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 Plactyleidini. Classical (C-banding, DAPI/CMA3-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.

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/CMA3 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 coverslip 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 A3 (CMA3) 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-
resentance 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 WARCHALOWSKA-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 CMA3), as well as cytogenetic mapping of 18S rDNA and Ag-staining are shown. After both C-staining and fluorochrome DAPI/ CMA3 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>
<thead>
<tr>
<th>Species</th>
<th>2n (male), FN; chromosome morphology and B</th>
<th>C-bands: thick pc, i, d</th>
<th>Position of fluorochrome bands</th>
<th>rDNA FISH/NOR</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Parnassiana chelmos chelmos</em></td>
<td>29, 31; 1 sm</td>
<td>3pc, 4i, 7i, 8d</td>
<td>3pc, 8d</td>
<td>3pc+d, 4i, 8d</td>
</tr>
<tr>
<td><em>Parnassiana chelmos unicolor</em></td>
<td>29, 31; 1 sm</td>
<td>3pc, 4i, 8d</td>
<td>3pc, 8d</td>
<td>3pc+d, 4i, 8d</td>
</tr>
<tr>
<td><em>Parnassiana coracis</em></td>
<td>29, 31; 1 sm</td>
<td>3pc, 4i, 8d</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td><em>Parnassiana fusca</em></td>
<td>31, 31; all a</td>
<td>3pc+d, 4i, 5i, 6i, 7p*, 8pc*, 9pc*</td>
<td>most pc+i (excluding 4i)</td>
<td>most pc+i+d, 4i</td>
</tr>
<tr>
<td><em>Parnassiana gionica</em></td>
<td>29, 31; 1 sm</td>
<td>3pc, 4i, 5i</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td><em>Parnassiana menalon</em></td>
<td>29, 31; 1 sm</td>
<td>3pc, 4i, 5i, 8d</td>
<td>3pc, 8d</td>
<td>3pc, 4i, 8d</td>
</tr>
<tr>
<td><em>Parnassiana pannassica</em></td>
<td>29, 31; 1 sm</td>
<td>3pc, 4i, 5i</td>
<td>3pc, 5i</td>
<td>3pc+i, 4i</td>
</tr>
<tr>
<td><em>Parnassiana temuis</em></td>
<td>31, 31; all a</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td><em>Parnassiana tymphrestos</em></td>
<td>29, 31; 1 sm</td>
<td>3pc, 4i, 8d</td>
<td>3pc, 8d</td>
<td>3pc, 4i, 8d</td>
</tr>
<tr>
<td>Genus aff. <em>Parnassiana</em> sp. 1</td>
<td>31, 31; all a</td>
<td>3pc+d*, 4i, 5i+d*, 7p*</td>
<td>3d*, 4d*, 5d</td>
<td>3d*, 4i+d, 5d</td>
</tr>
<tr>
<td>Genus aff. <em>Parnassiana</em> sp. 2</td>
<td>29, 31; 1 sm</td>
<td>3pc, 4i</td>
<td>no</td>
<td>no</td>
</tr>
</tbody>
</table>

FN = fundamental number of chromosome arms; = published by WARCHALOWSKA-ŚLIWA et al. 2005; a = acrocentric; sm = submetacentric; pc = paracentromeric; i = interstitial; d = distal; no = not stained by fluorochrome techniques or FISH; *intraspécific variation of heterochromatin; X = X chromosome; B = B chromosome.
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, 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 CMA3 positive (CMA3+, GC-rich) signals (e.g. Fig. 1b, c, f, g). The DAPI-/CMA3+ 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/CMA3 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.

**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 CMA3 (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-/CMA3+ 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/CMA3 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.
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 tettigonid chromosomal evolutionary history (e.g. WHITE 1973; HEWITT 1979; WARCHALOWSKA–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-SLIWA 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-SLIWA 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 CMA3 and DAPI positive. In most of the latter cases both the DAPI and CMA3 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-SLIWA 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 CMA3 (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 Brachyporinae taxa that possess a reduced chromosome number resulting from tandem fusion or Rb-translocation (WARCHAŁOWSKA-SLIWA 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 the 18S rDNA cluster found in the paracentromeric/interstitial/distal regions in different sized chromosomes was previously observed in European representatives of Saginæ (WARCHAŁOWSKA-SLIWA et al. 2009) and both European (e.g. WARCHAŁOWSKA-SLIWA 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-SLIWA 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-SLIWA 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-SLIWA et al. 2008). In the tribe Platypleini, 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 chromosomes and reorganization and evolution within the tribe Platypleini 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-SLIWA 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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