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Extensive fragmentation of the X chromosome in the bed bug *Cimex lectularius* Linnaeus, 1758 (Heteroptera, Cimicidae): a survey across Europe

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Abstract

Variation in the number of chromosomes was revealed in 61 samples of *Cimex lectularius* Linnaeus, 1758 from the Czech Republic and other European countries, hosted on *Myotis* Kaup, 1829 (4) and *Homo sapiens* Linnaeus, 1758 (57). The karyotype of all the specimens of *C. lectularius* analysed contained 26 autosomes and a varying number of the sex chromosomes. The number of sex chromosomes showed extensive variation, and up to 20 fragments were recorded. Altogether, 12 distinct karyotypes were distinguished. The male karyotypes consisted of 29, 30, 31, 32, 33, 34, 35, 36, 37, 40, 42 and 47 chromosomes. The females usually exhibited the number of chromosomes which was complementary to the number established in the males from the same sample. However, 11 polymorphic samples were revealed in which the karyotypes of females and males were not complementary each other. The complement with 2n = 26+X_1X_2Y was found in 44% of the specimens and 57,4% samples of bed bugs studied. The karyotypes with higher chromosome numbers as well as individuals with chromosomal mosaics were usually found within the samples exhibiting particularly extensive variation between individuals, and such complements were not found within samples contaning a few or single specimen. The occurrence of chromosomal mosaics with the karyotype constitution varying between cells of single individual was observed in five specimens (4.3%) from five samples. We assume that polymorphism caused by fragmentation of the X chromosome may result in meiotic problems and non-disjunction can produce unbalanced gametes and result in lowered fitness of individuals carrying higher numbers of the X chromosome fragments. This effect should be apparently enhanced with the increasing number of the fragments and this may be

the reason for the observed distribution pattern of individual karyotypes in the studied samples and the rarity of individuals with extremely high chromosome numbers. The assumed lowering of the fitness of individuals carrying higher numbers of the X chromosome fragments could affect population dynamics of variable populations.

Keywords
Cimex lectularius, *C. pipistrelli*, cytogenetics, chromosome number variation, X chromosome

Introduction

The genus *Cimex* Linnaeus, 1758 is the best known taxon of the family Cimicidae (Heteroptera) which contains up to 110 described species of haematophagous ectoparasites exploiting mostly bats and birds as hosts (Usinger 1966, Péricart 1996, Henry 2009). The human bed bug *Cimex lectularius* Linnaeus, 1758, one of the two most important *Cimex* species parasiting on humans, is a temporal haematophagous ectoparasite usually found in human dwellings and bat roosts as well as on domestic and synanthropic vertebrates (Usinger 1966, Schuh and Slater 1995, Reinhardt and Siva-Jothy 2007). The bed bug was practically eradicated by a mass use of DDT in the 1940s and 1950s but it has re-started new expansion in all developed countries of the Temperate Zone during the last ten years (Hwang et al. 2005, Romero et al. 2007). Due to its reemerging history as a human pest the species has been intensively studied (e.g. Reinhardt and Siva-Jothy 2007, Szalanski et al. 2008, Balvín et al. 2012).

Karyotypic variation within the family Cimicidae and the genus *Cimex* is believed to be frequently related to the sex chromosomes. The XY sex determination system was proposed as ancestral in 53 species of cimicids that have been studied cytogenetically so far, and the diploid number in male complements varies from 2n=10 to 47, with the modal number of 31 (Ueshima 1979, Kuznetsova et al. 2011). Systems including multiple sex chromosomes were revealed in various species. The X_1X_2Y constitution prevails but several species showed karyotypes with three, four or even more X chromosomes (Ryckman and Ueshima 1964, Ueshima 1966, 1979, Manna 1984, Grozeva and Nokkala 2002, Poggio et al. 2009, Simov et al. 2006, Kuznetsova et al. 2011). Intraspecific variation in the number of sex chromosomes was also reported in two species of the genus *Paracimex* Kiritshenko, 1913 parasiting in birds (Ueshima 1968).

The bed bug, *C. lectularius,* shows combination of unusual cytogenetic characteristics, partly common for all Heteroptera. The chromosomes are holokinetic, with completely achiasmatic male meiosis of collochore type and inverted meiosis of the sex chromosomes. A particularly remarkable feature is numerical variation in the number of the sex chromosomes. The standard karyotype of the bed bug contains 26 autosomes and a varying number of supernumerary chromosomes which is supposed to originate after fragmentation of the X chromosome (e.g. Ueshima 1966, Grozeva et al. 2010). Variation in the chromosome number in the bed bug karyotype was first reported by Darlington (1939) and Slack (1939) from Great Britain. Darlington

(1939) distinguished in natural populations 13 karyotypes containing two up to 14 X chromosomes. Most of the specimens examined possessed complements with higher chromosomal number. Slack (1939) revealed the presence of 13 karyotypes with the number of the X chromosomes varying between three and 15. Ueshima (1966, 1967) studied nine samples of bed bugs collected in various continents and he was able to recognize five karyotypes with the number of the X chromosomes varying from two to nine. Grozeva et al. (2010, 2011) examined small samples of bed bugs originating from Russia (St Petersburg) and Bulgaria (Sofia) and recorded the standard karyotype only ($2n=26+X_1X_2Y$).

The related species *C. pipistrelli* Jenyns, 1839 is known as an obligate parasite of bats which may share its hosts with *C. lectularius*. The karyotype of *C. pipistrelli* is similar to the standard complement of *C. lectularius* but contains a higher number of autosomes ($2n=28+X_1X_2Y$; Ueshima 1966). No variation in the chromosome number has been recorded in this species.

The recent expansion is a reason why cytogenetic analysis of this species starts to be more important in respect of recent findings indicating that karyotypic divergences could have evolved faster than DNA sequences (e.g. Britton-Davidian et al. 2007, Horn et al. 2012). This is another piece of evidence that initial evolution at the genomic, karyotypic and organismal level can proceed rather independently, as is apparently the case of the bed bug. The intraspecific karyotypic variation may be associated with segregation irregularities resulting in possible lowering of the fitness. Research of this variation can thus provide more understanding of reproductive biology and population dynamics of the bed bug.

This study reports cytogenetic findings in *C. lectularius* and *C. pipistrelli* based on large samples of studied individuals from the Czech Republic and other European countries. We aim to investigate karyotypic variation reported previously in the bedbug and to obtain data revealing possible temporal and geographic pattern of this variation. Another goal of this study is to contribute to better understanding of the mechanisms underlying this variability.

Material and methods

The studied specimens of *C. lectularius* and *C. pipistrelli* were collected from bat roosts and human dwellings in 2010–2012 (Fig. 1). The karyotype was determined in 116 specimens of *C. lectularius* from 61 localities within 10 European countries and in five specimens of *C. pipistrelli* from two localities in Slovakia. The live individuals of synantropic bed bugs from humans were mostly collected by pest exterminators in flats, hotels and hostels. The studied samples originated from individual collecting sites which were localized with varying levels of precision, particularly in the synathropic habitats (flat, house, town, city) depending on information available from the collectors. Individual sites within a single city are differentiated by numerals (e.g., Prague 1, Prague 2). Bugs identified as *C. lectularius* were also collected at four sites

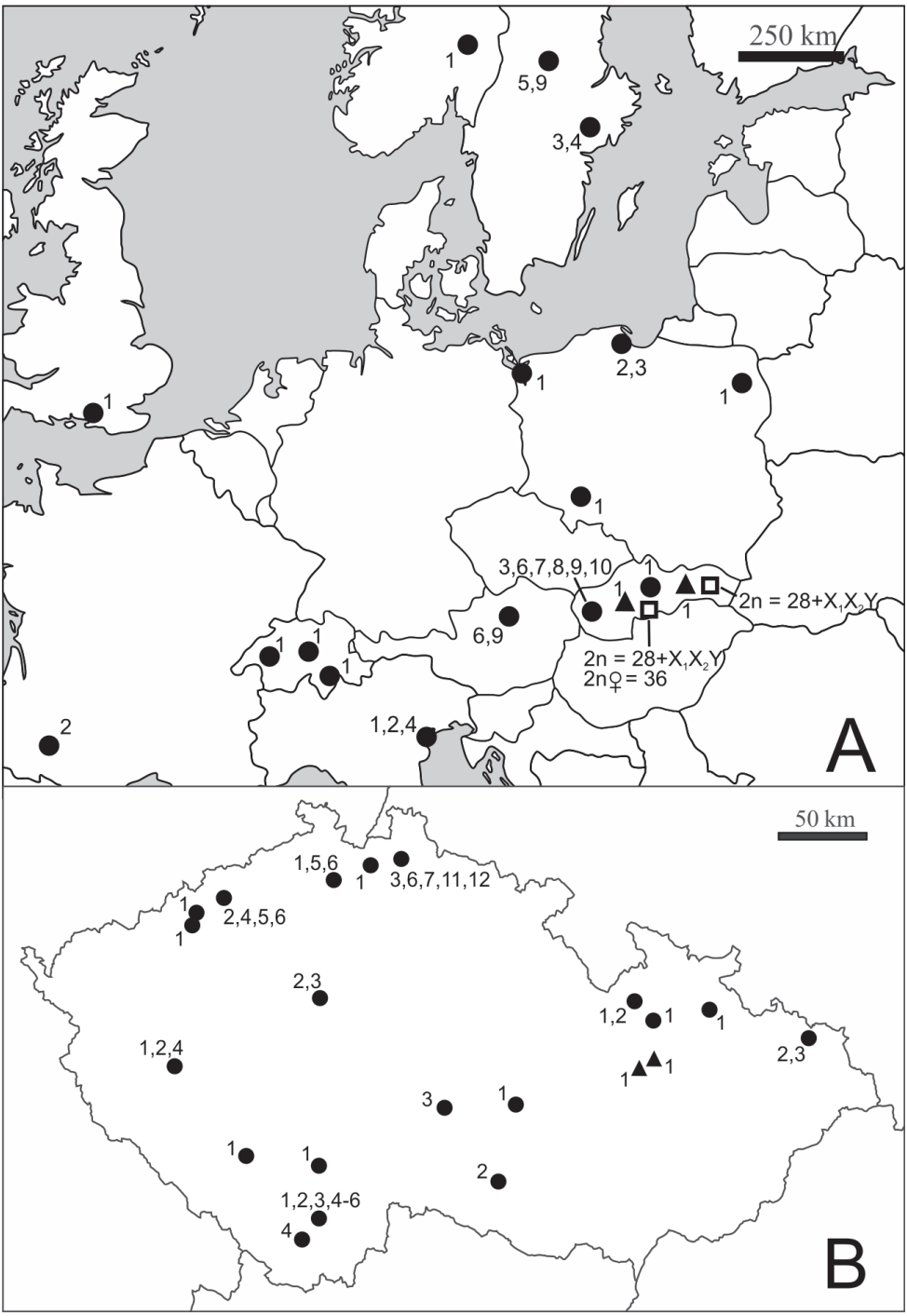

Figure 1. Geographical distribution of the sites studied. **A** Samples of *Cimex lectularius* and *C. pipistrelli* from Europe **B** Samples of *Cimex lectularius* from Czech Republic. ● *C. lectularius*, human habitats, ▲ *C. lectularius*, bat roosts, □ *C. pipistrelli*. Numbers refer to karyotypes 1–12 described in Results.

of bat roosts in the Czech Republic and Slovakia. The complete list of the collecting sites is shown in Table 1.

The chromosome preparations were made from gonads or midgut using the spreading technique described by Traut (1976) modified after Šťáhlavský and Král (2004). Briefly, the tissues were dissected and hypotonised in 0.075 M KCl solution for 25 minutes and then fixed in glacial acetic acid:methanol (1:3) for 15–25 minutes. The fixed material was suspended in a drop of 60% acetic acid on a microscope slide and the slide was placed on a warm histological plate (temperature 40–45°C). The drop was than moved on the slide until it evaporated. The chromosome preparations were stained in a 5% Giemsa solution in Sörensen phosphate buffer (pH = 6.8) for 30 minutes. The chromosome slides were examined with the use of the Olympus Provis AX 70 microscope and selected cells and stages of division were documented by the digital imaging system Olympus DP 72 and software QuickPHOTO CAMERA 2.3. The diploid chromosome complements of males were described by the formula $2n=26+X_{1-n}Y$ where n stands for the additional X chromosomes. The corresponding karyotypes of females were characterized by the formula $2n=26+2X_{1-n}$.

After withdrawing of tissues for cytogenetic methods, the material was preserved in 96% ethanol and used in parallel molecular studies. Their results have approved the original specimens determination according to morphological characters (Balvín et al. 2012, 2013). The material is deposited in collections of the Department of Zoology, Charles University in Prague.

Results

The karyotype of all the specimens of *C. lectularius* analysed contained 26 autosomes and a varying number of the sex chromosomes. The relative length of chromosomes in the complement was successively diminishing from 5.3 to 1.7%. No distinct size groups of chromosomes could be differentiated; however, the largest and the smallest autosomal pair could be usually recognized according to their size. The original sex chromosomes X_1X_2Y were medium-sized whereas their supposed fragments occurring in the karyotypes with higher chromosome numbers were the smallest elements of the set.

In the samples of *C. lectularius* studied, 12 distinct karyotypes were differentiated (Table 2). These karyotypes were distinguished according to the varying diploid chromosome number (2n=29–37, 40, 42, 47 in the male complement) and the varying number of the X chromosomes (2–20).

The identical karyotype was found in all the specimens studied in 46 monomorphic samples, whereas karyotype differences were recorded between individuals in 15 polymorphic samples. We should note, however, that about half of the studied samples (26) consisted of a single specimen only. The results recorded in individual collecting sites are summarized in Table 1.

The most common karyotype 1 was characterized by the standard complements with two X chromosomes; 2n=29 in males ($2n=26+X_1X_2Y$) and 2n=30 in females

Table 1. The list of the collecting sites and a summary of primary results. A = Austria, CH = Switzerland, CZ = Czech Republic, F = France, GB = Great Britain, I = Italy, N = Norway, PL = Poland, S = Sweden, SK = Slovakia. Specimens: left column males, right column females. Designation of the type of karyotype in the last column is the same as in the text and Table 2.

Sample Code	Country	Locality	Specimens		Karyotype
Cimex pipistrelli			♂	♀	
190	SK	Hontianske Nemce	1	2	see text
191	SK	Ľubovec	2		see text
Cimex lectularius					
Host: *Myotis myotis* (Borkhausen, 1797), *Myotis emarginatus* (E. Geoffroy, 1806)					
417	CZ	Bílá Lhota		1	1
418	CZ	Moravičany	1		1
421	SK	Krásnohorské Podhradie	2		1
423	SK	Hosťovce	2	1	1
Cimex lectularius					
Host: *Homo sapiens*					
609	CZ	Bruntál	1		1
610	CZ	Plzeň (1)	2	1	1
612	CZ	Chomutov – Dřínovská	1	1	1
613	CZ	Liberec (1) – Krejčího	2	1	3, 6
614	CZ	Liberec (2)- Krejčího	3		7, 11, 12
615	CZ	Jirkov - Na Borku	1	1	1
617	CZ	Štědrákova Lhota	1	1	1,2
618	CZ	Stráž pod Ralskem	1		1
619	CZ	Bohumín – Studentská	3		2,3
621	CZ	Plzeň (2) – Na Vinicích		2	1
623	CZ	Šumperk	1	1	1
624	CZ	Plzeň (3) – Na Slovanech	1	1	1
625	CZ	Plzeň (4) – Na Slovanech	2	1	1
629	CZ	České Budějovice (1) – Puklicova		1	3
632	CZ	Janov	1	2	2, 4, 5, 6
633	CZ	Jaroměřice nad Rokytnou	1		2
634	CZ	Plzeň (5)	1		2
640	CZ	Plzeň (6) – Na Slovanech	2		1
642	CZ	Praha (1)	2		2
643	CZ	Praha (2)	1		3
644	CZ	České Budějovice (2)		2	1
645	CZ	České Budějovice (3) - Okružní		1	3
647	CZ	Praha (3)		1	2
648	CZ	Praha (4)	3		2
657	CZ	Plzeň (7)	2		1, 4
658	CZ	Humpolec	2	1	3
659	CZ	Praha (5) – Křížíkova		1	2
661	CZ	Česká Lípa – Svárovská	3	1	1, 5, 6
662	CZ	České Budějovice (4) – Netolická	1	1	2, 4-6
665	CZ	Chvalšiny		2	4

Sample Code	Country	Locality	Specimens		Karyotype
667	CZ	Týn nad Vltavou – Hlinecká	1		1
668	CZ	České Budějovice (5) – J. Bendy	1		1
669	CZ	Strakonice – Bezděkovská	1		1
670	CZ	České Budějovice (6) – M. Chlajna	2		1
671	CZ	Žďár nad Sázavou	1		1
707	SK	Banská Bystrica	2		1
708	SK	Trnava	5	1	3, 6, 7, 8, 9, 10
719	GB	Brighton		1	1
720	A	Melk	1	2	6, 9
732	CH	Luzern	1		1
737	CH	-		1	1
745	CH	Fribourg – Rue de l´Hôpital	1	1	1
750	I	Mestre	1	2	2
751	I	Venezia (1)	1		1
752	I	Venezia (2)	2	1	1, 2
753	I	Venezia (3)	1		1, 4
789	N	Ottestad	1		1
795	S	Borlänge (1)	2		5
796	S	Borlänge (2)	1	1	5, 9
798	S	Stockholm – Vårber	1	2	3, 4
817	F	Aire/Adour		2	2
831	PL	Świnoujscie		1	1
838	PL	Gdansk (1)	1		2
840	PL	Gdansk (2)	2	1	2, 3
843	PL	Wroclav – Grabiszynska		1	1
844	PL	Białystok (1)	1		1
845	PL	Białystok (2)	1		1

(2n=26+$X_1X_1X_2X_2$) (Fig. 2a, b). This complement was found in 51 specimens (33 males and 18 females) and in 31 monomorphic and four polymorphic samples. Seven monomorphous samples of this karyotype included females only. The monomorphic samples from synanthropic habitats were collected in the Czech Republic, Great Britain, Italy, Norway, Poland, Slovakia and Switzerland. This karyotype was further recorded in some individuals from the polymorphic samples collected in the Czech Republic and Italy and in the all samples of *C. lectularius* collected in bat roosts (Fig. 1).

Karyotype 2 included complements with three X chromosomes; 2n=30 in males (2n=26+X_{1-3}Y) and 2n=32 in females (2n=26+2X_{1-3}) (Fig. 2c, d). This chromosome constitution was recognized in 24 specimens (15 males and 9 females) from 15 samples. The karyotype was recorded in both the monomorphic and polymorphic samples. The monomorphic samples from synanthropic habitats in the Czech Republic and Poland included males only, the sample from Italy included males and females, and other samples from the Czech Republic and France included females only. This karyotype was further found in polymorphic samples from the Czech Republic, Italy and Poland.

Table 2. The distribution of samples studied in individual karyotypes characterized in the text. A = Austria, CH = Switzerland, CZ = Czech Republic, F = France, GB = Great Britain, I = Italy, N = Norway, PL = Poland, S = Sweden, SK = Slovakia. Single female possessing the odd number of chromosomes is not included.

Karyotype	2n	Sex chromosomes	No. of samples	%	No. of specimens	%	Country
1	29	2XY	35	57.4	51	44.0	CZ, GB, CH, I, N, PL, SK
2	30	3XY	15	24.6	24	20.7	CZ, F, I, PL
3	31	4XY	9	14.8	13	11.2	CZ, S, SK
4	32	5XY	4	6.6	5	4.3	CZ, S
5	33	6XY	3	4.9	5	4.3	CZ, S
6	34	7XY	3	4.9	3	2.5	A, CZ, SK
7	35	8XY	2	3.3	2	1.7	CZ, SK
8	36	9XY	1	1.6	1	0.9	SK
9	37	10XY	3	4.9	3	2.5	A, S, SK
10	40	13XY	1	1.6	1	0.9	SK
11	42	15XY	1	1.6	1	0.9	CZ
12	47	20XY	1	1.6	1	0.9	CZ
mosaic	-	-	5	8.2	5	4.3	A, CZ, I, SK

Karyotype 3 included complements with four X chromosomes; 2n=31 in males (2n=26+X_{1-4}Y) and 2n=34 in females (2n=26+2X_{1-4}) (Fig. 2e, f). This complement was found in 13 specimens (8 males and 5 females) from nine samples. The karyotype was recorded in monomorphic samples from the synathropic habitats collected in the Czech Republic and in polymorphic samples from the Czech Republic, Slovakia and Sweden.

Karyotype 4 included complements with five X chromosomes; 2n=32 in males (2n=26+X_{1-5}Y) and 2n=36 in females (2n=26+2X_{1-5}) (Fig. 2g, h). It was found in five specimens (2 males and 3 females) from four samples. This complement was recorded in a monomorphic sample from the Czech Republic and in polymorphic samples from the Czech Republic and Sweden.

Karyotype 5 included complements with six X chromosomes; 2n=33 in males (2n=26+X_{1-6}Y) and 2n=38 in females (2n=26+2X_{1-6}) (Fig. 2i, j) and it was found in five specimens (4 males and 1 female) from three samples. This complement was identified in a single monomorphic sample including two males collected in Sweden and in polymorphic samples from the Czech Republic and Sweden.

Karyotype 6 included complements with seven X chromosomes; 2n=34 in males (2n=26+X_{1-7}Y) and 2n=40 in females (2n=26+2X_{1-7}) (Fig. 2k, l) and it was found in three specimens (1 male and 2 females) from three polymorphic samples collected in Austria and the Czech Republic.

Karyotype 7 included a complement with eight X chromosomes and 2n=35 in males (2n=26+X_{1-8}Y) (Fig. 2m) and it was found in two male specimens collected in polymorphic samples from two sites in the Czech Republic and Slovakia, respectively.

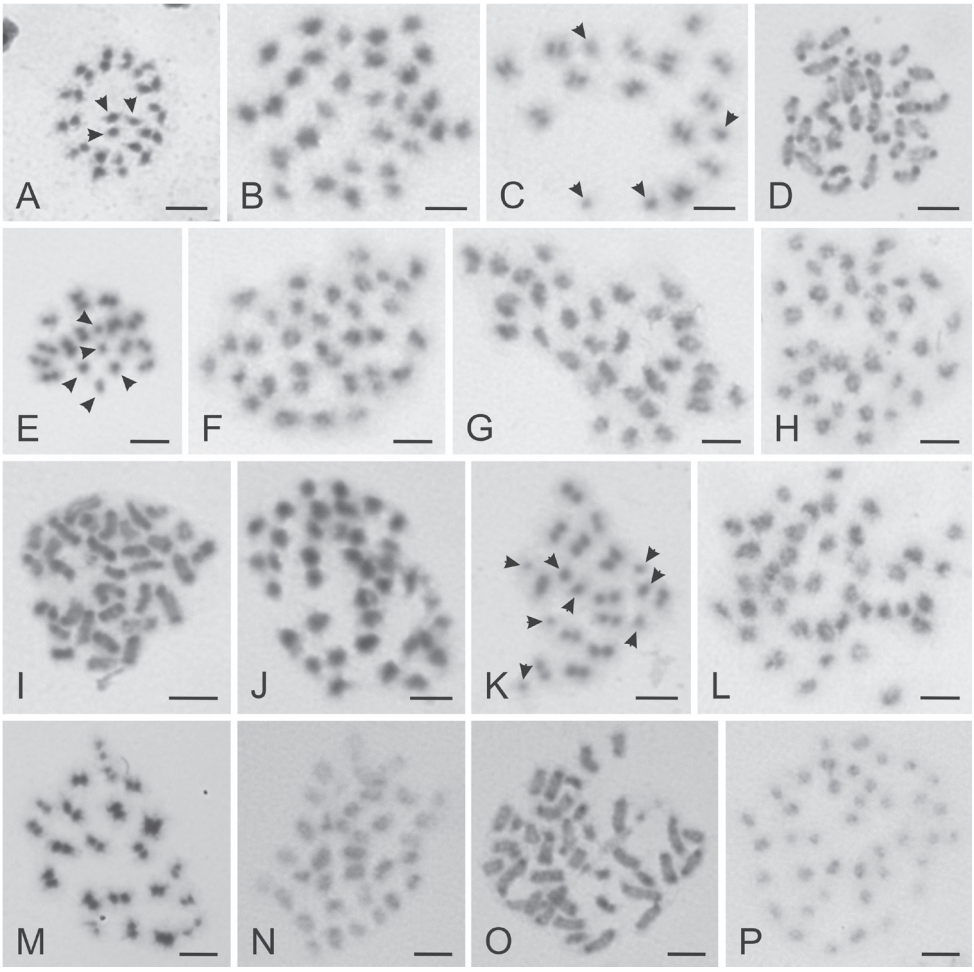

Figure 2. Examples of chromosomes of *C. lectularius* from various stages of cell division stained with Giemsa. **A** Metaphase II ♂, 2n=29 **B** Mitotic metaphase ♀, 2n=30 **C** Metaphase II ♂, 2n=30 **D** Mitotic prometaphase ♀, 2n=32 **E** Metaphase II ♂, 2n=31 **F** Mitotic metaphase ♀, 2n=34 **G** Mitotic metaphase ♂, 2n=32 **H** Mitotic metaphase ♀, 2n=36 **I** Mitotic prometaphase ♂, 2n=33 **J** Mitotic metaphase ♀, 2n=38 **K** Metaphase II ♂, 2n=34 **L** Mitotic metaphase ♀, 2n=40 **M** Metaphase I ♂, 2n=35 **N** Mitotic metaphase ♂, 2n=36 **O** Mitotic prometaphase ♂, 2n=37 **P** Mitotic metaphase ♂, 2n=40. Arrows indicate sex chromosomes. Bar = 5 μm.

Karyotype 8 included a complement with nine X chromosomes and 2n=36 in males (2n=26+$X_{1\text{-}9}$Y) (Fig. 2n) and it was recorded in a single male collected in the Slovakia.

Karyotype 9 included a complement with ten X chromosomes and 2n=37 in males (2n=26+$X_{1\text{-}10}$Y) (Fig. 2o) and it was recorded in three males collected in the Austria, Slovakia and Sweden.

Karyotype 10 included a complement with 13 X chromosomes and 2n=40 in males (2n=26+$X_{1\text{-}13}$Y) (Fig. 2p). This karyotype was identified in a single male collected in Slovakia.

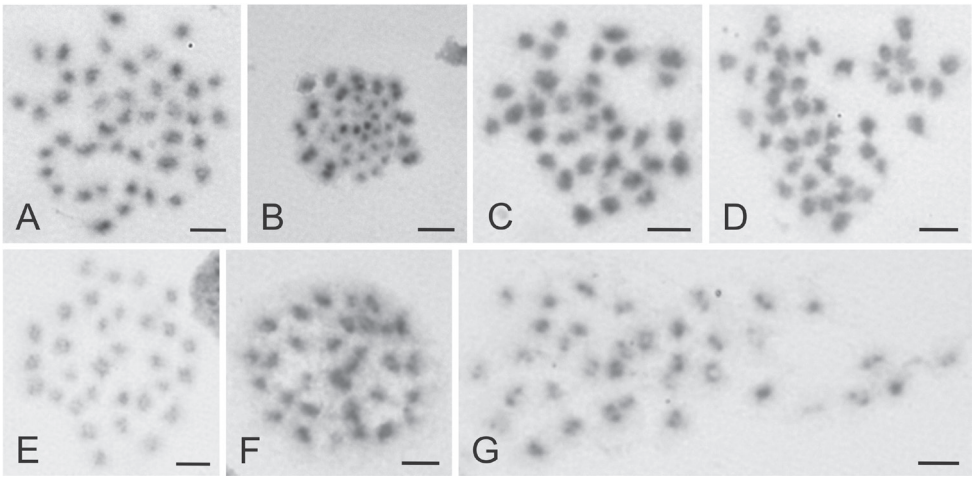

Figure 3. Examples of chromosomes of *C. lectularius* (**A–D**) and *C. pipistrelli* (**E-G**) from various stages of cell division stained with Giemsa. **A** Mitotic metaphase ♂, 2n=42 **B** Metaphase II ♂, 2n=47 **C** Mitotic metaphase ♀, 2n=33 **D** Mitotic metaphase ♀, 2n=43 **E** Mitotic metaphase ♂, 2n=31 **F** Mitotic metaphase ♀, 2n=32 **G** Mitotic metaphase ♀, 2n=36. For more details see text. Bar = 5 μm.

Karyotype 11 included a complement with 15 X chromosomes and 2n=42 in males (2n=26+$X_{1\text{-}15}$Y) (Fig. 3a). This karyotype was identified in a single male collected in the Czech Republic.

Karyotype 12 included complements with 20 X chromosomes and 2n=47 in males (2n=26+$X_{1\text{-}20}$Y) (Fig. 3b). This complement was identified in a single male from the Czech Republic.

The females exhibited the number of chromosomes which was usually complementary to the number established in the males from the same sample. However, 11 polymorphic samples were revealed in which the karyotypes of females and males were not complementary one another. Two females showing karyotypes with odd numbers of X chromosomes (7 and 17; 2n=33 and 43, respectively) were recorded (Fig. 3c, d).

The occurrence of chromosomal mosaics with the karyotype constitution varying between cells of single individual was observed in five specimens (2 males and 3 females) from five samples. A female from Slovakia (Trnava) had two karyotypically different cell types. The complement with 14 X chromosome fragments (2n=40) was found in mesenteron cells, whereas 17 X chromosome fragments (2n=43) were observed in germinal cells from ovarium. In other individuals showing mosaic, variation was recorded between germinal cells derived from gonads only. In a single female from Austria (Melk), the karyotypes with 12 and 14 X chromosome fragments were recorded (2n=38 and 40, respectively). A male from Janov in the Czech Republic showed cells with six or seven X fragments (2n=33 and 34, respectively). A similar mosaic constitution was recorded in a female from České Budějovice (4) (2n=36 and 40,

respectively). Mosaicism with two and five X chromosome fragments was also revealed in a male from Italy (Venezia 3) (2n=29 and 32, respectively).

The karyotypes with higher chromosome numbers as well as individuals with chromosomal mosaic were usually found within the samples exhibiting particularly extensive variation between individuals. A sample from Liberec contained two males with karyotypes 2n=26+X_{1-4}Y and a single female with 2n=26+2X_{1-14}. The other sample from Liberec, collected in another flat in the same house, included three males with distinctly different karyotypes (2=26+X_{1-8}Y, 2n=26+X_{1-15}Y, 2n=26+X_{1-20}Y). The Trnava sample contained five males with different karyotypes (2n=26+X_{1-4}Y, 2n=26+X_{1-8}Y, 2n=26+X_{1-9}Y, 2n=26+X_{1-10}Y, 2n=26+X_{1-13}Y) and a female showing a mosaic karyotype with different chromosomal numbers observed in both examined tissues. The Melk sample included a male with 2n=26+X_{1-10}Y, and two females, one with 2n=26+2X_{1-7} and another with a mosaic karyotype constitution 2n=26+2$X_{1-12/14}$.

The karyotype of *C. pipistrelli* included 28 autosomes and the sex chromosome trivalent X_1X_2Y (males 2n=28+X_{1-2}Y=31, females 2n=28+2X_{1-2}=32; Fig. 3e, f). This complement was found in four specimens examined. The complement of a female from Slovakia (Hontianske Nemce) contained eight X chromosomes (2n=28+2X_{1-4}=36; Fig. 3g).

Discussion

Our data confirm considerable variation in the karyotype of the bed bug and further extend its range (Table 3). The distribution of the karyotypes in various Czech and European localities appeared random, and did not show any consistent geographic pattern. Therefore, no reliable information concerning the historical or current dispersal of bed bugs can be derived.

We have obtained certain findings that are at variance with the previously published results. The distribution pattern of incidence of the X chromosome fragments reported by Darlington (1939) is different from that revealed in our study. Darlington (1939) recorded mostly individuals with higher chromosome numbers and the numbers of the X chromosomes higher then six prevailed in his samples (23 specimens out of the 25 examined ones). In our study, individuals with lower numbers clearly prevailed. In the samples examined, 89 out of the 116 specimens studied had less then five X chromosomes in their complements and the karyotype containing only two X chromosomes was recorded in approximately a half of the specimens studied. This pattern is fairly congruent with data obtained by Slack (1939), Ueshima (1966, 1967) and Grozeva et al. (2010, 2011). We have not recorded some of the karyotypes reported by Darlington (1939) and Slack (1939) in the studied European samples. This absence is apparently related to random sampling. On the other hand, our study has revealed the highest known chromosome number in the male bedbug karyotype with 47 chromosomes (2n=26+X_{1-20}Y). This represents the highest X chromosome number recorded within Cimicidae, Heteroptera and probably also Insecta.

Table 3. A synopsis of known karyotypes in *C. lectularius*. References: 1 - Darlington 1939, 2 - Slack 1939, 3 - Ueshima 1966, 4 - Grozeva et al. 2010, 5 - Grozeva et al. 2011. BG = Bulgaria, ET = Egypt, J = Japan, MEX = Mexico, RUS = Russia, USA = United States of America. See Tables 1 and 2 for explanation of other countries abbreviations. The samples reported in this study in bold.

Karyotype	X, Y	2n	Country	References
1	2XY	29	**CH**, **CZ**, BG, F, **GB**, **I**, J, MEX, **N**, **PL**, RUS, **SK**, USA	1, 3, 4, 5, this study
2	3XY	30	**CZ**, **F**, GB, **I**, **PL**	1, 2, this study
3	4XY	31	**CZ**, GB, **S**, **SK**	1, 2, this study
4	5XY	32	**CZ**, GB, **I**, **PL**, **S**	1, 2, this study
5	6XY	33	**CZ**, ET, GB, **PL**, **S**, USA	1, 2, 3, this study
6	7XY	34	**A**, **CZ**, GB, **PL**, **SK**, USA	1, 2, 3, this study
7	8XY	35	**CZ**, GB, **SK**, USA	1, 2, 3, this study
8	9XY	36	GB, **SK**, USA	1, 2, 3, this study
9	10XY	37	**A**, GB, **S**, **SK**	1, 2, this study
10	11XY	38	GB	1, 2
11	12XY	39	GB	1, 2
12	13XY	40	GB, **SK**	1, 2, this study
13	14XY	41	GB	1, 2
14	15XY	42	**CZ**, GB	2, this study
15	20XY	47	**CZ**	this study

The results obtained in *C. pipistrelli* confirm the previously published data (Ueshima 1966) in respect of the standard karyotype but the finding in a single female indicate that variation in the number of the X chromosomes may rarely occur also in this species.

There are various possible explanations of the origin of extensive variation in the chromosome number in the karyotypes of bed bugs. The elements responsible for numerical variation in bed bugs could belong to a specific chromosomal type known in other heteropterans. In 14 families of this order, a special pair of chromosomes occurs called the m-chromosomes (e.g. Ueshima 1979). The size of these chromosomes is distinctly smaller than that of other chromosomes, and their meiotic behaviour is unusual. The origin and significance of these elements remain unknown (Ueshima 1979, Ituarte and Papeschi 2004, Rebagliati et al. 2005, Papeschi and Bressa 2006, Kuznetsova et al. 2011). It is quite improbable that the supernumerary chromosomes producing the numerical variation between karyotypes of bed bugs are related to the m-chromosomes. The small supernumerary elements in the bed bug complement are rarely negatively heteropycnotic and they enter the reductional division as late as in the metaphase II, similarly as typical sex chromosomes. There is no evidence of the presence of the m-chromosomes in karyotypes of bed bugs.

B chromosomes were reported in species from various bug families including Cimicidae (Ueshima 1966, Grozeva and Nokkala 2001, Pérez et al. 2004, Panzera et al. 2010). The characteristic of the B chromosomes is different compared to the supernumerary elements from the bed bug complements. These additional chromosomal

fragments are not distributed randomly, they are mainly isochromatic and they do not show any signs of heterochromatinization.

Therefore, the most plausible explanation of the origin of the supernumerary elements in the bed bug complements remains fragmentation of the X chromosome. This mechanism produces a complicated system of multiple sex chromosomes and it was proposed already in previously published papers (see Ueshima 1966, 1979 for review). This explanation was supported by the observed behaviour of the fragments in meiosis and also by comparisons with other related species of the genus *Cimex*. Similar systems have been commonly found in some other heteropteran species but the extent of variation is usually limited (Papeschi and Bressa 2006). In the bed bug, the supposedly original fragmentation of the X chromosome into two segments (X_1X_2) has already become widely fixed in the extant populations. However, it is not sure that the assumed original fission resulted always in the formation of the same fragments. Similarly, the nature of subsequent fissions producing successively other fragments is not clear and may vary. The karyotypes of females with higher numbers of the X chromosome fragments could be heterozygous with varying constitution of fragments derived from parents. This possible variation cannot be evidenced with the use of classical cytogenetic techniques and molecular approach should be employed in clarifying this question.

The causes of the origin and maintenance of extensive fragmentation of the X chromosome of bed bugs remain unclear. Populations of bedbugs have been exposed to various insecticides all over the world for decades (Romero et al. 2007, Weeks et al. 2010). Potential mutagenetic effects of these toxic substances might have increased the rate of chromosomal rearrangements in bed bugs. The increased incidence of the X chromosome fragments in synanthropic populations seems to support this explanation as well as the absence of the multiple X chromosome fragments found in populations parasiting bats. However, variation resulting from this mechanism was recorded in the related species *C. pipistrelli* which does not occur in man and, rarely, also in other species of the family Cimicidae (genus *Paracimex*) not related to humans (Ueshima 1968).

We found an extraordinarily wide extent of karyotype variation between specimens in a few population samples only. We assume that this extreme variation might result from random mixing of individuals of different origin at a single site. Mating between geographically unrelated individuals can easily be imagined in a parasite such as the bed bug transmitted by migrating people. However, it is difficult to explain why these highly variable samples usually included specimens with an extreme karyotype constitution and the highest numbers of the X chromosome fragments. It is obvious that mating of parents with different karyotypes can produce great variety of recombinant complements in offspring, particularly in females. Variation in the number of chromosome fragments may be associated with abnormalities occurring in chromosome segregation during the cell division. The regular course of meiosis in the bed bug may be influenced by the holokinetic nature of chromosomes, completely achiasmatic male meiosis and inverted meiosis of the sex chromosomes. Slack (1939) and Ueshima (1964, 1966, 1967) recorded varying chromosome numbers among germ cells of single individual. We found similar mosaics in five specimens examined.

The irregular meiotic division or meiotic drive may enhance segregational variation in the chromosome number in progeny as well as mitotic segregation disturbances may contribute to the origin of mosaics in somatic cells. Mating of individuals of different origin and possibly different genetic constitution may initiate and increase the occurrence of segregation problems. We can assume that non-disjunction can produce unbalanced aneuploid gametes and result in lowered fitness of individuals carrying higher numbers of the X chromosome fragments. This effect should be apparently enhanced with the increasing number of the fragments and this may be the reason for the observed distribution pattern of individual karyotypes in the studied samples and the rarity of individuals with extremely high chromosome numbers. On the other hand, meiotic drive could cause preferential transmission of certain karyotype variants to the offspring. Ueshima (1966) investigated experimentally the transmission of different parental karyotypes to hybrids but did not report any evidence of aneuploidy or meiotic drive. We have not found any indication of such abnormalities of the cell division also in our study.

We can only speculate about relationships between the system of transmission of the fragmented sex chromosomes and the unusual features of reproductive biology of bed bugs. The assumed lowering of the fitness of individuals carrying higher numbers of the X chromosome fragments could potentially affect population dynamics of variable populations. It is apparent that more intensive cytogenetic screening combined with data on molecular variation in DNA sequences might shed light to this question.

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RESEARCH ARTICLE

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Cytological investigations and new chromosome number reports in yarrow (*Achillea millefolium* Linnaeus, 1753) accessions from Iran

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Abstract

In this study, a new chromosome number for Iranian yarrow (*Achillea millefolium* L.) accessions was reported. Cytological analyses on four *A. millefolium* accessions, indicated that two accessions were diploids (2n=2x=18) and two tetraploids (2*n*=4*x*=36). Cluster analysis based on chromosomal characteristics and karyotype asymmetry, categorized the four accessions separated into two groups. In terms of the Stebbins' system, the karyotype of diploid accessions grouped in 2A class. The average value of the total form percentage (TF%) in the group one (diploid accessions) and two (tetraploid accessions) were 40.85 and 41.15, respectively. The group one had the highest mean value for the symmetry index (S%=57.5). Consequently, it can be inferred that diploids belonging to the group one are the earlier evolutionary forms.

Keywords

Achillea millefolium, karyotype, chromosome number, ploidy level

Introduction

Achillea is one of the most recent genera of the Asteraceae family which exists throughout the world (Rechinger 1963). More than 100 species have been identified in this genus. Many of those who used these plants reported properties such as anti-inflammatory, anti-rheumatic, antiseptic, antispasmodic, analgesic, astringent, carminative, diaphoretic, digestive, expectorant, hypotensive, stomachic and etc. (Balbir et al. 2012). These plants are native to Europe and Western Asia but are also found in Australia, New Zealand, and North America (Rechinger 1963).

Achillea millefolium has a high genetic and phenotypic variation in Iran (Farajpour et al. 2012, Ebrahimi et al. 2012). The basic chromosome number is often reported in different species of *Achillea* is x = 9; however, the diversity in chromosome numbers and ploidy levels are frequently occurring in the genus (Ebrahim et al. 2012). Polyploid taxa have originated in many clades including 4*x*, 6*x* and 8*x* species, and as a result, several *Achillea* species show high morphological variability (Sheidai et al. 2009). Biste (1987) explained worthy diversity in leaf width, height, shoot number, and stomata length in different populations of the same species.

In most of the chemotypes in *Achillea* sp, camphor, borneol (Rohloff et al. 2000 and Mockute and Judzentiene 2003) and 1.8-Cineole (Saeidnia et al. 2004; Barghamadi et al. 2009 and Azizi et al. 2010) have been detected. Among a number of data that can be obtained by chromosome studies: karyotype structure, karyotype asymmetry, chromosome banding, FISH, GISH and chromosome painting (Stace 2000, Levin 2002, Graphodatsky et al. 2011, Guerra 2012), the most popular, cheap and widely used approaches is that concerning karyotype asymmetry (Peruzzi and Eroğlu 2013).

Achillea millefolium has been cytologically analyzed extensively in different regions of the world (Felfoldy 1947, Mizianty and Frey 1973, Pireh and Tyrl 1980, Lavrenko et al. 1991, Guo et al. 2012, Bala and Gupta 2013). Three cytological studies have been reported in Iran and showed the following ploidy levels: tetraploid 2n = 4x = 36, hexaploid 2n = 6x = 54 and octoploid 2n = 8x = 72 (Farsi et al. 2000, Ebrahim et al. 2012, Sheidai et al. 2009). The aims of this study were (1) to determine the chromosome numbers of four *A. millefolium* accessions and (2) to find any relationship between the karyotype characteristics and asymmetrical index with ploidy levels.

Material and methods

The aerial parts of the four *Achillea millefolium* accessions were collected from three provinces in north, west and south of Iran (Table 1). Voucher samples were deposited at the herbarium of Research Institute of Forests and Rangeland (RIFR) of Tehran, Iran. Seeds were germinated on moist filter paper in Petri dishes. Actively growing root tips, 1 to 2 cm length were cut from the germinating seeds and pretreated with 8-hydroxyquinoline (0.002M) for 2 to 4 hours and fixed in Farmer (1:3, glacial acetic acid : ethanol 95%) for 24 hours at 4° C. Thereafter, the root tips were hydrolyzed in

Table 1. Accessions of *Achillea millefolium* studied.

Accessions no.	Location	Latitude	Longitude	Elevation (m.a.s.l.)
Am1	Iran, Ardabil, Ardabil	38°15'N	48°17'E	1332
Am2	Iran, Ardabil, Meshkin-Shahr	37°58'N	48°58'E	1723
Am3†	Iran, Ilam, Ilam	34°27'N	46°25'E	1387
Am4	Iran, Fars, Estahban	53°04'N	29°12'E	1767

†some of the characteristics of this accession were reported by Farajpour et al. (2012) in table 1 (Am30)

1 N NaOH at 60° C for 5-10 minutes, stained for 45 minutes in esterase stain at 30° C, and squashed in 45% glacial acetic acid. Finally, the chromosome images were obtained with photomicroscope.

Karyotypec characteristics such as differences of range relative length (DRL), mean chromosome length (MCL), and mean arm ratio (MAR) were calculated using MICROMEASURE (Version 3.3) Software (Reeves 2001). Stebbin's classification was calculated (Stebbins 1971). Cluster analysis was performed to differentiate the accessions according to the Ward's method SPSS software for Windows 20.0 (SPSS Inc., Chicago, IL, USA).

Results and discussion

Karyological data

Am1 and Am2 accessions were diploids (2n = 2x = 18) whereas the two other accessions (Am3 and Am4) showed tetraploid (2n = 4x = 36) level (Figure 1). According to previous studies, Farsi et al. (2009) and Khaniki (1995), reported $2n = 4x = 36$ chromosomes, while Sheidai et al. (2009) and Ebrahim et al. (2012) reported hexaploid and octoploid cytotypes. In our findings, we have observed a new ploidy level (2n = 2x = 18) for two Iranian accessions of *A. millefolium* (Am3, Am4) that were collected in northern parts of Iran.

Karyotypic analysis indicated asymmetrical pattern in the four accessions of *A. millefolium* (Table 2). Mitotic metaphases and karyograms of the four accessions are shown in Figure 1. The highest TCL value was found in Am3 (60.9 μm) and the lowest was found in Am2 (24.5 μm) (Table 3). The lowest and the highest DRL values were found in Am3 and Am2 accessions, respectively (Table 3). High DRL value leads to more changes in the construction of chromosomes. DRL values in the two diploid accessions were higher than the tetraploid ones; it can be a relationship between ploidy level and DRL value. The tetraploid accessions had the most symmetric karyotypes.

Other parameters that indicate karyotype asymmetry are total form percentage (TF %; Huziwara 1962) and symmetrical index (S% or S/L%; Battaglia 1955) (Table 2). Group one (Am1 and Am2) had the highest mean value for the symmetrical index (S%=57.5) than the group two (S%= 50) (Table 2). It can be inferred that the group one, as diploids,

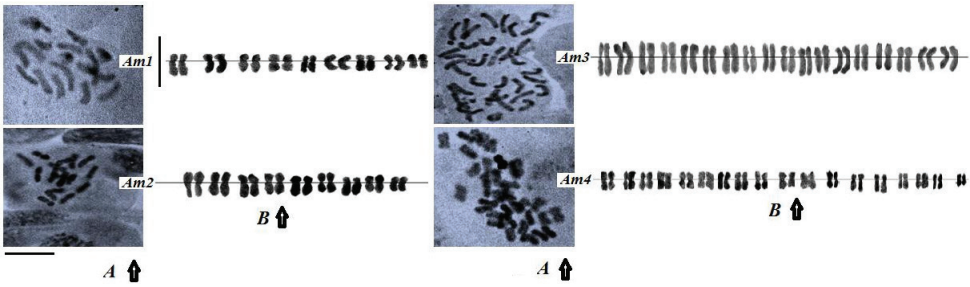

Figure 1. A–B Mitotic metaphases (**A**) and karyograms (**B**) of four*Achillea millefolium* accessions (Am1-Am4). Bars = 5μm.

Table 2. Karyotype features of four *Achillea millefolium* accessions.

Accession	2*n*	Ploidy level	TF%	S%	Karyotype formulae
Am1	18	2*x*	40.9	64	1M+8m
Am2	18	2*x*	40.8	51	1M+6m+2sm
Am3	36	4*x*	40.5	63	16M+2sm
Am4	36	4*x*	41.8	37	17M+1sm
Mean ofgroup1	-	-	40.85	57.5	-
Mean ofgroup2	-	-	41.15	50	-

‡ TF%=total form percentage (sum of short arm lengths of individual/total haploid length of the complement chromosomes), S% -symmetry index (shorter chromosome length / longer chromosome length), karyotype formula (m, median region; sm, submedian region; M, median point).

Table 3. Total chromosome length (TCL), mean chromosome length (MCL), mean arm ratio (MAR), difference of range relative length (DRL), chromosome length range (CLR), Symmetry Classes of Stebbins (SC) of four *Achillea millefolium* accessions.

Accession	TCL(μm)	MCL (μm) (±SD)	MAR(μm)	DRL (μm)	CLR (μm)	SC
Am1	26.4	2.93(±0.13)	0.71	4.92	2.4-3.7	2A
Am2	24.5	2.72(±0.17)	0.69	6.9	1.8-3.5	2A
Am3	60.8	3.37(±0.09)	0.68	2.2	2.6-4.1	1A
Am4	41	2.27(±0.11)	0.73	4.49	1.1-2.9	1B

are the earlier evolutionary form. The average value of the total form percentage (TF%) in the group one and two were 40.85 and 41.15, respectively. The TF% index has frequently been used to explain karyotype asymmetry (Mercado-Ruaro and Delgado-Salinas 1998, Ruas et al. 2000). In terms of the Stebbins' system, the karyotype of Am1 and Am2 grouped in 2A class, and it can be ancient evolutionary origin of *A. millefolium* species.

Cluster Analysis

Cluster analysis was done based on karyotypic characteristics (TCL, MCL, MAR and DRL) and karyotype asymmetry (TF% and S%) (Figure 2) and agrees with Ebrahim

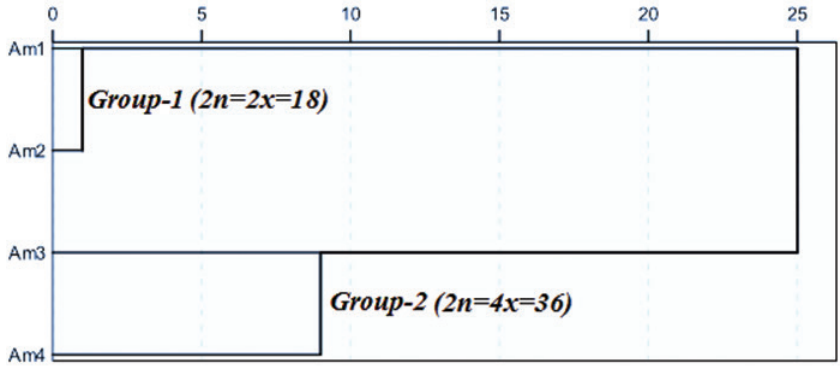

Figure 2. Dendrogram of four *Achillea millefolium* accessions based on the karyotype characteristics and asymmetry.

et al. (2012). The results of cluster analyses divided the four accessions in two groups (Figure 2); based on ploidy levels. The first group included diploid accessions (Am1 and Am2), while the second group comprised tetraploid accessions (Am3 and Am4). In the dendrogram, distance between diploid accessions is lower than tetraploid accessions that confirm the result of Stebbins' system that both of diploid accessions grouped in 2A class.

Conclusion

The results of the present study illustrated a new ploidy level (2n = 2x = 18) in Iranian *Achillea millefolium* accessions. Cluster analysis indicated that accessions can be classified based on ploidy levels.

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RESEARCH ARTICLE

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Karyotypic similarities between two species of *Rhamphichthys* (Rhamphichthyidae, Gymnotiformes) from the Amazon basin

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Abstract

The family Rhamphichthyidae includes three genera: *Rhamphichthys* Müller et Troschel, 1846, *Gymnorhamphichthys* M. M. Ellis, 1912 and *Iracema* Triques, 1996. From this family, only the species *Rhamphichthys hanni* Meinken, 1937 has had its karyotype described. Here, we describe the karyotypes of two additional *Rhamphichthys* species: *R. marmoratus* Castelnau, 1855 from the Reserva de Desenvolvimento Sustentável Mamirauá, Amazonas state and *R.* prope *rostratus* Linnaeus, 1766 from Pará state, both in Brazil. Our karyotypic analyses demonstrated that the diploid number is conserved for the genus (2n = 50), but the karyotypic formulas (KFs) differed between *R. marmoratus* (44m/sm+6a) and *R.* prope *rostratus* (42m/sm+8a). In both species, the constitutive heterochromatin (CH) was located in the centromeric region of most chromosomes. Large heterochromatic blocks were found on the long arms of pairs 4 and 14 in *R. marmoratus* and on chromosomes 3, 4 and 19 in *R.* prope *rostratus,* which also has a heteromorphism in chromosome pair 1. The CH was DAPI positive, indicating that it is rich in AT base pairs. The Nucleolus Organizer Region (NOR) showed staining at a single location in both species: the long arm of pair 1 in *R. marmoratus* and the long arm of pair 12 in *R.* prope *rostratus*, where it showed a size heteromorphism.

CMA_3 staining coincided with that of Ag-NOR, indicating that the ribosomal genes contain interspaced GC-rich sequences. FISH with an 18S rDNA probe confirmed that there is only one NOR site in each species. These results can be used as potential cytogenetic markers for fish populations, and comparative analysis of the karyotypes of *Hypopygus* Hoedman, 1962, *Rhamphichthys* and *Steatogenys* Boulenger, 1898 suggests that the first two genera diverged later that the third.

Keywords
Gymnotiformes, Rhamphichthyidae, Cytogenetics, FISH

Introduction

The family Rhamphichthyidae comprises three genera: *Rhamphichthys* Müller et Troschel, 1846, with eight described species, *Gymnorhamphichthys* Ellis, 1912, with six species, and *Iracema* Triques, 1996, with only one species (Ferraris 2003, Lundberg 2005, Triques 2005, Carvalho et al. 2011) (Table 1). These numbers are likely to be an underestimate, since the number of species described in Gymnotiformes has increased over the last 15 years (Albert and Crampton 2005).

The species of *Rhamphichthys* have a long and narrow body, a long tubular snout, no teeth in the jaw, and an anal fin with more than 300 rays. They are slow swimmers and spend most of their time at the bottoms of rivers (Mago-Leccia 1994, Ferraris 2003, Triques 2005). Among the Gymnotiformes, *Rhamphichthys* has the largest diversity and abundance in the Amazon basin, and the species *Rhamphichthys rostratus* Linnaeus, 1766 has the largest geographic distribution when compared with the other species of this genus (Ferraris 2003). All *Rhamphichthys* species generate electrical pulses that are used to communicate and identify mating partners and other species. This trait allows them to be nocturnal and live in rivers with dark waters (Kawasaki et al. 1996, Crampton 1998, Nanjappa et al. 2000, Gouvêa et al. 2002).

The phylogeny of the Gymnotiformes proposed by Albert (2001) was based on morphophysiological, behavioral and DNA sequence analyses by Alves-Gomes et al. (1995). In it, the families Rhamphichthyidae and Hypopomidae form a monophyletic group (Rhamphichthyoidea) that is separated from the clade that includes the families Sternopygidae and Apteronotidae. Among the Rhamphichthyoidea, the tribe Steatogenini (*Steatogenys* Boulenger, 1898, *Hypopygus* Hoedman, 1962 and *Stegostenopos* Triques, 1997) is accepted as monophyletic (Albert and Campos-da-Paz 1998, Crampton et al. 2007), but there is some debate as to whether this tribe belongs to the Rhamphichthyidae (Alves-Gomes et al. 1995) or the Hypopomidae (Albert 2001).

Relatively few cytogenetic studies have been performed in Gymnotiformes. According to Oliveira et al. (2009), only 48 species of this order have had their karyotypes described. The genera *Gymnotus* Linnaeus, 1758 and *Eigenmannia* Jordan et Evermann, 1896 have the most available information on their karyotypic diversity (Almeida-Toledo et al. 2001, 2002, Lacerda and Maistro 2007, Milhomem et al. 2007, 2008, Silva et al. 2009, Nagamachi et al. 2010).

Table 1. Species of Rhamphichthyidae (According to Ferraris 2003 and Albert and Crampton 2005).

Species	Locality
Gymnorhamphichthys hypostomus Ellis, 1912	São Joaquim, Bolivia
G. rondoni Miranda Ribeiro, 1920	17 de Fevereiro River, Amazonas, Brazil
G. petiti Géry et Vu-Tân-Tuê, 1964	Bananal Island, Araguaia River, Brazil
G. rosamariae Schwassmann, 1989	Negro River, Amazonas, Brazil
G. bogardusi Lundberg, 2005	Orinoco River, Delta Amacuro State
G. britskii Carvalho et al., 2011	Paraná- Paraguay System
Iracema caiana Triques, 1996	Jauaperi Beach, Negro River, Amazonas, Brazil
Rhamphichthys apurensis Fernández-Yépez, 1968	Bucaral River, a tributary of Apure River, Venezuela
Rh. atlanticus Triques, 1999	Viana Lake, Amazonas, Brazil
Rh. drepanium Triques, 1999	Janauari Lake, confluence of the Negro and Solimões Rivers, Amazonas, Brazil
Rh. hahni Meinken, 1937	Paraná River basin, next to Corrientes, Argentina
Rh. lineatus Castelnau, 1855	Ucayali River basin, Peru
Rh. longior Triques, 1999	Paru Lake, confluence of the Trombetas River, Para, Brazil
Rh. marmoratus Castelnau, 1855	Araguaia River, Brazil; Ucayali River, Peru
Rh. rostratus Linnaeus, 1766	South America

In Rhamphichthyoidea, the available chromosome information comes from only six species (Table 2): *Hypopomus artedi* Kaup, 1856 with diploid number (2n) = 38, Fundamental Number (FN) = 70 and Karyotypic Formula (KF) = 32m/sm+6st/a; *Hypopygus lepturus* Hoedman, 1962 with 2n = 50, FN = 86 and KF = 36m/sm+10st+4a; *Brachyhypopomus brevirostris* Steindachner, 1868, with 2n = 36, FN = 42 and KF = 6m/sm+30st/a (Almeida-Toledo et al. 2000); *B. pinnicaudatus* Hopkins, 1991, with 2n = 41 in males and 42 in females (X_1X_2Y sex system) and FN = 42, with all acrocentric chromosomes except the Y (Almeida-Toledo 1978); *Steatogenys elegans* Steindachner, 1880, with 2n = 50 (ZZ/ZW sex system), FN = 62 and KF = 12m/sm+38st/a; *S. duidae* La Monte, 1929, with 2n = 50, FN = 100 and KF=50m/sm (Cardoso et al. 2011); and *Rhamphichthys hahni* Meinken, 1937, with 2n = 50, FN = 94 and FK = 44m/sm+6st/a (Mendes et al. 2012).

In the present work, we studied the karyotypes of two species of *Rhamphichthys* from the Amazon region in an effort to better define the boundaries between the species, and compared our findings with those from the single previously described species of *Rhamphichthys* to better understand the phylogenetic relationships in this genus.

Material and methods

Fishes were collected using a bioamplification device that detects electric fields and translate them into sounds (Crampton et al. 2007). We analyzed 13 animals (seven males and six females) of *Rhamphichthys marmoratus* Castelnau, 1855, collected from

Table 2. A review of the cytogenetic information in Rhamphichthyoidea from Cardoso et al. (2011) with modifications.

Family / Species	2n	KF	Sex system	CB	NOR	References
Hipopomidae						
Hypopomus artedi Kaup, 1856	38	32m-sm / 6st-a	Absent	-	-	Almeida-Toledo (1978) in Oliveira et al. (2009)
Brachyhypopomus brevirostris Steindachner, 1868	36	6m-sm / 30st-a	Absent	-	-	Almeida-Toledo (1978) in Oliveira et al. (2009)
B. pinnicaudatus (Hopkins, 1991)	41♂ / 42♀	1m/41a♂ / 42a♀	X1X2Y	Centromeric region of most chromosomes	Multiple	Almeida-Toledo et al. (2000)
Hypopygus lepturus Hoedeman, 1962	50	36m-sm / 14st-a	Absent	-	-	Almeida-Toledo, (1978) in Oliveira et al. (2009)
Steatogenys elegans (Steindachner, 1880)	50	12m-sm/ 38st-a	ZZ/ZW	Centromeric region of all chromosomes and interstitial (1q and 2 blocks in Wq)	Single	Cardoso et al. (2011)
Steatogenys duidae (La Monte, 1929)	50	50 m-sm	Absent	Centromeric and pericentromeric region of all chromosomes and interstitial (2q , 3q, 5q and 7q)	Single	Cardoso et al. (2011)
Rhamphichthyidae						
Rhamphichthys hahni (Meinken, 1937)	50	44m-sm / 6a	Absent	Centromeric region of most chromosomes and blocks of CH in three chromosomes (SM)	Single	Mendes et al. (2012)
R. marmoratus Castelnau, 1855	50	44m-sm / 6st-a	Absent	Centromeric region of most chromosomes and interstitial blocks (4q and 14p)	Single	Present work
R. prope *rostratus* (Linnaeus, 1766)	50	42m-sm / 8a	Absent	Centromeric region of most chromosomes and interstitial blocks (3q, 4q and 19p)	Single	Present work

rivers in the Reserva de Desenvolvimento Sustentável Mamirauá (Mamirauá Sustainable Development Reserve, RSDM), Amazonas state, Brazil (03°07'32.5"S / 064°46'47.3"W). The sample was deposited in the museum of the RSDM (IDSMIctio000735 and IDSMIctio000750). The two individuals of *Rhamphichthys* prope *rostratus* Linnaeus, 1766, one male and one female, came from the Parú River, Pará state, Brazil (01°31'13.39"S / 52°38'49.00"W). This sample was deposited in the Museu Paraense Emílio Goeldi (MPEG 18347). Figure 1 shows the collection sites.

Metaphase chromosomes were obtained according to the method described by Bertollo et al. (1978) and analyzed by Giemsa staining, C-banding (Sumner 1972), Ag-NOR staining (Howell and Black 1980), CMA_3 banding (Schweizer 1980) and DAPI

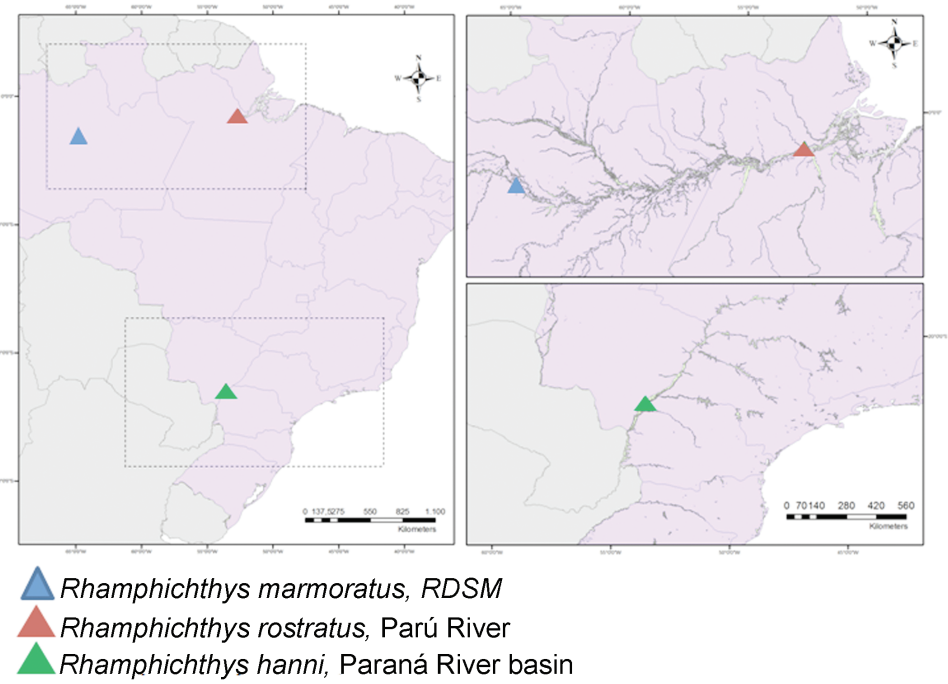

Figure 1. A map with the location of the *Rhamphichthys* species with cytogenetic descriptions. *R. marmoratus* and *R. rostratus* were analyzed in the present work.

banding (Pieczarka et al. 2006). Fluorescent *In Situ* Hybridization (FISH) was performed using 18S rDNA probes from *Prochilodus argenteus* Spix et Agassiz, 1829 (Hatanaka and Galetti Jr 2004). Microscopic images were obtained using a Zeiss Axiophot 2 microscope and a Zeiss Axiocam Mrm controlled by the Zeiss Axiovision software. Metaphase organization was performed following the method of Levan et al. (1964).

Results

Rhamphichthys marmoratus

All samples of *R. marmoratus* (Fig. 2) had 2n = 50 and a karyotypic formula (KF) consisting of 44 metacentric/submetacentric (m/sm) and 6 acrocentric chromosomes (Fig. 2a), with no evidence of any sex-determination chromosome system. Ag-NOR staining showed that the NOR is located in the interstitial region of the long arm of pair 1, in a secondary constriction (Fig. 2b, box). Constitutive heterochromatin (CH) was found in the centromeric regions of all chromosomes (Fig. 2c). Pair 4 was notable for a large heterochromatic block running from the proximal region across most of the long arm, while pair 14 had a CH block covering most of its short arm. CH was also found in the

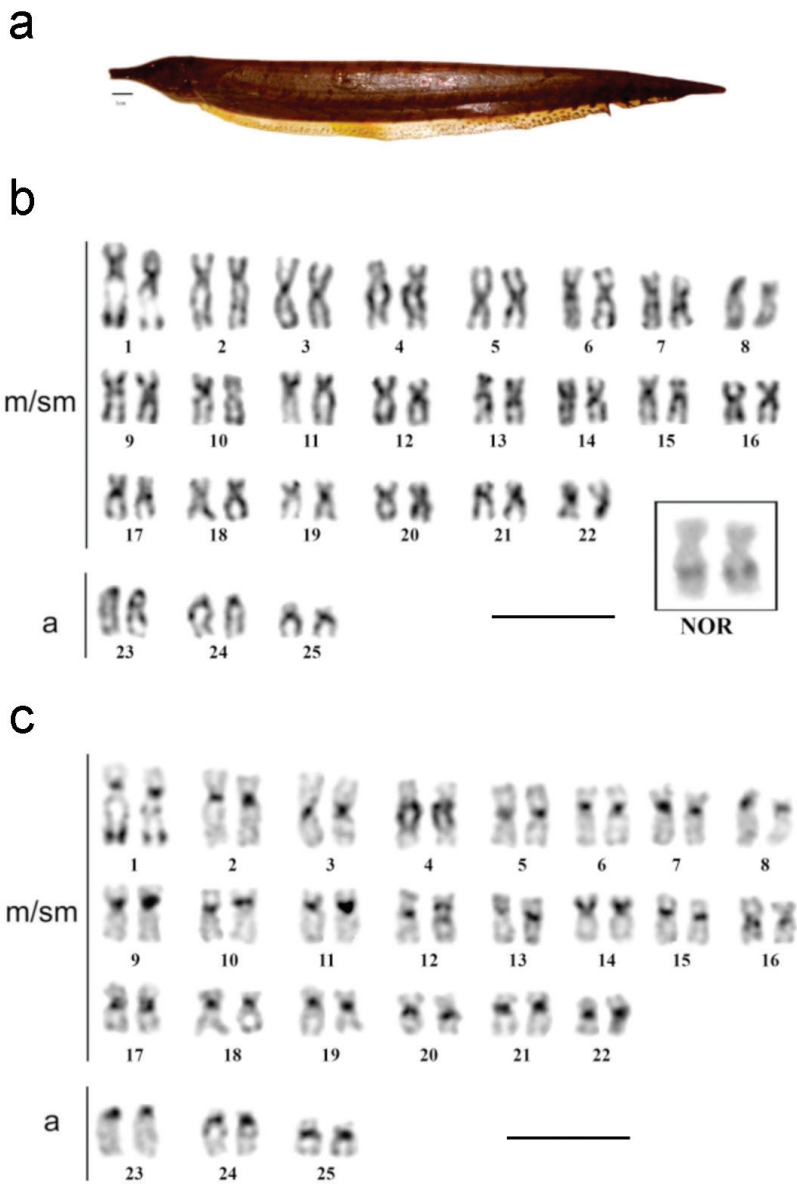

Figure 2. a *R. marmoratus* **b** Giemsa stained karyotype with the NOR bearer pair into the box **c** C-banded sequenced karyotype (m/ms- metacentric/submetacentric, a- acrocentric). Scale bar: **a**) 1 cm, **b**) and **c**) 10 μm.

distal region of the long arm of pair 1 (Fig. 2c). DAPI fluorochrome banding coincided with positive C-banding in all centromeres, and was especially strong in pairs 4 (Fig. 3a). The CMA_3 fluorochrome banding localized to the same region as the NOR, suggesting that this region is GC-rich (Fig. 3b). FISH with 18S rDNA probes confirmed that the NOR is located in the interstitial region of the long arm of pair 1 (Fig. 3c).

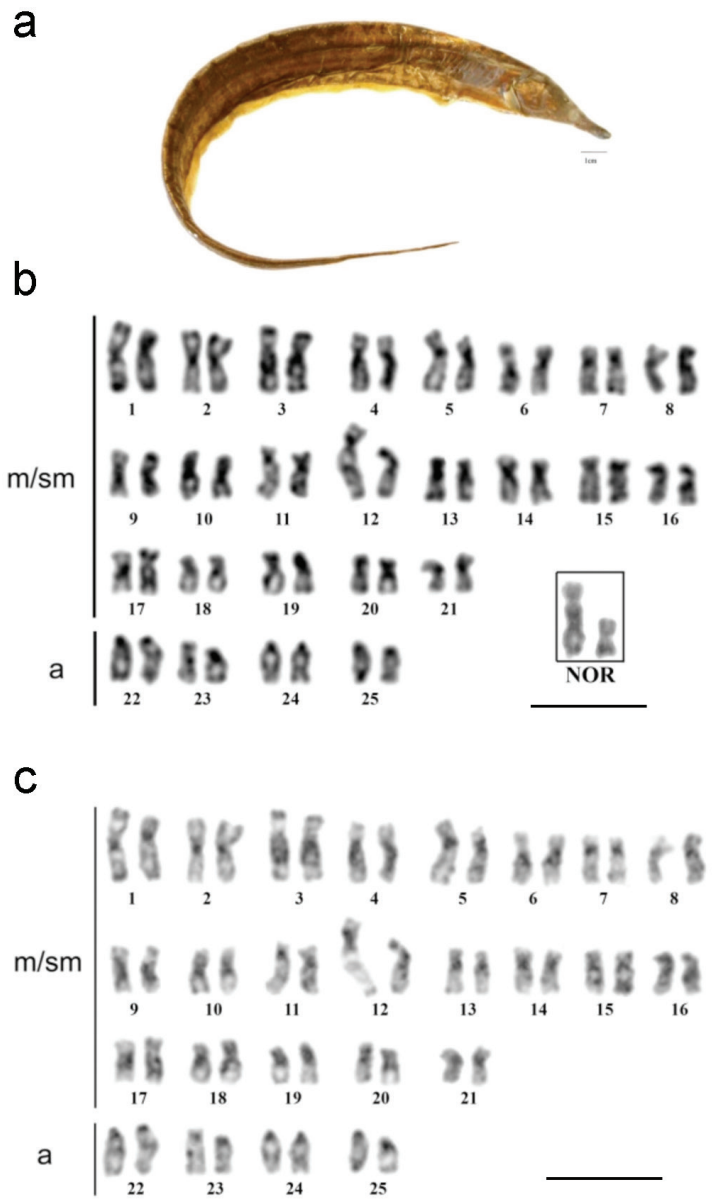

Figure 3. a *R.* prope *rostratus* **b** Giemsa stained karyotype with the NOR bearer pair into the box **c** C-banded sequenced karyotype; (m/ms- metacentric/submetacentric, a- acrocentric). Scale bar: **a**) 1 cm, **b**) and **c**) 10 µm.

Rhamphichthys prope *rostratus*

R. prope *rostratus* (Fig. 4a) had 2n = 50 and a KF of 42m/sm+8a, with no evidence of a sex-determination system (Fig. 4b). Ag-NOR staining was noted in the interstitial region of the long arm of pair 12 (Fig. 4b, box). CH was found in the pericentromeric

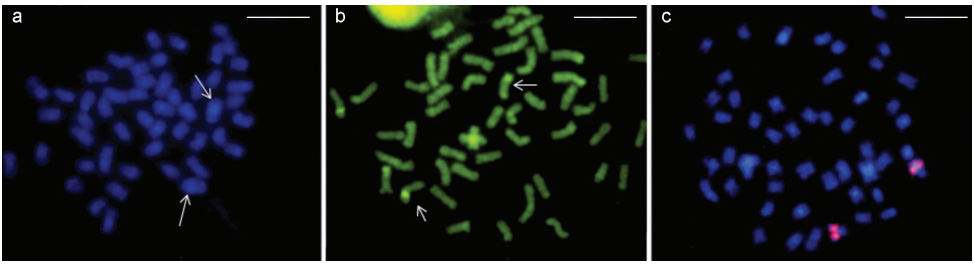

Figure 4. *R. marmoratus* - **a** DAPI staining. Arrows: pair 4 with a large CH block **b** CMA_3 staining, arrows designate NOR pair **c** FISH with rDNA probe. Scale bar: 10 μm.

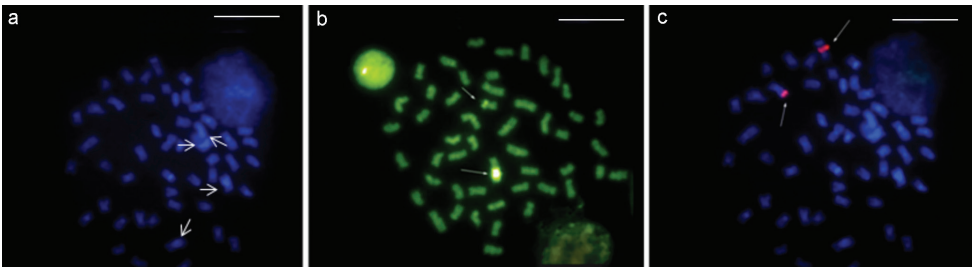

Figure 5. *R. rostratus* - **a** DAPI staining, arrows designate pairs 3 and 4 with large CH blocks **b** CMA_3 staining, arrows designate NOR pair **c** FISH with rDNA probe. Scale bar: 10 μm.

regions of most chromosomes, and large CH blocks were found in the proximal regions of the long arm of pairs 3, 4 and 9. Pair 1 had a heteromorphism in both males and females, probably because of a heterochromatin block, as did pair 12 (Fig. 4c). DAPI banding was positive in the CH regions, suggesting that these regions are AT-rich (Fig. 5a). CMA_3 banding showed size differences between the homologs, suggesting the presence of a size difference in this GC-rich region (Fig. 5b). Finally, FISH against the 18S rDNA hybridized to the same region that was positive for Ag-NOR staining (Fig. 5c).

Discussion

Both *Rhamphichthys marmoratus* and *Rhamphichthys* prope *rostratus* had 2n = 50, but differed in their KFs, with *R. marmoratus* having 44m/sm+6a and *R.* prope *rostratus* having 42m/sm+8a. Previously, *Rhamphichthys hanni* was described as having 2n = 50, but 20m+24sm+6a (Mendes et al. 2012). These differences can be explained by chromosome rearrangements that have altered the chromosome morphology but not the diploid number (e.g., pericentric inversions). These rearrangements can be sufficient to act as a post-mating reproductive barrier (King 1993). A more refined analysis, such as the use of chromosome painting, will be necessary for the precise determination of the rearrangements that differentiate the karyotypes of these three species. In a similar situ-

ation in Gymnotiformes, Nagamachi et al. (2010) demonstrated that two cytotypes of *Gymnotus carapo* Linnaeus, 1758 (2n = 42 and 2n = 40) differed not just by the fusion event suggested by the conventional analysis, but also by many rearrangements.

The CH in *R.* prope *rostratus* and *R. marmoratus* is AT-rich (i.e., DAPI banding-positive), which is consistent with other species of Gymnotiformes (Milhomem et al. 2007, 2008, Silva et al. 2008, Silva et al. 2009). The CH blocks found in pairs 4 and 12 of *R. marmoratus* and in pairs 3, 4 and 9 of *R.* prope *rostratus* can be used as cytogenetic markers for these species, as suggested for other Neotropical fish species (Almeida-Toledo 1998, Silva et al. 2008). Mendes et al. (2012) found only three submetacentric pairs with heterochromatin blocks in *Rhamphichthys hanni*. This is an important trait and can be used along with other characteristics to differentiate populations of these species, since there is some debate regarding their interspecific boundaries.

The NOR was found on a secondary constriction and stained positive with CMA_3 as previously observed on other species (Pendás et al. 1993, Fernandes et al. 2005, Milhomem et al. 2007, Silva et al. 2008, De Souza et al. 2009). Each of the species studied herein had a single NOR, but *R.* prope *rostratus* had a size heteromorphism in this region. The 18S rDNA probe hybridized to a similar-sized segment in both homologs, suggesting that the size difference is not likely to be the result of an in-tandem duplication of the ribosomal genes (Martins-Santos and Tavares 1996), as described in *Eigenmannia* sp.1 by Almeida-Toledo et al. (1996). Instead, the heteromorphism found by CMA_3 banding can be explained by a variation in the amount of GC-rich sequences interspersed among the ribosomal genes in this region. In *R. hanni* (Mendes et al. 2012), the results of the Ag-NOR staining and 18S rDNA probe hybridization were very similar to our findings in *R. rostratus.*

The phylogeny proposed by Albert (2001) places the families Rhamphichthyidae and Hypopomidae into a monophyletic group (Rhamphichthyoidea) that is only distantly related to the clade that joins the families Sternopygidae and Apteronotidae. The monophyly of Rhamphichthyoidea was supported by the synapomorphic characteristics described by Triques (2005).

However Alves-Gomes et al. (1995) suggested that Hypopomidae is not monophyletic, in that the genera *Hypopygus* and *Steatogenys* are more closely related to Rhamphichthyidae. The cytogenetic data described herein, as well as the recent work of Cardoso et al. (2011), seem to support the latter phylogenetic arrangement, since all the *Rhamphichthys* karyotypes described to date have 2n = 50. Among the Hypopomidae, *Hypopygus* and *Steatogenys* have 2n = 50, but all of the other genera have lower diploid numbers (2n = 26 to 42, Table 2). However, while the *Rhamphichthys* have karyotypes with KFs similar to those of *Hypopygus* and *Steatogenys* (42-44 bi-armed and 6- 8 mono-armed chromosomes) the KFs diverge considerably into *Steatogenys*, ranging from all bi-armed chromosomes (*Steatogenys duidae*) to mostly mono-armed chromosomes (*Steatogenys elegans*). Conversely, the karyotype of *Hypopygus* has a KF similar to those of *Rhamphichthys*. These differences seem to indicate that the genera *Hypopygus* and *Steatogenys* split from *Rhamphichthys* at an earlier date than the *Rhamphichthys* species split from one another, which is consistent with the phylogeny of Alves-Gomes et al.

(1995). The chromosome similarity between *Hypopygus* and *Rhamphichthys* suggests that these genera separated more recently than *Steatogenys*, or that chromosome evolution proceeded more quickly in the latter genus, with a buildup of autoapomorphies.

The available cytogenetic information on Gymnotiformes may be sparse (of eight species of this genus, only three have had their karyotypes analyzed), but the existing data show an important variability in this group. More cytogenetic investigations on the family Rhamphichthyidae are warranted, as they will help us better understand the chromosomal evolution of these fishes for use in other fields of science, and assist us in defining the boundaries of the *Rhamphichthys* species.

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SHORT COMMUNICATIONS

COMPARATIVE Cytogenetics
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Bibliography of studies on hybrid zones of the common shrew chromosome races distributed in Russia

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Abstract

The common shrew, *Sorex araneus* Linnaeus, 1758, has become a model species for cytogenetic and evolutionary studies after discovery of extraordinary Robertsonian polymorphism at the within-species level. Development of differential staining techniques (Q-, R-and G-banding) made it possible to identify the chromosomal arms and their combination in racial karyotypes. Entering into contact with each other, the chromosomal races might form hybrid zones which represent a great interest for understanding of the process of speciation. Until recently all known hybrid zones of *S. araneus* were localized in Western Europe and only one was identified in Siberia (Russia) between Novosibirsk and Tomsk races (Aniskin and Lukianova 1989, Searle and Wójcik 1998, Polyakov et al. 2011). However, a rapidly growing number of reports on discovery of interracial hybrid zones of *Sorex araneus* in the European part of Russia and neighboring territories appeared lately. The aim of the present work is to compile the bibliography of all studies covering this topic regardless of the original language and the publishing source which hopefully could make research data more accessible to international scientists. It could also be a productive way to save current history of *Sorex araneus* researches in full context of the ISACC (International *Sorex araneus* Cytogenetics Committee) heritage (Searle et al. 2007, Zima 2008).

Keywords

Chromosome races, Hybrid zones, Robertsonian variation, *Sorex araneus*

Introduction

The common shrew, *Sorex araneus* Linnaeus, 1758, displays exceptional variability of karyotype derived from intraspecific chromosome rearrangements of the Robertsonian type. Metacentric pairs of *S. araneus* are formed by fusion of originally acrocentric chromosomes at their centromeres in different combinations of arms. As a result, the chromosomes number (2n) varies from 20 to 33, the odd number is due to the presence of karyotype of the Robertsonian heterozygote with one metacentric and two acrocentrics, instead of two homozygous metacentrics or four acrocentrics. At the same time the fundamental number of chromosome arms (FN) remains unchanged and is equal to 40. As far as this process takes place within populations, we could talk about Robertsonian polymorphism which occurs in the vast range of *S. araneus* species.

After the pioneer analysis in Western Europe in the 1950s and 1960s, the studies of Robertsonian polymorphism in *S. araneus* populations started in Russia, widening the area of cytogenetic investigations to include European and Asian parts of the former USSR (Orlov 1974). The observed variations in chromosome arm lengths led to conclusion that Robertsonian fusions might involve different arms in different populations, which resulted in widely varying non-homologous metacentrics (Orlov and Kozlovsky 1969, Ford and Hamerton 1970, Hausser et al. 1985).

Introduction of new methods of chromosome identification (Q-, R- and G-banding) improved the karyotype definition and increased the interest in the common shrew chromosome evolution. The International *Sorex araneus* Cytogenetics Committee, ISACC was founded at Oxford University in 1987 and until recently international meetings were held every 3 years. The results of its activity were summarized in 2007 by Searle et al. Based on chromosome specific G-banding patterns, Searle et al. (1991) established the standard nomenclature for chromosomes of *S. araneus*. Later rules for differentiation of the intrapopulation variants (polymorphism) from the interpopulation ones (polytypy) as well as from individual karyotype forms were developed (Hausser et al. 1994). Chromosome identification made it possible to describe the chromosomal races of *S. araneus* (Halkka et al. 1974, 1987). Results of karyological studies over the full species range were successively summarized first by Zima et al. (1996) and then by Wójcik et al. (2003). In Russia G-banded chromosomes of the common shrew were first described for a Siberian (Novosibirsk) population by Král and Radjabli in 1974. Results of further studies of high resolution G-banding and chromosome painting of race Novosibirsk represented the species in the international "Atlas of Mammalian Chromosomes" (2006) and in comprehensive comparative studies of *Sorex* (Biltueva et al. 2011).This race was also used for DAPI karyotyping of the common shrew (Minina et al. 2007).

Currently, no less than 72 chromosomal races are recognized in total (White et al. 2010). The number of Russian chromosomal races has already reached 25 (Orlov et al. 1996, 2007, Bulatova et al. 2000, Shchipanov et al. 2009, Pavlova 2010). Only four of these races are common for Russia and some neighboring areas. They include the following: 1) the Neroosa race which spreads over the southern regions of Russia and

Ukraine; 2) the West Dvina race which can be found in Russia – Belarus neighboring regions; 3) the Goldap race which inhabits the Baltic coast area of Poland and Kaliningrad region of western Russia; 4) the Ilomantsi race which occurs in the bordering areas of north-western Russia (Karelia) and Finland (Orlov et al. 1996, 2007, Bulatova et al. 2000, Shchipanov et al. 2009, Borisov et al. 2009a). As anticipated, regular studies of distribution of different races resulted in discoveries of interracial zones of contact in Russia (Shchipanov et al. 2009, Orlov et al. 2012, Pavlova 2013, Shchipanov and Pavlova 2013) and neighboring territories (Borisov et al. 2010, 2013). Due to ISACC activity, research that involves detection of the hybrid zones, as well as discovery and description of the chromosome races continues on a regular basis.

The first case of *S. araneus* interracial hybridization in Russia was presented by Aniskin and Lukianova (1989) for Tomsk and Novosibirsk races in Western Siberia. This hybrid zone is characterized by the high number of the chromosome arm combinations and remains one of the most complex and best studied *S. araneus* hybrid zones (Searle and Wójcik 1998, Polyakov et al. 2011). The hybrids here form a complex meiotic configuration, a long chain of 9 monobrachially homologous acrocentrics and metacentrics. Presumably, chromosome incompatibility proved by meiosis data may induce infertility in hybrids which, in turn, could contribute to promotion of the selection for assortative mating (Searle and Wójcik 1998). Given that racial karyotypes of *S. araneus* as a rule differ by 1–5 variable metacentrics, the hybrids should produce rings or chains of different numbers and length in meiosis. Thus, the simplest heterozygotes form the chain of three, CIII, or ring of four, RIV. The most complex heterozygote was registered in Moscow and Seliger races hybrids in European Russia, and represents the chain of eleven, CXI (Bulatova et al. 2007). As far as the meiotic complications may lead to reduced hybrid reproductive fitness, the incompatibility is to be considered as the first stage in reproductive isolation. There are indications that the Robertsonian rearrangements do not interrupt the existent gene flow in hybrid zones and could not promote speciation in *S. araneus*. Instead, races might be merely remnants of past allopatric differentiation followed by the loss of secondary contact (Horn et al. 2012, Polly et al. 2013), presenting in particular astonishing racial 'patchwork'.

As has been shown in a variety of recent studies, the number and diversity of the chromosome rearrangements along with the relative variety of hybrid zone types represent a great opportunity both for understanding of the aftereffects and possible connections of chromosome mutations with the morphological, ecological and genetic differentiation in wild populations of common shrews (see Bibliographic list). It seems quite appropriate to recall the forecast made the British cytogeneticists CE Ford and JL Hamerton in 1970 (p. 235): "… shrews displayed multiple patterns of chromosome variation predicting the problems essential for the interpretation of species evolution. Information about hybrid meiosis would be of outstanding value and studies of pregnant females and their embryos from polymorphic populations could give important information about the breeding system and relative fertility. At a more modest level there remain many parts of Europe from which simple identification of the karyotype in samples from the local population could at least help to fill in the still rather

fragmentary distribution map of Races A and B and might reveal further unsuspected chromosome variation". Till now only the second part of this task has been mostly accomplished, while our knowledge of the influence of chromosome rearrangements on cells, specimen and species is still too fragmentary.

The first tribute to the bibliography on the *S. araneus* cytogenetic model was paid by Prof. Jan Zima at the 8th ISACC meeting (2008). To support his idea, we compiled the bibliographical list which includes majority if not all of currently available papers devoted to interracial hybrid zones of *S. araneus* in Russia. The Bibliographic list presented here includes 43 full papers published in national and international scientific editions within the last 40 years. As it shown by the published data, hybrid karyotypes and true hybrid zones were reported for at least 14 out of 25 chromosome races (which are indexed below) of the common shrew that inhabit Russia. This index includes the names of the races and their standard abbreviations, karyotypic diagnosis and F1 hybrids meiotic formula followed by the reference number of the relevant papers from our Bibliographic list.

Bibliographic list *

*Papers from the Bibliographic list referred to in the Introduction and not included in the final References are marked with asterisks.

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Index

Moscow (Mo)
gm, hi, kr, no, pq
- Neroosa (F1: gm/go/no/mn, hi, kr, pq; RIV): 17, 24
- Seliger (F1: g/gm/mq/pq/pr/kr/ik/hi/hn/no/o; CXI): 1, 6, 8, 10, 15, 21, 23, 25, 26, 27, 29, 40, 41, 42
- West Dvina (F1: gm, hi/ip/pq/qr/kr/hk, no; RVI): 6, 18, 20, 21, 23, 40

Neroosa (Ne)
go, hi, kr, mn, pq
- Moscow (F1: gm/go/no/mn, hi, kr, pq; RIV): 17, 24

Novosibirsk (No)
go, hn, ik, mp, qr
- Tomsk (F1: o/go/gk/ik/hi/hn/mn/mp/p, qr; CIX): 1, 11, 15, 16, 28, 29, 30, 31, 32, 34, 35, 36, 38
- Serov (F1: go, hn, ik/ip/mp/km, qr; RIV): 28, 33

Petchora (Pt)
gi, hn, kq, mo, pr
- Kirillov (F1: gi/hi/hn/no/mo/gm, kq, pr; RVI): 39, 43
- Serov (F1: gi/go/mo/km/kq/qr/pr/ip, hn; RVIII): 43
- Sok (F1: gi/go/mo/mr/pr/ip, hn, kq; RVI): 43

Seliger (Sl)
g, hn, ik, mq, o, pr
- Moscow (F1: g/gm/mq/pq/pr/kr/ik/hi/hn/no/o; CXI): 2, 6, 8, 10, 15, 21, 23, 25, 26, 27, 29, 40, 41, 42
- West Dvina (F1: g/gm/mq/qr/pr/ip/ik/hk/hn/no/o; CXI): 20

Serov (Se)
go, hn, ip, km, qr
- Novosibirsk (F1: go, hn, ik/ip/mp/km, qr; RIV): 28, 33
- Petchora (F1: gi/go/mo/km/kq/qr/pr/ip, hn; RVIII): 43
- Sok (F1: go, hn, ip, km/mr/qr/kq; RIV): 43
- Yuryuzan (F1: go, hn, ip, km/mq/qr/kr; RIV): 40, 43

Sok (So)
go, hn, ip, kq, mr
- Manturovo (F1: go, kq, hi/ip/pr/mr/mn/hn; RVI): 43
- Petchora (F1: gi/go/mo/mr/pr/ip, hn, kq; RVI): 43
- Serov (F1: go, hn, ip, km/mr/qr/kq; RIV): 43

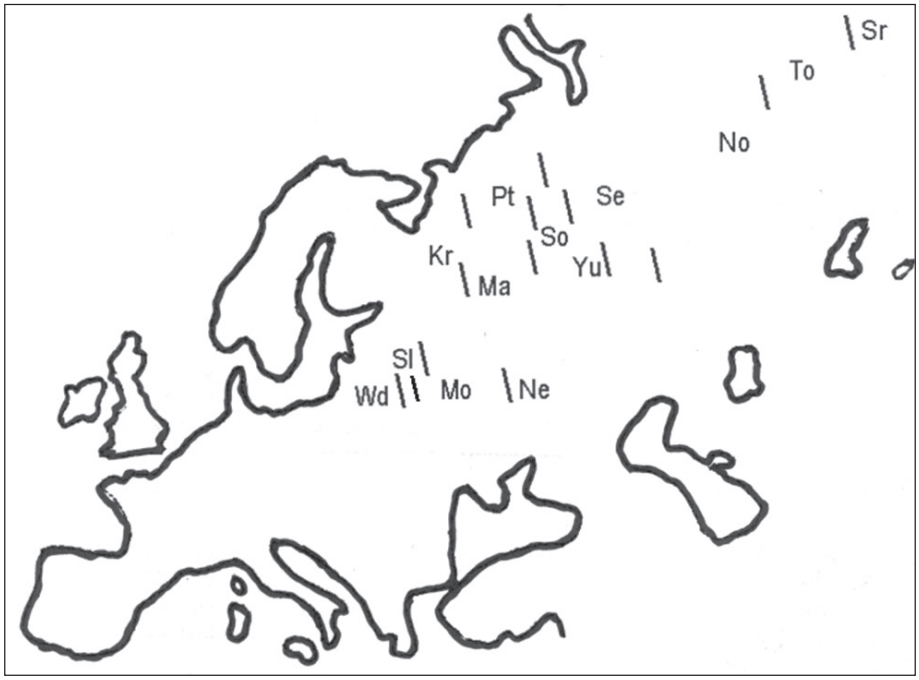

Figure 1. Schematic view of geographic distribution (slash) of hybrid zones between chromosome races of *Sorex araneus* in Russia. Standard abbreviations are used for the racial names (see Index).

Strelka (Sr)
go, hi, k, m, n, p, q, r
- Tomsk (F1: k/gk/go/o, hi, q/r, m, n, p; CIV): 28, 37

Tomsk (To)
gk, hi, mn, o, p, qr
- Novosibirsk (F1: o/go/gk/ik/hi/hn/mn/mp/p, qr; CIX): 1, 11, 15, 16, 28, 29, 30, 31, 32, 34, 35, 36, 38
- Strelka (F1: k/gk/go/o, hi, q/r, m, n, p; CIV): 28, 37

West Dvina (Wd)
gm, hk, ip, no, qr
- Moscow (F1: gm, hi/ip/pq/qr/kr/hk, no; RVI): 6, 19, 20, 21, 23, 40
- Seliger (F1: g/gm/mq/qr/pr/ip/ik/hk/hn/no/o; CXI): 20

Yuryuzan (Yu)
go, hn, ip, kr, mq
- Serov (F1: go, hn, ip, km/mq/qr/kr; RIV): 40, 43

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RESEARCH ARTICLE

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International Journal of Plant & Animal Cytogenetics, Karyosystematics, and Molecular Systematics

Karyotypes, B-chromosomes and meiotic abnormalities in 13 populations of *Alebra albostriella* and *A. wahlbergi* (Hemiptera, Auchenorrhyncha, Cicadellidae) from Greece

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Abstract

In this work 13 populations of the leafhopper species *Alebra albostriella* (Fallén, 1826) (6 populations) and *A. wahlbergi* (Boheman, 1845) (7 populations) (Cicadellidae: Typhlocybinae) from Greece were studied cytogenetically. We examined chromosomal complements and meiosis in 41 males of *A. albostriella* sampled from *Castanea sativa*, *Fagus sylvatica* and *Quercus cerris* and in 21 males of *A. wahlbergi* sampled from *C. sativa*, *Acer opalus* and *Ulmus* sp. The species were shown to share 2n = 22 + X(0) and male meiosis of the chiasmate preductional type typical for Auchenorrhyncha. In all populations of *A. albostriella* and in all but two populations of *A. wahlbergi* B chromosomes and/or different meiotic abnormalities including the end-to-end non-homologous chromosomal associations, translocation chains, univalents, anaphasic laggards besides aberrant sperms were encountered. This study represents the first chromosomal record for the genus *Alebra* and one of the few population-cytogenetic studies in the Auchenorrhyncha.

Keywords

Karyotype, meiosis, chromosomal associations, translocations, macrospermatids, B-chromosomes, Greek populations, *Alebra,* Cicadellidae, Auchenorrhyncha

Introduction

The leafhopper genus *Alebra* Fieber, 1872 (Cicadellidae: Typhlocybinae) comprises a complex of phytophagous species with several degrees of association to deciduous trees and shrubs. This Holarctic genus is represented in Europe by six valid species and several host associated populations of unknown taxonomic status. The taxonomy of *Alebra* is difficult due to the very slight morphological differences in male genital structures, a significant degree of intraspecific color pattern variation and the common occurrence of two or more species on the same food plant (Drosopoulos and Loukas 1988, Aguin-Pombo 2002). In the family Cicadellidae 387 species in 263 genera have been studied in respect to karyotype (Kuznetsova and Aguin-Pombo in press) but until now the genus *Alebra* remained totally untouched by chromosomal investigation.

Chromosomal polymorphisms in natural populations may play a significant role in speciation (White 1978, King 1993, Kawakami et al. 2011). This can easily be proven in cases in which chromosome rearrangements are easily detected as in some dipterans having giant polytene chromosomes in salivary glands. Quite the opposite situation is found in the Auchenorrhyncha with their rather small holokinetic chromosomes. Due to the absence of localized centromeres, the identification of rearrangements in holokinetic chromosomes is either difficult (e.g. translocations and deletions) or even completely impossible (duplications and inversions) if routine chromosome staining is applied. Despite the fact that approximately 820 auchenorrhynchan species have so far been karyotyped, all the data obtained concern almost exclusively chromosome numbers and gross karyotype morphology, and only few records of chromosomal polymorphisms have been published (for review see Kuznetsova and Aguin-Pombo in press).

In the present work, cytogenetic analysis of *Alebra albostriella* (Fallén, 1826) and *A. wahlbergi* (Boheman, 1845) was performed using routine chromosome staining. A study of 13 Greek populations of these species inhabiting different deciduous trees was undertaken to reveal whether the populations of these species display any polymorphism for chromosomal complements and meiotic patterns.

Materials and methods

Altogether, 41 males from 6 populations of *A. albostriella* and 21 males from 7 populations of *A. wahlbergi* inhabiting 5 different species of deciduous trees in Greece have been collected from 1989 to 1992 on plant foliage with a sweeping net. The locality names, altitude, data of collection and food plants are listed in Table 1, and the places of collection are also mapped on Fig. 1. For chromosome studies, adult males were fixed in Carnoy solution (3:1 ethanol and glacial acetic acid) and stored at -10°C. Chromosomal analysis was performed using conventional squashing procedure. Testes were dissected out, stained with 2% acetic orcein and squashed in a drop of 45% acetic acid under an 18-mm square coverslip. From 1 to 14 individuals in each population were examined. Chromosome preparations were analyzed under a Leica DM 4000B microscope (Leica

Table 1. Studied material.

Species	Population code	Locality	Altitude above sea level	Food plant	Data of collection	Number of studied males
A. albostriella	ASE*	Steni-Euboea Il.	440 m	*Castanea sativa*	8–9.07.1990	10
	AKA	Kastanitsa-Arkadia	850 m	*C. sativa*	25.06.1990 10.08.1989	2 3
	AACM	Anilio-Chania-Magnisia	990 m	*C. sativa*	23.07.1990	5
	AAPA	Agios Petros-Arkadia	990 m	*C. sativa*	15–16.7.1990	4
	AANE	Agios Nicolaos-Eurytania	1000 m	*C. sativa*	01.08.1991	2
	AATPF**	Agia Triada-Prespes-Florina	1200 m	*Fagus sylvatica*	14–21.08.1990	14
				Quercus cerris	20.08.1990	1
A. wahlbergi	WEDE	Evinos Delta-Etoloakarnania	20 m	*Ulmus* sp.	25.06.1991	5
	WSE	Steni-Euboea Il.	440 m	*C. sativa*	8–9.07.1990	2
	WKE	Kerasovo-Etoloakarnania	520 m	*Acer opalus*	14.06.1992	4
	WKA	Kastanitsa-Arkadia	850 m	*C. sativa*	25.06.1990	1
	WANE	Agios Nicolaos-Eurytania	1020 m	*C. sativa*	01.08.1991	3
	WCPF	Caries-Prespes-Florina	1100 m	*A. opalus*	19.08.1990	1
	WATPF	Agia Triada-Prespes-Florina	1200 m	*A. opalus*	14–21.08.1990	5

*Here and elsewhere we use abbreviations to refer to different populations of a species.

** Specimens of *A. albostriella* from the AATPF locality represent two different populations one occurring on *Fagus sylvatica* and the other on *Quercus cerris* (see Aguin-Pombo 2002 for details).

Microsystems Wetzlar GmbH, Germany) with a 100× objective. Images were taken with a Leica DFC 350 FX camera using Leica Application Suite 2.8.1 software with an Image Overlay module. The data obtained are presented in Tables 2–4.

Results

A. albostriella

Standard karyotype and meiosis

In males, the majority of cells showed 23 chromosomes at mitotic metaphases (Fig. 2a) and 12 units at meiotic metaphases I (MI) (Fig. 2b, c). The karyotype is asymmetric with two size groups of chromosomes. In mitosis six larger chromosomes and other chromosomes constituting a decreasing series in size were present. Chromosomes had no primary constrictions, i.e. centromeres and the sex chromosome could not be identified. At MI, 11 autosomal bivalents, including three larger, and a univalent X-chromosome were encountered (n=11 + X). Male karyotype formula of the species is thus as follows: 2n=22 + X(0). The univalent X-chromosome was similar in size to one of the larger half-bivalents within the group of smaller chromosomes and its location at MI was random. Bivalents mainly had a single terminal/subterminal or, rarer,

Figure 1. Map showing the collection localities of *Alebra albostriella* and *A. wahlbergi* in Greece.

interstitial chiasma, however in few nuclei up to four rings were present indicative of two terminal/subterminal chiasmata being formed in the larger bivalents (Fig. 2d, e). At anaphase I (AI), all the autosomes segregated to opposite poles and the X moved to one pole without dividing. The reductional division resulted thus in two daughter metaphase II (MII) cells with 11A+X and 11A, respectively (Fig. 2f).

B-chromosomes

In 4 out of 20 males analyzed in the populations ASE, AACM, AANE (sampled from *Castanea sativa*) and AATPF (sampled from *Fagus sylvatica*) one or two small B-chro-

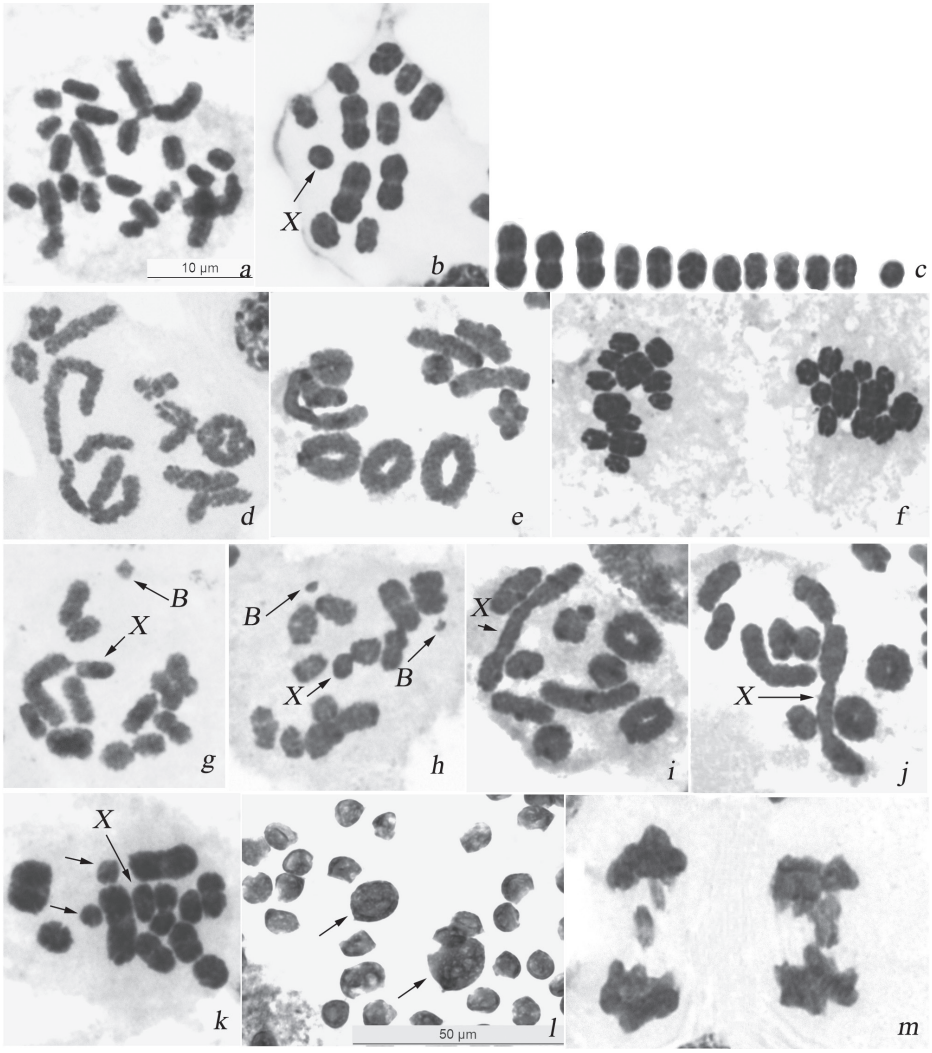

Figure 2. Karyotype and male meiosis in *Alebra albostriella*: **a** Mitotic metaphase showing 23 chromosomes **b** MI showing 11 bivalents and univalent X **c** karyogram prepared from MI (b) **d** diakinesis showing bivalents with one terminal/subterminal chiasma and a bivalent with two subterminal chiasmata **e** diakinesis showing bivalents with one terminal/subterminal chiasma, a bivalent with interstitial chiasma and 4 ring bivalents each with two terminal/subterminal chiasmata **f** two daughter AI with n=11 and n=12, respectively **g** diakinesis showing one B chromosome **h** diakinesis showing two B chromosomes **i** diakinesis with end-to-end association of two bivalents and X **j** diakinesis with end-to-end association of three bivalents and X **k** MI with one medium-sized bivalent as univalents (arrowed) **l** macrospermatids of different size (arrowed) among normal spermatids **m** AI with lagging chromosomes. Bar = 50 µm in **l** and 10 µm in other figures.

mosomes (additional to the standard complement) were found (Table 2). In the polymorphic populations, three males (1-AACM, 1-AANE and 1-AATPF) showed a single B-chromosome with the frequency of about 1% per specimen while male 10-ASE had

Table 2. B-chromosomes, meiotic abnormalities and macrospermatids in *A. albostriella*.

Populations (N=6)	Food plants	Males No (N=41)	Number of B-chromosomes	Meiotic abnormalities and macrospermatids
ASE	*Castanea sativa*	1	0	univalents
		2	0	end-to-end non-homologous associations anaphasic laggards macrospermatids
		3	0	end-to-end non-homologous associations macrospermatids
		4	0	end-to-end non-homologous associations macrospermatids
		5	0	anaphasic laggards macrospermatids
		6	0	macrospermatids
		7	0	macrospermatids
		8	0	macrospermatids
		9	0	-
		10	2	-
AKA	*C. sativa*	1	0	univalents
		2	0	-
		3	0	-
		4	0	-
		5	0	-
AACM	*C. sativa*	1	1	anaphasic laggards macrospermatids
		2	0	macrospermatids
		3	0	macrospermatids
		4	0	macrospermatids
		5	0	-
AAPA	*C. sativa*	1	0	macrospermatids
		2	0	-
		3	0	-
		4	0	-
AANE	*C. sativa*	1	1	end-to-end non-homologous associations univalents anaphasic laggards
		2	0	end-to-end non-homologous associations anaphasic laggards macrospermatids
AATPF	*F. sylvatica*	1	1	macrospermatids
		2	0	univalents macrospermatids
		3	0	end-to-end non-homologous associations
		4	0	macrospermatids
		5	0	macrospermatids
		6	0	macrospermatids
		7	0	macrospermatids

Populations (N=6)	Food plants	Males No (N=41)	Number of B-chromosomes	Meiotic abnormalities and macrospermatids
		8	0	macrospermatids
		9	0	macrospermatids
		10	0	macrospermatids
		11	0	macrospermatids
		12	0	macrospermatids
		13	0	macrospermatids
		14	0	-
	Q. cerris	15	0	anaphasic laggards

a pair of B-chromosomes in about 80% of MI (Table 4). B-chromosomes were different in size in different males while always appreciably smaller than the X-univalent and negatively heteropycnotic at late prophase and MI (Fig. 2g, h). At MI, B-chromosome(s) showed random distribution relative to autosomal bivalents and X-chromosome. In the case of two B-chromosomes, they did not show any connection to each other (Fig. 2h).

Meiotic abnormalities

Different kinds of meiotic abnormalities were encountered in 12 males (29% of the total number of males) sampled from all the 6 populations (Table 2). In males 2, 3 and 4 of ASE (from *C. sativa*), in both studied males of AANE (from *C. sativa*), and in male 3 of AATPF (from *F. sylvatica*) two to four bivalents were occasionally associated by ends. The univalent X-chromosome was very often involved in these associations. Non-homologous telomeres did not touch intimately each other but unstained gaps were seen between the bivalents (Fig. 2i, j). In some males (Table 2), one or two middle-sized bivalents were seen as univalents in some cells at diakinesis and MI (Fig. 2k). In addition, populations ASE, AACM, AANE and AAPA (from *C. sativa*) and AATPF (from *F. sylvatica*) the majority of studied males (61%; N=25) showed macrospermatids coexisting with normal spermatids within a cyst. Macrospermatids were different in size being either approximately twice larger or much larger than the normal spermatids (Fig. 2l). Some males with aberrant spermatids displayed also one or other type of meiotic abnormalities, including lagging chromosomes at anaphases (Fig. 2m) (Table 2).

A. wahlbergi

Standard karyotype and male meiosis

In sampled males, 11 autosomal bivalents and the univalent X-chromosome were present in cells at diakinesis and MI (Fig. 3a, b), male karyotype formula of this species

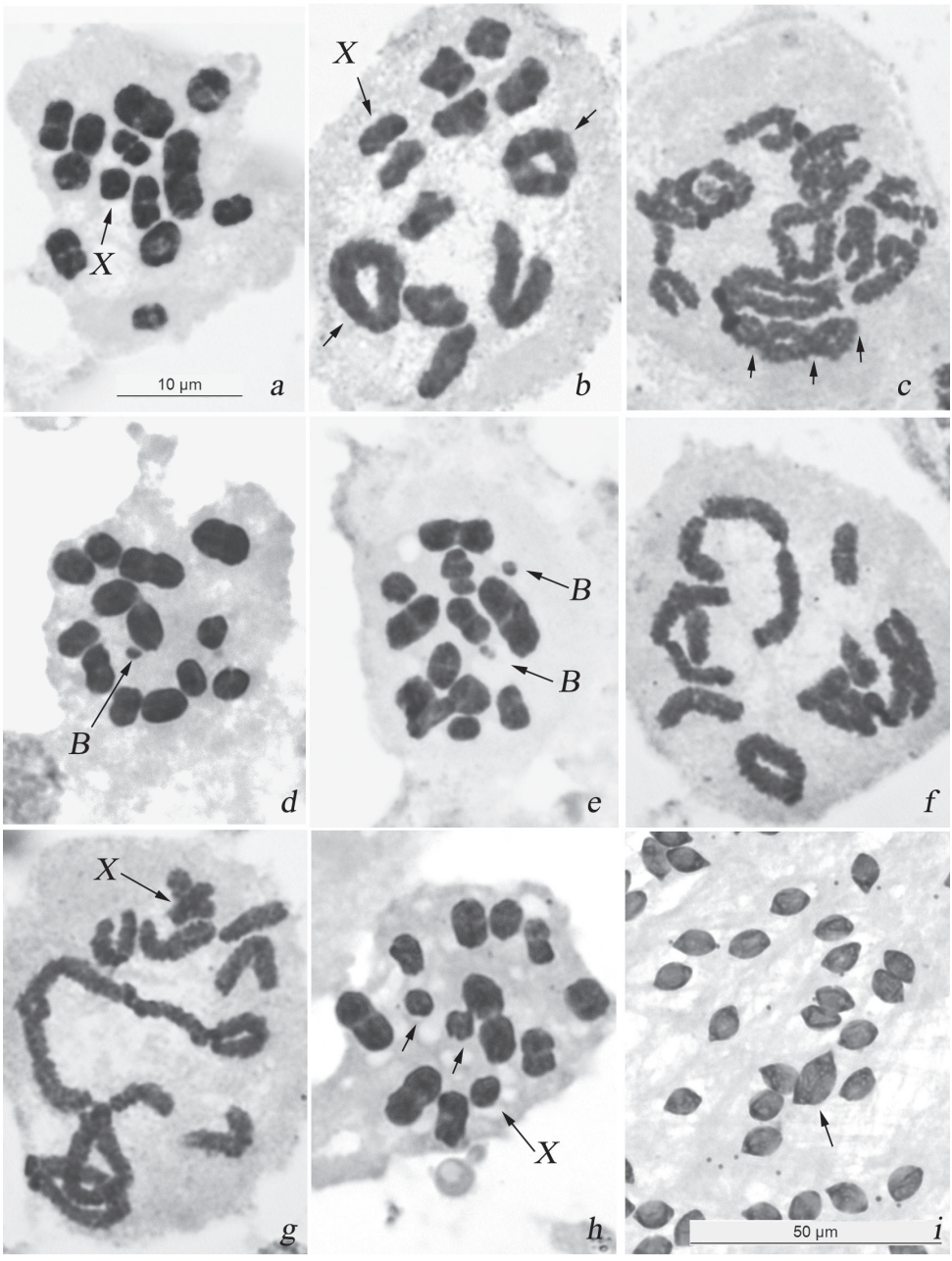

Figure 3. Karyotype and male meiosis in *Alebra wahlbergi*: **a** MI showing 11 bivalents and univalent X **b** diakinesis showing bivalents with one terminal/subterminal chiasma, a bivalent with interstitial chiasma and 2 ring bivalents each with two terminal/subterminal chiasmata **c** diplotene/diakinesis showing a bivalent with three (at least) chiasmata (arrowed) **d** MI with one B chromosome **e** MI with two B chromosomes **f** diakinesis with end-to-end association of three bivalents **g** diplotene/diakinesis showing translocation chain involving 4 bivalents **h** MI with one medium-sized bivalent as univalents (arrowed) **i** macrospermatid (arrowed) among normal spermatids. Bar = 50 μm in **i** and 10 μm in other figures.

being thus 2n = 22 + X(0). Much as in *A. albostriella*, this karyotype was asymmetric with three larger bivalents and 8 smaller bivalents, the X-chromosome being similar in size to one of the larger half-bivalents in this second group. The chromosomes had no centromeres. The univalent X-chromosome was located randomly at diakinesis and MI. The bivalents mainly had a single terminal/subterminal or rarely interstitial chiasma, however, two chiasmata (rings on Fig. 3b) and occasionally three chiasmata (arrowed on Fig. 3c) could be formed in larger bivalents. In few cells at diakinesis and metaphase I four bivalents or (in male 1-WNE) even six bivalents with two chiasmata each were observed (not shown). Both autosomes and X-chromosome separated reductionally during the first division and divided equationally during the second division of meiosis.

B-chromosomes

In 3 out of 9 males analysed in the populations WEDE (sampled from *Ulmus* sp.) and WKE (sampled from *Acer opalus*) one or two B-chromosomes were encountered (Table 3). The polymorphic male 3-WKE showed a single B-chromosome in about 1% of MI (Fig. 3d, Table 4). Males 1-WEDE and 1-WKE showed each a pair of Bs at MI with frequencies of approximately 60% and 80%, respectively (Fig. 3e, Table 4). In every case B-chromosomes were very small, negatively heteropycnotic and distributed randomly with reference to each other, to the bivalents and to the X-chromosome.

Meiotic abnormalities

Meiotic abnormalities were encountered in 11 males (52% of the total number of males) sampled from 5 out of 7 studied populations. Populations WKA and WCPF showed no meiotic disturbances however in our study they were represented by only one male each (Table 3). In males 2-WEDE, 2-WSE, 1- and 2-WANE the bivalents occasionally formed associations involving two or three bivalents connected by telomere ends. Non-homologous telomeres did not touch intimately each other but unstained gaps were seen between the bivalents (Fig. 3f). In addition, male 1-WANE had nuclei at diakinesis with X-chromosome, 7 bivalents and a translocation chain of four bivalents united by chiasmata (Fig. 3g). The chromosomal complement of these cells was in fact n = 7AA + 1(4AA) + X. Unfortunately, because of poor spreading of chromosomes in the slide no statistical analysis of the occurrence of translocation chains in this male was possible. Further still, we failed to detect the number of bivalents involved into certain translocation chains. In males 1- WEDE, 1-WSE, 2-WKE and 3-WANE, one of the middle-sized bivalents was present as univalents at MI (Fig. 3h). In populations WEDE, WKE and WATPF, 4 out of 14 males showed macrospermatids which were of approximately twice the normal size and coexisted with normal spermatids within a cyst (Fig. 3i). Among males with macrospermatids, only that 2-WEDE displayed meiotic disturbances namely the end-to-end non-homologous associations of bivalents (Table 3).

Table 3. B-chromosomes, meiotic abnormalities and macrospermatids in *A. wahlbergi.*

Populations (N=7)	Food plants	Males No (N=21)	Number of B-chromosomes	Meiotic abnormalities and macrospermatids
WEDE	*Ulmus* sp.	1	2	univalents
		2	0	end-to-end non-homologous associations macrospermatids
		3	0	macrospermatids
		4	0	-
		5	0	-
WSE	*C. sativa*	1	0	univalents
		2	0	end-to-end non-homologous associations
WKE	*A. opalus*	1	2	macrospermatids
		2	0	univalents
		3	1	-
		4	0	-
WKA	*C. sativa*	1	0	-
WANE	*C. sativa*	1	0	end-to-end non-homologous associations multiple translocation chains
		2	0	end-to-end non-homologous associations
		3	0	univalents
WCPF	*A. opalus*	1	0	-
WATPF	*A. opalus*	1	0	macrospermatids
		2	0	-
		3	0	-
		4	0	-
		5	0	-

Table 4. Frequency of B chromosomes in *A. albostriella* and *A. wahlbergi.*

Male N=7	Number of B chromosomes per cell	Total number of MI studied	Number of MI with B chromosomes	Frequency of B chromosomes per individual, %
A. albostriella				
1-AACM	1	460	3	0,65
1-AANE	1	370	4	1,08
10-ASE	2	98	82	83,7
1-AATPF	1	112	2	1,8
A. wahlbergi				
1-WEDE	2	28	17	60,7
1-WKE	2	107	84	78,5
3-WKE	1	180	2	1,1

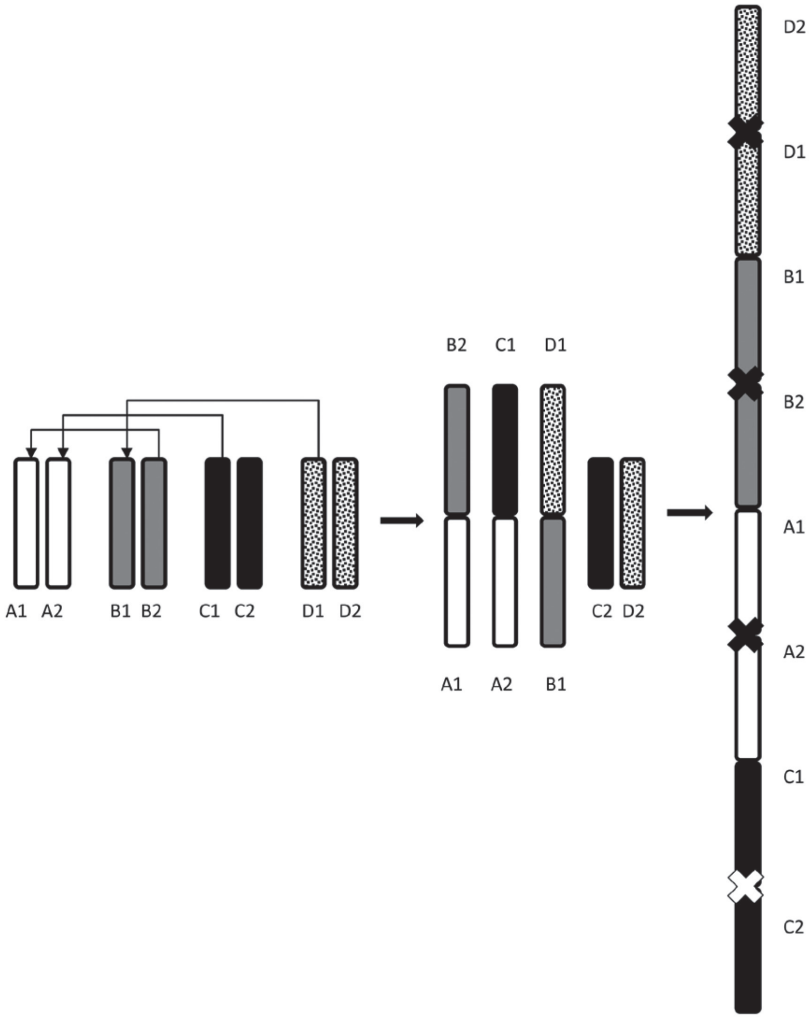

Figure 4. Schematic representation of the possible formation of a multiple translocation chain of four bivalents in meiosis of 1-WANE male. A1A2, B1B2, C1C2 and D1D2 are autosomal bivalents consecutively involved in translocation. Chiasmata in a translocation chain are shown by crosses.

Discussion

Standard karyotypes and meiosis

A. albostriella and *A. wahlbergi* were found to have the same karyotype of 2n = 22 + X(0) encountered without variation in 30,6% of studied males (N=19) and with some variation due to polymorphism in the rest of males (N=43) . The species share likewise a similar gross morphology of karyotypes. Both karyotypes are asymmetric in terms of the heterogeneity of chromosome size: one size group includes three pairs of larger chromosomes and the other group includes 8 pairs of smaller chromosomes. Within

every group, chromosomes represent continuous gradation in size and therefore can not been reliably distinguished by conventional cytogenetic approaches. The X-chromosome is close by size to one of the larger chromosomes within the smaller-sized group. Chromosomes are holokinetic as in other Hemiptera; that is, the centromeric activity is dispersed along the length of each chromosome rather than concentrated at one point (Schrader 1935).

In spite of holokinetic nature of chromosomes, there are only a few Cicadellidae genera in which chromosome number has been sufficiently liable to change in the course of speciation whereas most genera have a stable number of chromosomes (Kuznetsova and Aguin-Pombo in press). As mentioned above, *A. albostriella* and *A. wahlbergi* are the first representatives of the leafhopper genus *Alebra* studied in respect to karyotype. The chromosome complement of 2n = 22 + X(0) found in these species is fairly common in the Cicadellidae (Kuznetsova and Aguin-Pombo in press) but has never been recorded for the subfamily Typhlocybinae in which approximately 90 species in 35 genera are presently known cytologically (Kirillova 1987, Aguin-Pombo et al. 2006, Juan 2011). The only exception is probably a species referred to as Gen. nov. 6 for which Juan (2011) recorded n=12 (suggesting thus 2n = 22 + X).

Cytological analysis of male meiosis in *A. albostriella* and *A. wahlbergi* revealed that it was of the typical auchenorrhynchan type where all the chromosomes undergo segregation at anaphase I and chromatids separate at anaphase II (Halkka 1959). The small bivalents invariably had only one terminal/subterminal or, rarely, interstitial chiasma. In the larger bivalents one-two chiasmata were formed; in separate diakinesis cells up to six ring bivalents with two terminal/subterminal chiasmata were present. Halkka (1964) was the first to show that only one-two chiasmata are typically formed in male meiosis in Auchenorrhyncha species. Quite recently, Nokkala et al. (2004) have proved that the low number of chiasmata (one-two in a bivalent from a cytological standpoint) is characteristic of holokinetic chromosomes as such. The authors showed that holokinetic bivalents with multiple chiasmata entered AI but were unable to complete it as a result of wrong separation of homologues. It is noteworthy however that in our material the largest bivalents showed occasionally three and even four chiasmata. Multiple (more than two) chiasmata have also been described in other groups with holokinetic chromosomes, including in the Auchenorrhyncha (e.g. Kuznetsova et al. 2009a, b, Maryańska-Nadachowska et al. 2012), however, the work by Nokkala et al. (2004) still remains the only one in which the further fate of cells with such bivalents in meiosis was traced in detail.

In the majority of auchenorrhynchans, at least in all hitherto studied planthopper species (Fulgoroidea), the univalent X-chromosome shows a clear tendency to be arranged at the periphery of MI plate presumably forming its own meiotic spindle (Halkka 1959, Kuznetsova et al. 1998, Maryańska-Nadachowska et al. 2006). In contrast to this, in both leafhopper species studied in the present work, the univalent X-chromosome tended to locate randomly among the bivalents at MI.

Polymorphism for chromosomal rearrangements, B-chromosomes and meiotic abnormalities are not rare in nature and has been recorded for numerous species of plants

and animals, including insects (White 1973). This kind of information is however very scarce in the Auchenorrhyncha. In this group, among approximately 820 species studied cytogenetically, B-chromosomes and different chromosomal rearrangements have been reported in several species only (Kuznetsova and Aguin-Pombo in press). In light of this, it is somewhat unusual to find so extensive variation which occurs in *A. albostriella* and *A. wahlbergi* from Greece. A total of 62 individuals from 13 population samples belonging to both species were examined. The studied populations inhabited different food plants (*F. sylvatica*, *C. sativa*, *Q. cerris*, *A. opalus*) and different altitudes ranging from 20 m to 1200 m above sea level (Table 1). In all 6 populations of *A. albostriella*, 29 males (71%; N=41) showed meiotic abnormalities and of these 4 males displayed additionally B-chromosomes. In 5 populations of *A. wahlbergi*, 11 males (52%; N=21) showed meiotic abnormalities, and of these 2 males displayed also B-chromosomes. In addition, one further male showed B-chromosomes while no meiotic abnormalities. The remaining two populations of *A. wahlbergi*, each with the only studied specimen, showed neither B-chromosomes nor meiotic abnormalities.

B-chromosomes

B-chromosomes are accessory genomic elements that are known to occur approximately in 15% of living species (Beukeboom 1994). In the Auchenorrhyncha, B-chromosomes were described in several species of planthoppers (Fulgoroidea) (Halkka 1959, Booij 1982, Kirillova and Kuznetsova 1990, den Bieman 1988) but have never been found to date in any species of leafhoppers (Cicadellidae). In leafhoppers *A. albostriella* and *A. wahlbergi* studied herein, B-chromosomes were found in low numbers (0-2) in 4 males of the first species (in 4 populations) and in 3 males of the second species (in 2 populations). In both species, B chromosomes were fairly small with the exception of male 1-AANE in which a single B-chromosome was about two times larger compared to Bs in other males (Fig. 2g). In every case however the Bs were much smaller than the univalent X-chromosome and conspicuous during meiotic prophase and metaphase I because of their negative heteropycnosis. When Bs were two in number, they did not pair and passed randomly through meiosis as univalents being still maintained in populations. Also, B-chromosome(s) did not connect to the univalent X-chromosome at MI as it was observed by Kirillova and Kuznetsova (1990) in several planthopper species from the family Delphacidae. The inter-population differences in B-chromosome distribution is believed to depend on different selective factors (Beukeboom 1994, Camacho 2004). It is interesting to note in this connection that in delphacid species *Javesella pellucida* (Fabricius, 1794) B-chromosomes were present only in populations inhabiting Northeast Siberia and Kamchatka whereas individuals sampled from different populations in European Russia lacked B-chromosomes (Kirillova and Kuznetsova 1990). In our study, the occurrence and frequency of B-chromosomes in males showed no relation with particular food plants on which populations inhabit. For example, *A. albostriella* males with Bs were collected both from *C. sativa* and *F. sylvatica*; *A. wahl-*

bergi males with Bs were sampled both from *Ulmus* sp. and *A. opalus*. Similarly, no relationship was established between the occurrence of Bs and the habitat altitude above sea level: in *A. albostriella* Bs were present in populations inhabiting at 440 m and higher altitudes, while in *A. wahlbergi* they were found at lower altitudes (20m–520m).

In studied populations, the frequency of B chromosomes was rather low. Thus, in *A. wahlbergi* they were present in 14% and in *A. albostriella* in only 10% of specimens studied. The frequency of individuals with 1B and 2Bs differed between the species. Thus, in four *A. albostriella* populations with B-chromosomes, 9,6% of males carried 1B and 3,2% carried 2B, whereas in two *A. wahlbergi* populations with Bs, 11% of males had 1B and 22% had 2B. The data concerning the frequency of B chromosomes in natural populations of these species are too scarce to draw any firm conclusions. Noteworthy that the frequency of cells with B-chromosomes in 2B-males was markedly higher compared with that in 1B-carriers: 60,7%-83,7% against 0,65%-1,8% (Table 4). This observation suggests the existence of an accumulation mechanism responsible for maintaining the 2Bs in studied populations. Interesting, no males with more than two Bs were found in studied specimens. One can suppose that in *Alebra* populations, 1 or 2 B-chromosomes are tolerable to B-carriers and that natural selection operates by eliminating individuals with more than two Bs. In contrast, in the aforesaid planthopper species *J. pellucida*, males with up to four B-chromosomes were found (Kirillova and Kuznetsova 1990). However, in this species males with larger number of Bs were less frequent: males with 1B predominated (89%), males with 2Bs and 3Bs occurred with equal frequency (56%) whereas males with 4Bs were rare (11%).

The question of the adaptive significance of B-chromosomes in natural populations has been argued over for decades (Camacho et al. 2000), with the final position showing little if any substantial evidence to support such a role (Jones and Houben 2003). Among many others, the influence of B-chromosomes on recombination through the modulation of chiasma frequency and distribution in A-chromosomes has been recorded (e.g. in Orthoptera; Camacho et al. 1980, 2000). In *A. albostriella* and *A. wahlbergi*, intraindividual analysis demonstrated that chiasma frequency in a nucleus was independent of the occurrence and number of B-chromosomes that it contains. Similarly, there was no relationship between the occurrence of B-chromosomes and the occurrence, frequency and types of meiotic abnormalities in a male. As an example, B-chromosomes were absent in male 1-WNE which had bivalents with three chiasmata and in males 2, 3 and 4 of ASE with the highest percent of meiotic abnormalities (Tables 2 and 3). In some insects, B-carriers have shown a significant increase of the number of macrospermatids (Suja et al. 1986) however in our material such a correspondence was not observed.

Meiotic abnormalities

As noted above, information on chromosome rearrangements and meiotic disturbances in auchenorrhynchan species is very scarce. A number of meiotic abnormalities

including agmatoploidy (a result of fission of holokinetic chromosomes), aneuploidy, loose pairings of sex chromosomes and shrinkage of cytoplasm (changes in cytoplasmic volume) were described in three biotypes of the brown planthopper *Nilaparvata lugens* (Stål, 1854) from the family Delphacidae (Goh et al. 1992). A translocation polymorphism was encountered in the Australian leafhopper *Alodeltocephalus draba* Evans, 1966 (Whitten and Taylor 1969). Some other examples are presented in a review of Kuznetsova and Aguin-Pombo (in press). In *A. albostriella* and *A. wahlbergi*, one or another of meiotic abnormalities were found such as univalents, bivalent chains resulting from end-to-end non-homologous achiasmate associations, multiple translocation chains, anaphasic laggards and macrospermatids.

End-to-end non-homologous associations

In males originating from different populations of *A. albostriella* and *A. wahlbergi*, end-to-end associations between bivalents were found at different stages of meiosis. The chains, involving up to four bivalents and occasionally (in *A. albostriella*) also the X-chromosome, were formed during prophase and were still intact at MI. In these cases, the persistent association was certainty non-chiasmate. Non-homologous telomeres did not touch intimately each other but unstained gaps were present between bivalents. Since in both species chromosomes display distal heterochromatic blocks (our unpublished data), one can suggest that the formation of artificial bivalent chains (pseudomultiples) is caused by heterochromatin adhesion due to which non-homologous chromosomes easily attract to one another.

Terminal associations of non-homologous bivalents without chiasma formation have been described in many plants and animals (White 1973, John and King 1982, 1985). These associations may involve from two up to all bivalents of a species. For instance, in the "holokinetic" moth species *Sphinx ligustri* (Linnaeus, 1758) (Lepidoptera, Sphingidae), all of the 28 bivalents were non-homologously attached to each other throughout prophase until prometaphase in females. Late in meiosis, the chains were broken down sequentially however short chains of two or three bivalents were still present at metaphase I. Like in the two *Alebra* species, in this moth non-homologous telomeres did not touch intimately each other but an unstained gap or some chromatin threads were seen between bivalents (Nokkala 1987).

Translocation chains

In male 1-WANE sampled from *C. sativa*, part of nuclei had chains of several bivalents, non-homologous chromosomes showing the apparent intimate contacts by terminal chiasmata (Fig. 3g). In this male, the occurrence of heterozygotes for translocations could account for the multivalent chains formation. Since this chromosome rearrangement was observed only in some of meiotic cells, it must have happened after germi-

nal cell development. Unfortunately, using only classical cytogenetic methods it was possible neither to affirm which chromosomes formed the chains nor to identify the orientation of separate chromosomes within a chain. Schematic representation of the possible formation of the chain-of-four caused by multiple translocations in meiotic cells is presented on Fig. 4.

In natural populations, chromosomal rearrangements arise as heterozygotes but their probability to establish is low (King 1993). Interchanges of small terminal segments of chromosomal pairs relatively frequently happen as spontaneous mutations (Hewitt 1979, Reed et al. 1992). Several studies have shown the chiasmate multivalent configurations, either rings or chains, in first meiosis as a result of heterozygosity for chromosomal fissions and fusions (e.g. in orthopterans; White 1973, John 1987, Bidau and Mirol 1988, Mirol and Bidau 1994, Colombo 2013). These configurations can lead to irregular segregation and nondisjunction in meiosis, with a consequent reduction in reproductive potential (White 1973), although sometimes they have no apparent negative influence on fertility (Mirol and Bidau 1994). Regarding insects with holokinetic chromosomes, the rearrangements can be even less deleterious since diffuse kinetochore is spreading through the length of their chromatids and products of rearrangements are able to be transmitted to daughter cells at successive cell divisions. Heterozygous translocations have occasionally been recorded in natural populations of "holokinetic" insects such as aphids (Blackman and Takada 1975, 1977, Blackman et al. 1995), heteropterans (Papeschi 1994, Bressa et al. 1998, Pérez et al. 2004) and ‚psyllids (Grozeva and Maryańska-Nadachowska 1995, Nokkala et al. 2006). Within Auchenorrhyncha, a fascinating case of translocation polymorphism was described by Whitten and Taylor (1969) in populations of the Australian leafhopper species *A. draba*. In all of the 8 studied populations, males showed one of the following chromosome complements: 3AA (three bivalents) + X; 4AA + X; 2AA + 1AAA (trivalent) + X; 1AA + 1AAAA (tetravalent) + X. A peculiar feature of this case is that the reduction in chromosome number has reached different stages in different localities. In one area (Lake Pedder), it was nearly or almost fixed (2 n= 7: 3AA + X), while in another area (Bruny Island) chromosome number decreased from north to south. Reduction of chromosome number following a latitudinal cline was caused by differences in the frequency of chromosome translocations. *A. draba* was proposed to be under a process of speciation driven by the reorganization of karyotype which was initiated in some populations by the fixation of a particular chromosome fusion (Whitten and Taylor 1969).

Univalents

In separate males of *A. albostriella* and *A. wahlbergi*, univalents of one-two chromosome pairs were observed at MI. The univalency involved either one of the larger or one of the smaller pairs of autosomes or occasionally both of these pairs. Although synaptic abnormalities can be responsible for the induction of abnormal chromosome segregation, no abnormal spermatids were observed in males showing univalents at

metaphase I cells. This observation suggests a regular segregation of univalents in meiosis as it has been demonstrated by Nokkala (1986) for holokinetic univalents in the true bug species *Rhabdomiris striatellus* (Fabricius, 1794) (originally listed by Nokkala as *Calocoris quadripunctatus* (Vil.)) (Miridae, Hemiptera).

Aberrant spermatids

Macrospermatids were encountered in males of both species. Aberrant spermatids occurred in small proportion in part of spermatocysts and were twice and sometimes several times as much as normal spermatids within the same cyst. In *A. albostriella*, abnormal spermatids were more abundant being found in four populations and in about 37% of the specimens studied. Chromosomal abnormalities that affect gametogenesis are known to be one of the principle causal factors in nonbalanced gametes appearance (White 1973). If the emergence of abnormal spermatids is a reflection of meiotic disturbances, one would expect a correspondence between the amount of macrospermatids and that of meiotic abnormalities in a male. Despite of this, there was a clear discrepancy between these two parameters. Macrospermatids instead of being abundant in males with numerous abnormalities were either occasional or absent and vice versa. For instance, in *A. albostriella*, out of 15 males displaying macrospermatids, 10 had no evident meiotic abnormalities at diakinesis and MI. Notice however that the fate of abnormal meiotic configurations was not traced in any of the individuals analyzed.

Conclusion

It is not known at this stage what are the primary causes of abnormal chromosome behavior in males of *A. albostriella* and *A. wahlbergi* from Greek populations i.e. whether these causes are male-specific meiotic mutants, some environmental mutagens or the result of hybridization events between coexisting species on the same tree. Also it is not known whether these meiotic abnormalities may play a role in the karyotype evolution and speciation of the genus *Alebra*. This genus seems to be prone to chromosomal rearrangements that makes it an interesting group for further studies. From the cytological viewpoint, a greater number of species and samples as well as more detailed analysis employing special techniques like chromosome bandings and fluorescent *in situ* hybridization may help in determining the actual variety and frequency of chromosomal abnormalities and their contribution into the karyotype differentiation in the genus *Alebra*.

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