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Conventional and Molecular Chromosome Study in the European Genus *Parnassiana* Zeuner, 1941 (Orthoptera, Tettigoniinae, Platycleidini)

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Cytogenetical studies were performed in nine taxa (eight species and one additional subspecies) of *Parnassiana* and two additional closely related taxa belonging to tribe Platyleidini. Classical (C-banding, DAPI/CMA_3-staining, Ag-NOR) and molecular (fluorescence *in situ* hybridization with 18S rDNA probe) methods were used for cytological discrimination of chromosomes in this genus. Both studied groups (*Parnassiana* and genus aff. *Parnassiana*) possess two karyotypes: 2n = 31 (male) and 2n =29 (male). In all species only one pair of 18S rDNA signals was observed. In *P. fusca* and *P. parnassica* B chromosomes (Bs) were detected. The results of the present study will be useful in discussions on the evolutionary trends of genome organization and karyotype evolution in the subfamily Tettigoniinae.

Key words: Tettigoniinae, karyotype, chromosome banding, Ag-NOR, FISH, 18S rDNA.

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The superfamily Tettigonioidea (common name bush-crickets or katydids) contains 500 species in Europe (HOCHKIRCH *et al.* 2016). Some species of this group are widespread with good capabilities for dispersal. However, the majority is flightless with restricted ranges. This refers especially to Phaneropteridae, a mainly plant feeding family. But even in the Tettigoniidae, many species and even genera are short-winged and unable to fly. This property has some important consequences. On the one hand, many species are endangered because of their local distribution, on the other hand these genera offer a fascinating area for studying speciation. Comparing species-specific characters and tracing their evolutionary history will allow us to understand the forces changing the genome and the gene pools.

The genus *Parnassiana*, endemic to the southern Balkan peninsula, a member of the subfamily Tettigoniinae and subject of the present study, is a good example of both aspects. Out of its 13 currently recognized species, five are critically endangered, three are endangered and the remaining five are classified as vulnerable (HOCHKIRCH *et al.* 2016). Concerning speciation processes, sexual selection on male genital organs seems to be very important, whereas the calling songs, essential for long distance communication, have changed very little (HELLER 2006). However, the exact relationships between the different forms are still unresolved.

Chromosomal analyses have produced data that could help shed light on the evolutionary and taxonomic relationships within the tettigoniid bush-

crickets. Chromosomes within taxa of tettigoniids (e.g. genera) may vary in number, morphology, and staining properties (e.g. WARCHAŁOWSKA-ŚLIWA *et al*. 1992, 2009, 2013a, b). The diploid chromosome number (2n), chromosome morphology (FN), and type of sex determination systems of Tettigoniinae in Europe have been described only for 60 species in 11 genera including 32 species belonging to tribe Platycleidini. The majority of Platycleidini have acrocentric chromosomes with a chromosome number of 2n = 31 in males, except for three species belonging to the genera *Metrioptera* and *Montana* (see review WARCHAŁOWSKA-ŚLIWA 1998; WARCHAŁOWSKA-ŚLIWA *et al*. 2005). Chromosomes of this tribe have been occasionally examined using only the C-banding technique and NOR Ag-staining (WARCHAŁOWSKA-ŚLIWA *et al*. 2005).

The present study reports the cytogenetic characterization of 11 species and subspecies (further referred to as taxa) representing the genus *Parnassiana* and a closely related group of presently undescribed taxa. The physical characteristics of their karyotypes were here analyzed for the first time using classical methods of C-banding, silver impregnation (Ag-NOR), fluorochrome DAPI/CMA_3 staining and molecular fluorescence *in situ* hybridization (FISH) with 18S rDNA. *Parnassiana* species were cytologically examined in order to extend knowledge on their cytogenetics and provide taxonomically useful information. This is the first step towards a better understanding of the chromosomal organization within the tribe Platycleidini. Previous comparative cytogenetic studies on Orthoptera (e.g. CABRERO & CAMACHO 2008; LORETO *et al*. 2008; CABRERO *et al*. 2009; WARCHAŁOWSKA-ŚLIWA *et al*. 2009, 2013a, b; ROCHA *et al*. 2011) showed that chromosomal organization is a useful marker for understanding species relationships and routes of speciation in this group (JETYBAYEV *et al*. 2012).

Material and Methods

We investigated 24 specimens of 11 species and subspecies of *Parnassiana,* including one previously described species (WARCHAŁOWSKA-ŚLIWA *et al*. 2005), and closely related taxa collected in Greece and Albania. The species studied and respective collection sites are shown in Table 1.

Gonads were excised, incubated in a hypotonic solution (0.9% sodium citrate), fixed in ethanol : acetic acid (3 : 1), and stored at 2°C until use. After fixation testes and ovarioles were briefly macerated in a drop of 45% acetic acid, then covered with a coverslip and squashed. The cover slip was removed after freezing on dry ice and slides were air-dried.

The distribution of heterochromatin was analyzed by Giemsa C-banding (SUMNER 1972), the GC- and AT- rich bands were detected with chromomycin A_3 (CMA_3) and 4'-6-diamino-2-phenylindole (DAPI), respectively, according to SCHWEIZER (1976). The silver staining method for nucleolar organizer region (NOR) was performed as previously reported (WARCHAŁOWSKA-ŚLIWA & MARYAŃSKA-NADACHOWSKA 1992). For some specimens, fluo-

Table 1

Localities of taxa used in this study

Species	Collection sites	Geographical coordinates
Parnassiana chelmos chelmos (Zeuner, 1941)	Greece: Peloponnesus, Achaia, Chelmos Mts	37°59'N, 22°12'E
Parnassiana chelmos unicolor (Willemse, 1973)	Greece: Peloponnesus, Achaia, Panachaikon Mt.	38°12'N, 21°51'E
Parnassiana coracis (Ramme, 1921)	Greece: Central Greece, N. Fokidas	38°42'N, 22°8'E
Parnassiana fusca (Brunner v. Wattenwyl, 1882)	Greece: Peloponnesus, Lakonia, Taigetos Mt. Greece: Peloponnesus, Lakonia, Taigetos Mt., 1520-1950 m alt., nymphs	37°00'33"N, 22°20'44"E 37°00'04"N, 22°20'29"E
Parnassiana gionica La Greca & Messina, 1976	Greece: Central Greece, Fokis, Giona Mt., 1670-2200 m alt., nymphs	38°35'20"N, 22°16'50"E
Parnassiana menalon Willemse, 1975	Greece: Peloponnesus, Arkadhia, Mainalo Mt., 1650-1900 m alt., nymphs	37°38'42"N, 22°16'18"E
Parnassiana parnassica (Ramme, 1926)	Greece: Central Greece, N. Viotias Greece: Central Greece, N. Viotias, 1834 m alt., nymphs	38°33'N, 22°36'E 38°33'51"N, 22°34'35"E
Parnassiana tenuis (Heller & Willemse, 1989)	Greece: Arta, Tsoumerka Mt.	39°24'N, 21°9'E
Parnassiana tymphrestos Zeuner, 1941	Greece: Central Greece, N. Evritanias	38°56'N, 21°48'E
Genus aff. *Parnassiana* sp. 1	Albania: Berat, Tomorr Mt., 1880 m alt., nymphs	40°36'58"N, 20°11'09"E
Genus aff. *Parnassiana* sp. 2	Albania: Gjirokastra, Mali i Nemerckes (Nemerchka) Mt., 1800 m alt., nymphs	40°07'56"N, 20°23'50"E

rescence *in situ* hybridization (FISH) with ribosomal 18S rDNA probe containing 1.8 kb fragments amplified from the genomic DNA of *Isophya pavelii* (Orthoptera), was conducted following the protocol described by WARCHAŁOWSKA-ŚLIWA *et al*. (2009). Chromosomes were analyzed and documented using a Nikon Eclipse 400 with CCD DS-U1 camera and a set of standard filters. An NIS-Elements BR 3.0 image-analyzing system (Nikon) was used and images were processed and arranged with Adobe Photoshop.

For each individual, at least 15 meiotic divisions (from diplotene to metaphase I) per male and at least five spermatogonial and/or oogonial metaphases were analyzed using some techniques (detailed information in Table 2). Relative chromosome lengths of the diploid complement including the X chromosomes, based on five mitotic metaphase plates from females of *P. fusca*, *P. chelmos chelmos* and a male of *P. coracis*, were calculated as a percentage of the total chromosome length (% TCL) according to KRÁL *et al*. (2006).

Results

The chromosome number (2n) and chromosome morphology (FN = the fundamental number of chromosome arms including the X chromosome) for all studied taxa are shown in Table 2. Two different karyotypes were observed. In the first case, the mitotic metaphase and meiotic plates showed 2n = 31 in males (Fig. 1b-h) and 32 in females (Fig. 1a). Fifteen pairs of acrocentric autosomes could be classified into three groups according to their size: one long (8.6% TCL), seven medium (4.6 to 2.3% TCL) and seven small pairs (1.8 to 0.9% TCL). The acrocentric X chromosome (9.1% TCL) is the largest in the set. The second type of karyotype is characterized by 2n = 29 (males) and 30 (females), in this case the bivalents could be classified into one large submetacentric pair (11.7% TCL), six medium sized (5.1 to 2.4 % TCL) and seven small acrocentric pairs (1.9 to 1.0% TCL). The X chromosome (8.9% TCL) is the second largest element in the complement (Fig. 2 a-d). Sometimes, minor length differences in chromosome pairs caused problems with precise identification. Both karyotypes showed the same sex determination system, X0 (male) and XX (female).

In Table 2, a comparison of the C- and fluorochrome banding patterns (DAPI and CMA_3), as well as cytogenetic mapping of 18S rDNA and Ag-staining are shown. After both C-staining and fluorochrome DAPI/ CMA_3 double-staining, chromosome regions showed some quantitative and qualitative variation between the analyzed taxa in terms of the base composition of the DNA molecule. All species had paracentromeric C-bands, which varied in size; in most cases, these C-bands were restricted to the centromere (thin C-bands), in other cases C-bands occupied the region next to

Table 2

Comparison of number and morphology of chromosomes, distribution of heterochromatin bands (C-staining and base specific fluorochromes) and the occurrence of NORs and rDNA on the chromosomes in species of the genus *Parnassiana* and related taxa

Species	2n (male), FN; chromosome morphology and B	C-bands: thick pc, i, d	Position of fluorochrome bands		rDNA FISH/ NOR
			DAPI+	CMA_3+	
Parnassiana chelmos chelmos	29, 31; 1 sm	3pc*,4i, 7i, 8d	3pc, 8d	3pc+d, 4i, 8d	4i
Parnassiana chelmos unicolor	29, 31; 1 sm	3pc, 4i, 8d	3pc, 8d	3pc+d, 4i, 8d	4i
Parnassiana coracis	29, 31; 1 sm	3pc, 4i, 8d	no	no	4i
Parnassiana fusca	31, 31; all a	3pc+d, 4i, 5i, 6i, 7pc*, 8pc*,9pc	most pc+i (excluding 4i)	most pc+i+d, 4i	4i
Parnassiana gionica	29, 31; 1 sm	3pc, 4i, 5i	no	no	4i
Parnassiana menalon	29, 31; 1 sm	3pc, 4i, 5i 8d	3pc, 8d	3pc, 4i, 8d	4i
Parnassiana parnassica	29, 31; 1 sm	3pc, 4i, 5i	3pc, 5i	3pc+t, 4i	4i
Parnassiana tenuis	31, 31; all a **?**	no	no	no	no
Parnassiana tymphrestos	29, 31; 1 sm	3pc, 4i, 8d	3pc, 8d	3pc, 4i, 8d	4i
Genus aff. *Parnassiana* sp. 1	31, 31; all a	3pc+d*,4i,5i+d*, 7pc*	3d*, 4d*, 5d	3d*, 4i+d, 5d	4i
Genus aff. *Parnassiana* sp. 2	29, 31; 1 sm	3pc, 4i	no	no	4i

FN = fundamental number of chromosome arms; ≈ published by WARCHAŁOWSKA-ŚLIWA *et al*. 2005; a = acrocentric, sm = submetacentric; pc = paracentromeric, i = interstitial, d = distal; no = not stained by fluorochrome techniques or FISH; *intraspecific variation of heterochromatin; X = X chromosome; B = B chromosome.

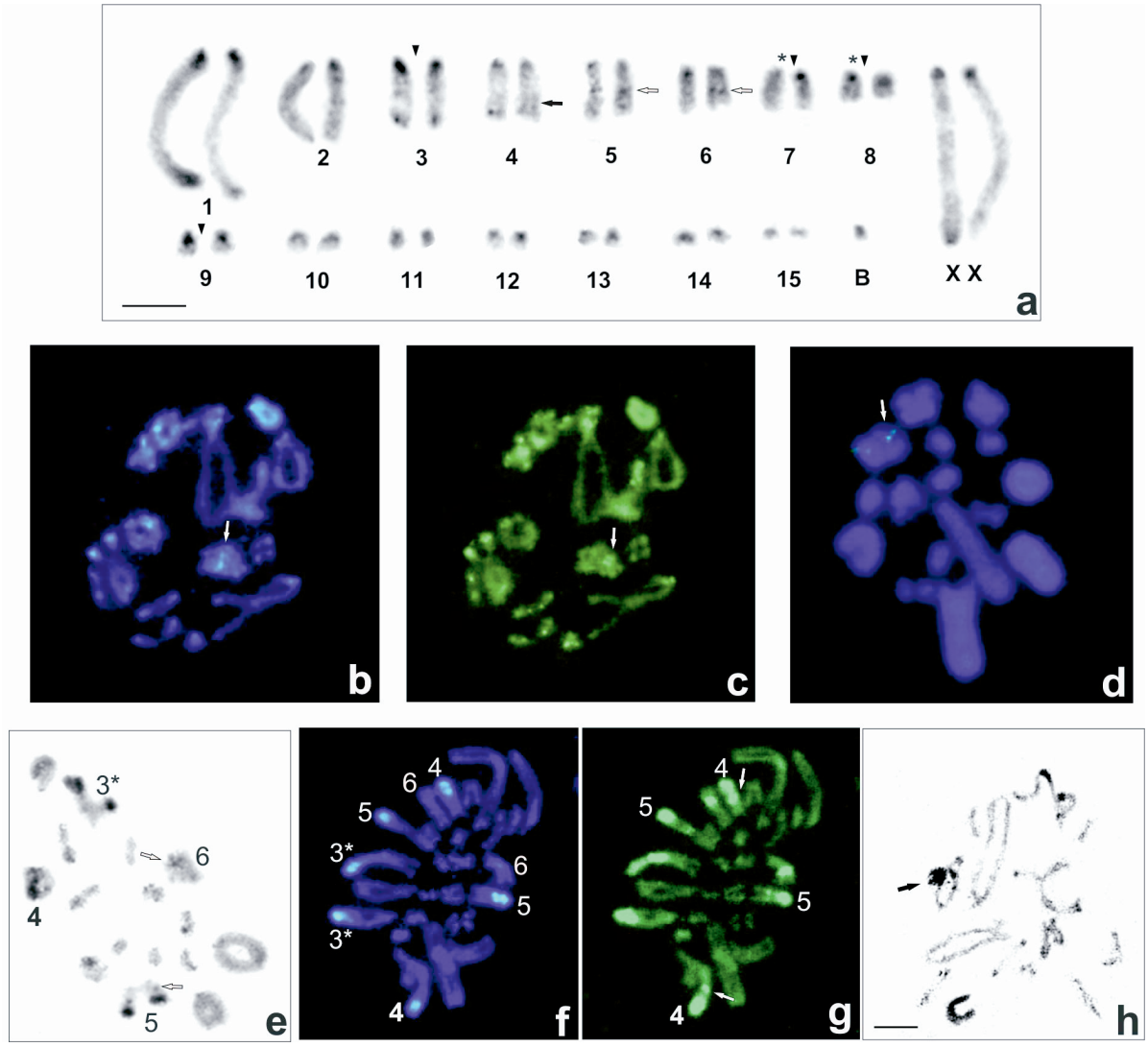

Fig. 1. Examples of karyotypes with 31 (male), 32 (female) chromosomes in *Parnassiana* species: *P. fusca* (a-d) and Genus aff. *Parnassiana* sp. 1 (e-h); C-banding pattern of female chromosome complement (a) and diplotene (e); fluorochrome staining heterochromatin with DAPI (blue) and CMA_3 (green) bands in diakinesis (b, c) and spermatogonial metaphase (f, g); FISH with 18S rDNA probe (green) (d); silver staining (h). Open arrows indicate interstitial C-bands on the fifth and sixth pair (a, e), whereas black arrowheads thick paracentromeric C-bands (a, e). The arrows indicate the presence of DAPI-/CMA_3+ bands (b, f and c, g respectively), a GC-rich band coincident with the rDNA-FISH signal (d) and active NOR (h) located on an interstitial region of a medium pair/bivalent. C/DAPI/CMA_3 blocks vary in size between homologous chromosomes (marked by an asterisk in a, e, f). B, supernumerary chromosome (a); X, sex chromosome. Bars = 10 µm.

the centromere (thick C-bands) as in the medium and small-sized chromosome pairs (3, 7-9) of *P. fusca* (Fig. 1a). In most species, interstitial thin C-bands were located in the medium-sized chromosomes, whereas a distal band was clearly seen on the small pair (the eighth) in species with 29 chromosomes (Fig. 1a, 2b, respectively). Generally, heterochromatin in the form of thick paracentromeric, interstitially and distally located thin/thick C-bands was visualized with bright homogeneous DAPI positive (DAPI+, AT-rich) and CMA_3 positive (CMA_3+, GC-rich) signals (e.g. Fig. 1b, c, f, g). The DAPI-/CMA_3+, thin C-band was located probably on the fourth pair in all analyzed specimens, independently of the number of chromosomes in the karyotype, and contained GC-rich band (Fig. 1c,g) coinciding with the position of 18S rDNA and active NOR (Fig. 1h, 2c). Thus, in all analyzed individuals a single FISH signal was observed, detecting a low-intensity cluster of 18S rDNA located interstitially probably on the fourth

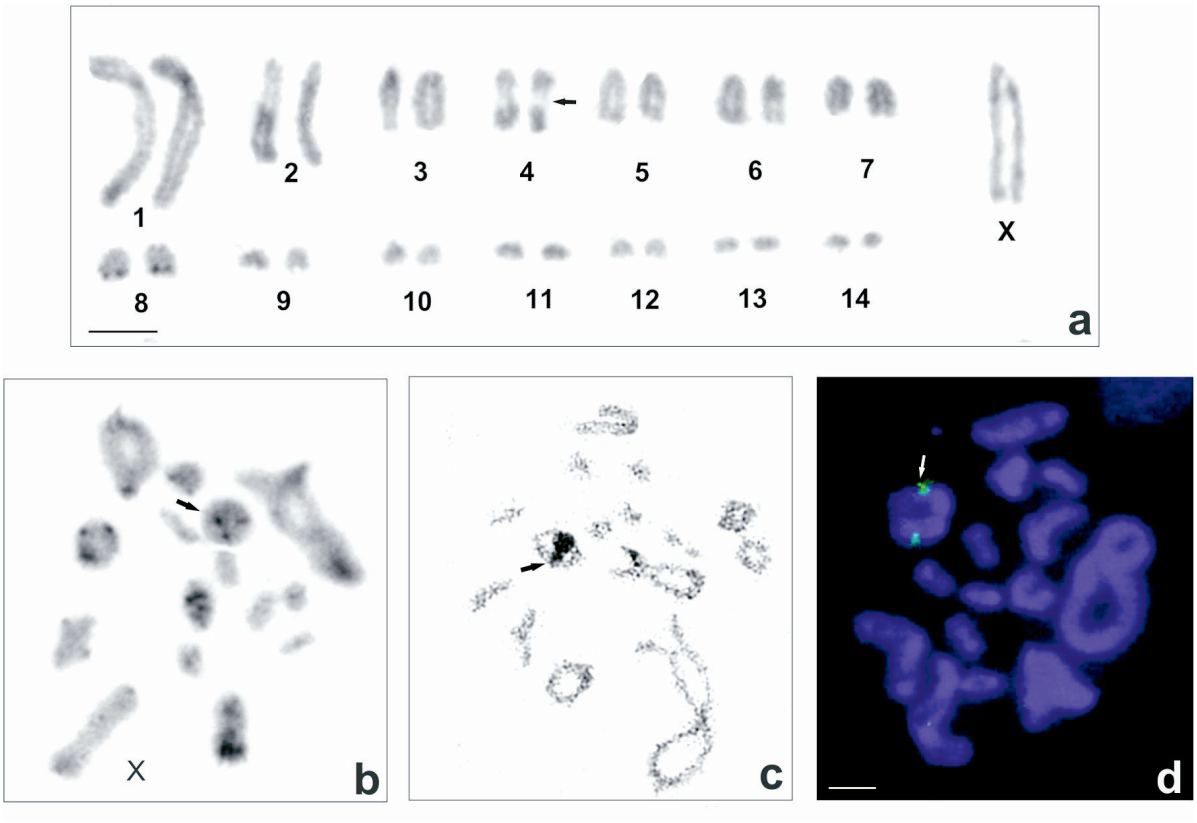

Fig. 2. Examples of karyotypes with 29 chromosomes (male) in *Parnassiana* species: *P. ch. chelmos* (a) and *P. menalon* (b-d); C-banding in male complement (a) and diakinesis (b); silver staining (c) and FISH with 18S rDNA (green) (d). Secondary constriction (located interstitially on a medium pair and C-bands (b) correspond to active NOR (c) and a cluster of 18S rDNA (d) marked by arrows. X, sex chromosome. Bars = 10 μm.

chromosome pair (Fig. 1d, 2d). In this pair a secondary constriction in the same place was rarely observed (Fig. 1a, 2a). In some species heteromorphism of both C-bands and fluorochrome bands was observed (Table 2 – indicated with an asterisk; Fig. 1a, e, f) in terms of the size/intensity of bands on homologous arms in a chromosome.

B chromosomes representing supernumerary elements to the standard chromosome set were found in one *P. fusca* female (Fig. 1a) and *P. parnassica* male (not shown). In both individuals, the B chromosome was similar in size to the small-sized chromosome pair, acrocentric, mitotically and meiotically stable, with thick paracentromeric C-bands (they were not examined using fluorochromes and FISH/NOR techniques).

Discussion

The taxa examined in this study form two groups according to their karyotype. The first group includes species with ancestral chromosome number 2n = 31 (male). The second group shows a reduced chromosome number 2n = 29 (FN = 31) as a result of one Rb-translocation (submetacentric large pair). A Robertsonian (Rb) translocation or centric fusion is the most common chromosomal rearrangement frequently found in the tettigoniid chromosomal evolutionary history (e.g. WHITE 1973; HEWITT 1979; WARCHAŁOWSKA-ŚLIWA 1998). This type of translocation occurs between two acrocentric chromosomes and reduces the diploid chromosome number.

In this case, one fusion changed the basic karyotype forming a biarmed large autosome pair. Analysis of the main relative lengths of autosomes shows that the change in chromosome number in the second group of taxa is the result of a centric fusion between the first and fifth or sixth medium pair of autosomes; the telomeres appeared to be lost during the chromosomal rearrangement or eliminated during chromosome differentiation. Only one species belonging to the tribe Platycleidini, *Metrioptera saussuriana*, has the same karyotype of 2n = 29 (FN = 31). *Montana daghestanica* also has one pair of biarmed autosomes and biarmed X chromosome (FN = 32), whereas in *M. tomini* with the same chromosome number the karyotype was formed as a result of one Rb-translocation and two

pericentric inversions in one pair of autosomes and X (FN = 34) (see review in WARCHAŁOWSKA-ŚLIWA 1998).

The application of classical and molecular methods enables a better characterization of the karyotype in different subfamilies of tettigoniids, as well as identification of genus-specific patterns (e.g. GRZYWACZ *et al*. 2011, 2014a, b; WARCHAŁOWSKA-ŚLIWA *et al*. 2005, 2009, 2011, 2013a, b). A comparison of the C-bands, fluorochrome staining and the FISH signal with the 18S rDNA probe and NOR sites distinguished two types of heterochromatin, depending on the base composition of the DNA molecule in species of the genus *Parnassiana* (see Table 2). The thick paracentromeric bands in the third autosomal pair and most of the thin interstitial (except for one medium pair) and distal C-bands were CMA_3 and DAPI positive. In most of the latter cases both the DAPI and CMA_3 staining suggest the occurrence of a high concentration of AT- and CG-base pairs of DNA situated near each other in two or three chromosome pairs. In those cases the staining did not detect NOR/rDNA clusters but a special type of GC-rich heterochromatin associated with this region. Similar results have been described for some tettigoniids (e.g. WARCHAŁOWSKA-ŚLIWA *et al*. 2013a; GRZYWACZ *et al*. 2014b), grasshoppers (e.g. ROCHA *et al*. 2011) and coleopterans (e.g. SCHNEIDER *et al*. 2007). However, in all analyzed taxa, only the interstitial region of the fourth autosome pair showed thin C-bands and bright CMA_3 (DAPI negative) CG-rich segments. Therefore, different heterochromatin types suggest the occurrence of specific rearrangements of repetitive DNA families resulting from processes that occurred during the diversification of the analyzed species groups.

In both types of karyotype described herein, one (per haploid genome) 18S rDNA locus is coincident with a single active NOR and GC-rich heterochromatin located interstitially on one medium acrocentric bivalent. The presence of interstitial rDNA loci on a single bivalent of acrocentric or bi-armed autosomes has previously been observed in some Bradyporinae taxa that possess a reduced chromosome number resulting from tandem fusion or Rb-translocation (WARCHAŁOWSKA-ŚLIWA *et al*. 2013a). It can not be excluded that in *Parnassiana* and related species the presence of a secondary constriction and location of an rDNA/NOR in the same region on the medium autosome indicates a rearrangement that has been fixed in this group. A single bivalent carrying the18S rDNA cluster found in the paracentromeric/interstitial/distal regions in different sized chromosomes was previously observed in European representatives of Saginae (WARCHAŁOWSKA-ŚLIWA *et al.* 2009) and both European (e.g. WARCHAŁOWSKA-ŚLIWA *et al*. 2013b) and African Phaneropterinae (HEMP *et al*. 2010, 2013, 2015). In addition one active NOR seems to be a typical feature of karyotypes with the ancestral chromosome number in European Tettigoniinae (WARCHAŁOWSKA-ŚLIWA *et al*. 2005). In Genus aff. *Parnassiana* sp. 1 (only one individual examined), the pattern of heterochromatin distribution revealed size heteromorphism in C- and fluorochrome-positive bands in some chromosomes and in the intensity of the rDNA hybridization signals and NOR on homologous pair/s of autosomes (indicated by an asterisk in Table 2). Similar heteromorphism has been observed in other tettigoniids (e.g. WARCHAŁOWSKA-ŚLIWA *et al*. 2013a, b; GRZYWACZ *et al*. 2014a, b) as a result of different mechanisms, i.e. homologous translocation, unequal crossing-over, or specific rearrangements of repetitive DNA families.

The occurrence of B chromosomes has been previously noted in some tettigoniid species (for a review see WARCHAŁOWSKA-ŚLIWA *et al*. 2008). In the tribe Platycleidini, supernumerary chromosomes were not found up to now. In the present study, we found the same type of Bs in *P. fusca* (female 2n = 32) and *P. parnassica* (male 2n = 29) being both mitotically and meiotically stable. However, the origin of Bs is currently unclear as this question requires a comparison of the DNA sequences shared by both autosomes and Bs.

Finally, the results described herein constitute the first step towards a better understanding of the chromosomal reorganization and evolution within the tribe Platycleidini and thus also within the subfamily Tettigoniinae. Besides changes in chromosome number and morphology (by Rb-translocation), interspecific autosomal differentiation has involved minor differences concerning the heterochromatin composition and distribution obtained by C-banding and fluorochrome staining. However, rDNA/NOR distribution has not proven to be a good cytogenetic marker for distinguishing taxa in the genus *Parnassiana*. Among other tettigoniids, chromosomal variability for the 18S rDNA was noticed, with species characterized by multiple clusters, including species in which this cytogenetic marker seems to be a good tool for distinguishing species/genera and phylogenetic lineages (e.g. GRZYWACZ *et al*. 2011; WARCHAŁOWSKA-ŚLIWA *et al*. 2013a, b). Future cytogenetic and molecular studies involving larger samples and more species of *Parnassiana* and related genera are needed in order to gain a more comprehensive view of the chromosome evolution in this group.

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