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Physical mapping of 5S and 18S ribosomal DNA in three species of *Agave* (Asparagales, Asparagaceae)

Victor Manuel Gomez-Rodriguez[1], Benjamin Rodriguez-Garay[1], Guadalupe Palomino[2], Javier Martínez[2], Rodrigo Barba-Gonzalez[1]

1 *Centro de Investigación y Asistencia en Tecnología y Diseño del Estado de Jalisco A.C., Unidad de Biotecnología Vegetal. Av. Normalistas No. 800. C.P. 44270. Guadalajara, Jalisco. Mexico* **2** *Instituto de Biología, Jardín Botánico, Universidad Nacional Autónoma de México, México D. F., C.P. 04510, Mexico*

Corresponding author: *Rodrigo Barba-Gonzalez* (rbarba@ciatej.net.mx)

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Abstract

Agave Linnaeus, 1753 is endemic of America and is considered one of the most important crops in Mexico due to its key role in the country's economy. Cytogenetic analysis was carried out in *A. tequilana* Weber, 1902 'Azul', *A. cupreata* Trelease et Berger, 1915 and *A. angustifolia* Haworth, 1812. The analysis showed that in all species the diploid chromosome number was 2n = 60, with bimodal karyotypes composed of five pairs of large chromosomes and 25 pairs of small chromosomes. Furthermore, different karyotypical formulae as well as a secondary constriction in a large chromosome pair were found in all species. Fluorescent *in situ* hybridization (FISH) was used for physical mapping of 5S and 18S ribosomal DNA (rDNA). All species analyzed showed that 5S rDNA was located in both arms of a small chromosome pair, while 18S rDNA was associated with the secondary constriction of a large chromosome pair. Data of FISH analysis provides new information about the position and number of rDNA *loci* and helps for detection of hybrids in breeding programs as well as evolutionary studies.

Keywords

Agave, Fluorescent *In Situ* Hybridization, Ribosomal DNA, Karyotype, Physical mapping

Introduction

Agave Linnaeus, 1753 is a genus of the monocotyledonous family Asparagaceae, belonging to the subfamily Agavoideae (APGIII 2009). It is distributed from southern U.S.A. to Colombia and Venezuela, including the Caribbean Islands (García-Mendoza 2002). The genus has a basic chromosome number x = 30 (Doughty 1936, Brandham 1969, Ruvalcaba-Ruiz and Rodriguez-Garay 2002) and diploid to hexaploid species have been reported (Banerjee and Sharma 1987, Castorena-Sánchez et al. 1991, Palomino et al. 2005, Palomino et al. 2012). Species of this genus are characterized by asymmetric and highly conserved bimodal karyotypes, which consist in five pairs of large chromosomes and 25 pairs of small chromosomes, maintaining the same karyotype structure (Castorena-Sánchez et al. 1991, Brandham and Doherty 1998, Moreno-Salazar et al. 2007, Palomino et al. 2010).

Fluorescent *in situ* hybridization (FISH) is a very useful technique in plant cytogenetics for the physical mapping of multigene families (Mukai et al. 1991) and DNA sequences to plant chromosomes (Rayburn and Gill 1985) as well as chromosome identification (Brown et al. 1999, Hizume et al. 2002, Koo et al. 2004, Kato et al. 2004). The ribosomal RNA (rRNA) genes have been used as probes in FISH because of the high copy number of repeat units, specific position in chromosomes and highly conserved sequences (Liu and Davis 2011). Plant rDNA consists of the 18S, 5.8S and 26S (45S) and 5S genes; in yeasts, these genes are juxtaposed in the same *locus,* whereas in higher eukaryotes, they are organized as families of tandemly repeated units located at one or a few chromosomal sites (Lavania et al. 2005, Garcia et al. 2009). 45S rRNA genes are clustered in tandem arrays of repeat units of 18S, 5.8S and 26S genes, internal transcribed spacers (ITS) and external non-transcribed spacers (NTS), with an approximate size of 7.5–18.5 Kb in plants (Mizuochi et al. 2007). 5S rRNA genes also occur in high numbers as tandem repeats, usually independent of 45S rDNA, however, co-localization of 45S and 5S rDNA have been reported in some angiosperms as *Silene chalcedonica* E.H.L. Krause, 1901 (Siroky et al. 2001) and *Artemisia* Linnaeus, 1753 (Garcia et al. 2007); 5S rDNA repeat unit size ranges between 0.2-0.9 Kb, with a highly conserved region (120 bp in length) separated by a NTS (Specht et al. 1997). These genes are highly conserved, so they have been used as molecular markers in a large number of plant species, such as *Triticum* Linnaeus, 1753 (Jiang and Gill 1994), *Gossypium hirsutum* Linnaeus, 1763 (Ji et al. 1999), *Hordeum vulgare* Linnaeus, 1753 'Plaisant' (Cuadrado and Jouve 2010); however, comparative studies using rDNA as markers in *Agave* have been limited, such as those by Robert et al. (2008), where they reported the number of rDNA *loci* in a few species and demonstrated the existence of additivity in the number of *loci* with increasing ploidy.

The aim of this work was to identify the number and chromosomal location of rDNA sites in three different species of the genus *Agave* including *A. tequilana* Weber, 1902 'Azul', *A. angustifolia* Haworth, 1812 'Lineño' and 'Cimarron' and *A. cupreata* Trelease et Berger, 1915 by physical mapping of 5S and 18S rDNA from *A. tequilana* 'Azul'.

Methods

Plant material

Plants were collected in the Denomination of Origin Zone for *Agave tequilana* 'Azul' and in southern Jalisco, México (municipality of Tolimán) for *A. angustifolia* 'Lineño' and 'Cimarron' and in Miraval, Guerrero for *A. cupreata*. Three accessions of each species and varieties were used in this work; the accessions were planted in pots containing a mixture of organic soil:sand:vermiculite (3:3:1) and kept under standard greenhouse conditions.

Mitotic chromosome counts

Elongating secondary root tips were treated with 2 mM 8-hydroxyquinoleine for 6 hours at 18 °C, in darkness. Later, root tips were fixed in ethanol:acetic acid (3:1) for 24 hours. Root tips were hydrolyzed with 1 N HCl for 15 minutes at 60 °C, transferred to Schiff's reagent for 1 hour, and then to 1.8% propionic orcein to stain chromosomes (Moreno-Salazar et al. 2007). Slides were frozen with dry ice (Conger and Fairchild 1953), and mounted in Canada balsam. Twelve of the best cells of each population were photographed by using Technical Pan Film and a Zeiss photomicroscope II (Carl Zeiss AG, Germany).

Karyotype analysis

A negative film was used to draw and measure the chromosome arms and the total genome length. The centromere position was obtained following Levan et al. (1964); arm ratio (r = long arm/short arm) was calculated for each chromosome. Chromosome homology was assigned according to similarities in length and centromere position. In addition, secondary constrictions were useful to distinguish homologous pairs in all populations. Idiograms were constructed according to the arm ratio of the chromosomes, and then grouped in metacentric (m), submetacentric (sm), subtelocentric (st) and telocentric (t) chromosomes. The number of homologous chromosomes was sequentially assigned following chromosome length, for a total number of 30.

Chromosome preparations

Root tips of each three accessions of *A. tequilana* 'Azul', *A. angustifolia* 'Lineño' and *A. angustifolia* 'Cimarron' and *A. cupreata* were collected early in the morning, pretreated with satured α-bromonaphthalene solution and kept in ice water overnight, then fixed in ethanol:acetic acid (3:1), for at least 12 hours and stored at -20 °C until use. Root

tips were incubated in a pectolytic-enzyme mixture, containing 0.2% (*w*/*v*) pectolyase (Sigma, USA), 0.2% (*w*/*v*) cellulase Onozuka RS (Yakult, Japan), and 0.2% (*w*/*v*) cytohelicase (Sigma) in 10 mM citrate buffer (pH 4.5), at 37 °C for approximately 2 hours. Squash preparations were made in a drop of 45% acetic acid and frozen in liquid nitrogen; the cover slips were removed with a razor blade and slides were dehydrated in absolute ethanol and then air-dried. The best slides were stored at 2–3 °C for up to 1 month.

Amplification and cloning of rDNA from *A. tequilana* 'Azul'

Total genomic DNA from *A. tequilana* 'Azul' was extracted from fresh young leaves using the CTAB method (Murray and Thompson 1980). The 5S and 18S rRNA genes were amplified by PCR using the following set of primers as follows: 5SF (5'-CACCAGATCCCATCAGAACT-3'); 5SR (5'-TTAGTCTGGTATGATCGCAC-3'); 18SF (5'-CAAAGATTAAGCCATGCATG-3') and 18SR (5'-CCCAGAACATCTAAGGGCAT-3') (Integrated DNA Technologies, USA). Both PCR reactions were performed in 20 µl reactions containing: 5.2 µl mQ water, 2µl *Taq* buffer 10×, 1 µl 50 mM $MgCl_2$, 1.6 µl 2.5 mM dNTPs, 2 U *Taq* polimerase (Life Technologies Corporation, USA), 2.5µl 1 mM of each primer and 50 ng DNA (5µl). Cycling conditions for 5S rDNA were: 94 °C for 4 minutes; 35 cycles of 94 °C for 30 s, 55 °C annealing temperature for 30 s and 72 °C for 30 s, followed by a final extension of 72 °C for 10 minutes. Cycling conditions for 18S rDNA were: 94 °C for 5 minutes; 35 cycles of 94 °C for 30 s, 60 °C annealing temperature for 30 s and 72 °C for 90 s, followed by a final extension of 72 °C for 10 minutes. PCR products were separated by 1% agarose gel electrophoresis in 1× TAE running buffer. Products were visualized by staining with ethidium bromide and the most prominent bands (~1400 bp for 18S and 300-500 bp for 5S) were purified by QIAquick Gel Extraction kit (Qiagen, Germany) according to the manufacturer's instructions. The purified bands were cloned into pGem®-T Easy Vector System I (Promega, USA), incubated overnight at 4 °C. Ligation products were transformed into electrocompetent *E. coli* DH5α cells (Life Technologies Corporation). The recombinant clones were sequenced by LANGEBIO (Cinvestav, Irapuato, Mexico). The sequences were edited with BioEdit version 7.0.9 (Ibis Biosciences, USA) and compared with other sequences available in GenBank (http://www.ncbi.nlm.nih.gov/).

Probe labeling

5S and 18S rDNA probes were isolated with the High Pure Plasmid Isolation kit (Roche Diagnostics GmbH, Germany) and labeled with biotin-16-dUTP by nick translation according to the manufacturer's instructions (Roche Diagnostics GmbH).

Fluorescent *in situ* hybridization

Slide pretreatment. Slides were incubated in RNase A (100 μg ml^{-1} in 2× SSC) for 1 hour at 37 °C, and washed with 2× SSC for 15 minutes. Then, the slides were incubated in 0.01 M HCl for two minutes and followed by treatment in pepsin (5 μg ml^{-1}) in 0.01M HCl for 10 minutes at 37 °C. Afterwards, the slides were washed in 2× SSC for 10 minutes and incubated in 4% paraformaldehyde for 10 minutes at room temperature. Finally, the slides were dehydrated in ethanol series (70%, 90%, and absolute ethanol for 3 minutes each), and air-dried.

Probe hybridization. Hybridization was carried by using a mixture consisting of 20× SSC, formamide, 50% sodium dextran sulphate, 10% sodium dodecyl sulphate, and 25-50 ng/slide of each probe. DNA probes were denatured by heating the hybridization mixture at 70 °C for 10 minutes and then placing it on ice for at least 10 minutes. For each slide, 40 μl of the hybridization mixture were used. Slides were denatured at 80 °C for 5 minutes. The slides were then placed in a pre-warmed humid chamber and incubated overnight at 37 °C. Slides were washed at 37 °C in 2× SSC for 15 minutes, 0.1× SSC at 42 °C for 30 minutes, and 2× SSC at room temperature for 10 minutes.

Signal detection. Biotin-labeled probes were detected with streptavidin-Alexa Fluor ^{546} conjugate (Life Technologies Corporation) and amplified with biotinylated goat-antistreptavidin (Vector Laboratories, USA). Chromosomes were counterstained with DAPI solution (1 μg ml^{-1}), and one drop of Vectashield antifade (Vector Laboratories) was added before examination under a Leica DMRA2 microscope (Leica Microsystems, Germany) equipped with epifluorescent illumination and coupled to an Evolution QEi Camera (Media-Cybernetics, USA), and the images were analyzed with the Image-Pro software (Media-Cybernetics) and enhanced with Photoshop (Adobe Systems Incorporated, USA).

Results

Agave tequilana 'Azul' rDNA cloned sequences

The partial amplification of 18S rDNA generated one band, which was cloned into electrocompetent *E. coli* DH5α cells and a single clone was isolated, which after sequencing showed a fragment of 1424 bp (GenBank: KF159807) and a maximal identity of 100 % with *A. tequilana* cultivar Azul (GenBank: GU980213.1) and *A. ghiesbreghtii* K.Koch, 1862 voucher Chase 3467(K) (GenBank: HM640709.1) according to BLASTn analysis (nucleotide blast) at the NCBI database. The partial amplification of 5S rDNA generated one band, which was cloned into electrocompetent *E. coli* DH5α cells and one clone was isolated, which after sequencing showed a fragment of 436 bp (GenBank: KF159808) and a maximal identity of 97% with *Arabidopsis thaliana* (Linnaeus, 1753) clone CIC YAC 9A12 and 9A5 5S ribosomal RNA gene (GenBank: AF198223.1), according to BLASTn analysis (nucleotide blast) at the NCBI database.

In situ hybridization

The physical mapping of 5S and 18S rDNA from *A. tequilana* 'Azul' were investigated by fluorescent *in situ* hybridization (FISH) (Fig. 1). FISH experiments with both probes labeled with biotin and detected as a red signals, showed that the number of sites of rDNA were constant among all the species under study. 5S rDNA *loci* were located in both arms of small chromosome pair in each species (Fig. 1). The hybridization sites of cloned 18S rDNA were associated with the secondary constriction of a large chromosome pair in each species, being a subtelocentric chromosome pair in *A. tequilana* 'Azul' and a telocentric chromosome pair in *A. cupreata* and *A. angustifolia* 'Lineño' and 'Cimarron' (Fig. 1).

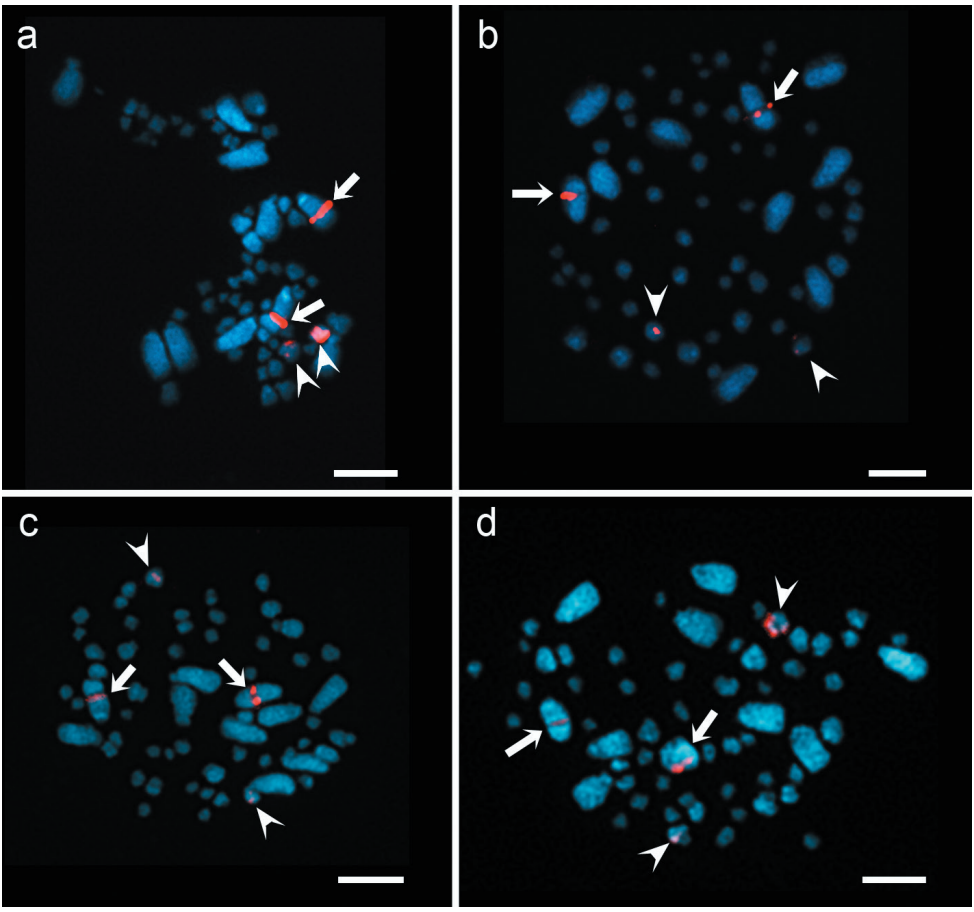

Figure 1. FISH of 5S and 18S rDNA in *Agave* species. Two hybridization sites of 18S rDNA (arrows) and 5S rDNA (arrowheads) in: **a** *A. tequilana* 'Azul' **b** *A. cupreata* **c** *A. angustifolia* 'Lineño' **d** *A. angustifolia* 'Cimarron'. Bars = 10 μm.

Karyotype analysis

All the studied species were diploids with 2n = 2x = 60, confirmed by chromosome counting, considering the basic chromosome number x = 30 for the genus, and showed a bimodal karyotype with five pairs of large chromosomes and 25 pairs of small chromosomes. Karyotype analysis of *Agave* species is summarized in Table 1, and where it can be seen that all species showed different karyotypic formulae as well as a secondary constriction in one large chromosome pair; in *A. tequilana* 'Azul' it was observed in pair 1, in *A. cupreata* in pair 3, in *A. angustifolia* 'Lineño' in pair 5 and in *A. angustifolia* 'Cimarron' in pair 2.

FISH data were integrated in idiograms, indicating the number and position of rDNA *loci* (Fig. 2). 5S rDNA *loci* always were located in a proximal region on both arms of a small chromosome in each species, whereas 18S rDNA *loci* always were located in the interstitial region of a large chromosome. Fig. 2a shows a hybridization signal of 18S rDNA in *A. tequilana* 'Azul' on pair 1, while the 5S rDNA signals are on both arms of pair 10; in *A. cupreata* (Fig. 2b), the hybridization signal of 18S rDNA is on pair 3, while the 5S rDNA signals are on both arms of pair 8; in *A. angustifolia* 'Lineño' and 'Cimarron' (Fig. 2c-d), the hybridization signal of 18S rDNA is on pair 5 and 2, respectively, while the 5S rDNA signals are on both arms of pair 11 in both varieties.

Discussion

Cytogenetic analysis showed the diploid chromosome number 2n = 60 in all species, which is in agreement with previous reports in the genus (Palomino et al. 2005, Palo-

Table 1. Karyotypes in *Agave* species (2n= 2x= 60).

Taxa and origin	Collector and voucher information	Karyotype formula	Secondary constriction
A. angustifolia 'Cimarron' Tolimán, Jalisco State, México. 19°32'06"N; 103°53'44"W (DMS).	Rodríguez JM C	42m + 4sm + 6st + 8t†	2t
A. angustifolia 'Lineño' Tolimán, Jalisco State, México. 19°32'06"N; 103°53'44"W (DMS).	Rodríguez JM L	48m + 2sm+ 2st + 8t†	2t
A. cupreata Miraval, Guerrero State, México. 17°43'00"N; 99°45'00"W (DMS).	Trinidad RA 573	42m + 2sm + 8st + 8t‡	2t
A. tequilana 'Azul' CIATEJ, Jalisco State, México. 20°41'39"N; 103°20'47"W (DMS).	Rodríguez JM A, C, D	42m + 12st + 6t§	2st

† = Palomino et al. unpublished data.

‡ = Karyotype published by Palomino et al. (2012).

§ = Karyotype published by Palomino et al. (2008).

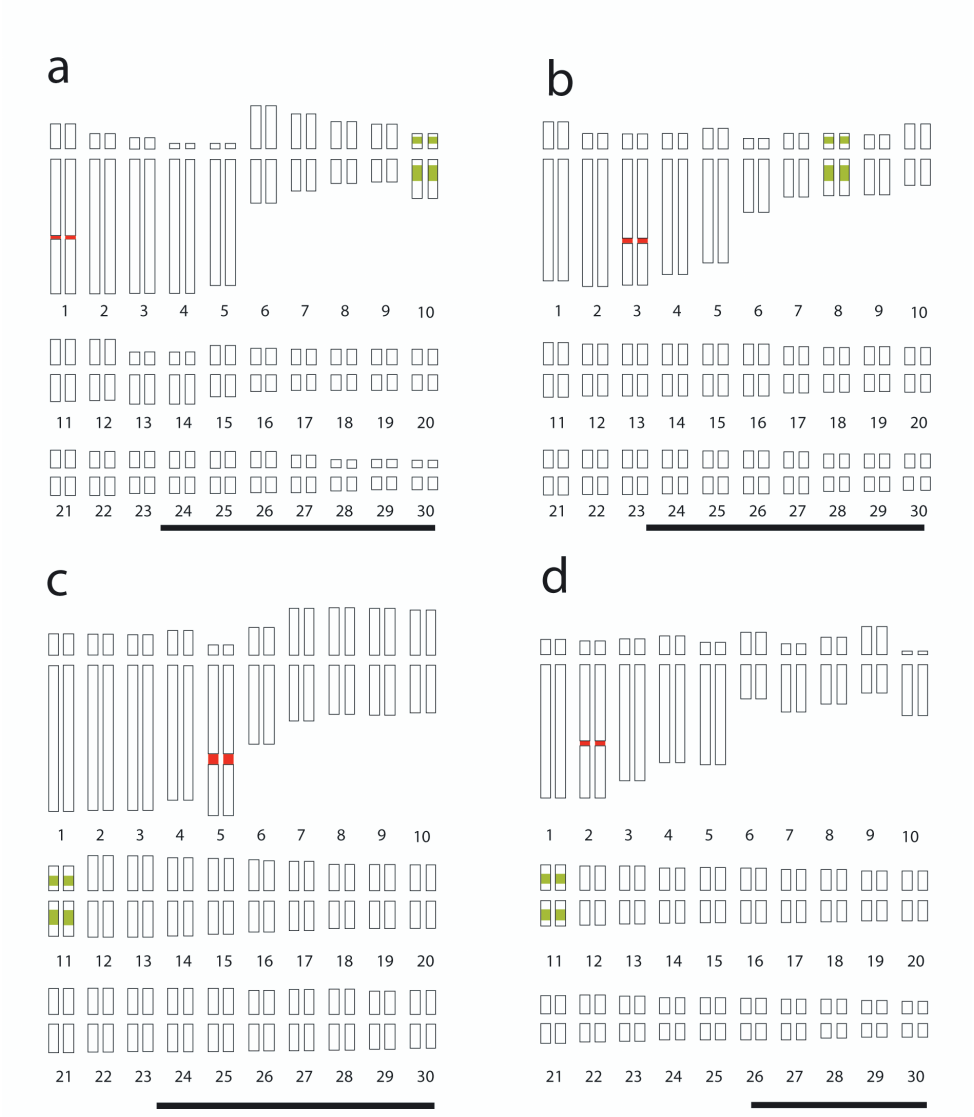

Figure 2. Idiograms of *Agave* karyotypes showing the 5S (green) and 18S (red) rDNA *loci*. **a** *A. tequilana* 'Azul' **b** *A. cupreata*; **c** *A. angustifolia* 'Lineño' **d** *A. angustifolia* 'Cimarron'. Bars = 10 μm.

mino et al. 2008, Palomino et al. 2010). All species showed a bimodal karyotype with small and large chromosomes (n = S + L); this bimodal karyotype is shared among multiple genera in Asphodeloideae (Brandham and Doherty 1998, Adams et al. 2000, Vosa 2005) and Agavoideae (McKelvey and Sax 1933, Brandham 1969) and recently, McKain et al. (2012) demonstrated that the Agavoideae bimodal karyotype was originated by an allopolyploid event, where the progenitor species seems to be extinct. Despite maintaining the same karyotype in all species, it was also found different karyo-

type formulae. This inter- and intraspecific variation shown here has been reported in other species and varieties in the genus (Banerjee and Sharma 1988, Moreno-Salazar et al. 2007, Palomino et al. 2008), leading to the formation of different cytotypes. Moreno-Salazar et al. (2007) studied three wild populations of *A. angustifolia* and found two different cytotypes; Palomino et al. (2008) analyzed eight varieties of *A. tequilana* and reported the same number of cytotypes. The presence of different cytotypes in *Agave* genus could be originated by heterozygous chromosomal exchange (Moreno-Salazar et al. 2007, Palomino et al. 2008, Palomino et al. 2010), which can modify the structure of chromosomes and maintaining at the same time their diploid number (Lima-Cardoso et al. 2013).

FISH with rDNA probes showed that *loci* of 18S and 5S rDNA in *Agave* species were located in different chromosomes and on similar position in all species; this finding suggests that the chromosomes bearing the rDNA *loci* are homeologous and the difference in numerical assignment is due to chromosomal rearrangements as mentioned before. 18S rDNA *locus* always was located in the interstitial region on the large arm of a large chromosome and associated to the secondary constriction, whereas the 5S rDNA *loci* were located in a proximal region on both arms of a small chromosome in all species. These results differ from Robert et al. (2008) because they reported that *Agave* species have one *locus* of 5S rDNA by monoploid genome in some diploid and polyploid species in the genus, including *A. tequilana* 'Azul' and *A. angustifolia* 'Letona' (tetraploid) and *A. angustifolia* 'Chelem ki' (hexaploid). The presence of 5S rDNA *loci* on both arms of a small chromosome in all species can be resulted from an unequal recombination or an event of transpositions; the latter have been reported previously in other monocots such as *Allium* Linnaeus, 1753 (Schubert and Wobus 1985), *Oryza* Linnaeus, 1753 (Shishido et al. 2000) and *Alstroemeria* Linnaeus, 1762 (Chacón et al. 2012). Recently, Khaliq et al. (2012), reported that Ty1-Copia retrotransposons are a major component of the *A. tequilana* genome (approximately 32 %) and might played a vital role in the organization and evolution of it, which could explain the results reported here.

To the best of our knowledge, here we reported the number and location of rDNA *loci* in two species with no previous report, *A. cupreata* and *A. angustifolia* 'Lineño' and 'Cimarron' as well as a different *locus* of 5S rDNA in all species studied. Data of FISH analysis provides new information about physical mapping of rDNA in *Agave* and such identified sites can be useful as chromosome markers for chromosome identification in hybrids in breeding programs as well as in evolutionary studies.

Conclusions

Despite the great diversity of the genus *Agave* which includes 166 species, the physical mapping of rDNA or other molecular markers are scarce, since just about five species have been described. The different karyotype formulae found in all species indicated the presence of cytotypes and data of FISH of rDNA allowed the physical mapping of *A. cupreata* and two new varieties of *A. angustifolia*. This work provides new informa-

tion about the position and number of rDNA *loci* in *Agave* species through comparative karyotype analysis, however, further cytogenetic research must be conducted to understand the evolution of this genus and develop breeding programs to preserve its biodiversity.

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RESEARCH ARTICLE

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Karyotype and chromosome banding of endangered crucian carp, *Carassius carassius* (Linnaeus, 1758) (Teleostei, Cyprinidae)

Martin Knytl[1], Lukáš Kalous[1], Petr Ráb[2]

1 *Department of Zoology and Fisheries, Faculty of Agrobiology, Food and Natural Resources, Czech University of Life Sciences Prague, 16521 Praha 6 - Suchdol, Czech Republic* **2** *Laboratory of Fish Genetics, Institute of Animal Physiology and Genetics, AS CR v.v.i., 277 21 Liběchov, Czech Republic*

Corresponding author: *Lukáš Kalous* (kalous@af.czu.cz)

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Abstract

The karyotype and other chromosomal characteristics the crucian carp (*Carassius carassius* (Linnaeus, 1758)) were revealed by means of conventional banding protocols (C, CMA_3, AgNOR). The diploid chromosome number (2n) in this species was 100. Its karyotype was composed of 10 pairs of metacentric, 18 pairs of submetacentric and 22 pairs of subtelo- to acrocentric chromosomes without any microchromosomes. C-banding identified blocks of telomeric heterochromatin on seven chromosome pairs. The NORs were situated on the p arms of the 14^{th} pair of submetacentric chromosomes and on the p arms of the 32^{nd} pair of subtelo-acrocentric chromosomes; AgNOR-positive signals corresponded to the CMA_3-positive signals. These chromosome characteristics may suggest a paleo-allotetraploid origin of *C. carassius* genome.

Keywords

Fish cytogenetics, paleotetraploid, heterochromatin, metaphase chromosomes

Introduction

The crucian carp, *Carassius carassius* (Linnaeus, 1758), is a cyprinid fish that inhabits densely vegetated backwaters and oxbows of lowland rivers, shallow lakes and ponds. It is a native species to Europe with a distribution extending eastwards from the River Rhine to the River Kolyma in Siberia (Szczerbowski 2002, Kottelat and Freyhof 2007). Despite its ability of "tissue breathing" (Blažka 1958) which helps it to survive in unfavourable conditions, the crucian carp has undergone a substantial decline in many localities during the last decades (Navodaru et al. 2002, Kottelat and Freyhof 2007, Sayer et al. 2011). Indisputable disappearance from nature resulted in the inclusion of the crucian carp in the list of endangered species by authorities of several EU countries (Economidis 1995, Schiemer and Spindler 2006, Copp et al. 2008, Sayer et al. 2011).

There is a number of factors that may have contributed to the disappearance of *C. carassius*, including habitat loss and degradation (Copp 1991, Holopainen and Ikari 1992, Wheeler 2000), displacement via competition with introduced species such as the polyploid biotype of the Prussian carp *Carassius gibelio* (Bloch, 1782), the Amur sleeper *Perccottus glenii* (Dybowski, 1877), feral goldfish *Carassius auratus* (Linnaeus, 1758) and the common carp *Cyprinus carpio* (Linnaeus, 1758) (Tarkan et al. 2012, Litvinov and O'Gorman 1996, Copp et al. 2005, Lusk et al. 2010). Moreover, all species of *Carassius* Nilsson, 1832 present in Europe (Rylková et al. 2013), including the crucian carp (*C. carassius*), Prussian carp (*C. gibelio*), ginbuna (*Carassius langsdorfii* Temminck & Schlegel, 1846) and goldfish (*C. auratus*) are often confused due to their morphological similarity (Hensel 1971, Kalous et al. 2007). Such confusion may lead to inappropriate stocking of wrong species instead of intended support of a local endangered population of crucian carp with negative consequences (Sayer et al. 2011).

Genetic contamination seems to be a very important but hidden threat to *C. carassius* that has been recently discovered. Hybridization occurs between *C. carassius* and *C. gibelio* (Prokeš and Baruš 1996). This type of hybridization was later confirmed using molecular (Papoušek et al. 2008, Wouters et al. 2012) and cytogenetic techniques (Knytl et al. 2013) in Sweden and the Czech Republic. Hybrids between *C. carassius* and *C. auratus* (Hänfling et al. 2005, Smartt 2007) and intergeneric hybrids between *C. carassius* and *Cyprinus carpio* (Hänfling et al. 2005) were discovered in England also by using microsatellite analysis. We believe that these processes also take place in other localities where *C. carassius, C. auratus* and/or *C. gibelio* co-occur. Moreover, molecular data suggest that these hybrids are able to reproduce and form filial generations by backcrossing (Hänfling et al. 2005, Wouters et al. 2012).

The cytogenetics of *C. carassius* is still poorly understood, since only a few studies of this species based on Giemsa-stained chromosomes are known (Table 1). Interestingly, two different diploid chromosome numbers 2n = 50 and 2n = 100 were reported.

Such an unclear situation encourages us to present cytogenetic analyses of *C. carassius* with respect to ongoing hybridization processes and threats in European waters. The present study deals with chromosomal characteristics of crucian carp (*C. carassius*)

Table 1. Chromosome numbers and karyotypes of *Carassius carassius* reported from Europe; NA= not available.

2n	Diploid karyotype	Locality	Source
104	20m+72sm+12a	NA	Chiarelli et al. 1969
100	20m+44sm+36a	France	Hafez et al. 1978
100	52m-sm+48 st-a	Drina R., Ukrinski Lug (Bosnia)	Sofradžija et al. 1978
100	20m+40sm+40a	the Netherlands	Kobayasi et al. 1970
50	20m+12sm+18s-ta	lower Danube R. (Romania)	Raicu et al. 1981
100	48m-sm+52st-a	Russia	Vasilev and Vasileva 1985
100	NA	Elbe R. System (Czech Republic)	Mayr et al. 1986
100	NA	Vistula R. System (Poland)	Boroń et al. 2010
100	20m+36sm+44st-a	Elbe R. System (Czech Republic)	This study

from the locality Byšičky in vicinity of the Elbe River (Czech Republic). Prussian carp (*C. gibelio*) and crucian carp co-occur in this place and the a hybrid allopolyploid female with 206 chromosomes was recently discovered there (Knytl et al. 2013). In this paper, we have used Giemsa staining as well as banding techniques like C, CMA_3, AgNOR and DAPI (4', 6-diamino-2-phenylindole) banding.

Material and methods

Fish sampling

Four females and one male were collected during a field survey of ichthyofauna in alluvial ponds and old oxbows of the Elbe River close to the city of Lysá nad Labem (GPS: 50°10.75' N, 14°47.62' E). All five individuals were identified morphologically as common *Carassius carassius* (not the dwarf form) according to Szczerbowski (2002) and Kottelat and Freyhof (2007). This material is deposited as voucher specimens in the collection of the Department of Zoology and Fisheries, Czech University of Life Sciences Prague under number KZR141083Cc.

Chromosome preparation and staining

All collected fish were subjected to a non-destructive procedure for chromosome preparation from fin clips developed by Völker and Kullmann (2006) and modified by Kalous et al. (2010); 50 metaphases from each individual were analyzed. Metaphase chromosomes stained in 4 % Giemsa-Romanowski solution in phosphate buffer (pH = 7) were counted with PC software QuickPhoto Micro. Karyotypes were arranged using PC software Ikaros (karyotyping system), version V 3.4.0 and Adobe Photoshop, version CS7. Chromosome morphology was determined according to Levan et al. (1964). Analyzed slides with recorded co-ordinates of selected metaphases were cleaned in xy-

lene for 2 minutes, then in benzoin for 2 minutes and finally destained in fixative (methanol: acetic acid; 3:1, v/v) for 3 minutes. Chromosome slides were then stored at +4°C for 12 hours before banding experiments. Chromosome banding (CMA_3, DAPI, C and AgNOR) was carried out according to Rábová et al. (2013). Different slides were used for each banding method (non-sequential chromosome banding), except for the sequential DAPI + CMA_3. Valid Animal Use Protocols were in force at the Institute of Animal Physiology and Genetics and Czech University of Life Sciences Prague during this study.

Microscopy and image processing

CMA_3, DAPI, C-banding and AgNOR images were captured with a cooled CCD camera Olympus DP30BW (equipped with a black-and-white (B&W) CCD-Chip Sony ICX285-AL) coupled to an epifluorescence microscope Olympus AX70 equipped with a set of 3 narrowband fluorescent filters. Micrographs were captured with the Olympus Acquisition Software and B&W images were processed with the software Micro Image. Altogether 200 images (metaphases), i.e. 50 images for each banding type (CMA_3, DAPI, C and AgNOR) were taken and analyzed.

Results

Karyotype

The diploid chromosome number of the examined individuals was invariably 2n = 100 (75 % investigated metaphases). The karyotype consisted of 10 pairs of metacentric (m), 18 pairs of submetacentric (sm) and 22 pairs of subtelo- (st) to acrocentric (a) chromosomes without any microchromosomes (Fig. 1).

Chromosome banding and AgNOR staining

Sequential banding (DAPI + CMA_3) revealed four CMA_3-positive bands situated at the sites of the secondary constrictions on the p arms of the 14^{th} pair of sm chromosomes and on the p arms of the 32^{nd} pair of st-a chromosomes (Figs 2b, c, e, f). DAPI uniformly stained all chromosomes (Figs 2a, d). AgNOR analysis revealed four positive signals (Figs 3a, b) which corresponded to four CMA_3 positive signals. C-banding detected blocks of constitutive heterochromatin at the telomeric and pericentromeric chromosome regions (Figs 4a, b). Telomeric signals were more intensive than pericentromeric ones. C-banded chromosomes were arranged in an karyotype (Fig. 5). Seven chromosome pairs had conspicuous C-banded arms.

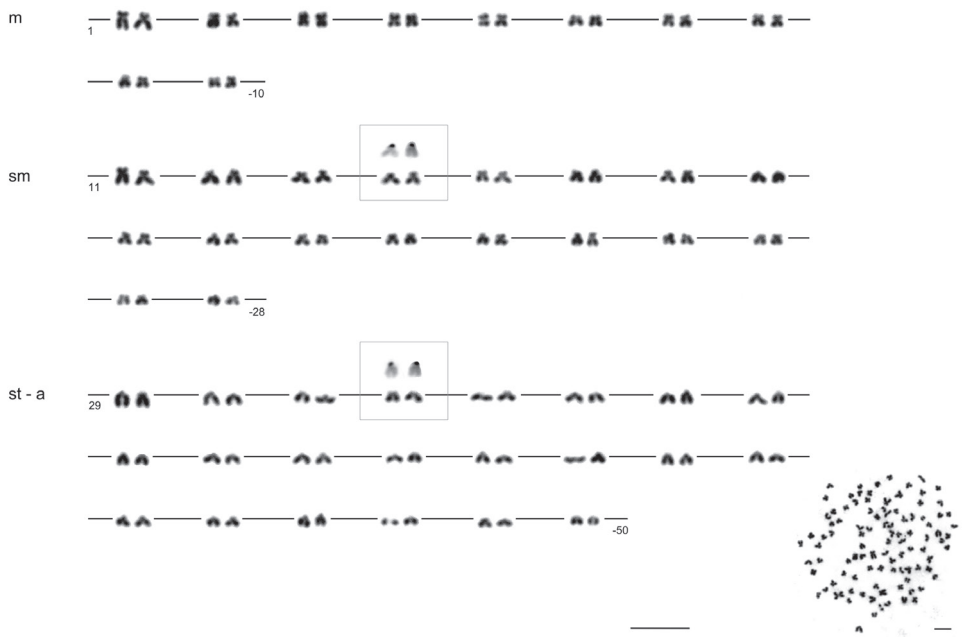

Figure 1. Karyotype of *C. carassius* female arranged from Giemsa-stained chromosomes (shown as inlay); m – metacentric, s – submetacentric, st – subtelocentric, a – acrocentric chromosomes. Four CMA_3-positive (color-inverted) chromosomes (14th pair of sm chromosomes and 32nd pair of st-a chromosomes) are additionally shown in the frames. Bar = 10 μm.

Discussion

The karyotype of all the five individuals of crucian carp from Byšičky ox-bow had the same diploid chromosome number 2n = 100. This number equalled the value reported in other previous studies (Table 1) except those by Raicu et al. (1981) and Chiarelli et al. (1969). Interestingly, Raicu et al. (1981) found the diploid chromosome number 2n = 50 in individuals from the Danube Delta. Although this report might be a result of a laboratory-generated error (slide mix-up), our closer inspection of the published karyotype did not provide any obvious answer. Vasilev and Vasileva (1985) discussed the finding of Raicu et al. (1981) and suggested that the presented karyotype belonged to a member of the genus *Gobio* Cuvier, 1816. At present, it is difficult to speculate more about the observed difference between the reported chromosome numbers unless detailed population screening of this species will be available. In contrast to the results obtained by Raicu et al. (1981), the diploid number of 104 chromosomes presented by Chiarelli et al. (1969) could be most likely attributed to preparation artifact.

The present study demonstrated that karyotype of individuals of *C. carassius* under study possessed 10 pairs of metacentric, 18 pairs of submetacentric and 22 pairs of subtelo- to acrocentric chromosomes, already reported by Knytl et al. (2013) as a haploid

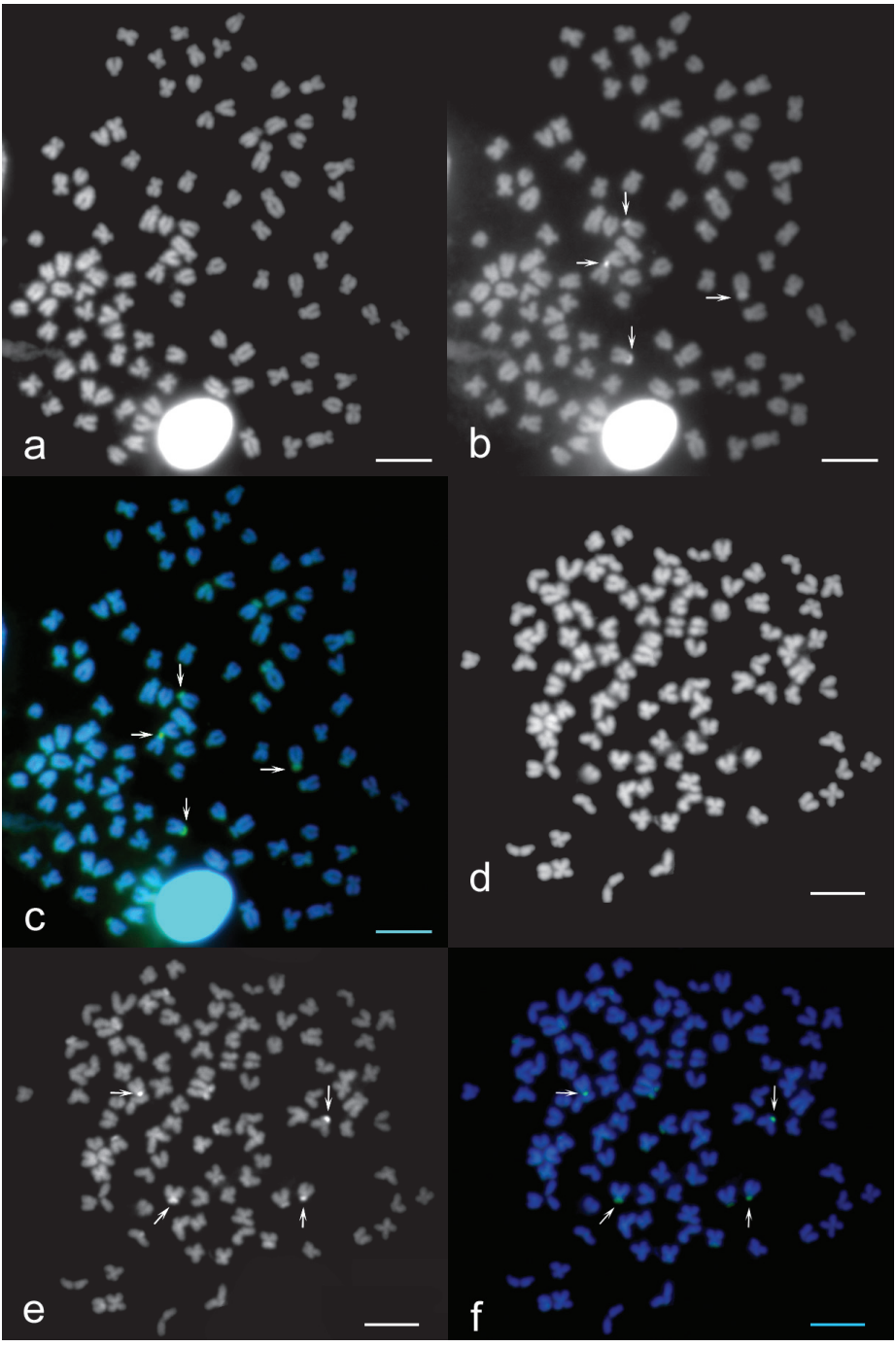

Figure 2. a–f Sequential chromosome banding of *C. carassius* female chromosomes. Metaphases counterstained by DAPI show all 100 chromosomes (**a**, **d**), metaphases stained by CMA_3 show 4 NORs (**b, e** white arrows) and the combination of these bandings show 4 identical NORs (**c, f** white arrows; green signals). Bar = 10 μm.

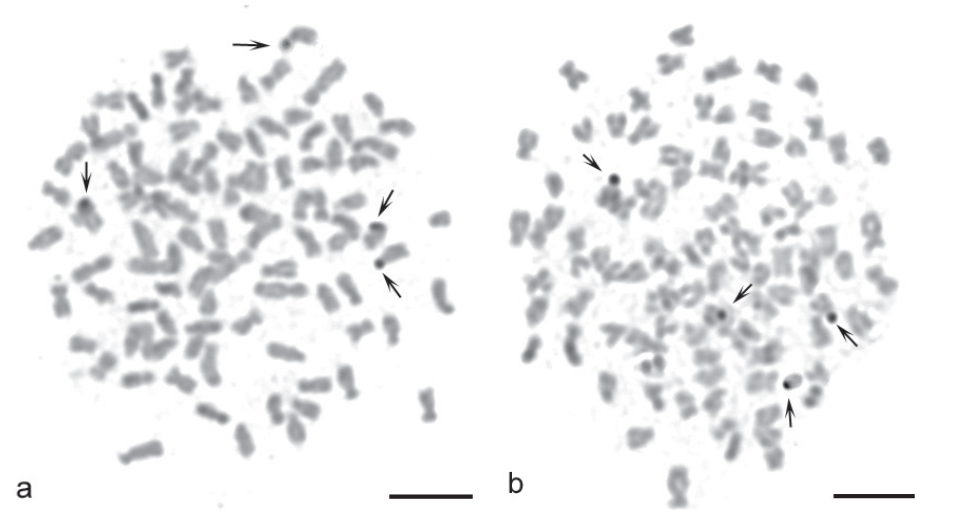

Figure 3. a–b AgNOR staining metaphases of *C. carassius* female (**a, b** black arrows) indicate 4 NOR-positive sites. Bar = 10 μm.

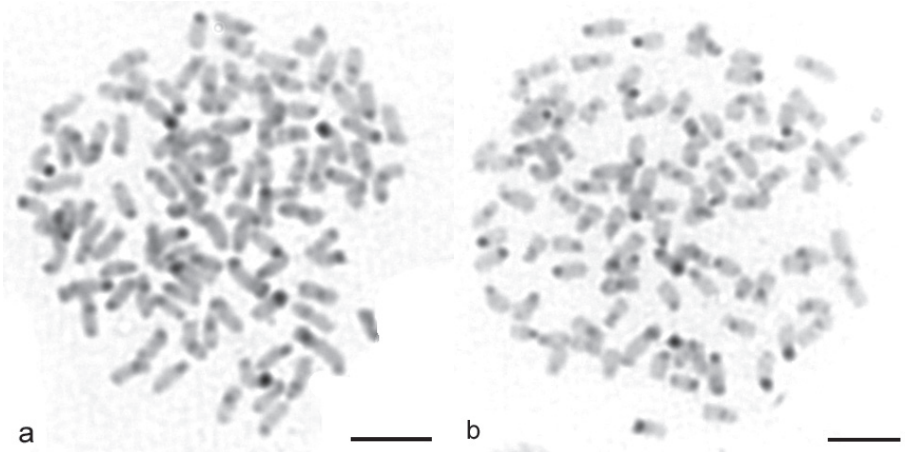

Figure 4. a–b C-banded metaphases of *C. carassius* female (**a, b**) show signals localized in the telocemeric and pericentromeric chromosome regions. Bar = 10 μm.

Figure 5. karyotype of *C. carassius* female arranged from C-banded chromosomes. Seven pairs of chromosomes show significant signals (black arrows). Bar = 10 μm.

component of the genome of the allopolyploid female of *C. gibelio*. Arrangement of chromosomes within the karyotype was different compared with other findings (i.e. Hafez et al. 1978, Sofradžija et al. 1978), probably due to a different level of chromosome spiralization (Ráb and Collares-Pereira 1995). Two other available studies dealing with the number, location and chromosomal characteristics of the major rDNA sites (Mayr et al. 1986, Boroń et al. 2010) showed four chromosomal sites on two different sm pairs of chromosomes. We also observed this pattern, i.e. four mutually corresponding CMA_3 and AgNOR signals respectively, on the secondary constrictions on the short arms of a single pair of sm chromosomes and another pair of st-a chromosomes. Though this chromosomal pattern is very common, it represents an additional evidence in favor of paleotetraploidy of the crucian carp genome as suggested by Vasilev and Vasileva (1985). This hypothesis must be examined using other techniques, since it was proven in other similar cases when common carp *Cyprinus carpio* (Larhammar and Risinger 1994, David et al. 2003, Zhang et al. 2008) as well as various species of *Barbus* Cuvier, 1816 (*sensu lato*) (Chenuil et al. 1999) were also revealed as evolutionary tetraploids based on sequences and substitutions analyses, as well as microsatellite analyses respectively.

DAPI-counterstained chromosomes did not provide any useful information since the observed signals were uniform throughout the chromosomes. Similar results were reported for *C. gibelio* by Zhu and Gui (2007).

We have performed C-banding on chromosomes of *C. carassius* for the first time. Constitutive heterochromatin blocks detected by C-banding method were located in telomeric regions of 7 pairs of chromosomes. Number of these signals can be a species-specific marker, especially in paleotetraploid forms.

Although there is no information about sex differences between *C. carassius* karyotypes, we have to point out that only one male specimen was included in this study

In respect to its status of a highly endangered fish species and unclear distribution of possible diploid and/or paleotetraploid forms as well as ongoing hybridization process with other species of this genus across its range of distribution, the present study is a moderate but important contribution to the cytogenetics and cytotaxonomy of *C. carassius*.

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Karyological evidence for diversification of Italian slow worm populations (Squamata, Anguidae)

Marcello Mezzasalma[1], Fabio Maria Guarino[1], Gennaro Aprea[1], Agnese Petraccioli[1], Angelica Crottini[2], Gaetano Odierna[1]

1 *Dipartimento di Biologia, Università di Napoli Federico II, Via Cinthia I- 80126 Napoli, Italy* **2** *CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Campus Agrário de Vairão, R. Padre Armando Quintas, 4485-661 Vairão, Vila do Conde, Portugal*

Corresponding author: *Marcello Mezzasalma* (marcello.mezzasalma@unina.it)

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Abstract

A karyological analysis on six Italian populations the slow worm (*Anguis veronensis* Pollini, 1818) was performed and their genetic differentiation at the mitochondrial 16S rRNA gene fragment from a Spanish sample has been assessed. The Italian populations were karyologically uniform, all showing 2n= 44 elements, of which 20 were macrochromosomes and 24 microchromosomes. Comparison with literature data on Central European populations showed a difference on the morphology of the 10th chromosome pair: submetacentric in Italian populations and telocentric in the Central European ones. Our analysis showed the presence of a fragile site on chromosomes of this pair, suggesting its propensity for structural rearrangements. Analysis of the 16S rRNA gene fragment showed uniformity among Italian populations (uncorrected genetic distance of 0.4%), and their genetic distinctness from the Spanish individual (uncorrected genetic distance of 4.2%). Our results confirm the existence of two different *Anguis fragilis* Linnaeus, 1758 lineages, each one characterized by a different cytotype.

Keywords

Karyotype, chromosome banding, 16S rRNA, *Anguis*, Italian Peninsula

Introduction

Until recently there were only two recognized species of the genus *Anguis* Linnaeus, 1758 in the Palaeartic region: *A. cephallonica* Werner, 1894 and *A. fragilis* Linnaeus, 1758, commonly known as slow worms. The first species was considered a Mediterranean endemic restricted to the Peloponnese and some Ionian islands, while the second (*A. fragilis*) was considered a widespread taxon distributed from Western Europe to NW Iran and from the Mediterranean coast to Scandinavia, with a broad altitudinal distribution, ranging from the sea level up to 2300 m above sea level (Gasc et al. 1997, Sindaco et al. 2006). Two recent studies (Gvoždik et al. 2010, 2013) based on the analysis of mitochondrial and nuclear genes and morphology redefined the distribution and the phylogenetic relationships of various populations of the genus *Anguis* and identified five main distinct evolutionary lineages: *A. fragilis* sensu stricto (present in Austria, Bosnia and Herzegovina, Bulgaria, Croatia, Czech Republic, France, Germany, Great Britain, Greece, Hungary, Italy, Macedonia, Montenegro, Norway, Poland, Portugal, Serbia, Slovakia, Slovenia, Spain, Sweden and Switzerland), *A. graeca* Bedriaga, 1881 (in Albania, Greece and Montenegro), *A. cephallonica* (limited to the Peloponnese), and *A. colchica* (Nordmann, 1840) (widely distributed from Eastern Europe to Iran) and subdivided into three main lineages (*A. c. colchica*, *A. c. incerta* and *A. c. orientalis*) each with a geographically distinct distribution; and more recently *A. veronensis* Pollini, 1818 (present in Italian peninsula and some areas from south-eastern France). Both the mitochondrial (mtDNA) and the nuclear (nuDNA) analyses analyses identified *A. cephallonica* as the sister lineage of a wider clade comprising *A. fragilis, A. colchica* and *A. graeca* (Gvoždik et al. 2010), while the position of the Italian lineage still remains unresolved (Gvoždik et al. 2013). The proposed species overall parapatric distributions, excluding some apparently isolated populations of *A. fragilis* sensu stricto from Greece (Gvoždik et al. 2010). In a previous karyological study (Gigantino et al. 2002) on the herpetofauna from the Matese Regional Park (Campania, Southern Italy) showed that local slow worm populations had a different chromosome formula when compared with available karyological data of *A. fragilis* (Dalq 1921, Margot 1946). Those karyological data, indeed scarce and dated, referred to referred to Central European populations of *A. fragilis* and described a karyotype of 2n=44 elements, of which 20 were macrochromosomes (the first, the fourth and the fifth pairs shaped as metacentric and the rest as telocentric elements) and 24 microchromosomes, arm number (A.N.) = 48. The populations of the Matese Regional Park differed in showing as submetacentric the elements of the tenth macrochromosome pair (A.N.= 50). On the basis of the recent finding of Gvoždik et al. (2013) and our previous karyological data (Gigantino et al. 2002), we extended the chromosome analysis to other Italian populations. The results of this comparative karyological study are here presented together with the re-worked analysis on populations from Matese Regional Park. In addition, since in Balkan Peninsula two endemic *Anguis* species were found (Gvoždik et al. 2013), we also perfomed a molecular analysis of a fragment of the mitochondrial 16S rRNA gene in order to test the genetic uniformity of the studied Italian specimens.

Material and methods

Eleven individuals from six geographically distinct Italian localities were analysed in this work: Travacò Siccomario (Pavia, northern Italy), one male; Ancona (central Italy), two males; Valle Agricola (Caserta, southern Italy), one male and one female; Giffoni Valle Piana (Salerno, southern Italy), one male and one female; Ruvo del Monte (Potenza, southern Italy), two males and one females; Monte Cocuzzo (Cosenza, southern Italy), one males. As already successfully performed (Gigantino et al. 2002), adult individuals were sexed in the field by means of hemipenis extroversion before their release. For the samples of the Matese Regional Park we benefitted from stored chromosome suspensions obtained from organs (see Odierna et al. 2004 for details) kept in the herpetological collection of one of the co-author (G.F.M.). For all other samples, chromosomes were obtained by establishing blood cultures using a freshly collected blood sample (100–500 µl), drawn on the field from the caudal vein, incubated for 5 days in 3 ml of peripheral blood karyotyping medium (Biological Industries). Colcemid (100 ng/ml) was added to culture two hours before harvesting. After washing in Phosphate Buffer Saline (PBS) 1x, and incubation in hypotonic solution (KCl 0,075 M) per 30 min, cells were fixed in 3:1 methanol-acetic acid. This non-invasive method, useful for both karyologycal and molecular investigations, allows the avoidance of specimen sacrifice and animals were released at capture sites immediately after the sampling. In addition to standard staining method (5% Giemsa solution at pH 7) we performed various chromosome banding techniques: Ag-Nor banding (Howel and Black 1980), C-banding (Sumner 1972), C-banding+CMA_3+DAPI staining (Odierna et al. 2004), Chromomycin A_3-methyl green staining (Odierna et al. 2007); G banding performed on 10 days old slides by a brief incubation (10–30 sec) in a 0,05% trypsin solution (Odierna et al. 1994). For Late Replicating banding pattern (LR), bromedeoxyuridine (BrdU; 35 µg/ml) was added to blood cultures during the last six hours and differential staining was revealed by staining chromosomes with 4% Giemsa solution in 2% 4Na-EDTA for two minutes (Odierna et al. 2004).

Total genomic DNA was extracted from blood cells using conventional phenol-chloroform method (Sambrook et al. 1989). A fragment of ca. 320 bp of the mitochondrial 16S rRNA gene was amplified for one individual for each studied population using the primers 16Sa 5' - CGCCTGTTTACCAAAAACAT - 3' and 16Sb 5'-CCGGTCTGAAACTCAGATCAGT- 3' (Palumbi 1996). Amplification consisted of an initial denaturation step at 94°C for 5 min, followed by 36 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 30 s, followed by a final extension of 72°C for 7 min. Amplicons were sequenced on an automated sequencer ABI 377 (Applied Biosystems). Sequences were blasted in GenBank and chromatograms were checked by eye and edited, when necessary, using Chromas Lite© and the BioEdit sequence alignment editor (version 7.0.5.3; Hall 1999). Newly provided sequences were compared with a homologous sequence of a Spanish individual from Vilarmiel (Lugo province, Galicia, Spain) available in GenBank (Albert et

al. 2009; NC012431). A homologous sequence of *Ophisaurus attenuatus* Baird, 1880 (EU747729) from Castoe et al. (2008) was add to the alignment and used as outgroup in the phylogenetic analysis. The alignment of all sequences required the inclusion of gaps to account for indels in only a few cases. All newly determined sequences were submitted to the European Nucleotide Archive (ENA) (accession numbers: HG003678-HG003683). Uncorrected pairwise distances (*p*-distances transformed into percent) within individuals of *A. veronensis* and between species were computed using MEGA, version 5.05 (Tamura et al. 2011). Bayesian analyses were performed in MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003). The HKY model was determined by AIC in jModeltest (Posada 2008) as the best-fitting model of substitution. We performed two runs of 10 million generations (started on random trees) and four incrementally heated Markov chains (using default heating values), sampling the Markov chains at intervals of 1,000 generations. Stabilization and convergence of likelihood values was checked by visualizing the log likelihoods associated with the posterior distribution of trees in the program Tracer (Rambaut and Drummond 2007). The first three millions of generations were discarded and seven thousand trees were retained post burn-in and summed to generate the majority rule consensus tree (Fig. 1).

Results and discussion

The alignment of the analysed 16S rRNA gene fragments showed a minimum of 14 nucleotidic substitutions (11 transitions and 3 transversions) and 2 insertion/deletions between samples from Italy and Spain, corresponding to an average uncorrected genetic distance of 4.2%, thus confirming the genetic distinctiveness of the Italian populations. On the contrary, the analyzed Italian specimens were genetically very uniform and showed an intraspecific uncorrected divergence of 0.4%, whereas their uncorrected genetic distance from the outgroup, *O. attenuatus*, was 12.8%. According to Gvoždik et al. (2010) the average genetic divergence at the ND2 mitochondrial locus among the several *Anguis* species is about 7%, while the maximum intraspecific distance within the various *A. colchica* clades is 4.4%. Unfortunately, we did not use the same molecular marker, but the observed 4.2% distance at the more conserved 16S rRNA gene fragment among Spanish and Italian samples provide further evidenced for the differentiation of these two lineages. Even if the phylogenetic analyses resulted in a tree (Fig. 1) with largely unresolved relationships, we could recover a good support for the monophyly of the Italian specimens, as well as for a clear mithocondrial segregation between them and the extra-Italian *Anguis* samples analysed in this study. Metaphase plates suitable for chromosome analysis were obtained from four out the six investigated populations (Travacò Siccomario, Valle Agricola, Ruvo del Monte and Monte Cocuzzo). All individuals showed a karyotype of 2n = 44 elements, comprising ten pairs of macrochromosomes and twelve pairs of microchromosomes. The first macrochromosomes pair was metacentric, the fourth and fifth were submetacentric and the others were telocentric with the exception of the tenth pair (Fig. 2A; Table

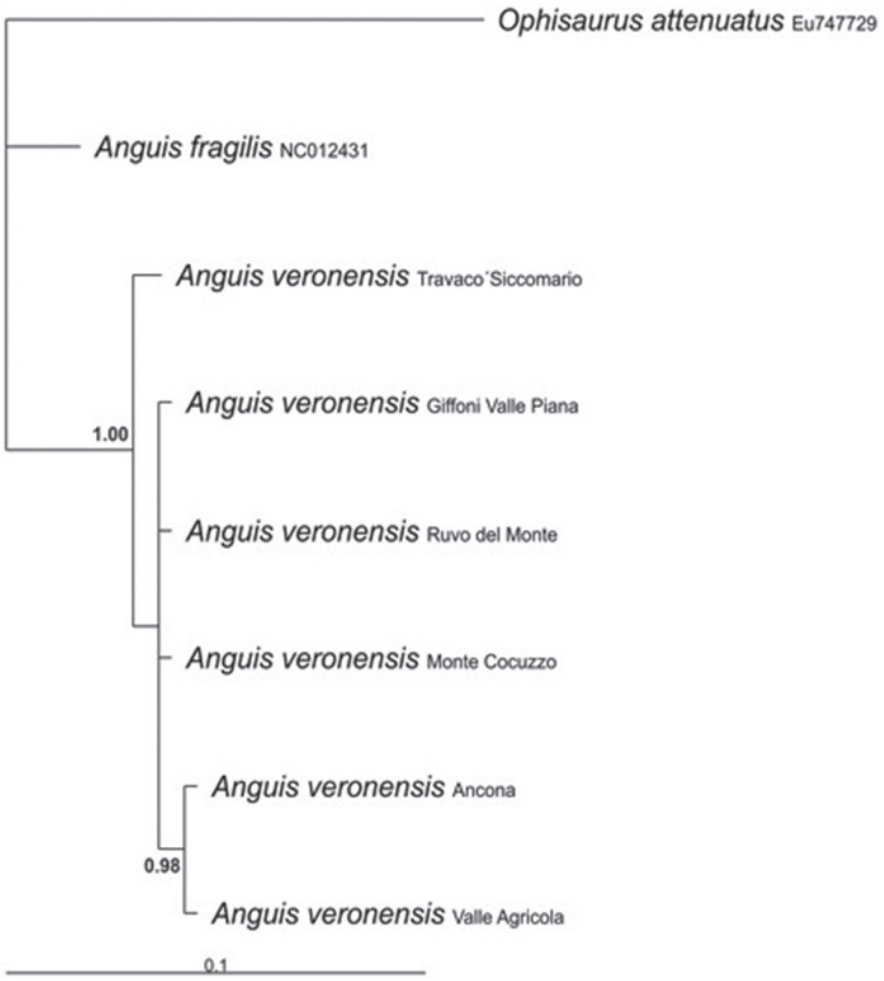

Figure 1. 70%-majority consensus tree derived from a Bayesian inference analysis of 321 bp of the mitochondrial 16S rRNA gene. *Ophisaurus attenuatus* was used as outgroup. Sequences retrieved from GenBank are marked with their accession numbers.

1). Chromosomes morphology did not differ between sexes and in any of the various Italian localities studied. The Ag-NOR banding revealed the presence of NOR loci on three microchromosome pairs in all examined specimens (Fig. 2B, arrows). This condition is considered an apomorphic character in Squamata, derived from a plesiomorphic single NOR locus (Porter et al. 1994, Aprea et al. 1996). The C-banding + Giemsa and the C-banding+fluorochromes colorations rrevealed small pericentromeric C-bands on almost all macrochromosomes and on three pairs of microchromosomes, namely those NOR bearing ones (Fig. 2C, D, E). Both G and LR-banding did not reveal any difference among analysed populations or between sexes (Fig. 3A, B). However, LR banding highlighted the presence of a uncondensed trait in three out

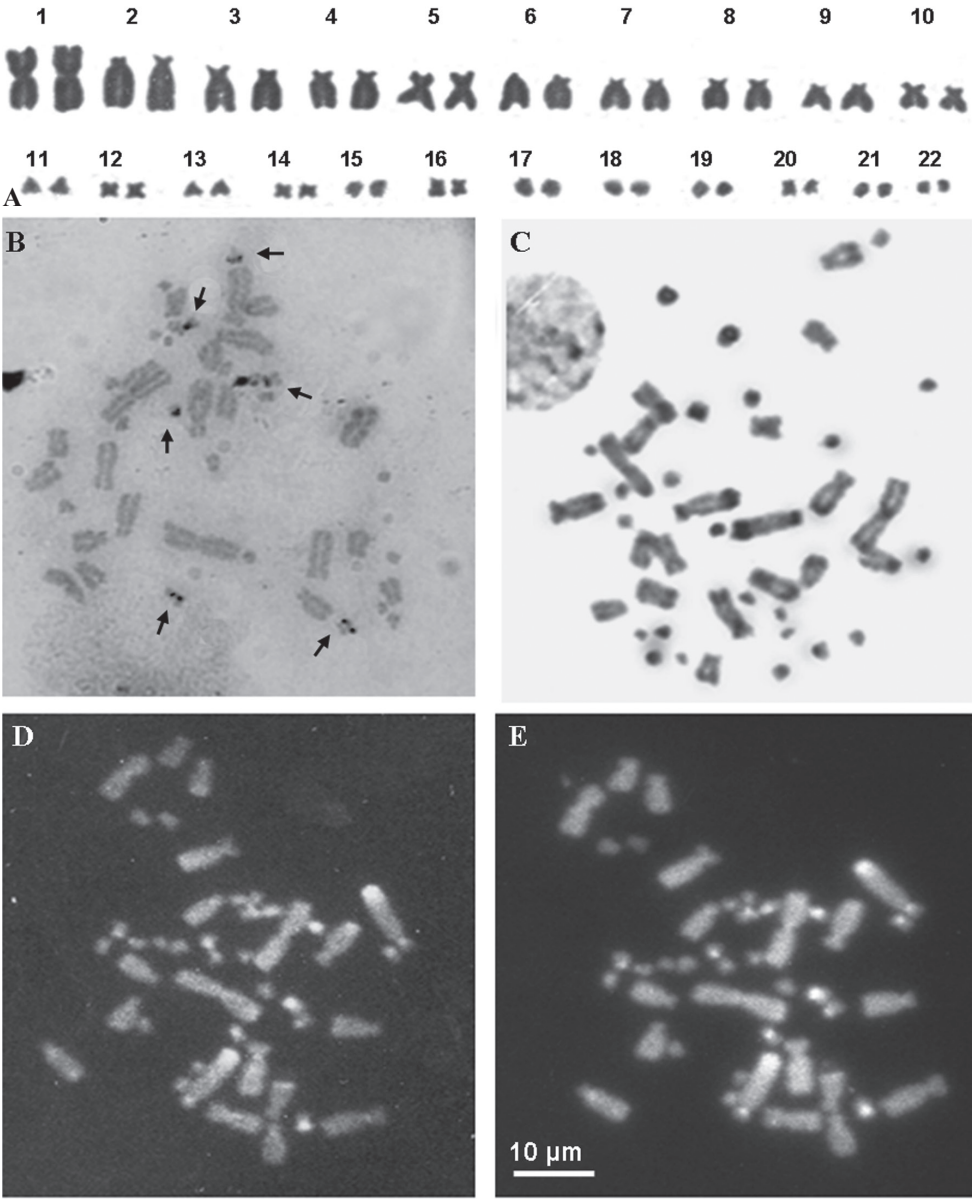

Figure 2. A Giemsa stained karyotype of an individual from Valle Agricola **B** Ag-NOR-banding stained metaphase plate of an individual from Ruvo del Monte **C**, **D**, **E** metaphase plate of an individual from Valle Agricola stained with C-banding (**C**) and C-Banding+ CMA_3 (**D**)+ DAPI (**E**). Arrows in (**B**) point at NOR loci.

of 20 examined metaphase plates (15% on the short arms of one chromosome of the tenth pair). This uncondensed trait was observed only on metaphase plates from cultures with BrdU addition and probably is a fragile site. In fact, it is known that BrdU promotes the expression of fragile sites (e.g. Sutherland 1979, Stone and Stephens

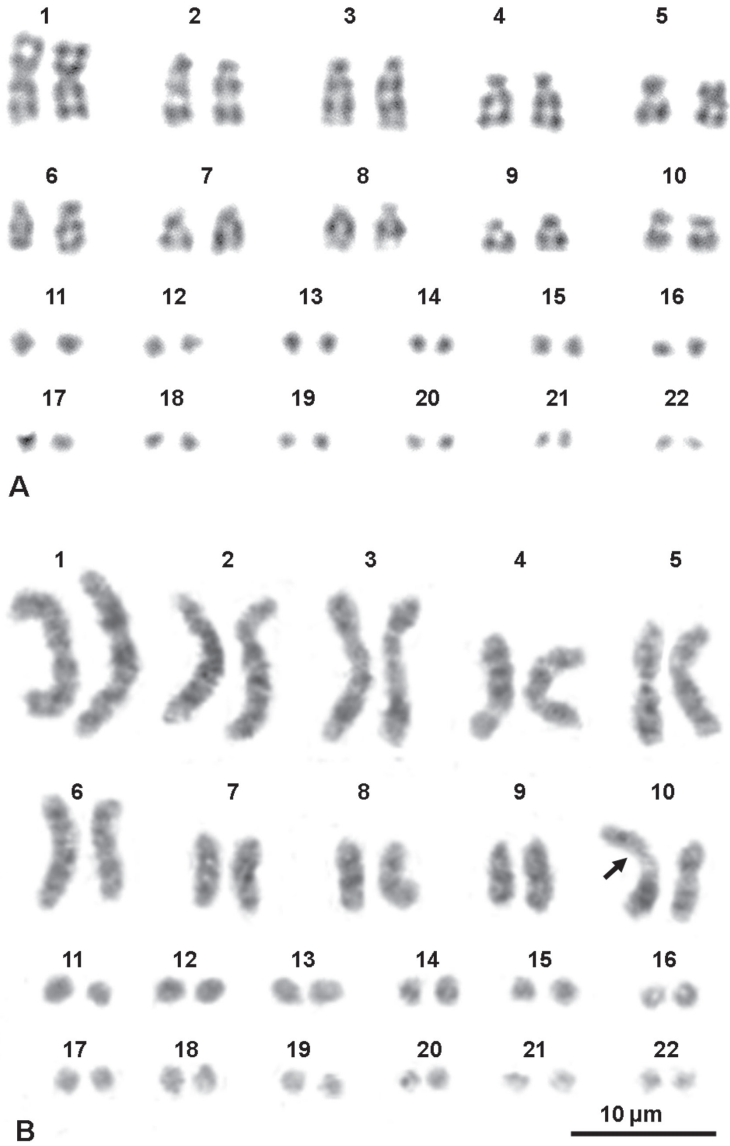

Figure 3. Karyotypes of a male from Travacò Siccomario (**A**) and of female from Ruvo del Monte (**B**) stained with G-banding and replication pattern, respectively. The arrow in (**B**) points at a fragile site.

1993) (Fig. 3B). Fragile sites are usually due to a DNA strand breakage and represent hot spots for recombination (Glover and Stein 1988). The presence of a fragile site on the tenth chromosome suggests the propensity for this chromosome for structural rearrangements. By chance, this chromosome pair karyologically differentiates Italian slow worm populations from Centro-European ones, this pair being shaped as submetacentric in the former populations and as telocentric in the Centro-European ones. A pericentric inversion can account for the different morphology of the tenth chromo-

Table 1. Chromosome relative length (RL), centromeric index (CI) and chromosome shape (CS) of studied samples of *A. veronensis*. The values of RL and CI are expressed as mean ± standard deviation. Chromosome morphology was measured according to Levan et al. (1964). For the microchromosomes 11–22 only their complessive RL value is provided.

chrom.	RL	CI	CS
1	15,2 ±3,5	0,45±3,6	metacentric
2	11,7 ±2,7	0,10±3,8	telocentric
3	9,7 ±3,3	0,08±4,4	telocentric
4	7,6±3,1	0,07±3,5	telocentric
5	7,4±3,4	0,42±3,6	metacentric
6	7,1±3,1	0,07±4,1	telocentric
7	6,2 ±3,1	0,10±3,1	telocentric
8	5,7 ±2,8	0,08±2,9	telocentric
9	5,1 ±2,7	0,07±4,0	telocentric
10	4,6 ±3,2	0,34±3,8	submetacentric
11–22	19,7 ±6,8	–	–

some pair. Due to the absence of karyological data on other congeneric species, the polarity of chromosome rearrangements cannot be unambiguously assessed. The role of chromosome rearrangements in speciation is heavily debated (White 1978, Odierna et al. 1996, Simard et al. 2009), as it is difficult to ascertain if chromosome changes occur after species diversification or if they are involved in the speciation process in itself, acting as proximate cause of diversification. Chromosome polymorphism as well as interspecific differences characterized by a single chromosome inversion are known in lizards (Odierna et al. 2004, Kupriyanova et al. 2008, Aprea et al. 1996). Thus, the difference in the chromosome 10th pair might have occurred during or after the diversification between Italian and central European lineages. It should be also taken into consideration that the different morphology of the 10th pair may be a consequence of a centromere repositioning, so far observed only among different species using comparative genome analysis (Montefalcone et al. 1999, Cardone et al. 2007).

To conclude, our karyological study is consistent and provides further support for the recently discovered molecular and morphological differentiation of the Italian slow worm lineage (Gvoždik et al. 2013), who ascribe the Italian populations to the recently resurrected *Anguis veronensis*.

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RESEARCH ARTICLE

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A chromosomal investigation of four species of Chinese Panorpidae (Insecta, Mecoptera)

Bo Xu[1], Yankai Li[2], Baozhen Hua[2]

1 *College of Life Sciences, Northwest A&F University, Yangling, Shaanxi 712100, China* **2** *Key Laboratory of Plant Protection Resources and Pest Management, Ministry of Education, Entomological Museum, Northwest A&F University, Yangling, Shaanxi 712100, China*

Corresponding author: *Baozhen Hua* (huabzh@nwsuaf.edu.cn)

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Abstract

The male adults of four species of the Chinese Panorpidae in Mecoptera were cytogenetically studied using conventional squashing procedures. The results show that their sex-chromosome system belongs to the XO type, with n = 19 + X(O) in *Panorpa emarginata* Cheng, 1949 and *Panorpa dubia* Chou & Wang, 1981, n = 23 + X(O) in *Panorpa* sp., and n = 20 + X(O) in *Neopanorpa lui* Chou & Ran, 1981. X chromosomes of these species usually appear dot-shaped in late prophase I and are easily differentiated from autosomal bivalents. Meiosis in these Panorpidae lacks typical diplotene and diakinesis. In late prophase I, pairs of homologous chromosomes remain parallel in a line and show no evidence of crossing-over. Some of them even appear as a single unit because of extremely intimate association, all with a tendency of increasing condensation. The evolutionary significance of their chromosomal differences and the achiasmatic meiosis of Panorpidae are briefly discussed.

Keywords

Mecoptera, Panorpidae, chromosome, XO sex-chromosome system, achiasmatic male meiosis

Introduction

Mecoptera are one of the minor orders of holometabolous insects with approximately 650 described species worldwide (Bicha 2010). They are unique in Holometabola because many taxa of them possess a pair of compound eyes on the head in their larval stages (Byers and Thornhill 1983). In this respect, Mecoptera may represent one of the basal lineages in the Holometabola, or more specifically the most basal taxon of Antliophora (Kristensen 1999).

Panorpidae are the most species-rich family in Mecoptera, with over 420 described species assigned to six genera (Ma et al. 2009, Ma and Hua 2011, Zhong and Hua 2013). They are commonly called scorpionflies because the male genital bulb (the ninth abdominal segment) is enlarged and recurved upward, superficially resembling the stinger of a scorpion (Esben-Petersen 1921). *Panorpa* Linnaeus, 1758 is the largest genus of Panorpidae and is such a diverse taxon that it is often subdivided into different species groups based on external morphological characters for regional faunas (Ma and Hua 2011). The genus *Neopanorpa* Weele, 1909 is an Oriental group in Panorpidae with more than 130 known species in the world to date (Cai and Hua 2009).

The cytogenetics of Mecoptera received a passing interest from the 1930s to the 1970s. To date, only some European and American species have been cytogenetically studied. Species of *Panorpa* were first reported to have an XO sex determination mechanism in males and to have a fairly high complement number (more than 40) by Naville and de Beaumont (1934). Subsequently, male hangingfly *Bittacus italicus* (Müller, 1766) was also reported to have XO sex chromosomes with 13 pairs of autosomes (Matthey 1950). Cooper (1951, 1974) found a different sex determination system in the family Boreidae: *Boreus brumalis* Fitch, 1847 possesses compound sex chromosomes X_1X_2Y with 11 pairs of autosomes in males and *Boreus notoperates* Cooper, 1972 possesses XO sex chromosomes with 9 pairs of autosomes in males. Ullerich (1961) found achiasmatic meiosis in three species of *Panorpa*. Later, to elucidate achiasmatic meiosis in *Panorpa,* Gassner (1969) investigated the synaptonemal complex and chromosome structure in the achiasmatic spermatogenesis of *Panorpa communis* Linnaeus, 1758. *Chorista australis* Klug, 1838 (Choristidae) was also found to possess XO sex-chromosome system (Bush 1967). According to cytological observations of spermatogenesis, Atchley and Jackson (1970) found that male scorpionflies of *Panorpa anomala* Carpenter, 1931 and *Panorpa acuta* Carpenter, 1931 have both achiasmatic meiosis and $2n = 45$ chromosomes, but male hangingflies of *Bittacus pilicornis* Westwood, 1846 and *Bittacus stigmaterus* Say, 1823 (Bittacidae) have chiasmatic meiosis with relatively low chromosome numbers ($2n = 29$ and 31, respectively).

To increase our knowledge of the cytogenetic nature and chromosomal evolution in Mecoptera, we studied meiosis in four species of the Chinese Panorpidae, including three species of *Panorpa* and one more species of *Neopanorpa*.

Materials and methods

Male adults of *Panorpa emarginata* Cheng, 1949*, Panorpa dubia* Chou & Wang, 1981*, Panorpa* sp.*,* and *Neopanorpa lui* Chou & Ran, 1981 were investigated using conventional squashing procedures. At least three specimens of each species were sampled. The examined species and their localities are listed in Table 1.

Testes of these species were extracted from ethyl ether anaesthetized specimens and subjected to hypotonic treatment in 0.48% solution of potassium chloride for 15 min, then fixed in a mixture of methanol and acetic acid (3:1) for 2 h. The fixed testes were squashed and stained with 1% Giemsa in Sörense buffer solution (0.067 mol/L, pH 6.8) for 10 min except for *P. emarginata*, which was stained with 2% hematoxylin solution for 10 sec. Photographs were taken with a Nikon DS-Fil digital camera equipped with a Nikon Eclipse 80i microscope.

Results

Panorpa emarginata Cheng

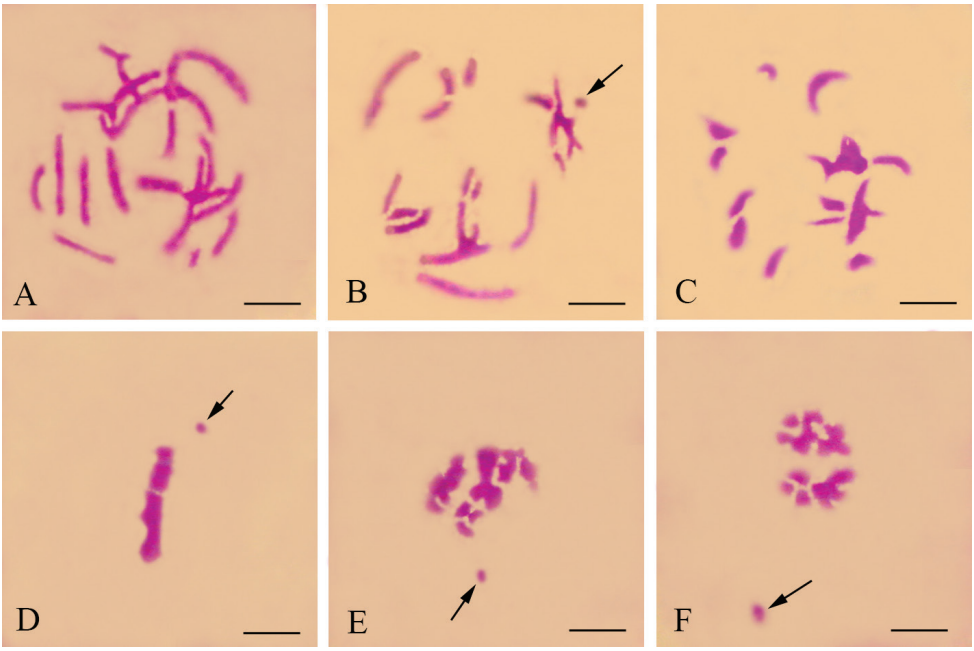
The males have a meioformula of n = 19 + X(O) (Fig. 3A). In pachytene (Fig. 1A), the X univalent and autosomal bivalents are all strip-shaped. It is difficult to distinguish the X univalent from autosomal bivalents. In late prophase I (Fig. 1B), the X univalent is dot-shaped and the homologous chromosomes of each bivalent are closely associated. No traces of crossing-over were observed. In pre-metaphase (Fig. 1C), the chromosomes become much more condensed. In the lateral view of metaphase I (Fig. 1D), all bivalents are located in the equatorial plate with the X univalent being precocious. In the lateral view of anaphase I (Figs. 1E, 1F), the X can also move ahead of other bivalents.

Panorpa dubia Chou & Wang

The males also have a meioformula of n = 19 + X(O) (Fig. 3B). In pachytene (Fig. 2A) these bivalents are rod-shaped and almost of the same size, but the X

Table 1. The examined species and their localities.

Species	Localities	Collection date
Panorpa emarginata	Taibai Mountain, Shaanxi	Early June 2007
Panorpa dubia	Huoditang Forest Farm, Shaanxi	Early June 2012
Panorpa sp.	Tongbai Mountains, Henan	Late July 2012
Neopanorpa lui	Nangong Mountain, Shaanxi	Middle June 2012

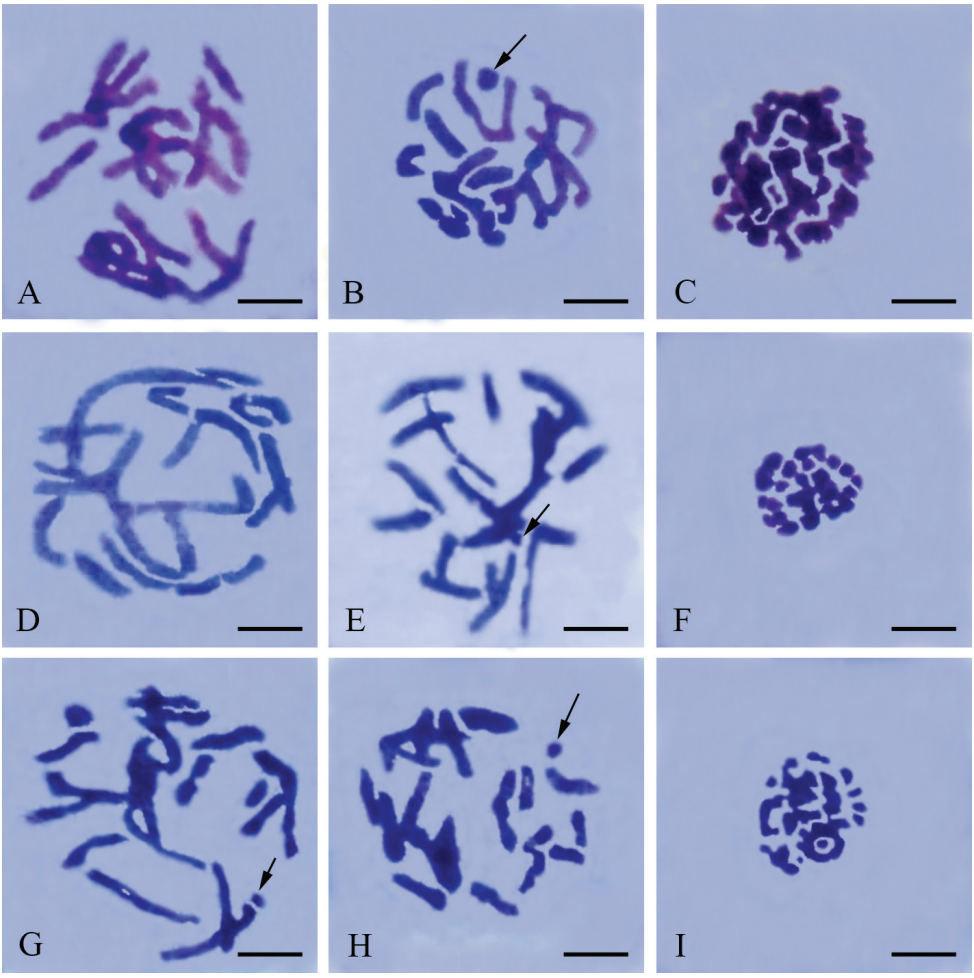


Figures 1. Meiotic chromosomes of male *Panorpa emarginata* subjected to hematoxylin staining. **A** pachytene **B** late prophase I showing the dot-shaped X univalent (arrow) and achiasmatic bivalents **C** metaphase I showing substantially more condensed chromosomes **D** lateral view of metaphase I showing congression of autosomal bivalents and precocity of X univalent (arrow) **E, F** lateral view of anaphase I showing precocity of X univalent (arrow). Bars = 10 μm.

univalent is difficult to observe. In late prophase I (Fig. 2B), the bivalents become more condensed and the X univalent appears dot-shaped. As in *P. emarginata*, homologous chromosomes are associated with each other so intimately that they appear as single units. No indication of crossing-over was observed. In the polar view of metaphase I (Fig. 2C), the majority of these bivalents present parallel-arranged homologous chromosomes.

Panorpa sp.

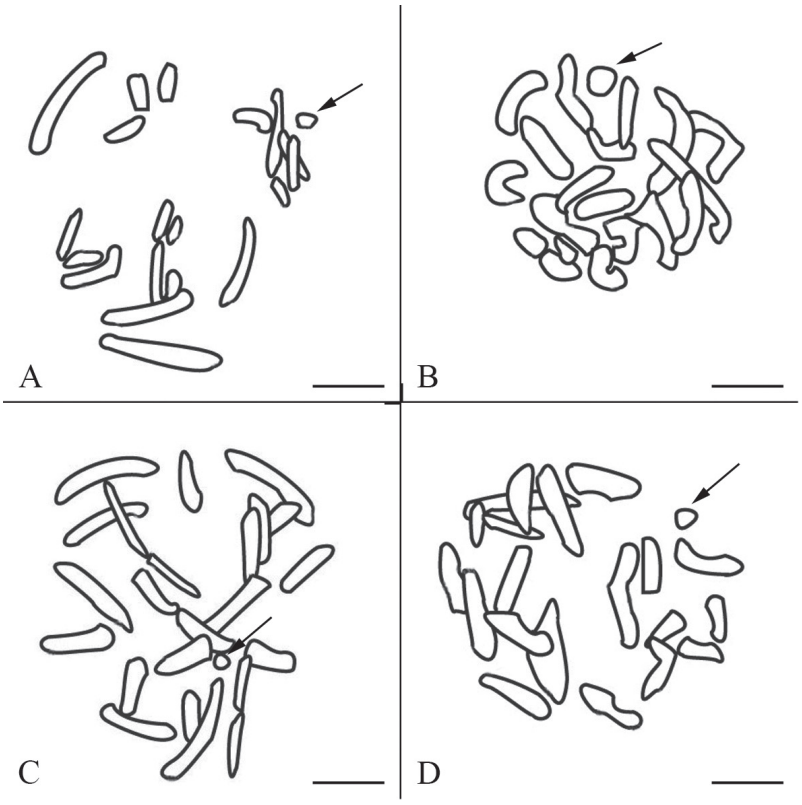
The males have a meioformula of n = 23 + X(O) (Fig. 3C). In pachytene (Fig. 2D) the X univalent is difficult to observe. In late prophase I (Fig. 2E), these bivalents become condensed and the X univalent is usually dot-shaped. In particular, some bivalents exhibit two parallel-arranged homologous chromosomes without chiasmata. In the polar view of metaphase I, only a few bivalents present parallel-arranged homologous chromosomes (Fig. 2F).



Figures 2. Meiotic chromosomes of male Panorpidae. **A–C** *Panorpa dubia* **D–F** *Panorpa* sp. and **G–I** *Neopanorpa lui* subjected to Giemsa staining **A, D** pachytene **B, E, G, H** late prophase I showing more condensed bivalents than in pachytene and the dot-shaped X univalent (arrow). **C, F, I** polar view of metaphase I. Bars = 5 μm.

Neopanorpa lui Chou & Ran

In males, the late prophase I reveals a meioformula of n = 20 + X(O) (Figs 2G, 3D). There are 20 bivalents of different sizes. Some of the bivalents present two parallel homologous chromosomes. The X univalent is usually dot-shaped. A more condensed stage without chiasmata was observed near the end of prophase I (Fig. 2H). In the polar view of metaphase I (Fig. 2I), only a few bivalents present parallel-arranged homologous chromosomes.



Figures 3. Schematic drawings of late prophase I chromosomes. **A** *Panorpa emarginata* **B** *Panorpa dubia* **C** *Panorpa* sp. and **D** *Neopanorpa lui* corresponding to Fig. **1B**, Fig. **2B**, Fig. **2E** and Fig. **2H**, respectively. The X univalents are indicated by arrows. Bars = 5 μm.

Discussion

The present work is the first cytogenetic description of the Chinese mecopteran species. In particular, we obtained first cytogenetic data for a *Neopanorpa* species. All the four examined species of Panorpidae possess a relatively high chromosome number, the males with an XO sex-chromosome system and achiasmatic meiosis.

Based on our study, the meioformula of males is $n = 19 + X(O)$ in *P. emarginata* and *P. dubia*, $n = 23 + X(O)$ in *Panorpa* sp., and $n = 20 + X(O)$ in *N. lui*. Their chromosome numbers are very similar to those of the European and American species of Panorpidae, whose meioformula is $n = 22 + X(O)$ in *P. communis*, *P. anomala* and *P. acuta*, $n = 21+ X(O)$ in *Panorpa cognata* Rambur, 1842, and $n = 20 + X(O)$ in *Panorpa germanica* Linnaeus, 1758 (Naville and de Beaumont 1934, Atchley and Jackson 1970). This stability implies a model diploid karyotype of Panorpidae of about 40 chromosomes. The existing small differences in chromosome number between these species likely result from chromosomal rearrangements in the evolutionary history of Panorpidae.

Compared with Panorpidae, other families of Mecoptera possess relatively low chromosome numbers. In Boreidae, males of *B. brumalis* have a meioformular of n = 11 + X_1X_2Y (Cooper 1951) and males of *B. notoperates* have a meioformula of n = 9 + X(O) (Cooper 1974). In Bittacidae, males of *B. italicus*, *B. pilicornis* and *B. stigmaterus* have meioformulas of n = 13 + X(O), n = 14 + X(O) and n = 15 + X(O), respectively (Matthey 1950, Atchley and Jackson 1970). In Choristidae, males of *C. australis* have a meioformula of n = 14 + X(O) (Bush 1967). This implies that fissions or duplications could play a significant role in the divergence of Panorpidae.

The precocity of X chromosome in anaphase I and the presence of dot-shaped X chromosome in late prophase I imply an XO sex-chromosome system in the four studied species. The XO sex-chromosome system also occurs in five European and American species of Panorpidae, three species of Bittacidae (Naville and de Beaumont 1934, Matthey 1950, Atchley and Jackson 1970), a species of Choristidae (Bush 1967) as well as in a species of Boreidae (Cooper 1974). *B. brumalis* has an extraordinary sex-chromosome system X_1X_2Y in males (Cooper 1951). This system could originate via translocation between the X chromosome and one autosome in a species with XO sex-chromosome system. Since multiple sex chromosomes are shared by many species of the order Siphonaptera (Rothschild 1975), which is considered as the sister group of Boreidae (Biliński et al. 1998, Whiting 2002), it is also possible that the ancestors of the Mecoptera had multiple sex-chromosome system as in the Boreidae and Siphonaptera, and the XO sex-chromosome system represents a derived character state.

It is generally acknowledged that the formation of the XO systems is ascribed to Y chromosome degeneration from the XY system in Orthoptera and Heteroptera (White 1951, Grozeva and Nokkala 1996). The process of degeneration of Y chromosome has been well studied in some dipteran and orthopteran species, which have a neo-Y chromosome in the process of differentiation (Castillo et al. 2010, Kaiser and Bachtrog 2010). To date, no XY sex-chromosome system has been found in Mecoptera, and therefore it is premature now to conclude that this process also occurs in this order. If the ancestral sex-chromosome system was of the multiple chromosome type, the formation of XO sex-chromosome system in Mecoptera would not appear so simple. Some complicated rearrangements of chromosomes may have been present during the evolution of sex chromosomes in Mecoptera.

The achiasmatic meiosis in male Panorpidae has been reported several times to date (Ullerich 1961, Atchley and Jackson 1970, Welsch 1973). However, this is not the case in Bittacidae (Ashley and Moses 1980) and Boreidae (Cooper 1974), implying that achiasmatic meiosis is a derived character and can be used for phylogenetic analysis in Mecoptera. Achiasmatic meiosis is not limited to Mecoptera, but has also been discovered in male Orthoptera, Mantodea, Heteroptera, Coleoptera as well as in male Diptera and female Lepidoptera (White 1965, Gassner 1969, Traut 1977, Serrano 1981, Grozeva et al. 2008, McKee et al. 2012), implying its polyphyletic origin in Insecta.

Chiasmata are manifestations of meiotic crossovers, which not only facilitate the exchange of DNA between maternal and paternal chromosomes but also perform the

important function of securing physical connections between homologous chromosomes that are essential for their co-orientation and proper disjunction at the first meiotic division (Carpenter 1994, Jones and Franklin 2006). Although some forms of crossover may not result in chiasmata (Moens 1996, Sybenga 1996), two inevitable problems arise in most cases when achiasmatic meiosis occurs: the proper segregation of homologous chromosomes in prophase I and the adaptive significance of these species lacking recombination in one sex.

To solve the first problem, some different modes have been proposed to facilitate the segregation of non-exchange homologous chromosomes. For example, in meiosis of male *Drosophila*, adhesion of homologous chromatids probably contributes to the association of homologous chromosomes (Wolf 1994). The associated chromosomes can be sequestered to discrete pockets of the prophase nucleus to ensure their segregation at meiosis I (Hawley 2002, Vazquez et al. 2002). In Panorpidae, male *Panorpa* has a modified synaptonemal complex with all four chromatid axes being connected by transverse filaments from pachytene to metaphase I, similar to female silk moth *Bombyx mori* Linnaeus, 1758, in which the synaptonemal complex is maintained in an apparently expanded form till metaphase I by a compact layer between homologs, while the oocyte of *Panorpa* contains only two transverse filaments between the axes of the homologous chromatids which disappear before diakinesis (Welsch 1973, Rasmussen 1977).

As far as the second problem is concerned, sexual recombination is generally considered as an adaptive advantage of sexual organisms (Eshel and Feldman 1970, Rice 2002, Carvalho 2003), but many species with halved capacity of recombination exist in the world biota for millions of years. Some mechanisms must therefore counterbalance the seeming disadvantages. White (1973) proposed two alternative explanations: selection for a lower level of recombination or facilitation of paracentric inversion heterozygosity. Altiero and Rebecchi (2003) sustained the first explanation in tardigrades. Serrano (1981) argued that male achiasmatic meiosis in several phylogenetic lineages of Caraboidea (Coleoptera) represented the final step towards coadapted gene blocks that must be preserved from recombination. In Saldidae and Miridae (Heteroptera), achiasmatic meiosis was considered as one of the mechanisms by which regular segregation of homologous chromosomes was achieved, and the reduction of recombination was only a side effect (Nokkala and Nokkala 1983, 1986). In Panorpidae, however, the adaptive significance of the absence of recombination in males remains unclear and needs further investigation.

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RESEARCH ARTICLE

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Meiosis in rare males in parthenogenetic *Cacopsylla myrtilli* (Wagner, 1947) (Hemiptera, Psyllidae) populations from northern Europe

Christina Nokkala[1], Valentina G. Kuznetsova[2], Seppo Nokkala[1]

1 *Laboratory of Genetics, Department of Biology, University of Turku, FI-20014, Turku Finland* **2** *Department of Karyosystematics, Zoological Institute, Russian Academy of Sciences, St. Petersburg 199034, Russia*

Corresponding author: *Christina Nokkala* (chrinok@utu.fi)

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Abstract

For studying meiosis in males, large samples of *Cacopsylla myrtilli* (Wagner, 1947) (Hemiptera, Psyllidae) were collected in Norway, Sweden, Finland and northwest Russia. In addition to all-female populations, males were present in 10 out of 47 populations; still, all populations were highly female-biased, the proportion of males varying from 0.1% to 9.1%. These males are thus rare or so-called spanandric males. Males in northern Norway, Finland and northwest Russia showed normal chiasmate meiosis, while complete absence of chiasmata due to asynapsis was found in males collected in Norway and northern Sweden. In asynaptic meiosis, all univalent chromosomes divided during the first meiotic division resulting in incomplete second meiotic division and formation of diploid sperms. Hence, males in these populations are nonfunctional and do not contribute to the genetic constitution of the population, but appear in every generation as reversals from apomictic parthenogenesis and the mode of parthenogenesis is of obligatory type.

Keywords

Cacopsylla myrtilli, parthenogenesis, spanandric males, asynaptic meiosis, nonfunctional males

Introduction

For bisexual reproduction it is essential that the diploid chromosome complement is reduced to haploid during gametogenesis. This is achieved by a specialized cell cycle, meiosis, where the chromosome number of germ line cells is reduced during two rounds of cell divisions after just one round of chromosome replication. In the first, reductional division homologous chromosomes segregate from each other while in the other, equational division individual chromosomes divide. During early prophase of the first meiotic division, homologous chromosomes pair, undergo tight synapsis and form a bivalent. Bivalent integrity is retained throughout prophase with either of two ways. Most often a crossing-over occurs during synapsis creating a physical link or chiasma between the homologs, which are held together until anaphase I. Alternatively, in achiasmate meiosis, pairing, alignment and synapsis are normal, but no chiasmata are formed. In these cases, the paired condition of homologs is retained till the onset of anaphase I (for references see Nokkala and Nokkala 1983, Nokkala 1987, Grozeva et al. 2010).

In natural populations, the meiotic mechanism is under strong selection; all mutations causing disturbances in meiosis are quickly removed from the population. The only exceptions are thelytokous parthenogenetic populations, where males are occasionally found (Lynch 1984, Palmer and Norton 1990). In these rare males defects affecting normal meiotic pattern and making males nonfunctional in sexual reproduction have been described. In two oribatid mite species *Trhrypochthonius tectorum* (Berlese, 1896) and *Platynothrus peltifer* (CL Koch, 1839) (Acari: Oribatida: Desmonomata) male meiosis was normal, but spermiogenesis was aberrant; males produced few spermatophores only and females left them without attention (Taberly 1988). In the thelytokous false spider mite *Brevipalpus obovatus* Donnadieu, 1875 (Phytoptipalpidae) males had low sperm production and did not inseminate females despite copulation (Pinjacker et al. 1981). Spermatogonia are developed into sperm without meiosis in the spider mite *Tetranychus urticae* Koch, 1836 (Tetranychidae) (Pijnacker and Drenth-Diephuis 1972).

The object of our study is the Holarctic psyllid species *Cacopsylla myrtilli* (Wagner, 1947), which has long been considered as parthenogenetic (Linnavuori 1951, Lauterer 1963, Ossiannilsson 1975, 1992), since collections have been made from all-female populations (Ossiannilsson 1992). Recently, we established that parthenogenetic females in *C. myrtilli* were triploids and displayed parthenogenesis of an apomictic type (Nokkala et al. 2008). However, also males have been found in some populations in northwest Russia (Ossianilsson 1975, Labina et al. 2009), Norway (Hodkinson 1983), northern Sweden (Hodkinson and Bird 2006) and northern Finland (Labina et al. 2009). Based on the presence of males, either facultative parthenogenesis (Hodkinson 1983, Hodkinson and Bird 2006) or even bisexual reproduction (Labina et al. 2009) has been suggested.

For the present study we have collected *C. myrtilli* from many populations from Norway including recollection of Hodkinson (1983), Sweden including recollection of

Hodkinson and Bird (2006), and Finland. For northwest Russia, we have utilized the collection of Labina et al. (2009). We have analyzed populations quantitatively for the presence of males and when present studied male meiosis in detail to reveal possible aberrations and their significance for the meiotic mechanism in general. In addition we wanted to find firm evidence for the origin of males in populations.

Materials and methods

Adult *Cacopsylla myrtilli* specimens were collected in Norway, Sweden, Finland and northwest Russia. For the present study, we selected only those populations, where males were found (Table 1). The Rindhovda population in Norway has been earlier studied by Hodkinson (1983) and the Abisko population in Sweden by Hodkinson and Bird (2006).

Specimens were fixed immediately after collection in the field in freshly made 3:1 Carnoy and stored in fixative in the laboratory at + 6°C until slides were made according to the method by Nokkala et al. (2008). For males collected in alcohol in the field, a novel approach was used. The abdomen of a male was immersed in fresh 3:1 Carnoy fixative for two hours, testes were dissected in a drop of 45% acetic acid and squashed. This procedure allows both chromosomal and molecular analysis of the same individual.

For staining of chromosomes the Feulgen-Giemsa method by Grozeva and Nokkala (1996) was employed with extending staining in Schiff's reagent to 25 min and subsequent Giemsa staining in Sørensen's buffer, pH 6.8, to 30 min.

Table 1. Locations of *C. myrtilli* populations in Norway, Sweden, Finland and Russia. Number of cytologically studied males, total number of males, number of females and frequency of males.

Population	latitude	longitude	altitude	males		females	male freq.
				studied	total		
Norway							
Finnmark, Šuoššjávri	69°22'11"N	24°18'20"E		3	4	2950	0.001
Sjoa, Rudihøe	61°46'27"N	9°17'16"E	1000	1	3	1078	0.003
Sjoa, Kvernbrusætrin	61°42'27"N	9°19'25"E	950	15	22	1443	0.015
Sjoa, Stålane	61°41'15"N	9°14'27"E	1000	11	14	470	0.030
Sjoa, Kringlothaugen	61°43'06"N	9°22'40"E	700	1	1	277	0.004
Sjoa, Rindhovda	61°43'05"N	9°05'12"E	1080	30	38	379	0.091
Sweden							
Abisko, Lapporten	68°19'26"N	18°51'05"E	610	5	5	386	0.013
Finland							
Utsjoki	69°51'06"N	27°00'34"E		2	3	2603	0.001
Paltamo	64°33'28"N	27°43'41"E		6	10	1595	0.006
Russia							
White Sea, Sredny Island†	66°17'00"N	33°40'00"E		9	49	2946	0.016

†Data from Labina et al. (2009)

Chromosomes were photographed with BU4-500C CCD camera (BestScope International Limited, Beijing, China) mounted on Olympus BX51 microscope (Olympus, Japan) using ISCapture Sofware version 2.6 (Xintu Photonics Co LTD, Xintu, China). Photographs were processed with Corel Photo-Paint X5 software.

Results

We found males in ten (Table 1) out of a total of 47 populations collected. All populations studied were highly female-biased, male frequency varying from 0.1 % to 9.1 %. The highest proportion of males was found from Rinhovda population in Norway collected above tree line. This same population was earlier studied by Hodkinson (1983). All other populations showed a considerably lower proportion of males, less than 3 %. The lowest proportion of males or 0.1 % was found in Finnmark, Šuoššjávri in Norway and Utsjoki in Finland.

The testes in male psyllids are organized into lobed structures, the number of which varies in a species specific manner (Głowacka et al. 1995). In *C. myrtilli*, males have two lobed testes. The earliest meiotic stage found in an adult male is pachytene, while earlier meiotic stages are rare. The number of cells at pachytene in one primary spermatocyte cyst is 64.

Males collected in Finnmark, Šuoššjávri in Norway, in Paltamo and Utsjoki in Finland and the White Sea area in Russia (Labina et al. 2009), on one hand, and in Sjoa in Norway and in Abisko in northern Sweden, on the other hand, showed strikingly different course of meiosis.

Males from northern Norway, Finland and Russia showed meiotic stages typical for psyllids known to possess holocentric chromosomes. The most common stage in testes was the diffuse stage during which chromatin had a diffuse appearance covering the whole nucleus in a cell. During this stage the size of cells is increased considerably. When the chromosomes condensed out from the diffuse stage, diplotene stage with 12 autosomal bivalents with one chiasma in each and a univalent X chromosome were seen (Fig. 1a). At metaphase I, the bivalents showed axial orientation with homologous telomeres oriented to opposite poles and the univalent X chromosome lying at the equatorial plane (Fig. 1b). Segregation of bivalents at anaphase I resulted in half-bivalents moving towards the poles. The bi-oriented X chromosome was seen as a laggard at this stage. At telophase I, the X chromosome moved to one of the poles (Fig. 1c), resulting in two kinds of secondary spermatocytes, those with the X chromosome and those without it.

However, males collected near Sjoa, Norway (Rindhovda, Rudihøe, Kvernbrusætrin and Stålane) and northern Sweden (Abisko, Lapporten) showed 25 (24 + X) univalent chromosomes at diplotene and diakinesis stages, indicating a complete failure in chiasma formation (Fig. 2a). Univalent chromosomes oriented with sister chromatids to opposite poles at metaphase I (Fig. 2b). These bi-oriented chromosomes divided at anaphase I. No laggard was seen at anaphase I, indicating that the X chromosome also divided in the first meiotic division (Fig. 2c). Anaphase I resulted in secondary sper-

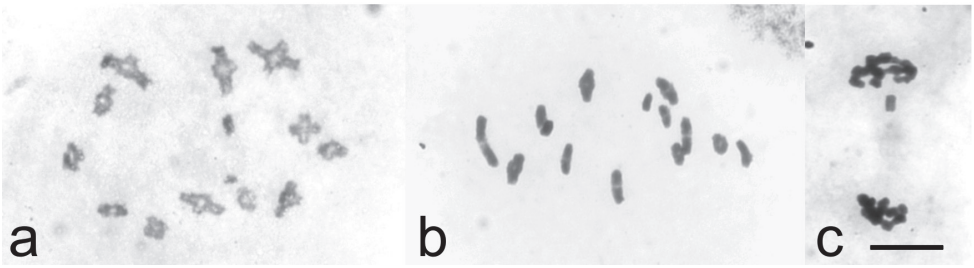

Figure 1. Chiasmate male meiosis in *C. myrtilli* (**a**) diakinesis with twelve autosomal bivalents an univalent X chromosome (**b**) metaphase I with twelve chiasmate bivalents and univalent X (**c**) telophase I, univalent X chromosome moving towards upper pole. Bar equals 10 μm.

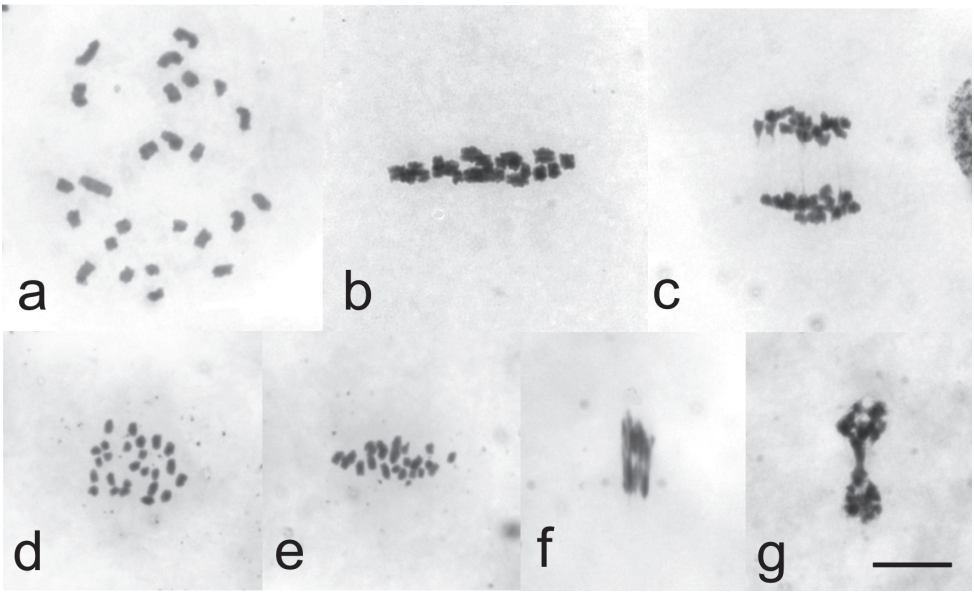

Figure 2. Male meiosis without chiasmata in *C. myrtilli* (**a**) Diakinesis with 25 univalent chromosomes (**b**) Metaphase I in side view with 25 bi-oriented univalents (**c**) Anaphase I, daughter chromosomes moving towards opposite poles. No laggard chromosomes present (**d**) Metaphase II in polar view showing 25 daughter chromosomes (**e**) Metaphase II in side view. All bi-oriented daughter chromosomes aligned with the equatorial plane (**f**) Anaphase II, daughter chromosomes stretched towards poles (**g**) Telophase II, daughter nuclei joined by stretched chromosomes. Bar equals 10 μm.

matocytes with 25 daughter chromosomes, oriented to both poles at metaphase II (Fig. 2d, e). As daughter chromosomes are unable to divide, anaphase II started but could not be completed (Fig. 2f, g). Consequently, diploid spermatids were produced. Meiosis resulted in a cyst of 128 developing spermatids instead of the 256 in normal chiasmate meiosis.

To find out reasons for the absence of chiasmata, early stages of meiosis were analysed in more detail. In chiasmate male meiosis pachytene stage (Fig. 3a) was easily

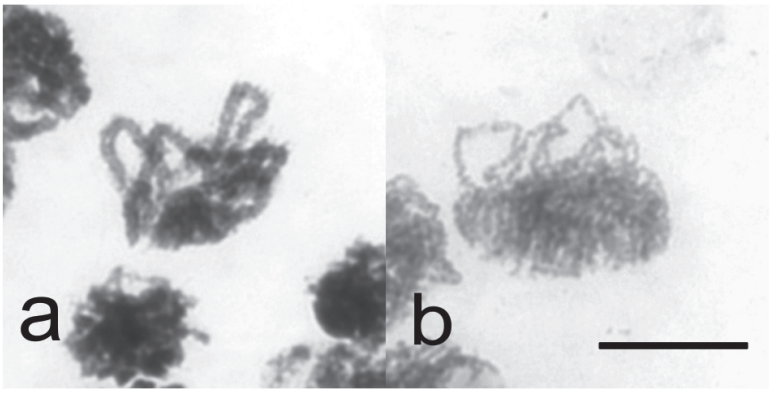

Figure 3. Synapsis and synaptic alignment of chromosomes in *C. myrtilli* (**a**) Pachytene, synapsed chromosomes in bouquet orientation in chiasmate male meiosis (**b**) Presynaptic alignment of leptotene chromosomes in male meiosis lacking chiasmata in bouquet orientation. Bar equals 10 μm.

found in all males studied. However, in males without chiasmata no pachytene cells were found in 61 males studied, but leptotene-like nuclei were abundant. When seen in side-view chromosomes showed distinct bouquet orientation (Fig. 3b), indicating that presynaptic alignment was normal, but synapsis did not occur. It seems apparent then that the failure in chiasma formation in these males is due to a mutation resulting in asynapsis of homologous chromosomes.

Discussion

Males in populations

In the present study we have established that none of the male-carrying populations in Norway, northern Sweden, Finland and northwest Russia were genuinely bisexual, but all were parthenogenetic with highly female-biased sex ratio. Variation of male frequencies from 0.1 to 9.1 % was quite similar to that previously found in parthenogenetic oribatid mites, in which frequencies are varying from 0.15 % to 6.3 % (Palmer and Norton 1990). On the other hand, in bisexual species of mites, the proportion of males is over 30 % (Maraun et al. 2003). Clearly, males in *C. myrtilli* populations represent rare or so-called spanandric males. Surprisingly, two kinds of males were found. Meiosis in males was normal in northern populations in Norway, Finland and Russia. Generally it is thought that rare males are nonfunctional (Lynch 1984, Palmer and Norton 1990, Maraun et al. 2003, Smith et al. 2006). However, the significance of rare males in these *C. myrtilli* populations is difficult to evaluate at present, as we do not know, if there are diploid females with normal meiosis within triploid parthnogenetic populations and if the males are able to make a distinction between triploid and diploid females. These problems are at present under investigation. The low frequencies

of males in the populations, 0.1% in Utsjoki, 0.6% in Paltamo and 1.6% in White Sea might indicate that they are not capable of independent bisexual reproduction in these populations. In the remaining populations, due to asynapsis, only univalents were present in meiosis resulting in the formation of diploid spermatids. Evidently, males in these populations are nonfunctional, not being able to produce diploid offspring with any kind of females and not contributing thus to the genetic constitution of the population. Apparently, males appear in every generation as reversals from apomictic parthenogenesis and reproduction in the populations is of obligatory parthenogenetic type.

Univalents and their meiotic behavior

Although complete failure in chiasma formation in male meiosis was found for the first time in a natural population in the present study, the phenomenon is well known in the holocentric laboratory model organism, the nematode *Caenorhabditis elegans* (Maups, 1900). Genetic dissection studies on early events in meiosis, presynaptic alignment, formation of double strand breaks, synapsis and crossing over, and chiasma formation, have revealed several gene loci, in which mutations result in complete lack of crossing over in an affected animal (for reviews see Garcia-Muse and Boulton 2007, Zetka 2009). Consequently, mutants show twelve univalents in diakinesis stage nuclei instead of six bivalents described in wild type male. Univalent chromosomes behave in two ways during meiotic divisions. In *spo-11* mutant, univalent chromosomes are unable to divide in the first meiotic division and distribute randomly to poles at first anaphase, resulting in highly aneuploid gametes. In mutants *rec8, htp1, htp2* and *htp3*, univalent chromosomes undergo equational first division. The daughter chromosomes cannot divide in the second division and are all included in the gametes formed. Consequently, if for example *rec8* mutants are mated with normal wild type animals, triploid offspring will be produced (Severson et al. 2009). It is obvious that univalent chromosomes in *C. myrtilli* male meiosis in Rindhovda population behave similarly as the univalents in the *rec8* and *htp* mutants in *C. elegans*.

Cell cycle checkpoints are not activated despite of extensive asynapsis

Both mitosis and meiosis include surveillance mechanisms to ensure regular behavior of chromosomes. In meiosis, homologous chromosomes pair and align with each other followed by their tight synapsis that allows exchange process or crossing over between homologs necessary for maintaining bivalent configuration later in meiotic prophase. These early stages are controlled by the pachytene checkpoint. Defects in synapsis or recombination result in meiotic arrest or apoptosis, thus preventing the formation of defective gametes (Li et al. 2009). The checkpoint is very robust in mammalian spermatogenesis, for example in male mouse asynapsis results in apoptosis or meiotic arrest (Hamer et al. 2008, Kouznetsova et al. 2011), but is less effective in female mouse

(Kouznetsova et al. 2007). There are some indications that asynapsis activates apoptosis in the nematode *C. elegans* (Bhalla and Dernburg 2005), but still the behavior of univalents in meiotic mutants in this species seems to be regular in meiotic divisions (Severson et al. 2009). As found in the present study, the pachytene checkpoint is totally inactive in *C. myrtilli* male meiosis despite extensive asynapsis. It appears to be inactive also in other insects. In *Drosophila melanogaster* Meigen, 1830, univalents resulted from an asynaptic mutation *c(3)G* behave in a regular way and reach anaphase I -like stage in mature eggs in female meiosis (Puro and Nokkala 1977). Univalents reach MI also in lepidopteran species (Nayak 1978) and plants (Couteau et al. 1999).

The orientation of chromosomes in metaphase spindle is monitored by the spindle assembly checkpoint. As far as even one chromosome does not reach a proper spindle microtubule attachment to the spindle pole, the onset of anaphase in mitosis or anaphase I or anaphase II in meiosis is inhibited. Once all the chromosomes display proper orientation, the onset of anaphase is triggered by anaphase promoting complex that activates separase enzyme that will degrade sister chromatid cohesion (Nasmyth 2001, Musacchio and Salmon 2007). In *C. myrtilli*, univalents behave in a regular way both in the first and second divisions in male meiosis without any disturbances. Apparently, stable bipolar orientations of univalents at MI and daughter chromosomes at MII allow them to evade the spindle assembly checkpoint. This observation is well in accordance with the behavior of univalents in mouse (Kouznetsova et al. 2007).

Segregation of univalent chromosomes in meiosis

In meiosis, there are mechanisms, which are responsible for the regular segregation of univalent chromosomes, especially in the case of specialized chromosomes like sex chromosomes (Nokkala 1986a), m-chromosomes (Nokkala 1986b) or B-chromosomes (Nokkala 1986c, Nokkala and Nokkala 2004). Typical for these mechanisms is that they can ensure the regular segregation of only one pair of univalents. If more than one pair of univalents are present, regular segregation is disturbed (Nokkala 1986c). Asynaptic meiosis in *C. myrtilli* male clearly indicates that if all chromosomes appear as univalents in meiosis there is no mechanism to ensure their segregation but asynapsis leads to highly uneuploid gametes or diploid gametes depending on the behavior of univalents in the first meiotic division. *Drosophila* female meiosis is an exception as asynaptic mutants like *c(3)G* can be maintained as homozygote mutant stocks, since segregation of homologous chromosomes is still highly regular. Regular segregation is ensured by distributive segregation mechanism operating in the female (Puro and Nokkala 1977, Hawley and Theurkauf 1993).

Conclusions

In this study we have established that males occurred in ten out of 47 *C. myrtilli* populations sampled in northern Europe. All populations were highly female-biased, the propor-

tion of males varying from 0.1 % to 9.1 %. Thus, all populations are in fact parthenogenetic and males are rare or so-called spanandric males. In northern Norway, Finland and Russia male meiosis was normal whereas in Norway and Sweden males displayed asynaptic meiosis leading to the formation of diploid spermatids. These males are nonfunctional in reproduction and appear in every generation as reversals from apomictic parthenogenesis.

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