 2008, Delgado-Salinas and Sotuyo 2012, Silva and Antunes 2014, Antunes and Silva 2017, Chaintreuil et al. 2018) and currently 170 scientific names are accepted according to The Plant List (http://www.theplantlist.org/tpl/search?q=Aeschynomene). The genus *Aeschynomene* has evolved in different ecological niches and includes herbaceous forms, annual and perennial shrubs as well as trees up to 8 meters high, with compound pinnate leaves and papilionoid flowers that are generally self-pollinated, although there is cross-pollination by bees (Arrighi et al. 2014, Carleial et al. 2015). Half of the species are found in the New World, the proposed center of origin of the genus (Chaintreuil et al. 2013, 2018), mainly in Mexico and South America (Rudd 1955); the other half is found in the tropical regions of Africa (center of secondary diversification), SE Asia, Australia and the Pacific Islands (Arrighi et al. 2013, Chaintreuil et al. 2013, LPWG 2013). The current infrageneric classification of New World *Aeschynomene* largely follows the taxonomic groups proposed by Rudd (1955, 1981) who recognized 67 taxa, although recent estimates suggest the existence of 86 species (Fernandes 1996, Klitgaard and Lavin 2005). Subgenus *Aeschynomene* Léonard, 1954 includes hydrophytes herbs and shrubs with peltate stipules, fruits with articles separated by septa, and bilabiate calyx, growing in swamps, wet meadows, river channels and streams. Species of subgenus *Ochopodium* (Vogel, 1838) J. Léonard, 1954 are terrestrial herbs, shrubs and trees with basifixed stipules, fruits with articles separated by an isthmus, and campanulate calyx with five subequal teeth, occur in savannahs, pine and oak groves, rocky slopes, sandy beaches and dry places (Rudd 1955, Fernandes 1996).

In Mexico grow 31 species and infraspecific taxa (including several endemisms) distributed in both Atlantic and Pacific slopes as well as in the center of the country. Those corresponding to subgenus *Aeschynomene* are included in three of five series that make up the group (Americanae- plants with flexible edaphic requirements; Sensitivae and Indicae- predominantly hydrophytic). Those corresponding to subgenus *Ochopodium* are included in three of the four series (Pleuronerviae, Scopariae and Viscidulae) and occupy mesic and subxeric habitats (Rudd 1955, Fernandes 1996).

Traditionally *Aeschynomene* was included in the tribe Aeschynomeneae, however molecular evidence place it in the most widely circumscribed tribe Dalbergieae *sensu lato* (Lavin et al. 2001, Wojciechowski et al. 2004), and show that it is a paraphyletic group with species that are nested separately in two well supported clades (Lavin et al.

2001, Ribeiro et al. 2007, Cardoso et al. 2012). These studies suggest that *Ochopodium* should be raised to the category of genus as a sister group of *Machaerium* Persoon, 1807. Morphological studies on floral ontogeny also support this proposal (Sampaio et al. 2013). Additionally, Chaintreuil et al. (2013) showed that the aquatic and semi-aquatic species of *Aeschynomene* (series Indicae and Sensitivae) form the monophyletic clade Nod-independent, whose taxa are nodulated on roots and stems by photosynthetic Bradyrhizobium strains lacking the nod ABC genes necessary for the synthesis of Nod factors (Giraud et al., 2007), while *A. americana* Linnaeus, 1753 and *A. villosa* Poiret, 1816 (series Americanae) are sisters within American clade Nod-dependent, whose hydrophytes do not nodulate on stems. However, the number of species sampled in previous studies is still limited, and the inclusion of African species and related genera indicates that subgenus *Aeschynomene* is also paraphyletic. In addition to this generic delimitation problem, there is a need to understand the biology and taxonomy of several polymorphic species (e. g., *Aeschynomene americana* and *A. villosa*), which justifies a more comprehensive taxonomic revision of the genus (LPWG 2013).

The cytogenetics studies of the genus showed that there is agreement on the basic number x = 10 (Bir and Kumari 1977, Coleman and Demenezes 1980, Bairiganjan and Patnaik 1989) and 2n = 20 for most species (Renard et al. 1983, Vanni 1983, Kumari and Bir 1990, Seijo and Vanni 1999). Arrighi et al. (2014) used molecular, cytogenetic methods and measure of nuclear DNA content, to analyze the role of polyploidy in *Aeschynomene* New World species of the Nod-independent clade from North America. In addition to providing new records of chromosome numbers, they revealed multiple hybridization/polyploidization events, highlighting the prominent role of allopolyploidy in the diversification of *Aeschynomene* Nod-independents. Chaintreuil et al. (2016a) studied African *Aeschynomene* species and their data support the idea that the whole African group is fundamentally tetraploid (4x) with a common AB genome structure, indicating that a single ancient polyploid event occurred that preceded its diversification. They also revealed the allopolyploid origin of *A. afraspera* J. Léonard, 1954 (2n = 8x = 76) and *A. schimperi* Hochstetter ex A. Richard, 1847 (2n = 8x = 56), where variations in the number of chromosomes also indicated possible dysploidy/aneuploidy events. Therefore, it is necessary to expand the sampling of some taxa or clades of *Aeschynomene* to delimit morphologically similar taxa that show geographically based intraspecific genetic diversity or that exhibit cytotypes (Brottier et al. 2018, Chaintreuil et al. 2018).

Although polyploidy and dysploidy play an important role in the evolution of genomes, chromosomal rearrangements also participate in the evolution of genome size and in the remodeling of its architecture, thus contributing to the diversification of genomes (Rieseberg 2001, Raskina et al. 2008, Faria and Navarro 2010, Chaintreuil et al. 2016a). In this sense the karyotypic analysis in *Aeschynomene* has been little explored, which makes it impossible to know the magnitude and direction of the karyological evolution, the mechanisms involved in the diversification of the genomes and their systematic and phylogenetic implications. This encouraged us to perform a cytogenetic analysis of selected species and infraspecific Mexican taxa of *Aeschynomene*,

together with *A. rudis* Bentham, 1843 from Argentina to investigate (1) its chromosomal and karyotype diversity (2) its relation to the current taxonomic classification and molecular phylogeny; (3) evaluate interspecific delimitations and infraspecific differences, particularly in taxa with taxonomic difficulties and (4) compare the cytogenetic information with the recent morphological and molecular evidence to improve the taxonomy and offer an opinion on the separation of *Ochopodium* as a genus.

Material and methods

Plant material

Together 17 accessions including ten species and four varieties of the genus *Aeschynomene*, as well as two populations that could potentially represent new species or varieties herein categorized as *Aeschynomene* sp. prope *americana* and *Aeschynomene* sp. prope *villosa* were examined in this study (Table 1). The vouchers of the studied specimens were deposited in the National Herbarium (MEXU) of the Instituto de Biología, UNAM, and in the Herbarium of the Facultad de Ciencias Naturales (MCNS), Universidad Nacional de Salta Argentina.

Chromosome and karyotype procedures

The mitotic cells were gathered from radicular meristems of seeds that come from at least six individuals per accession, germinated in Petri dishes lined with cotton moistened in distilled water. Chromosomes at metaphase and prometaphase were obtained following the splash method by Tapia-Pastrana and Mercado-Ruaro (2001) briefly described as follows: the meristems were separated from the root when it reached between 3–5 mm in length and were pretreated with fresh solution of 0.002M 8-hydroxyquinoline for 5 h at room temperature and fixed in the fixative Farmer´s solution (ethanol : acetic acid, 3 : 1). Then they were treated in a mixture of 2% cellulase (w/w, Sigma) and 20% pectinase (v/w, Sigma) in 75 mM KCl for 2 h at 37 °C. After centrifugation at 1500 rpm for 10 min, the cell pellet was transferred to 75 mM KCl solution for 20 min at 37 °C. After two successive rinses with KCl solution they were again fixed in Farmer´s solution and subsequently rinsed twice more. One or two drops of the suspension of pellet were placed on clean slides, air dried and stained in 10% Giemsa for 10 min. Preparations were made permanent using a synthetic resin. At least ten well spread metaphase plates were photographed (AxioCam ERc5s Zeiss) from each collection, using a Carl Zeiss Axioscope A1 and analyzed for chromosome number. Five photographs of metaphases with chromosomes having comparable degrees of contraction were utilised to obtain mean values in the following chromosomal parameters: the difference in length between the longest chromosome and shortest chromosome

Table 1. Geographical data on studied *Aeschynomene* accessions.

Species	Original location	Habitat	Latitude / Longitude
Aeschynomene americana Linnaeus, 1753	MEX, Jalisco, Municipio de la Huerta	Semiaquatic	19.4833333, -105.016667
A. americana var. *flabellata* Rudd, 1955	MEX, Guerrero, Municipio de Chilapa de Álvarez	Semiaquatic	17.9833333, -99.0333333
A. americana var. *glandulosa* (Poiret) Rudd, 1955	MEX, Guerrero, Municipio de Cocula	Semiaquatic	18.2333333, -99.15
Aeschynomene sp. prope *americana*	MEX, Oaxaca, Municipio de Santiago Pinotepa Nacional	Semiaquatic	16.35, -98.05
A. amorphoides Rose, 1894	MEX, Jalisco, Municipio de la Huerta	Terrestrial	19.4833333, -105.016667
A. ciliata Vogel, 1838	MEX, Veracruz, Municipio de Catemaco	Semiaquatic	18.4166667, -95.1
A. deamii Robinson et Bartlett, 1909	MEX, Tabasco, Municipio de Jonuta	Semiaquatic	18.0833333, -92.1333333
A. evenia C.Wright, 1869	MEX, Guerrero, Municipio de Coyuca de Catalán	Semiaquatic	18.3166667, -100.7
A. lyonnetii Rudd, 1989	MEX, Guerrero, Municipio de Tepecoacuilco de Trujano	Terrestrial	18.3, -99.15
A. paniculata Willdenow ex Vogel, 1838	MEX, Guerrero, Municipio de Chilpancingo de los Bravo	Terrestrial	-99, 17.55
A. rudis Bentham, 1843	ARG, Provincia de Salta	Semiaquatic	-64.05, -23.15
A. scabra G.Don, 1832	MEX, Guerrero, Municipio de Arcelia	Semiaquatic	18.3, -100.283333
A. sensitiva Swartz, 1788, I	MEX, Guerrero, Municipio de Atoyac de Álvarez	Semiaquatic	17.2, -100.416667
A. sensitiva Swartz, 1788, II	MEX, Veracruz, Municipio de Texistepec	Semiaquatic	17.8166667, -94.15
A. villosa var. *villosa* Poiret, 1816	MEX, Oaxaca, Municipio de Santiago Pinotepa Nacional	Semiaquatic	16.3333333, -98.05
A. villosa var. *longifolia* (Micheli) Rudd, 1955	MEX, Veracruz, Municipio de Jáltipan de Morelos	Semiaquatic	-94, 17
Aeschynomene sp. prope *villosa*	MEX, Oaxaca, Municipio de Santiago Pinotepa Nacional	Semiaquatic	16.3333333, -98.05

(Range), total haploid chromosome length (THC), average chromosomal size (AC) and ratio of the longest/shortest chromosome (Ratio, L/S). The index of asymmetry (TF) was obtained following Huziwara (1962) and the centromeric index (CI) was established by the formula CI = [SA/SA + LA)] × 100. The chromosomes were classified according to Levan et al. (1964) and the classification of the satellites followed Battaglia (1955). Only the preparations of two species, *Aeschynomene evenia* C.Wright, 1869 and *A. scabra* G.Don, 1832 were recorded in digital images and analyzed in free microscope software Zen lite (Zeiss Microscopy). Remaining taxa were recorded on photographs with the same magnification and the chromosome sizes were estimated using a digital calibrator Mitutoyo Digimatic Caliper CD-6" BS. In the estimation of chromosomal sizes the satellite size was not considered. Karyotypes were prepared from photomicrographs by cutting out individual chromosomes, arranging them in descending order of length and matching on the basis of morphology.

Data analysis and processing

To analyze the patterns of chromosomal variation in the studied taxa, grouping and sorting techniques were used through the NTSYS-PC program version 2.21 developed by Rohlf (2012). A basic data matrix was constructed with 10 chromosome characters, including the total number and number of particular types of chromosomes (m, sm and st), THC, AC, Range, Ratio, TF, and CI (Table 2) and standardized by the linear transformation method and a character correlation matrix was calculated. The variation patterns were evaluated by a principal component analysis (PCA) performed on the correlation matrix. The significance of the groupings was later proven by an analysis of discriminant functions (DFA).

Results

Karyotype diversity

All the taxa exhibited constancy in the chromosome number 2n = 20. Chromosome complements with metacentric (m) and submetacentric (sm) chromosomes and subtelocentric chromosomes (st, no more than two pairs per complement), predominated. Together 10 karyotypic formulae were found. The most frequent karyotype formulae were 8m + 1sm + 1st (studied taxa of series Americanae of *Aeschynomene*) and 9m + 1sm (both populations of *A. sensitiva* Swartz, 1788, and one species of the series Scopariae of *Ochopodium*) (Fig. 1, Table 2). Other taxa had its unique karyotype formula. In accordance with the above, both the CI (34.07 to 45.54) as well as TF (35.17 to 45.54) indicated slightly asymmetric karyotypes in *Aeschynomene* (Table 2). All the complements contained chromosomes with secondary constrictions in the short arms associated with microsatellites (all taxa in series Americanae; Fig. 1A–G) or with macrosatellites (series Sensitivae, Indicae, Pleuronerviae and Scopariae; Fig. 1H–P), that can be located in metacentric chromosomes (e.g., *Aeschynomene* sp. prope *americana*, *A. villosa* var. *villosa* Poiret, 1816 and *Aeschynomene* sp. prope *villosa*), submetacentric (e.g., *A. paniculata* Willdenow ex Vogel, 1838) or subtelocentric (e.g., *A. americana* and *A. rudis*), and with a maximum number of six in *A. villosa* var. *villosa*. Only *A. paniculata* (Fig. 1N) exhibited macrosatellites situated on the largest pair of chromosomes (sm). Thus SAT chromosomes varied both in number (1 to 3 pairs) and position within the karyotypes, commonly they were occurred in the smallest chromosomal pair but also in the first pair. Variation in the size of the satellites was also observed and the most notable case was *A. lyonnetii* Rudd, 1989 where their location in the smallest chromosomal pair was only achieved after clearly observing both the centromere and the secondary constriction, which prevented a misinterpretation due to its large size (Fig. 1O).

The chromosomal complements of the analyzed taxa were small sized chromosomes (Lima de Faria 1980) which can be separated into two groups based on their size: (i) complements with average chromosomal size (AC) close to 1.5 μm (e.g., series

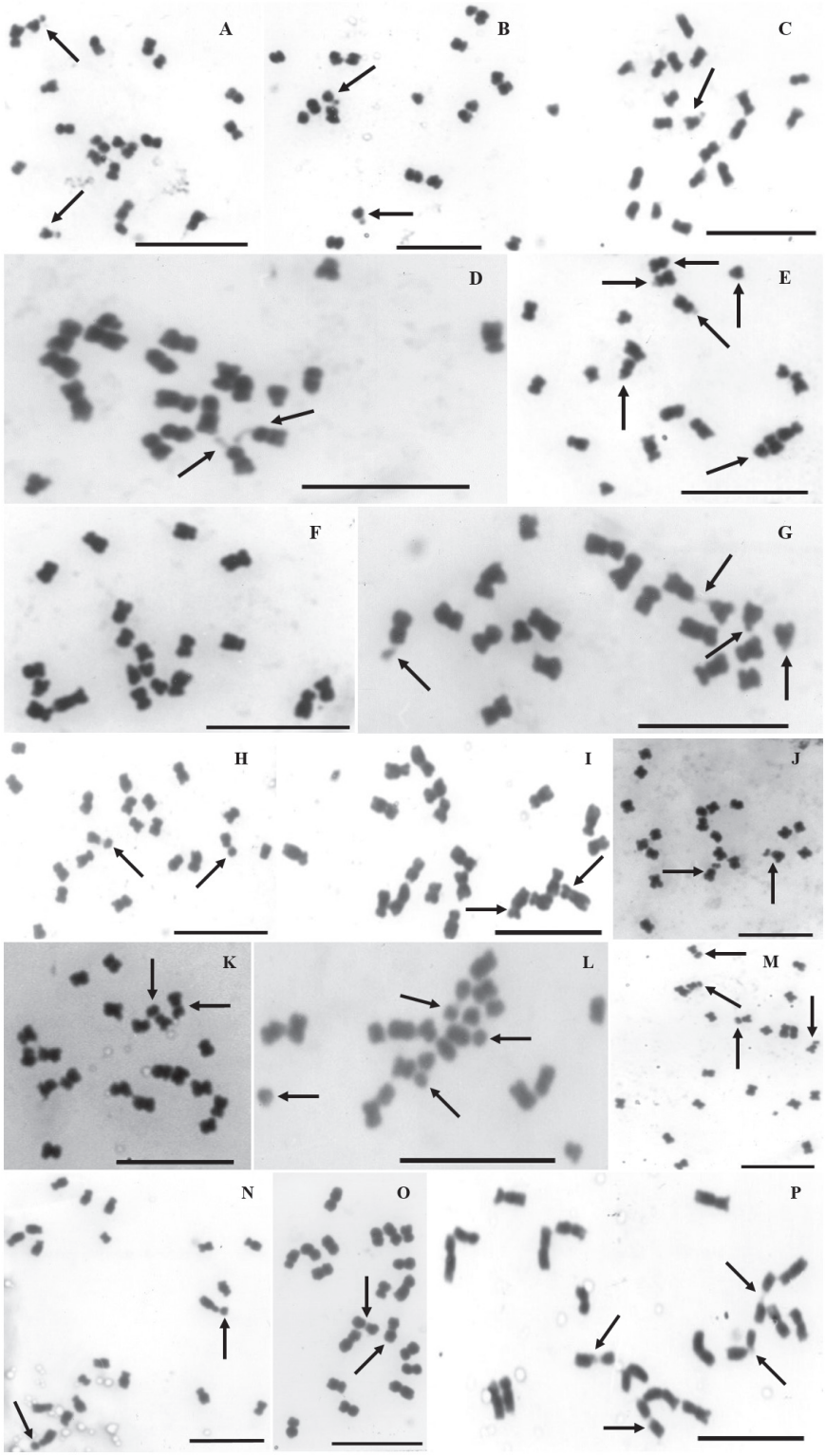
Table 2. Matrix of cytogenetic data on taxa under study.

Subgenus *Aeschynomene*	2n	Karyotype formula	THC (µm)	AC (µm)	Range (µm)	Ratio (L/S)	TF	CI
A. americana	20	8m + 1sm + 1st	12.85	1.28	0.86	1.99	40.75	39.88
A. americana var. *flabellata*	20	8m + 1sm + 1st	13.92	1.39	0.77	1.74	42.02	41.40
A. americana var. *glandulosa*	20	8m + 1sm + 1st	15.86	1.58	0.95	1.98	43.23	42.12
Aeschynomene sp. prope *americana*	20	8m + 1sm + 1st	16.54	1.64	0.98	1.86	43.06	42.13
A. villosa var. *villosa*	20	4m + 4sm + 2st	14.16	1.41	0.98	2.16	35.17	34.07
A. villosa var. *longifolia*	20	4m + 6 sm	13.68	1.36	0.91	2.01	36.98	36.79
Aeschynomene sp. prope *villosa*	20	7m + 2sm + 1st	15.90	1.58	1.09	2.06	40.40	39.79
A. sensitiva I	20	9m + 1sm	16.65	1.66	0.90	1.72	42.82	43.11
A. sensitiva II	20	9m + 1sm	15.66	1.56	0.77	1.63	43.25	42.97
A. deamii	20	8m + 2sm	20.82	2.07	1.01	1.65	41.35	41.48
A. scabra	20	10m	15.71	1.56	0.66	1.51	45.54	45.54
A. evenia	20	7m + 3sm	14.15	1.41	0.82	1.82	42.04	41.50
A. rudis	20	8m + 2st	11.39	1.13	0.60	1.74	39.64	39.13
A. ciliata	20	7m + 3sm	15.71	1.56	0.90	1.82	41.13	40.83
Subgenus *Ochopodium*								
A. paniculata	20	3m + 7sm	19.28	1.92	1.82	2.52	36.56	36.63
A. lyonnetii	20	9m + 1sm	21.86	2.18	1.67	2.33	43.78	41.88
A. amorphoides	20	8m + 2st	22.41	2.24	1.46	1.98	39.66	37.61

Americanae, Sensitivae and Indicae of subgenus *Aeschynomene*) and (ii) those with AC close to 2.0 µm (e.g., series Pleuronerviae and Scopariae of subgenus *Ochopodium*) (Table 2). Intriguingly *A. deamii* Robinson et Bartlett, 1909 with AC = 2.07 µm represented a notable case in the subgenus *Aeschynomene*.

Chromosomal comparisons within a phylogenetic framework

The members of clades distinguished by Chaintreuil et al. (2016a) had similar values in several parameters. It can be seen that within subgenus *Aeschynomene*, series Americanae includes *Aeschynomene americana* and its varieties, as well as populations labeled as *Aeschynomene* sp. prope *americana*, *A. villosa* var. *villosa*, *A. villosa* var. *longifolia* (Micheli) Rudd, 1955 and *Aeschynomene* sp. prope *villosa*. They share certain similarities in size and architecture and showed a common characteristic: microsatellites distributed in metacentric (m), submetacentric (sm) and subtelocentric (st) chromosomes. The smallest chromosome pair constantly showed a displaced centromere (sm/st) in this group. *A. americana* and *A. americana* var. *flabellata* Rudd, 1955 carried satellites in the last pair (st), while in *A. americana* var. *glandulosa* (Poiret) Rudd, 1955 the position alternated between penultimate pair (sm) and the smallest pair (st) however shared the same karyotype: 8m + 1sm + 1st. These taxa showed slight variations in parameters such as THC, range and ratio. Although *Aeschynomene* sp. prope *americana* exhibited a karyotype formula 8m + 1sm + 1st the satellites were in pair six (m), while in *Aeschynomene* sp. prope *villosa* (7m + 2sm + 1st) were in pairs five and ten (Fig. 2). In addition, both taxa exhibited the highest THC in the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series. On the other hand, *A. villosa* var. *villosa* showed two pairs of st chromosomes (4m + 4sm + 2st), consequently the most asymmetric karyotype of the group (CI = 34.07), but stood out for showing the greatest number of microsatellites (pairs 5, 6 and 10). In contrast *A. villosa* var. *longifolia*, without st chromosomes and with the largest number of sm chromosomes in the series (4m + 6sm) carried microsatellites in the smallest chromosomal pair (sm). In series Sensitivae, *Aeschynomene sensitiva* and *A. deamii* (Figs 1H, I, 2 and Table 2) shared relatively similar karyotypic formulae and exhibited only one pair of macrosatellites in the smallest chromosomal pair (sm) however in the latter the secondary constriction is so short that the associated satellite is almost imperceptible. The series Indicae, represented by *Aeschynomene evenia*, *A. scabra*, *A. rudis* and *A. ciliata* Vogel, 1838 showed three different chromosomal formulae in addition to one clear separation between karyotypes with one and two pairs of chromosomes with macrosatellites. The first two taxa: *A. scabra* and *A. evenia*, had a single SAT chromosomes pair, although in a different position, pairs 6 (m) and 10 (sm) respectively, while *A. rudis* and *A. ciliata* exhibited satellites in both pairs of smallest chromosomes (st and sm respectively) (Figs 1J–M, 2 and Table 2).

The species belonging to subgenus *Ochopodium* (Figs 1N–P, 2 and Table 2) showed, from the cytogenetic point of view, greater discrepancies. They presented well-differentiated karyotypic formulae and macrosatellites of variable aspect, position and number, in addition to having the highest values in THC and AC. These are species whose karyotypic asymmetry is related not only to the presence of displaced centromeres, but also to the greater differences in range and ratio (Table 2). This subgenus also includes *Aeschynomene paniculata*, the only taxon with satellites in the first pair, *A. amorphoides* Rose, 1894 with two pairs of satellites and *A. lyonnetii* whose macro-satellites are situated in the last pair, and for their volume and shape, makes it stands out from the rest of the taxa analyzed.

Chromosomal variability and relationship patterns.

The graphic model (PCA) explains most of the variation in chromosomal characters. The characters with the highest load and determinants in the grouping pattern of the taxa were: the number of metacentric chromosomes (41.2935%) and THC (31.9768%). Together, these characters accumulated 73.2703% of the total variation. The PCA separated taxa under study into three groups (Fig. 3). Group 1 is made up of species from series Americanae (*A. americana*, its varieties and *Aeschynomene* sp. prope *americana*),

Figure 1. Mitotic metaphase cells of *Aeschynomene*, all the taxa with 2n = 20. Subgenus *Aeschynomene* **A–G** series Americanae **A** *A. americana* **B** *A. americana* var. *flabellata* **C** *A. americana* var. *glandulosa* **D** *Aeschynomene* sp. prope *americana* **E** *A. villosa* var. *villosa* **F** *A. villosa* var. *longifolia* **G** *Aeschynomene* sp. prope *villosa* **H, I** series Sensitivae **H** *A. sensitiva* **I** *A. deamii* **J–M** series Indicae **J** *A. scabra* **K** *A. evenia* **L** *A. rudis* **M** *A. ciliata* Subgenus *Ochopodium* **N–P** series Pleuronerviae **N** *A. paniculata* **O, P** series Scopariae **O** *A. lyonnetii* **P** *A. amorphoides*. The arrows point to the chromosomes with satellites. Scale bars: 10 μm.

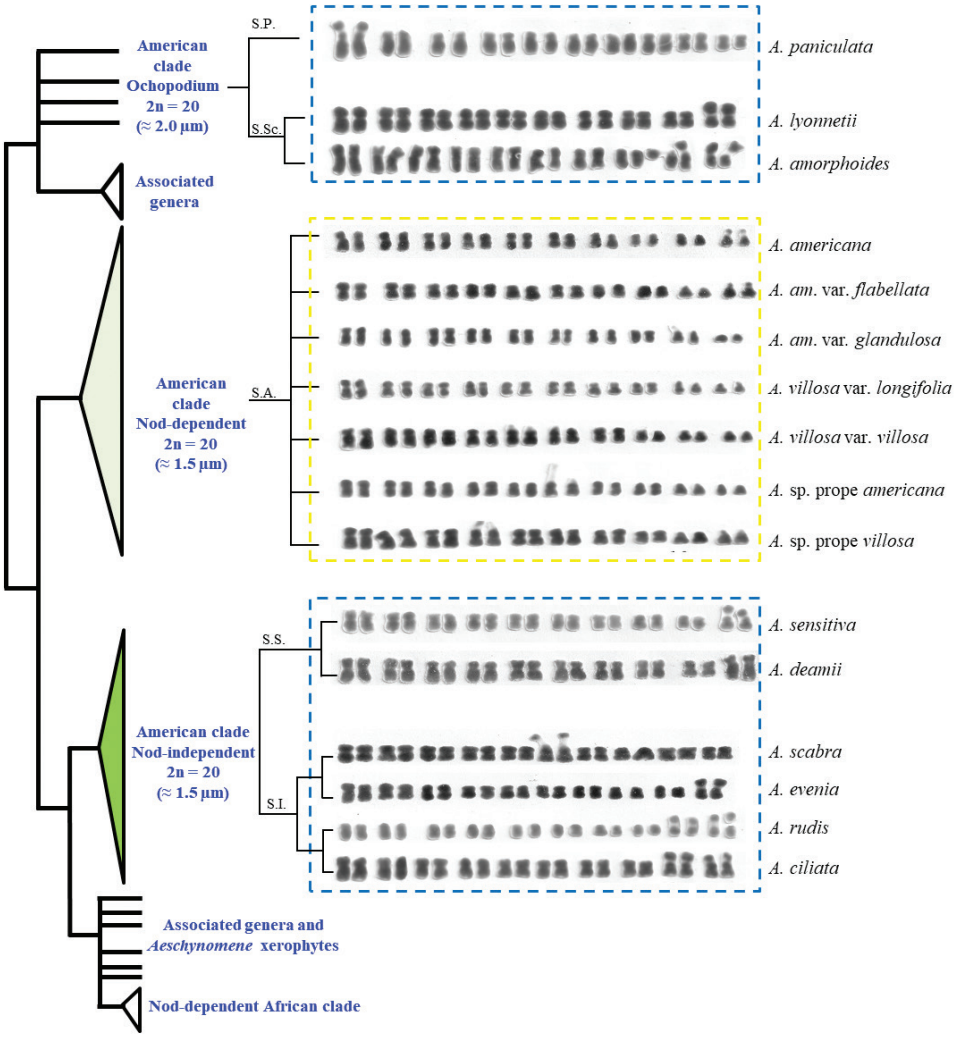

Figure 2. Karyotypes of the studied *Aeschynomene* taxa superimposed on a simplified and stylized phylogenetic tree (modified from Chaintreuil et al. 2016a). Abbreviations: S. P. – series Pleuronerviae; S. Sc. – series Scopariae; S. A. – series Americanae; S. S. – series Sensitivae; S. I. – series Indicae. Blue dashed lines frame the karyotypes exhibiting macrosatellites; the yellow ones, those with microsatellites.

Sensitivae and Indicae of subgenus *Aeschynomene*. This group is characterized by having a greater number of metacentric chromosomes, a higher centromeric index, higher values in asymmetric index and lower ratio values. Group 2 comprises *A. villosa* var. *villosa*, *A. villosa* var. *longifolia* and *Aeschynomene* sp. prope *villosa* of subgenus *Aeschynomene*; and is characterized by presenting lower centromeric indexes, lower values in TF, and higher ratio values. Group 3 includes species from series Pleuronerviae and Scopariae of *Ochopodium*, which are separated from the two previous groups, mainly because they

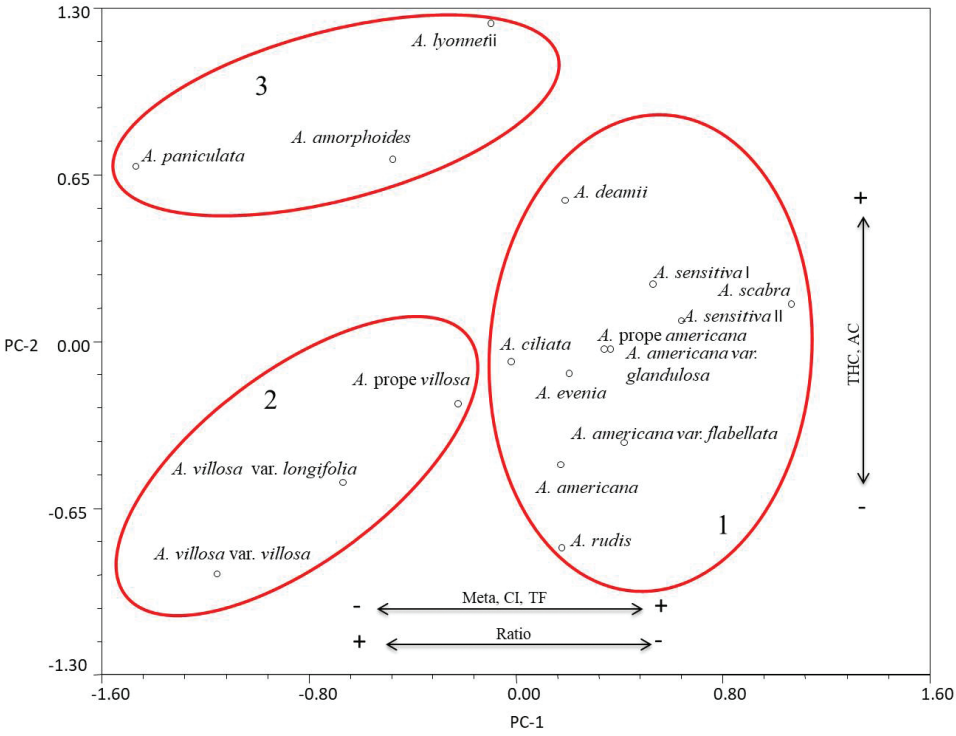

Figure 3. Projection of the 17 accessions of *Aeschynomene* onto the space of the first two principal components. Arrows indicate the patterns of variation in the characters with highest load. Abbreviations: AC = average chromosome size, CI = centromeric index, Meta = number of metacentric chromosomes, Ratio = major chromosome arm length/minor chromosome arm length, TF = index of asymmetry, THC = total haploid chromosomal length.

have a greater total haploid chromosomal length (THC) and higher average chromosome sizes (AC), as well as higher ratio values. Discriminant function analysis (DFA) reinforces the preview showing that the groups identified by PCA are statistically significant (Tables 3, 4). The number of metacentric (m) and submetacentric (sm) chromosomes separates *A. villosa* var. *villosa* and relatives from the rest of the taxa included in the subgenus *Aeschynomene* while THC and AC separate the *Ochopodium* group from the previous two. The centroids of the three groups were clearly separated and there was no overlap between the species that constitute them (Fig. 4), which excluded classification errors in the analysis (Table 4).

Identification of small isolated spherical structure and supernumerary NORs

In *Aeschynomene americana* var. *glandulosa* the localization of the satellites in the karyotypes was often a difficult task as their position was alternated between the last two

Table 3. Results of the discriminant Function Analysis.

Discriminant Function	Eigenvalues	% of Variance explained	% Cummulative	Canonical correlation
1	36.501	92.7	92.7	0.987
2	2.887	7.3	100.0	0.862
Derived Function	**Wilks Lambda**	***Chi square***	**d.f.**	**Significance**
1 to 2	0.007	52.311	16	0.000
2	0.257	14.255	7	0.047

Table 4. Classification of the 17 accessions of *Aeschynomene* according to Discriminant Function Analysis.

Actual groups	Predicted groups							
	1		2		3		Total	
	Number	%	Number	%	Number	%	Number	%
1	11	100	0	0	0		11	100
2	0	0	3	100	0		3	100
3	0		0	0	3	100	3	100

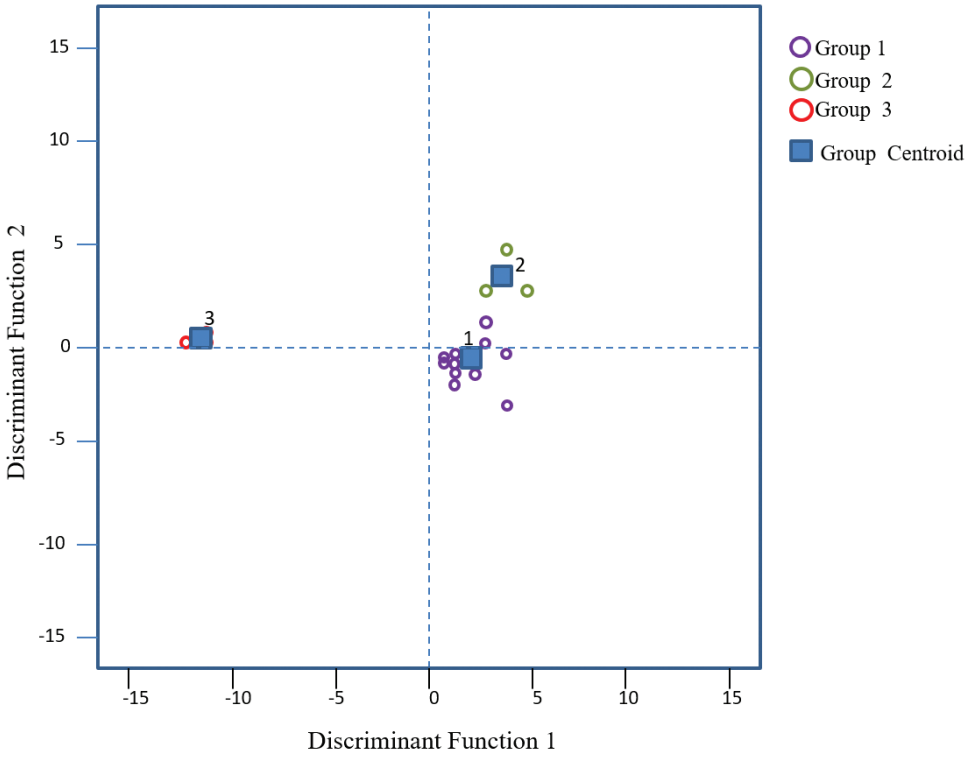

Figure 4. Groupings of the 17 accessions of *Aeschynomene* resulting from a Discriminant Function Analysis. Centroids indicate the average of the taxa in each group.

chromosomal pairs, sm and st respectively; representing a particular type of polymorphism that involves the secondary constriction and its satellite, although this transposition does not significantly alter the karyotype. In addition, nuclei in prometaphase and some metaphases frequently exhibited small isolated spherical structures with a density apparently different from that of the rest of the chromosomal complement. These structures of unknown nature were not found in the same position either associated or aligned with a particular chromosome and differ in size and shape from both the microsatellites described in series Americanae and the known chromosomal fragments (Fig. 5A–I). No similar structures were observed in any other taxon, even in *A. villosa* var. *villosa* where six satellites were found. Also, a complex sequence of rearrangements involving the presence of tiny chromosomal segments generally associated with one or two nucleoli or traces of these and apparently linked, without distinction of the arm, to chromosomes of different sizes by means of chromatin strands that were identified exclusively in nuclei in prometaphase (Fig. 5A–H).

Discussion

The genera included in the tribe Dalbergieae share the same basic chromosome number x = 10, which presupposes a certain uniformity (Goldblatt 1981, Lavin et al. 2001, Mendonça Filho et al. 2002). However, our results showed that the species and infraspecific taxa of *Aeschynomene* possess uniform chromosome number and exhibit a wide karyotypic diversity (Fig. 1; Table 2). We found 10 karyotype formulae and great variation in the total haploid chromosomal lengths (from 11.39 μm in *A. rudis* to 22.41 μm in *A. amorphoides*), in the range (0.60 μm in *A. rudis* up to 1.82 μm in *A. paniculata*), the ratio (1.51 in *A. scabra* to 2.52 in *A. paniculata*), and CI (34.07 in *A. villosa* var. *villosa* to 43.11 in *A. sensitiva*). Furthermore, the number, size and position of secondary constrictions and satellites (SAT chromosomes) confirm the karyotypic heterogeneity in this group and its usefulness as markers for taxa even below the species level, particularly for those taxa difficult to define (Palomino and Vázquez 1991, Solís Neffa and Fernández 2002, Tapia-Pastrana and Tapia-Aguirre 2018). Its role in the organization of the nucleolus is obvious since secondary constrictions and satellites were often associated with projections of nucleolar material or even were observed immersed in a single nucleolus or in several small nucleoli, so here they are considered as nucleolar organizer regions (NORs). In this sense, secondary constrictions have been identified in different plant genera by in situ hybridization with rDNA probes and due to their correspondence with the SAT chromosomes it was possible to describe cytotypes in species and varieties with different levels of ploidy and even in hybrid taxa (Hasterok et al. 2001, Taketa et al. 2001, Kulak et al. 2002, Marasek et al. 2004, Hwang et al. 2011, Roa and Guerra 2012). In addition, the use of conventional cytogenetic techniques has proven its usefulness in the identification of SAT chromosomes for taxa discrimination that exhibit a high degree of intraspecific karyotype uniformity (Solís Neffa and Fernández 2002).

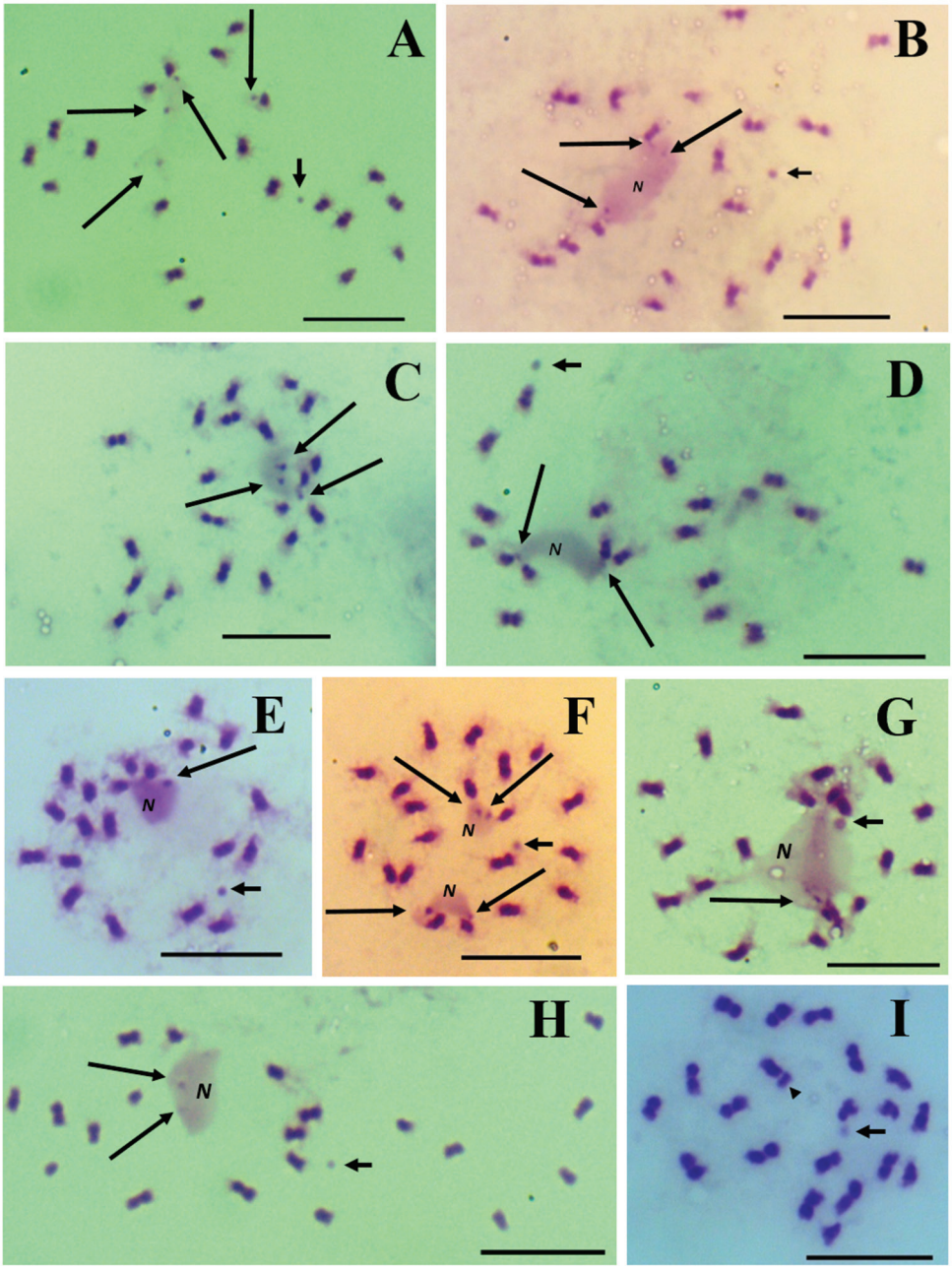

Figure 5. Chromosome rearrangements in *Aeschynomene americana* var. *glandulosa* (2n = 20). **A–H** Prometaphase. Chromosomal segments whose position suggests participation of the NOR function. The long arrows point to segments aligned or joined to the chromosomal arms by chromatin strands or embedded in one or two nucleoli (N) or in traces thereof. The short arrows highlight small isolated spherical structure. **I** Metaphase. The participation of the chromosomal segments decreases or ceases and only an isolated spherical structure is observed within the nucleus. The arrowhead points to a chromosomal fragment. Scale bars: 10 μm.

The above confirms the close association between major rDNA sites and SAT chromosomes (Pikaard 2000) and this agrees with the mapping of two 45S rDNA loci in the secondary constrictions of SAT chromosomes in *A. evenia*, particularly in the upper part of the AeLG10 linkage group (Chaintreuil et al. 2016b), that probably represents pair 10 in the karyotype of *A. evenia* obtained in our study (Figs 1K, 2).

The behaviour of the NORs in the form of secondary constrictions associated with satellites, as well as their size and position, has not been previously studied in species and infraspecific taxa in the genus *Aeschynomene*. Also, the location of the satellites, always in short arms, confirms a common tendency in the karyotypes of plant species where 86% of secondary constrictions are preferably located in short arms (Lima de Faria 1976, Lim et al. 2001) and particularly in Leguminosae (Biondo et al. 2006, Tapia-Pastrana 2012, Tapia-Pastrana and Tapia-Aguirre 2018 and literature therein cited).

Our results were in congruence with the classification based on morphological characters by Rudd (1955) for the New World species of the genus *Aeschynomene* and also with groupings based on phylogeny (Fig. 2). It is clear the presence of two groups that are separated by THC, AC, range and ratio; and whose entities correspond to the subgenera *Aeschynomene* and *Ochopodium*. It is likely that differences in THC, AC, and chromosome shape point to genomic differentiation processes through chromosomal evolution during speciation (Stebbins 1971, Kenton 1981, 1984, Grant 1989, Tapia-Pastrana et al. 2018). In our study, taxa having ACs about 2 μm, with the exception of *A. deamii*, series Sensitivae, belongs to the subgenus *Ochopodium*, which are perennial and occupy terrestrial habitat while those with ACs close to 1.5 μm belongs to the subgenus *Aeschynomene* and are annuals or short perennials and occupy semiaquatic habitats. Different investigations showed a close correlation between the life form, climatic and eco-geographic factors and genome size (Bennett 1972, 1976, Grime and Mowforth 1982, Ohri 1998, Bai et al. 2012 and literature therein cited). If considered that the THC expressed in μm is a good approximation to the size of the genome (Peruzzi et al. 2009, Harpke et al. 2015), then the subgenera *Aeschynomene* and *Ochopodium* could be another example in this regard.

In contrast, *A. deamii*, a perennial species, represents a particular case, because in spite of thriving in marshes and flooded areas and belonging to the group of species that nodulate in stem exhibits an exceptional THC (20.82 μm). Its chromosomal size, which corresponds to a high DNA content (1.93 pg) for a diploid species of the subgenus *Aeschynomene* (Arrighi et al. 2012) seems to correlate with large flowers (Rudd 1955) and with the height that exceeds 4 meters (Delgado-Salinas and Tapia-Pastrana, pers. obs.). *A. deamii* it was initially considered a tetraploid taxon, however, subsequent chromosomal counts corroborated a 2n = 20 (Arrighi et al. 2014). In addition to suggesting the existence of different chromosomal remodeling mechanisms involved in the evolution of its karyotype, our observations support its location as a monospecific lineage in an ITS-based phylogeny (Chaintreuil et al. 2018). Similar karyotype characteristics with *A. sensitiva*, the blackening of the stems and on drying fruits, as well as a calyx with whole or almost whole lips, justify so far, its location in the series Sensitivae (Fig. 2).

The karyotype analysis demonstrated being helpful in the infrageneric delimitation and exhibited a close association not only with the previous morphological and taxonomic groupings, but with phylogenetic trees obtained with molecular markers. Our results suggest the possibility of adding new taxonomic categories, particularly in the series Indicae, since it can clearly be separated into two subseries with species that exhibit one (*A. scabra* and *A. evenia*) and two (*A. rudis* and *A. ciliata*) pairs of chromosomes with satellites. This idea is corroborated by the complements of *A. denticulata* Rudd, 1955 (series Indicae) that also exhibit a pair of SAT chromosomes (data not shown).

In series Americanae taxa are morphologically related and difficult to identify, however the karyotypes of the species and infraspecific taxa show their own identity (Fig. 2) in accord to the Nod-dependent American clade recovered by Chaintreuil et al. (2013). Apparently, we detected a group of morphologically related taxa where the karyotypic differences observed between the species and their varieties in this series are consistent with the idea that we are dealing with a set of non-described taxa that require being review taxonomically. PCA on karyotype characteristics and the morphological differences observed in herbarium specimens, in the descriptions of habits and ways of life and discrepancies in both the floral morphotypes and the geometry of the maculae on the banner petal support this proposal (Fig. 6). In this sense, a more accurate evaluation of these floral morphotypes would provide valuable information for future taxonomic revisions.

The different location of NOR also suggests that *A. americana* var. *glandulosa* undergoes chromosomal remodeling *via* breaks in regions close to secondary constrictions and subsequent transposition of the nucleolus organizer regions; as well as the participation of tiny chromosomal segments whose location inside the nucleolus would indicate not only an active contribution of the NOR function, but also a dynamic state of chromatin remodeling. Such segments could be described as satellites except for the fact that they are not observed in metaphase nuclei or in corresponding stages in nuclei of closely related taxa. On the other hand, the presence of small isolated spherical structures of unknown nature, separated from both the nucleolus and chromosomes, frequently observed in the nuclear space of metaphase cells resembles extrachromosomal circular DNA (eccDNA) detected by electron microscopy in plants, and whose size ranges from 0.1 μm to more than 5 μm in contour length with an average of 1.7 μm for *Triticum aestivum* Linnaeus, 1753 and 1.5 μm for *Nicotiana tabacum* G.Don, 1838 respectively (Kinoshita et al. 1985) and containing sequences derived mainly from repetitive chromosomal DNA (Cohen et al. 2008). The contribution of eccDNA to the evolution and plasticity of plant genomes is unclear and, although there is currently no direct experimental evidence, it is speculated that it is involved in the evolution of B chromosomes and in the mobility of rDNA (Cohen et al. 2008). They also resembles the satellite-like structures recorded in chromosomes of prometaphase cells stained with Giemsa of *Nicotiana kawakamii* Y. Ohashi, 1976 (Nakamura et al. 2001) or well to the minichromosomes observed by fluorescent in situ hybridization (FISH) in metaphase chromosomes of interspecific marsupial hybrids (Metcalfe et al. 2007 in Fig. 5B). It is known that inter- or intraspecific hybridization events lead to genom-

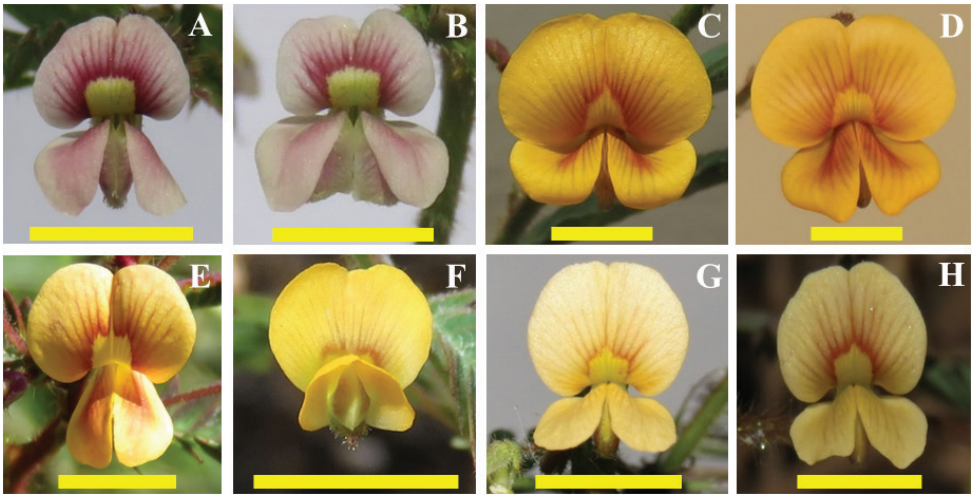

Figure 6. Floral morphotypes of taxa of the series Americanae. **A, B** *Aeschynomene americana* **C** *A. americana* var. *flabellata* **D** *A. americana* var. *glandulosa* **E** *Aeschynomene* sp. prope *americana* **F** *A. villosa* var. *villosa* **G** *A. villosa* var. *longifolia* **H** *Aeschynomene* sp. prope *villosa*. Scale bars: 5 mm.

ic instability, which results in *de novo* chromosomal rearrangements due to changes in chromatin structure among other aspects (Fontdevila 1992, 2005, Metcalfe et al. 2007). Thus, our evidence could indicate that *A. americana* var. *glandulosa* is actually a homoploid hybrid (Nieto Feliner et al. 2017). However, a more accurate interpretation of the nature and function of such structures will have to wait for the application of molecular cytogenetic methods.

Moreover, variations in the number and position of NORs (supernumerary NORs) without some other major karyotypic changes have been reported in *Allium cepa* Linnaeus, 1753 (Sato 1981), *A. fistulosum* Linnaeus, 1753 and its hybrids (Schubert et al. 1983, Schubert 1984, Schubert and Wobus 1985, Pich et al. 1996). Likewise, supernumerary NORs, all in subterminal position, have been found in *Allium flavum* Linnaeus, 1753 (Loidl and Greilhuber 1983) and in natural populations of *A. schoenoprasun* Linnaeus, 1753 (Bougourd and Parker 1976). In addition, *Turnera sidoides* Linnaeus, 1767, exhibits a high degree of intraspecific karyotype uniformity and the subspecies are distinguished only by the number, type and position of the satellites (Arbo 1985, Solís Neffa and Fernández 2002).

Thus, the genome plasticity exhibited in the nuclei of *A. americana* var. *glandulosa*, including the possible participation of supernumerary NORs, would explain the variability in karyotype morphology shown by a group of taxa identified as *A. americana*. It would also support the taxonomic proposal to recognize so-called *Aeschynomene americana* complex; however, this must also be confirmed with molecular cytogenetic studies in a greater number of populations and species.

In series Sensitivae, *Aeschynomene deamii* and *A. sensitiva* exhibit relatively similar karyotypes with macrosatellites in the last pair (Fig. 1H, I and Table 2). In addition,

between the two accessions of *A. sensitiva*, slight differences are observed in parameters such as THC, AC, range and ratio; however, they does not substantially affect neither karyotype nor CI, this suggests the loss of genetic interaction between these two populations or, adaptations to different eco-geographic factors.

Within series Indicae, both *A. ciliata* and *A. rudis* are easily identified by the presence of macrosatellites in both pairs of smallest chromosomes (Figs 1L, M, 2). Likewise, similarity in their CIs indicates a close relationship. The main difference was found in the chromosomal size, since *A. rudis* showed the lowest THC (11.39 μm) in our investigation (Fig. 1 and Table 2), which may be reflect to the eco-geographic characteristics of place of collection (the Salta Province, Argentina, the Southern Hemisphere). In this respect it is worth mentioning that although this species has been described with really large flowers, the Argentinian collections reviewed by Rudd (1955) had exhibited smaller flowers than those from latitudes farther north. The small size of their chromosomes should encourage population studies throughout their distribution to support a proposal that at that time lacked solid arguments about the inclusion of infraspecific categories in this taxon (Rudd 1955). Differences in flower sizes associated with changes in DNA contents were observed in the African *A. schimperi* (Chaintreuil et al. 2016a), while Verdcourt (1971) suggested that specimens of *Aeschynomene* with large flowers could be of polyploid origin. On the other hand, *A. scabra*, which exhibits the most symmetrical karyotype (TF = 45.54) within taxa under study differs from *A. evenia* (species of difficult morphological identification), not only by exhibiting larger chromosomes, but by the position of satellites in pair 6 (m) and not in pair 10 (sm), respectively (Fig. 2). It should be noted that in the Nod-independent nodulation clade (Chaintreuil et al. 2013) *Aeschynomene deamii* and *A. sensitiva* (series Sensitivae) appear as sister species of *A. ciliata*, *A. scabra* and *A. rudis* (series Indicae). Our results show that these five species, together with *A. evenia*, besides being associated by other cytogenetic parameters (Figs 1, 2; Table 2), share the characteristic of exhibiting macrosatellites in the short arms of generally small chromosomes, which clearly differentiates them from series Americanae (Nod-dependent American clade recovered by Chaintreuil et al. 2013) that exclusively exhibits microsatellites. In this sense, there is a concordance with the proposal derived from molecular studies.

A. paniculata (series Pleuronerviae) is the only species that exhibits macrosatellites in the short arms of the first chromosomal pair as well as the largest number of submetacentric chromosomes (seven), so it represents a distinctive case not only within subgenus *Ochopodium*, but throughout the genus *Aeschynomene*.

Series Scopariae includes *A. amorphoides* and *A. lyonnetii*, which bear little resemblance, judging from their different karyotype formulae, CI, and the number and shape of their satellites (Fig. 1O, P, Table 2).

It is pertinent to point out that the scarce chromosomal homology exhibited between the species of the two previous series seems to correspond to the polytomy observed in the Ochopodium clade in the phylogeny by Chaintreuil et al. (2013) and suggests the need for a new taxonomic revision.

Comparatively, our results show that the morphology and particularly the chromosomal size of the species included in the subgenus *Ochopodium* are more similar to those recorded in *Dalbergia spinosa* Roxburgh, 1814 (Jena et al. 2004) than to those of the subgenus *Aeschynomene*. However, the meaning of these types of comparisons should await the detailed karyotypic description in a greater number of species included in *Aeschynomene* and *Dalbergia* Linnaeus f., 1782. On the other hand, the fact that the higher THC, AC and ratio (Table 2) are found in the taxa included in *Ochopodium* indicate that it is a different group, which responds to different adaptations derived from its forms of life and/or the type of environment in which they are developed (Petrov 2001, Chaintreuil et al 2016a). Current phylogenies place *Ochopodium* close to *Machaerium* Persoon, 1807 and *Dalbergia* and propose their phylogenetic separation. However, the scarce reliable karyotypic information in these last two and the limited sampling in our study do not allow to support this proposal at this time from the cytogenetic perspective.

In this way, we show that karyotype information is useful in the taxonomic delimitation of the genus and its value can be extended to other genera of Dalbergieae *sensu lato* as research on chromosomal structure progresses.

Conclusions

The predominantly diploid species of the New World and the lack of an aneuploidy compared to the tetraploid and octoploid African species seem to confirm the New World origin of *Aeschynomene*. Although polyploidy has played an important role in the evolution of the genus, our results indicate that speciation in *Aeschynomene* has also been accompanied by chromosomal remodeling events, as well as subtle changes in the number and position of secondary constrictions and associated satellites, and that these changes preceded duplications and aneuploidies previously recorded in species distributed in the New and Old World. Therefore, the karyotype comparison is a reliable way in identification and classification in *Aeschynomene* since it generally agrees with the morphological series and even with the recent relationship hypotheses that indicate that *Ochopodium* should separate from *Aeschynomene* and constitute a new genus, although the latter must be corroborated by studies that include a greater number of species.

In addition, the identification of isolated small spherical structures and the finding of a complex sequence of rearrangements that could involve supernumerary NORs support the proposal that these elements model the chromosomal evolution of this subgroup in an unsuspected manner. *Aeschynomene* exhibits in both subgenera a high diversity of karyotypes that allow observing patterns of chromosomal evolution associated to important events in the divergence of lineages that have been detected in previous molecular studies. Such is the case of the species of the series Indicae which are grouped within Nod-independent clade and have been also proposed as parental taxa of allopolyploids, although attempts at hybridization have failed to form fertile individuals.

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