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Chromosomal differentiation among bisexual European species of *Saga* (Orthoptera: Tettigoniidae: Saginae) detected by both classical and molecular methods

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Key words. Orthoptera, Saginae, *Saga*, karyotype, Ag-NOR, FISH, chromosome, rDNA, telomeric repeats

Abstract. We report the karyotype characteristics including chromosome numbers of *Saga campbelli campbelli*, *S. c. gracilis*, and *S. rammei* using the following classical cytogenetic methods: C-banding, silver staining, and fluorochrome staining DAPI and CMA_3. We also present FISH data showing the distribution of telomeric repeats and 18S rDNA on the chromosomes of these species and the results of similar studies cited in the literature on *S. hellenica*, *S. natoliae*, and *S. rhodiensis*. The five European *Saga* species exhibit a high rate of karyotype evolution. In addition to changes in chromosome number and morphology (by chromosomal inversion and/or chromosome fusion), interspecific autosomal differentiation involved changes in the distribution and quantity of constitutive heterochromatin and GC-rich regions, as well as the number and location of NORs. In the present study we focused on testing a hypothetical model of karyotype evolution in *Saga*, with particular reference to the cytogenetic mapping of rDNA and telomeric sequences. Variation in the distribution of rDNA and location of Ag-NORs are novel phylogenetic markers for the genus *Saga*.

INTRODUCTION

The subfamily Saginae is an ancient, highly specialized group of carnivorous bush-crickets, the ancestors of which can be traced back to the mid-Jurassic and gave rise to several groups at present occupying arid regions of the Old World, Australasia, and Central America (partim. Kaltenbach in Harz, 1969; Gorochov, 1995; etc.). The subfamily itself (sensu Eades et al., 2007; OSF online) includes 4 genera, distributed over two disjunct regions – southern and southeastern Sub-Saharan Africa (3 genera) and part of the Western Palearctic (genus *Saga*).

The genus *Saga* Charpentier, 1825, comprised of 13 species, probably originated and underwent an early radiation in the Miocene of the Aegean, associated with the aridization and isolation of the area (for similar historic distributional patterns of other genera see e.g. La Greca, 1999; Ciplak, 2004). The centre of biodiversity includes Asia Minor and the Southern Balkans with most taxa having relatively restricted ranges. Nevertheless, the parthenogenetic species *Saga pedo* (Pallas) occurs over a territory several times larger than the total area of the ranges of all other species. In continental Europe, five species and two subspecies have been recorded so far (Kaltenbach, 1967). *S. rhodiensis* Salfi, although occurring on a Greek island and here also considered as "European", actually represents an element of the Mediterranean fauna of Asia Minor.

Most of the Palearctic tettigonnids of the family Tettigoniidae have a karyotype consisting of 2n = 31 acrocentric chromosomes in the male with an X0-XX sex chromosome mechanism. This karyotype was suggested as ancestral for most species of this family (e.g. Hewitt, 1979; Warchałowska-Śliwa, 1998; Warchałowska-Śliwa et al., 2005). The genus *Saga* is characterized by extreme karyotypic diversification. A previous study carried out on this genus by Matthey (1946) revealed that *S. cappadocica*, *S. ephippigera*, and *S. ornata* have the ancestral karyotype (2n = 31♂). The tetraploid, parthenogenetic species *S. pedo* is characterized by the karyotype 4n = 68 (Matthey, 1939, 1941, 1948; Goldschmidt, 1946). However, the chromosome complement of European species of *Saga*, based on an earlier study of *S. hellenica*, *S. natoliae*, and *S. rhodiensis*, is 2n = 29♂/30♀. Robertsonian centric fusion and tandem translocation are thought to be the main mechanisms responsible for the karyotypic variation observed within this group (Warchałowska-Śliwa, 1998; Warchałowska-Śliwa et al., 2007). In a previous paper, we investigated heterochromatin in three species using different staining methods, preliminary C-banding, silver impregnation (Ag-NORs), chromomycin A_3 (CMA_3) and 4-6-diamidino-2-phenylindole (DAPI). CMA_3 and DAPI are useful for detecting CG- and AT-enriched chromosome regions, respectively. Some similarities and differences within the C-positive constitutive heterochromatin regions were found, revealing that taxonomically closely related species with the same chromosome number show different patterns of C-bands. Cytogenetic differences between these species suggest that chromosomal divergence occurred during their speciation (Warchałowska-Śliwa et al., 2007).

Lemonnier-Darcemont et al. (2008) reached the same conclusion after studying of *S. campbelli* and *S. rammei*, which both have a chromosome number of 2n = 27♂.

In this study, five species (and one subspecies) from Europe were subjected to molecular cytogenetic analyses in order to clarify the mechanism of karyotype evolution within the genus *Saga*. This analysis included fluorescence in situ hybridization (FISH), which revealed presence of specific DNA within chromosomes (Nath & Johnson, 2000; Schwarzacher, 2003). Changes in the number and distribution of repetitive sequences within chromosomes provide excellent markers for chromosome evolution in many species. Ribosomal DNA (rDNA) genes are useful chromosome markers for interspecific comparative karyotyping in insects at the level of genera (Gallián et al., 1999; Martinez-Navarro et al., 2004; Zacaro et al., 2004) and population (Martinez-Navarro et al., 2004). FISH using rDNA of grasshoppers can be used on a wider range of taxa within an order (e.g. Bridle et al., 2002; Cabrero et al., 2003; Souza et al., 2003; Martinez-Navarro at al., 2004; Loreto et al., 2008). Other repetitive sequences, so called telomeric DNA, are located mainly at the chromosome termini. Telomeres play an important role in maintenance of chromosomal stability and preserve genome integrity. Telomeres were used as markers for identification of chromosome ends. In the majority of insect orders, including Orthoptera, the telomeres are composed of mutliple copies of short, tandemly arranged TTAGG sequences (Okazaki et al., 1993; Sahara et al., 1999; Frydrychová & Marec, 2002; Frydrychová et al., 2004; Vítková et al., 2005). The clusters of telomeric repeats and telomeric-like sequences were deemed useful in the identification of chromosomal rearrangements related to changes in chromosome number and evolution in insects (e.g., López-Fernández et al., 2004).

The present study reports the results of a cytogenetic analysis of *S. campbelli campbelli*, *S. campbelli gracilis*, and *S. rammei* using classical methods (C-banding, silver, DAPI, and CMA_3 staining). In addition, FISH with 18S rDNA and $(TTAGG)_n$-specific telomeric DNA (tDNA) probes for mapping these repeats within the chromosomes were applied to these species and the results compared with those obtained previously for *S. hellenica*, *S. natoliae* and *S. rhodiensis* (Warchałowska-Śliwa et al., 2007). Chromosomal localization of the clusters of these repeats, along with results of routine cytogenetic techniques, gave us an insight into chromosomal evolution in the bisexual European species of the genus *Saga*.

MATERIAL AND METHODS

A cytogenetic analysis of the following nymphs, adult males and female bush-crickets, collected in Bulgaria and Macedonia, was undertaken: *Saga campbelli campbelli* Uvarov, 1921, 3 male and one female nymph and imago, Bulgaria: Maleshevska Mt., July 2006 (41°43N, 23°06E) leg. Chobanov D; *Saga campbelli gracilis* Kis, 1962, 5 male nymphs, Bulgaria: E Rodopi Mts, north of Plevoun Vill., vi.2006 (41°27N, 26°01E), leg. Chobanov D., Warchałowska-Śliwa E.; *Saga rammei* Kaltenbach, 1965, 1 male, Macedonia, Bogoslovec Vill., July 2006 (41°46N, 22°01E), leg. Chobanov D. Specimens are deposited in the Institute of Systematics and Evolution of Animals, Polish Academy of Sciences (Kraków) and in the Institute of Zoology, Bulgarian Academy of Sciences (Sofia).

The testes and ovarioles were excised, incubated in a hypotonic solution (0.9% sodium citrate), and then fixed in ethanol : acetic acid (3 : 1). The fixed material was squashed in 45% acetic acid. Cover slips were removed by the dry ice procedure and then the slides were air dried. The C-banding was carried out according to Sumner (1972) with a slight modification. The silver staining method for nucleolar organizer regions (NORs) was performed as previously reported (Warchałowska-Śliwa & Maryańska-Nadachowska, 1992). In order to reveal the molecular composition of C-heterochromatin, some slides were stained with CMA_3 to reveal GC enriched regions and DAPI to reveal AT enriched regions (Schweizer, 1976). Chromosomes were classified on the basis of the criteria proposed by Levan et al. (1964).

DNA probe preparation

Three different 18S rDNA probes were used for the detection of rDNA sequences on chromosomes using FISH: (1) a 3.2 kb fragment of human 18S rDNA cloned in pHr13 (rDNA-probe) (Malygin et al., 1992), (2) and (3) about 1.8 kb 18S rDNA fragment amplified from genomic DNAs of *Isophya rammei* (Orthoptera) and *Philenus spumarius* (Homoptera), respectively, using polymerase chain reaction (PCR) and primers 18Sai forward (5'-CCT GAG AAA CGG CTA CCA CAT C-3') and 18Sbi reverse (5'-GAG TCT CGT TCG TTA TCG GA-3') (Whiting et al., 1997). The PCR reactions were carried out in 25 µl reaction volumes containing 1.5 mM $MgCl_2$, 2.5 mM dNTP, 10 µM of each of the two primers, 100 ng template DNA and 5 U *Taq* DNA polymerase (Qiagen, Hilden, Germany). An initial period of 3 min at 94°C was followed by 30 cycles of 60 s at 94°C, 60 s at 51°C, and 1.5 min at 72°C, and concluded by a final extension step of 10 min at 72°C. Probes were labelled with biotin-11-dUTP by nick translation according to the manufacturer's instructions (Invitrogen, Tokyo, Japan).

A $(TTAGG)_n$ probe, used to visualize clusters of telomeric repeats, was generated by a non-template PCR using a modified version of López-Fernández et al. (2004) technique. Briefly, PCR was carried out in a 50 µl reaction mixture containing 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 µM of each of the two primers (5'-GGTTA-GGTTA-GGTTA-GGTTA-GG-3' and 5'-TAACC-TAACC-TAACC-TAACC-TAA-3') and 2 U *Taq* DNA polymerase. The non-template PCR was performed with an initial cycle of 90 s at 94°C, followed by 30 cycles of 45 s at 94°C, 30 s at 40°C and 60 s at 72°C, and a final extension step of 10 min at 72°C. The PCR product was then labelled with digoxigenin-11-dUTP in additional PCR cycles to produce the $(TTAGG)_n$ telomeric probe.

Fluorescent in situ hybridisation

FISH of chromosomes using the rDNA and telomeric probes was performed according to a standard protocol (Lichter et al., 1988) with salmon sperm DNA as the carrier DNA. Slides were treated with RNase A for 1 h at 37°C (100 µg/ml 2 × SSC), rinsed in 2 × SSC, dehydrated in an ethanol series (70%, 85%, 100%) and air dried. For removal of cytoplasm, the slides were incubated in pepsin (1 mg/ml in 0.01 N% HCL) at 37°C for 20 min, fixed in 1% formaldehyde in phosphate buffered saline (PBS), 50 mM $MgCl_2$, dehydrated in an ethanol series (70%, 80%, 96%) and again air dried.

For each slide, 20 ng of the probe mixed with 10 µg of sonicated salmon sperm DNA (Invitrogen) were ethanol-precipitated, cooled to –20°C and resuspended in 15 µl of hybridization mix (50% formamide, 10% dextran sulphate, 1%

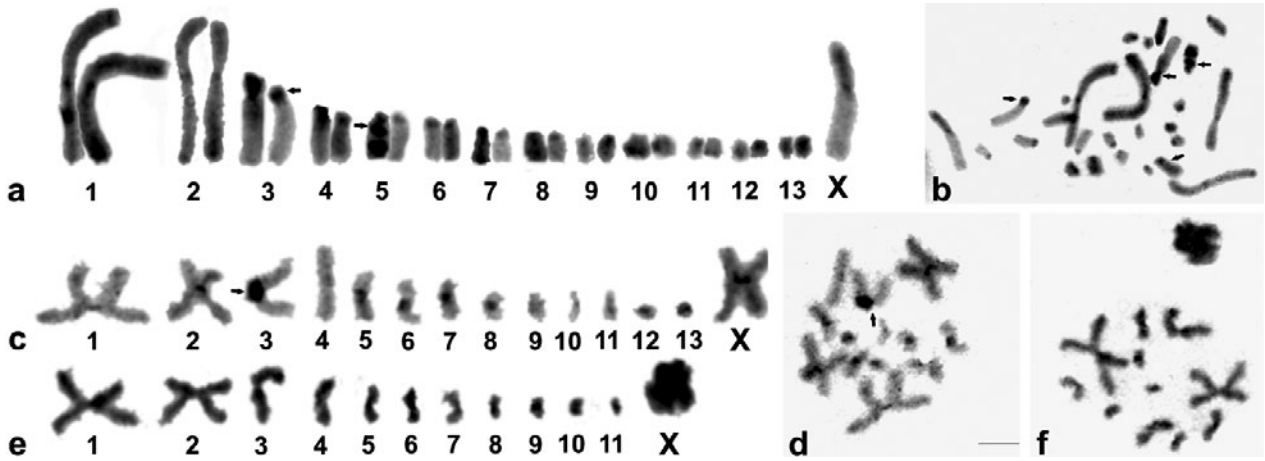

Fig. 1. C-banding staining of male chromosome complements of *Saga campbelli gracilis* (a–d), and *S. rammei* (e, f); a) *S. c. gracilis* – karyotype and b) spermatogonial metaphase (2n = 27), c) karyotype and d) metaphase II with 14 chromosomes, e) *S. rammei* – karyotype and f) metaphase II with 12 chromosomes. The arrows indicate large blocks of heterochromatin on M_3 (a–d) and an additional interstitial C-band occurring on the M_5 pair (a, b) of *S. c. gracilis*. X, sex chromosome. Bar = 10 µm.

TABLE 1. A comparison of the distribution of heterochromatin bands, NOR and rDNA on chromosomes of the European *Saga* species.

Species	2n (male), FN and chromosome morphology	C-bands on chromosomes * intraspecific variation of C-heterochromatin (thick/thin or present/absent)	Position of fluorochrome bands[1]		Position of NOR	rDNA -FISH signal	References
			bright DAPI	bright CMA_3			
S. campbelli campbelli	27, 32 X , L_2 submetacentric, L_1, metacentric, the remaining autosomes acrocentric	Paracentromeric L_1, L_2, M_4–S_{13}, X thin; M_3 thick $S_{8/9}$*subtelomeric all L, M and X telomeric	Paracentromeric: L_1, L_2, M_3	Paracentromeric M_3, M_4, telomeric in one arm L_1	Paracentromeric M_3, Subtelocentric S_9*	M_3	this study
S. campbelli gracilis	27, 32 X, L_2 submetacentric, L_1, metacentric, the remaining autosomes acrocentric	Paracentromeric L_1, L_2, M_4–S_{13}, X thin; M_3 thick; M_5* interstitial $S_{8/9}$* subtelomeric all L, M and X telomeric	Not analyzed	Not analyzed	Paracentromeric M_3, Subtelocentric S_9*	M_3	this study
S. rammei	23, 28 X, L_2 submetacentric, L_1, metacentric, the remaining autosomes acrocentric	Paracentromeric: L_1–M_8, S_{10}, S_{11} thin; S_9 thick*	Not analyzed	$S_{8/9}$	One per cell	$S_{8/9}$	this study
S. hellenica	29, 32 X submetacentric, L_1 metacentric, the remaining autosomes acrocentric	Paracentromeric: L_1, M_4–S_{14}, X thin; M_2 and M_3 thick M_6 interstitial L_1, M_2, M_3, M_6 telomeric	L_1, M_2, M_6	L_1, M_2, M_3	Paracentromeric M_3	M_3	Warchałowska-Śliwa et al., 2007
S. natoliae	29, 32 X submetacentric, L_1 metacentric, the remaining autosomes acrocentric	Paracentromeric: L_1, S_{11}–S_{14} thin; M_2 – S_{10}, X thick M_6 interstitial L_1, M_2–M_8 telomeric	$M_{8/9}$, X	$M_{8/9}$, X	Paracentromeric $M_{8/9}$	S_9	Warchałowska-Śliwa et al., 2007
S. rhodiensis	29, 32 X submetacentric, L_1 metacentric, the remaining autosomes acrocentric	Paracentromeric: most of autosomes and X thick, M_5, M_6 interstitial L_1, M_2–M_5 telomeric	$M_{8/9}$, X	S_9, X		S_9	Warchałowska-Śliwa et al., 2007

FN – fundamental number of chromosome arms; * intraspecific variation of heterochromatin; 1, 2, …, the number of autosome pair; X, sex chromosome. [1] The location of NOR and CMA_3 band on M_6 pair of *S. rhodiensis*, described by Warchałowska-Śliwa et al. (2007), is incorrect.

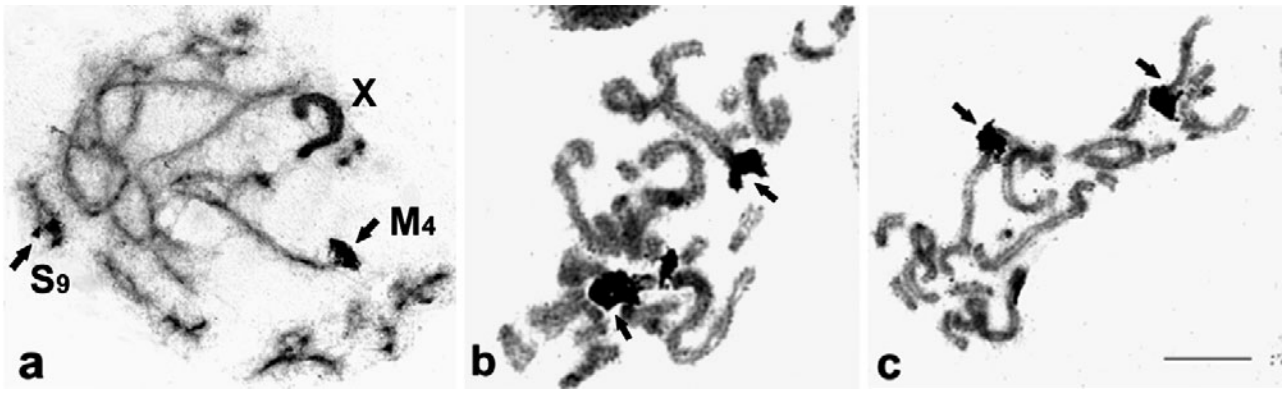

Fig. 2. Silver nitrate staining: (a) at male pachytene in *S. c. campbelli* and (b, c) spermatogonial prophase/metaphases in *S. c. gracilis*. NORs (arrows) were detected in the paracentromeric region of the M_3 (a, b) and in subtelocentric position of the S_9 (a). Note a heterochromatic univalent of the sex chromosome (X). Bar = 10 μm.

Tween 20, 2 × SSC). Metaphase spreads were denatured simultaneously with the DNA probe for 5 min at 75°C. Incubation was performed overnight at 37°C. After hybridization, slides were washed with 50% formamide in 2 × SSC (3×), 2 × SSC (2×), 0.2 × SSC (1×) at 45°C for 5 min. Biotin- and digoxigenin-labelled probes were visualized with avidin-Alexa 488 (Invitrogen) and mouse anti-digoxigenin antibodies conjugated to Cy3 (Sigma-Aldrich, Tokyo, Japan), respectively. Slides were counterstained with 2 μl/ml of DAPI (Sigma-Aldrich, Tokyo, Japan) for 15 min and then mounted in antifade solution with 1-4-diazabicyclo[2.2.2]octane (DABCO; Sigma) (see Sahara et al., 1999).

Microscopy was performed using an AxioPlan2 Imaging (Zeiss) equipped with a CCD camera (CV M300, JAI Corporation, Japan), Chroma filter sets and the ISIS5 image-processing package of MetaSystems GmbH (Altlussheim, Germany). In each experiment, at least one spermatogonial metaphase (with the exception of *S. rammei*) and 15–20 meiotic divisions (diplotene/diakinesis) were examined.

RESULTS

Karyotype description based on routine staining and banding techniques

The two subspecies of *Saga campbelli*: *S. c. campbelli* and *S. c. gracilis* have a similar karyotype, characterized by the same chromosome number, 2n♂ = 27 and 2n♀ = 28 and congruent chromosome morphology (FN = 32 and 34, respectively; FN is the number of chromosome arms, including X chromosome). Autosomes can be divided into three size groups: two large metacentric (L_1) and submetacentric (L_2) pairs, six medium sized pairs (M_3–M_8) and five small acrocentric pairs (S_9–S_{13}) (Fig. 1a–d). In *S. rammei*, the complement is reduced to 2n♂ = 23 and 2n♀ = 24 (FN = 28 and 30, respectively). For this species we examined five diakinesis and eight metaphase II. In this case the bivalents may be classified according to size into two large metacentric (L_1) and submetacentric (L_2) pairs, five medium sized (M_3–M_7) acrocentric pairs and four small (S_8–S_{11}) acrocentric pairs (Fig. 1e, f). In both species the submetacentric X chromosome is the largest in the set. These species show the X0♂ and XX♀ type of chromosome sex determination.

In Table 1, a comparison of the C-banding patterns, Ag-NOR localization, and fluorochrome banding patterns (DAPI and CMA_3) of chromosomes of these species and the results of a former study (Warchałowska-Śliwa et al., 2007) are shown.

C-banding of spermatogonial mitotic metaphase and meiotic cells of both subspecies of *S. campbelli* and of *S. rammei* revealed constitutive heterochromatin blocks (thin C-bands) in the paracentromeric region of most of the chromosomal elements, with the exception of M_3 in *S. c. campbelli* and *S. c. gracilis*. In this case, C-bands occupied the region next to the centromere (thick C-bands) (Fig. 1a, b). Additionally, in *S. campbelli*, a subtelomeric C-band occurs on the S_8 or S_9 ($S_{8/9}$) pair. Constitutive heterochromatin polymorphism involving interstitial C-bands on M_5 was noted in one out of three individuals of *S. c. gracilis* (Fig. 1a, b). Paracentromeric C-bands on S_9 were observed in an individual of *S. rammei*. Telomeric C-bands in *S. campbelli*, if present, are located in large and medium sized chromosomes, and in both arms of X.

$AgNO_3$ staining revealed the presence of two active NORs in the paracentromeric region of M_3 and a small NOR with a subtelocentric position on S_9 in both subspecies of *S. campbelli* (Fig. 2a, b). The latter is probably a "secondary NOR" carrying a small nucleolus seen only in some of the cells (Fig. 2c). In *S. rammei*, an NOR was revealed but not localized in diplotene in the studied cells, though in an early prophase cell one active NOR was observed. A previous description of the NOR-location in *S. rhodiensis* (Warchałowska-Śliwa et al., 2007) was incorrect because the nuclear remnants were associated with the $S_{8/9}$ bivalent (not M_6), because the determination of this particular pair of autosomes was wrong.

Bright DAPI signals in L_1, L_2, and M_3 of both subspecies of *S. campbelli* were observed in the paracentromeric region, clearly associated with C-bands (not shown). CMA_3 staining (not shown) of the male spermatogonial mitotic metaphase and meiotic cells in *S. c. campbelli* produced positive fluorescence signals on L_1, only in one arm, M_3 and M_4. However, a CMA_3-positive region was coincident with the active NOR visualized by $Ag\text{-}NO_3$ staining only on M_3. The NOR associated with the C-positive region of M_3 was formed by two proximate

regions of different structure: one positive band after DAPI and another after CMA_3 (not shown). Unfortunately, we were not able to conclusively confirm these positions in *S. c. gracilis* due to a low number of good-quality chromosome spreads. The results of DAPI and CMA_3 staining are summarized in Table 1.

Localization of rDNA and tDNA clusters

Hybridization with three different rDNA probes always produced the same results, i.e. their hybridization signals could not be distinguished from one another and resulted in the same signal on spermatogonial mitotic and meiotic chromosomes. Two colour FISH with two differently labelled probes, the Alexa 488-labelled 18S rDNA probe and Cy3-labelled $(TTAGG)_n$ probe, was performed in order to analyze the distribution of ribosomal and telomeric DNA on chromosomes of five species and one subspecies of the genus *Saga*. FISH revealed one cluster of rDNA, located on the autosomes of all species/subspecies (Table 1 and Fig. 3a–h). Their locations were observed at mitotic metaphases or on bivalents from diakinesis to metaphase I in the paracentromeric regions of the acrocentric chromosome M_3 of both subspecies of *S. campbelli* and *S. hellenica* (Fig. 3a–d), and also on a small chromosome, probably $S_{8/9}$, of *S. rammei*, *S. natoliae*, and *S. rhodiensis* (Fig. 3e–g). In all five species analyzed, FISH signals were coincident with the active NORs visualized by Ag-NOR staining. However, in both subspecies of *S. campbelli*, two NORs were observed on M_3 (cluster of labelled rDNA) and $S_{8/9}$ (low activity rDNA cluster in the subtelocentric region). However, FISH revealed rDNA presence only on M_3 , so that the NOR activity on $S_{8/9}$ is most likely due to only a few rDNA genes, i.e. less than the minimum number detectable by FISH (Cabrero & Camacho, 2008). The observed rDNA-FISH signal on $S_{8/9}$ of the three species varied in size (Fig. 3e–g), suggesting the occurrence of polymorphism in copy number of rDNA sequences.

FISH, using the $(TTAGG)_n$ probe, of spermatogonial mitoses and spermatocyte nuclei at different stages of meiosis was performed on all *Saga* species. On most chromosomes of all species, the FISH signal was detected at the distal ends of each autosome. Differences in the intensity of the hybridization signal were detected among most of the autosomes of each species, and in the X chromosome of some of them (Fig. 3a–h). Generally, the tDNA-FISH signals on chromosomes of *S. natoliae* and *S. hellenica* were smaller than those observed in other species. No tDNA-FISH signals were found in the centromeric region of metacentric/submetacentric chromosomes (Fig. 3a–h). Heterogeneity of the tDNA-FISH signals on the X chromosome was detected in all species.

DISCUSSION

The karyotype of bisexual species of the genus *Saga* differs extensively from the ancestral karyotype in terms of chromosome number and morphology, brought about by inversion of chromosomal regions and/or fusion of chromosomes in modern species. The ancestral chromosome number of 2n = 31 (FN = 31) is recorded in the male of *S. cappadocica* (Matthey, 1949), *S. ornata* (Matthey, 1946, 1948; Goldschmidt, 1946), *S. gracilipes*, and *S. ephippigera* (Matthey, 1946, 1948; Goldschmidt, 1946). Three European species, i.e. *S. hellenica*, *S. natoliae*, and *S. rhodiensis*, have a reduced chromosome number of 2n = 29 (FN = 32). The latter karyotype is as a result of one Robertsonian translocation (metacentric L_1 pair) and one pericentric inversion in the X chromosome (Warchałowska-Śliwa et al., 2007). For both subspecies of *S. campbelli* it is hypothesized that two Robertsonian translocations and one pericentric inversion, resulted in 2n = 27 (FN = 32) (two metacentric/submetacentric L_1, L_2 pairs and a subacrocentric X chromosome). The chromosome number of *S. campbelli* accords with the number recently reported by Lemonnier-Darcemont et al. (2008). However, in our study an unexpected chromosome number of 2n = 23 was revealed in *S. rammei*. Lemonnier-Darcemont et al. (2008) describe *S. rammei* as having 2n = 27. Our data indicate a tendency for a further reduction in chromosome number in this taxon. As mentioned above, two Robertsonian fusions and a pericentric inversion in the X were involved in the reorganization of the karyotype. The mechanism that led to the further reduction in the diploid chromosome number remains unclear. Such a significant difference in the chromosome number for a single species, bearing in mind the karyotype variation in the genus, cannot exclude the possibility that the specimens we studied and those studied by Lemonnier-Darcemont et al. (2008) belong to two different species. These differences may be caused too by inter-species hybridization or population polymorphism, but this remains to be resolved and additional samples from Macedonia need to be analyzed. Although this exceptionally low chromosome number is uncommon, it does occur within a few groups of Tettigoniidae (see Warchałowska-Śliwa, 1998). It should also be emphasized that the ancestor of the tetraploid, parthenogenetic species *S. pedo*, with 4n = 68 (Matthey, 1948; Goldschmidt, 1946; Cabrero et al., 2007), may be related to *S. ephippigera* (Warchałowska-Śliwa et al., 2007). Within the latter species (with 2n = 31) some individuals possessed a supernumerary pair of chromosomes (2n = 33). The occurrence of supernumerary chromosomes (B) has been previously noted in *S. hellenica* (Warchałowska-Śliwa et al., 2007).

Chromosome structure in Orthoptera has been thoroughly analyzed using the C-banding technique and NOR Ag-staining, including comparative studies of populations, species, and genera (e.g. Camacho et al., 1987; Warchałowska-Śliwa et al., 2005). Five species of the genus *Saga* revealed discrete differences not only in the amount of heterochromatin but also in the number of C-positive bands and their locations. *S. natoliae*, *S. rhodienis, S. campbelli*, and *S. rammei* showed similar C-banding patterns; *S. hellenica* exhibited a different pattern (Table 1). Interspecific polymorphism mainly associated with variable numbers of additional C-positive bands (indicated in Table 1 by an asterisk) was detected in some species of tettigonids (e.g. Warchałowska-Śliwa et al.,

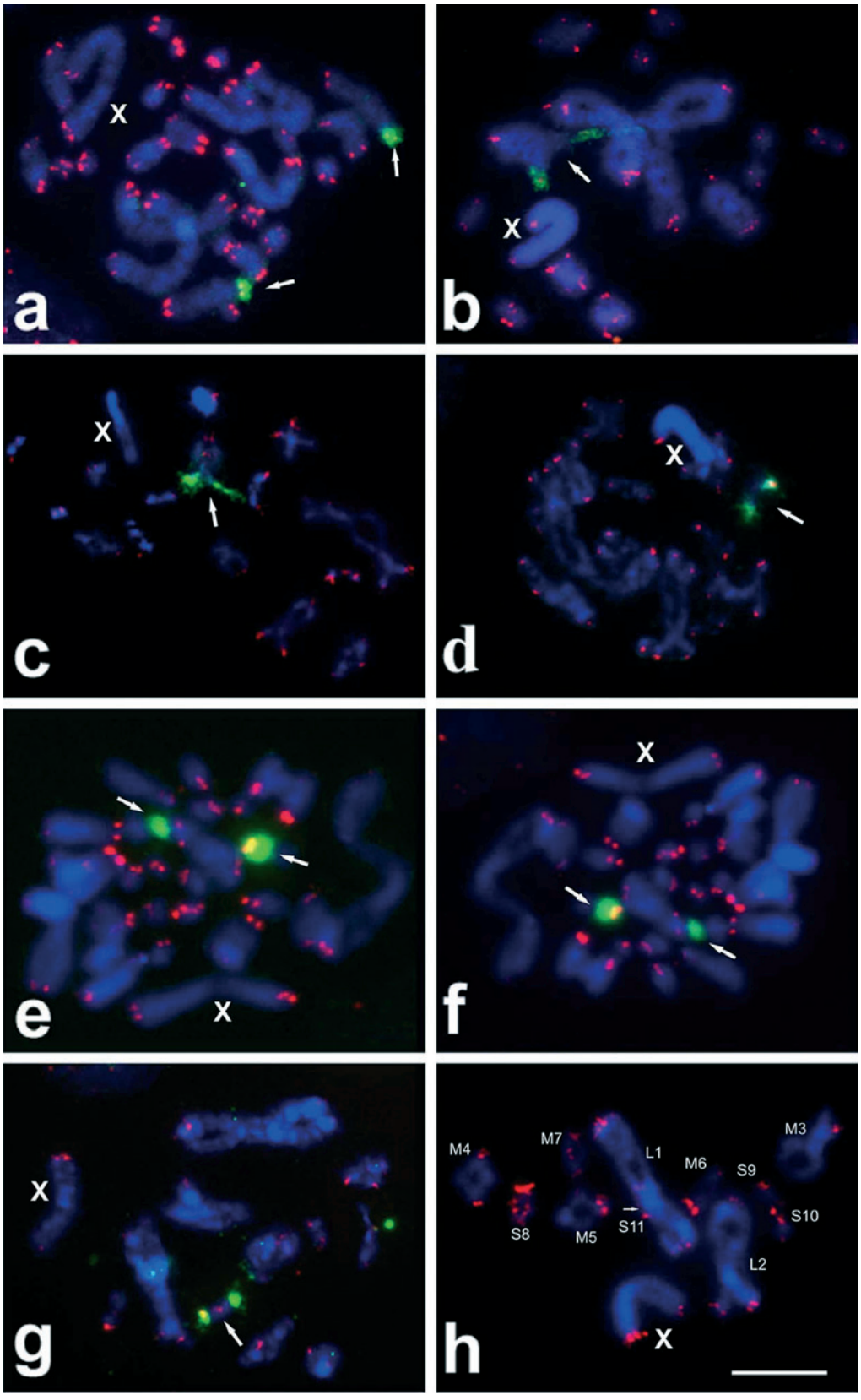

Fig. 3. FISH on male chromosomes of five species of *Saga* using both the 18S rDNA (green) and telomeric DNA (red) probes (a–g) or only the telomeric DNA probe (h). (a) Mitotic metaphase in *S. c. campbelli*; (b) diakinesis in *S. c. campbelli*; (c) diplotene in *S. hellenica*; (d) diplotene in *S. natoliae*; (e, f) mitotic metaphase in *S. rhodiensis*; (g, h) diplotene in *S. rammei*. Arrows indicate rDNA sites in paracentromeric regions of the acrocentric chromosome M_3 of *S. campbelli* and *S. hellenica* (a–c) and on a small chromosome (probably S_9) of *S. rammei*, *S. natoliae*, and *S. rhodiensis* (d–g). Hybridization areas on $S_{8/9}$ of these three species often varied in size between two homologous chromosomes (e–f). X – sex chromosome. Bar = 10 μm.

1992). The number of NORs and their locations are good markers for discriminating between related species of grasshoppers (e.g. Cabrero & Camacho, 1986; Rocha et al., 2004) and within Tettigonioidea (Warchałowska-Śliwa & Maryańska-Nadachowska, 1992: Warchałowska-Śliwa et al., 1992, 1994, 2005). The karyotypes of five *Saga* species described in this paper and an earlier study (Warchałowska-Śliwa et al., 2007) have NORs on two different chromosomes. In S. *hellenica* a single NOR occurs on the medium-sized bivalent M_3, whereas in S. *campbelli* two NORs were observed on M_3 and $S_{8/9}$. In contrast to these species, the only NORs in the karyotypes of *S. natoliae*, *S. rhodiensis*, and *S. rammei*, were associated with a small chromosome ($S_{8/9}$ according to size). Thus, this marker can successfully be used in the analysis of chromosomal stability within this group.

In species of the genus *Saga*, regions consisting of GC- or AT enriched DNA were visualized with the base-specific fluorochromes DAPI and CMA_3, respectively. NORs were associated with coincident C- and CMA_3-positive bands. However, the CMA_3 fluorochrome also revealed chromosomal regions that were not detected by silver impregnation (see Table 1 and Warchałowska-Śliwa et al., 2007). The data showed that C-positive regions had heterogeneous DNA sequences, i.e. not all were exclusively AT- or GC-rich. Furthermore, in the species investigated, the CMA_3 fluorochrome, as in other species, did not detect all NORs, (Vitturi et al., 1999, 2003; Colomba et al., 2004; Schneider et al., 2006).

Silver staining was used to evaluate the activity of rDNA clusters. We detected variation in the pattern of rDNA loci in the genus *Saga*. *S. campbelli* and *S. hellenica* (with different chromosome numbers), which have a M_3 autosomal pair with ribosomal loci, whereas in *S. rammei, S. natoliae*, and *S. rhodiensis* a small-sized S_9 bivalent (probably homologous in these species) showed a FISH signal. In some species with rDNA loci located on M_3 and S_9 autosomes, the size of the positive FISH fluorescent signals varies between homologous arms (Fig. 3e–f). The existence of the secondary NOR, carrying a small nucleolus in some cells in *S. campbelli* (located in $S_{8/9}$), might indicate that this NOR is either a new NOR (i.e., a part of rDNA copies moved to new locations) or it represents remnants of a nucleolus in the process of being eliminated (Cabrero & Camacho, 2008). Additionally, FISH analyses indicate a cluster of 18S rDNA repeats coincident with telomeric repeats at the end of chromosomes with active NORs, so the rDNA cluster is probably located near telomeres, in a subtelomeric region.

The $(TTAGG)_n$ sequence is found at chromosome ends in most arthropod clades, which supports the hypothesis that this is an ancestral sequence for telomere DNA in insects (Frydrychová et al., 2004; Vítková et al., 2005). To date these telomeric repeats have been reported in several species of Orthoptera such as the camel cricket *Diestrammena japonica*, locust *Locusta migratoria*, some grasshoppers and Taiwanese cricket *Teleogryllus taiwanemma* (Okazaki et al., 1993; López-León et al., 1999; Sahara et al., 1999; Kojima et al., 2002; López-Fernández et al., 2004; Vitturi et al., 2008). In this study we detected the "insect-type" telomeric sequence at the ends most of the chromosomes of European species of *Saga*. At the end of one arm of the X chromosome of *S. natoliae* and *S. hellenica*, the telomeric probe did not give a visible signal. Similar heterogeneity is recorded in the parthenogenetic *S. pedo* (Cabrero et al., 2007).The weak signal at the ends of the *Saga* chromosome arms could be due to low copy number of the telomeric repeats, too low for the detection by standard FISH techniques. Nevertheless, our results clearly demonstrate that the telomeric regions of autosomes and the submetacentric X chromosome are composed of the $(TTAGG)_n$ telomeric repeats typical of Orthoptera (López-Fernández et al., 2004).

The chromosomal characters discussed above may be partly considered as synapomorphies and characteristic of some groupings of possibly closely related species (Warchałowska-Śliwa et al., 2007). Though generally controversial, the characters investigated support a closer relationship between some taxa (e.g. *S. natoliae* and *S. rhodiensis*), while the position of others remain unclear. Based on the most conservative arguments, supported by the chromosome number, the following groups are suggested: (A) *S. ehippigera*, *S. ornata*, and *S. cappadocica*; (B) *S. natoliae*, *S. rhodiensis*, and *S. hellenica*; (C) *S. campbelli*; (D) *S. rammei*; (E) *S. pedo*. These relationships or differences are supported by their amplitude-temporal song structure, which has a very important role in sexual signalling duing mating (e.g. Heller, 1988; Warchałowska-Śliwa et al., 2007). The *S. natoliae* group is quite well defined by its song, consisting of long constant sequences of syllable groups (echemes), in which echemes are separated by intervals of several seconds. Within this group the song of *S. hellenica* differs in its fine syllable-structure, which is similar to that of *S. campbelli* (cf. Heller, 1988; Kolics et al., 2008). The song of *S. rammei* has shorter and more frequently repeated echemes (Kolics et al., 2008), thus it is somewhat transitional to the fast echeme repetitive song of *S. ephippigera* (D.P. Chobanov, unpubl. data; see also Warchałowska-Śliwa et al., 2007). However, this grouping is not well supported by morphology. On the basis of morphological characters, Kaltenbach (1967) outlines four groups of species within the genus *Saga*, which include the following: (A) *S. natoliae, S. rhodiensis, S. ehippigera*; (B) *S. cappadocica, S. campbelli, S. rammei, S. hellenica*; (C) *S. ornata*; (D) *S. pedo*. This classification needs to be revised taking into account not only the new data on chromosome characteristics but also a reconsideration of the morphological characters. One interesting point concerns the position of *S. pedo*. Though it is always ignored because of its unique parthenogenesis and tetraploidy, the question of its phylogenetic affinities persists. Morphology supports its close relationship with *S. campbelli*, while the chromosome set of *S. pedo* could be derived from that of *S. ephippigera*. It is worth mentioning that these are the northernmost distributed species and both *S. campbelli* and *S. ephippigera* are amongst the

few species that probably occur para- or syntopically with *S. pedo*.

In conclusion, the chromosomal analysis of five European *Saga* species suggests a high rate of karyotype evolution in these species. Besides changes in chromosome number and morphology (by chromosomal inversion and/or probably fusion), interspecific autosomal differentiation has involved the distribution and quantity of constitutive heterochromatin and GC- rich regions, as well as the number and location of NORs. The present study focused on testing the model of evolution in *Saga*, with particular reference to the cytogenetic mapping of rRNA coding genes and telomeric sequences, performed for the first time in tettigonids. Variation in the distribution of rDNA and/or Ag-NORs show the importance of these patterns as phylogenetic markers in the genus *Saga*. Future karyotype analyses using FISH with telomeric and rRNA genes should be carried out on more populations and individuals in order to gain a more comprehensive view of the chromosome evolution in this group. Combined interdisciplinary investigations (especially including DNA-sequencing) on larger samples would be helpful in revealing the phylogenetic relationships within *Saga* and the evolutionary history of this intriguing group.

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