Tribal affiliation of endemic Macaronesian bush-crickets similar to *Tettigonia* (Orthoptera: Tettigoniidae) based on their karyology and taxonomy

Elżbieta WARCHAŁOWSKA-ŚLIWA[1], Howon RHEE[2], Klaus-Gerhard HELLER[3] and Beata GRZYWACZ[1]

[1] Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Kraków, Poland;
e-mails: warchalowska@isez.pan.krakow.pl; grzywacz@isez.pan.krakow.pl

[2] Trier University, Department of Biogeography, Trier, Rheinland-Pfalz, Germany; e-mail: s6horhee@uni-trier.de

[3] Magdeburg, Germany; e-mail: heller.volleth@t-online.de

Abstract. Two endemic genera of large tettigoniids are found in the Canary Islands and Madeira, which are morphologically similar to the genus *Tettigonia*. In this study, the karyotypes of representatives of the Glyphonotini genera *Calliphona* and *Psalmatophanes* are described for the first time. Comparison of their chromosomes with that of members of Tettigoniini genus *Tettigonia* revealed that all of the species studied have the same karyotype (2n = 29 with sex determination system X0 in the male), which differs from the ancestral tettigoniid karyotype by one Robertsonian translocation. These results support not only the morphological and molecular genetic evidence but also the hypothesis of a close relationship between *Tettigonia* and the endemics *Calliphona* and *Psalmatophanes*. Cytogenetic data of Glyphonotini strongly indicate that the taxonomy of thes lineage needs to be re-evaluated in which evidence from multiple sources could help resolve the taxonomic problems.

INTRODUCTION

The Macaronesian region, situated in the North Atlantic Ocean west of North Africa and South West Europe, includes four large archipelagos. Canary Islands and Madeira are closest to the African mainland, while the Azores are further away in the ocean and Cape Verde is much further south than the other three archipelagos. All islands are of volcanic origin and have never been in contact with the mainland (true 'oceanic islands'). Therefore, all species living there must be descendants of immigrants and are expected to have their closest relatives in nearby mainland, except for newly introduced species.

Sometime after their arrival the immigrants may evolve into new species. Among the orthopteran species in the Macaronesian region, this change does not seem to have happened very often. Cape Verde has one endemic species (among 34 species occurring there; Harz, 1982; Mestre & Chiffaud, 1997; Buzzetti et al., 2005), the Azores one (among 14 species; De Sousa & Borges, 2005) and Madeira four (among 24 species; Lange, 1990). Only in the Canary Islands are a high percentage (about 45%) of all Orthoptera endemic (37 of 81 species; Bland et al., 1996, Bland, 2001; Hochkirch, pers. comm.). The number of endemic genera is of course smaller, with one in Madeira (*Psalmatophanes* Chopard, 1938) and seven (*Acrostira*

Enderlein, 1929; *Ariagona* Krauss, 1892; *Arminda* Krauss, 1892; *Calliphona* Krauss, 1892; *Canariola* Uvarov, 1940; *Evergoderes* Bolivar, 1936; *Purpuraria* Enderlein, 1929) in the Canary Islands, being only about one quarter to one fifth of the number of endemic species. These groups have diverged so much from their ancestors that their relationships are sometimes obscure or doubtful. This is the case for the tribal affiliation the tettigoniid genera *Calliphona* (Fig. 1a) and *Psalmatophanes* Chopard, 1938 (Fig. 1b), which is uncertain. Although both genera at present do not belong to the same tribe as *Tettigonia* Linneaus, 1758, they were considered as similar or closely related to this genus at the time of their description [(Krauss, 1892; Chopard, 1938; Bolívar, 1940 (revised 1991)] and later (e.g. Holzapfel & Cantrall, 1972; Pfau & Pfau, 2002). However, Rentz & Colless (1990) conclude after an extensive morphological review of the whole subfamily that they might belong to the tribe Glyphonotini, with relatives only in Central Asia, Australia and North West America, which is their currently accepted classification (Cigliano et al., 2020). Rentz & Colless (1990) were surprised by their conclusion because it was difficult to explain in terms of biogeography. Later Arnedo et al. (2008) confirmed the close relationship of *Psalmatophanes* to *Tettigonia* based on a detailed molecular phylogenetic study, but did not change the classification
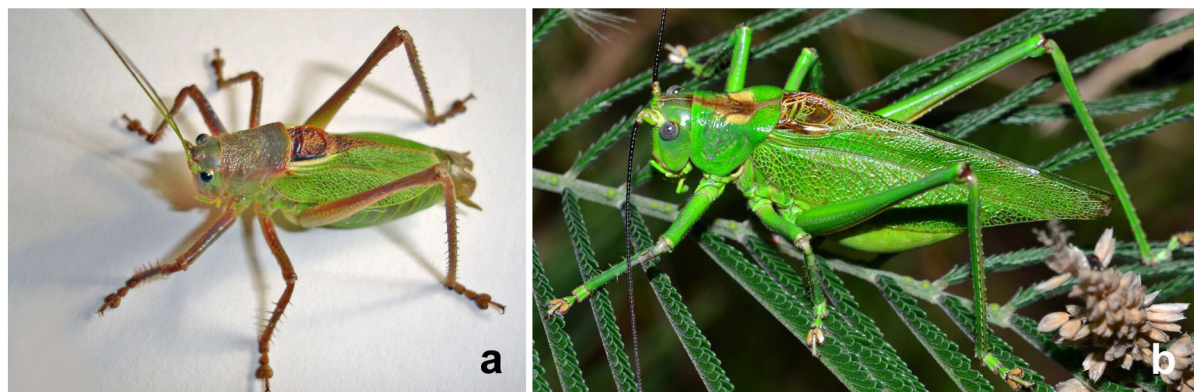
Fig. 1. Habitus of *Calliphona koenigi* (a) and *Psalmatophanes barettoi* (b) for comparison with *Tettigonia* see e.g. Fig. 5 in Grzywacz et al. (2017b).

of Rentz & Colless (1990). Since we had the opportunity to collect specimens of both genera and analyse their chromosomes, we used these new characters in a taxonomic re-evaluation of the situation, which included a comparison of several species of *Tettigonia*.

Comparative cytogenetic studies on more than 130 species from the subfamily Tettigoniinae (sensu Cigliano et al., 2020) reveal that diploid chromosome number (2n) varies from 15 to 33 in most cases with an X0 sex determination system. Most of the Old World species have 31 acrocentric chromosomes, but the few Australian and New World species diverge from the standard chromosome number with one or more bi-armed autosomes and often a metacentric X chromosome (e.g. reviewed Warchałowska-Śliwa, 1998). Until now, standard staining techniques (alcohol-carmine or Giemsa) have been used in studies of the tribe Glyphonotini (Bugrov, 1990; Ueshima & Rentz, 1990) and C-banding and the nucleolar organizer region (NOR) staining for those of the tribe Tettigoniini (Warchałowska-Śliwa & Bugrov, 1997; Warchałowska-Śliwa & Maryańska-Nadachowska, 1995a, b; Warchałowska et al., 2002).

In this study, nine taxa from two Tettigoniinae tribes, the Glyphonotini and Tettigoniini, were analysed using different cytogenetic techniques, such as C-banding, silver impregnation (Ag-NOR), fluorochrome staining and fluorescence in situ hybridization (FISH) using 18S rDNA and telomeric $(TTAGG)_n$ as probes. We aimed to describe the karyotypes and identify cytogenetic patterns as the first step towards a better understanding of the relationships at different taxonomic levels.

MATERIALS AND METHODS

This study included two species belonging to the tribe Glyphonotini: *Psalmatophanes barettoi* Chopard, 1938 (2 males) and *Calliphona koenigi* Krauss, 1892 (2 males) and seven taxa of *Tettigonia* (Tettigoniini): *T. armeniaca* complex (two males), *T. balcanica* Chobanov & Lemonnier-Darcemont, 2014 (one male), *T. cantans* (Fuessly, 1775) (one male), *T. caudata* (Charpentier, 1845) (one male), *T. viridissima* (Linnaeus, 1758) (four males), *T.* cf. *viridissima* (one male) and the *T. vaucheriana* complex (two males) (Table 1).

Gonads were incubated in a hypotonic solution (0.9% sodium citrate), then fixed in Carnoy's solution (ethanol and acetic acid, 3 : 1) and squashed in a drop of 45% acetic acid. The coverslip was removed using the dry ice procedure. Slides were dehydrated and then air dried. C-banding was done according to Sumner (1972). Constitutive heterochromatin was analysed qualitatively by CMA_3 (chromomycin A_3) and DAPI (4,6-diamidino-2-phenylindole) staining according to Schweizer (1976). Silver nitrate staining of active nucleolus organizing regions (NORs) was

Table 1. Localities at which the species analysed in this study were collected.

Species	Collection site and voucher ID	Geographical coordinates
Psalmatophanes barettoi Chopard, 1938	Portugal: Madeira; (1) CHX375, (2) CHX376	32.783°N, 16.883°W 32.817°N, 16.917°W
Calliphona koenigi Krauss, 1892	Spain: Canary Islands, Tenerife, Aguamansa, Caldera; CH8066, CH8067	28.35°N, 16.483°W
Tettigonia armeniaca complex	Turkey: (1) Horasan-Agri, Saclidag Pass, 2160 m; tam1a; (2) Horasan-Agri, Savsat-Ardahan road, 1630 m; tam2a	39.8747°N, 42.3856°E 41.2312°N, 42.4338°E
Tettigonia balcanica Chobanov & Lemonnier-Darcemont, 2014	Bulgaria: Pirin Mts, Predela Pass, 1020 m; (one male without ID)	41.8973°N, 23.3257°E
Tettigonia cantans (Fuessly, 1775)	Poland: Grębynice near Kraków; tca2a	50.0957°N, 19.5209°E
Tettigonia caudata (Charpentier, 1845)	Bulgaria: Byala; tct2	43.4717°N, 25.7696°E
Tettigonia viridissima (Linnaeus, 1758)	Bulgaria: (1) Haskovo, Perperikon Ruins; tvi5b; (2) Dobrich, Bolata Bay; tvi5c Morocco: (1) S Ajabo, 1360 m; tmo1b; (2) NW Khenifra, 1100 m; tmo2b	41.715°N, 25.4657°E 43.3838°N, 28.4715°E 33.0659°N, 5.4086°W 33.1377°N, 5.9235°W
Tettigonia cf. *viridissima*	Morocco: S Aïn Zora, 835 m; tmo5e	34.5708°N, 3.6657°W
Tettigonia vaucheriana complex	Morocco: (1) NW Khenifra, 1100 m; tmo2c; (2) Tilougguite Pass, 1570 m; tmo8a	33.1377°N, 5.9235°W 32.0852°N, 6.3003°W
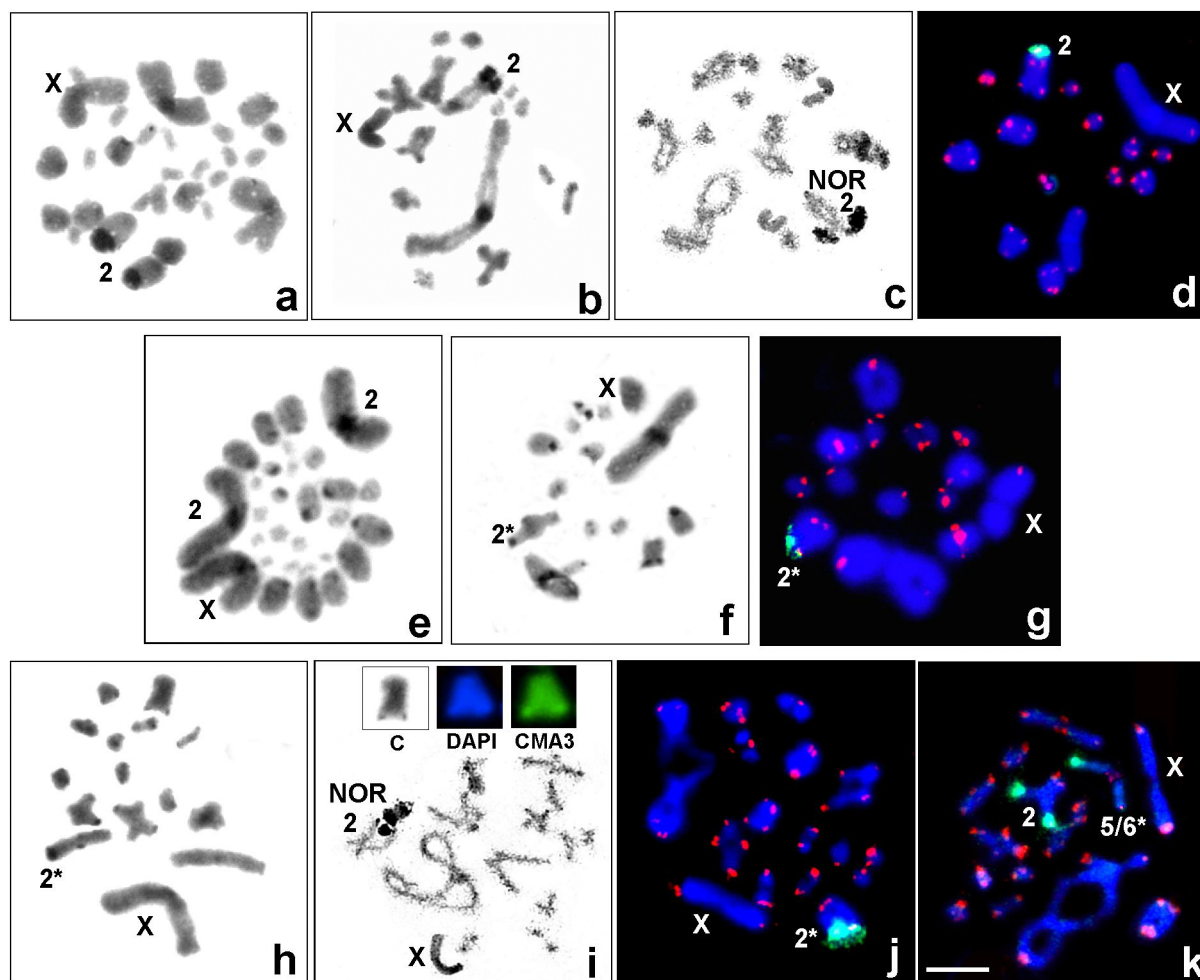
Fig. 2. Examples of chromosome banding in *Psalmatophanes barettoi* (a–d) and *Calliphona koenigi* (e–g), and in different species of the genus *Tettigonia*: *T. caudata* (h), *T. viridissima* (i), *T. cantans* (j) and *T. balcanica* (k) studied using different techniques. C-banding pattern of spermatogonial metaphases (a, e) and diakinesis (b, f, h); the thick (a, b) and thin (e, f, h) distal C-blocks on the second chromosomes/bivalent in the set. Diakinesis with one active NOR (c, i) and selected C+, DAPI- (blue) and CMA₃ (green) bands located in the distal region on the 2nd bivalent (on the top in i). Fluorescence in situ hybridization (FISH) with both 18S rDNA (green) and telomeric DNA (red) probes showing the chromosomal location of rDNA: diakinesis (d, g, j, k) with one rDNA sites located on 2nd bivalent, co-localized with NOR (c) and CMA₃ (green, i) bands. The second heteromorphic 18S rDNA-FISH signal on other medium sized bivalents (5 or 6) in *T. balcanica* (k). Heterochromatin and hybridization areas vary in size between homologous chromosomes (marked by an asterisk in f, g, h, j, k). X – sex chromosome. Bar = 10 µm.

done using the protocol of Warchałowska-Śliwa & Maryańska-Nadachowska (1992). The best chromosome preparations were used for fluorescence in situ hybridization (FISH) with 18S ribosomal DNA (rDNA) and telomeric DNA (TTAGG)$_n$ probes. FISH was carried out as described previously by Grzywacz et al. (2018). A probe containing a fragment of orthopteran 18S rDNA labelled with biotin-16-dUTP (Roche Diagnostics GmbH, Mannheim, Germany) was used to detect rDNA clusters in metaphase chromosomes. For the detection of telomeric repeats (TTAGG)$_n$ in metaphase chromosomes, a probe was generated using non-template PCR with Tel1 (5' GGT TAG GTT AGG TTA GGT TAG G 3') and Tel2 (5' TAA CCT AAC CTA ACC TAA 3') primers (Grozeva et al., 2011). Visualization of hybridized DNA labelled with biotin or digoxigenin was done using avidin-FITC (Invitrogen, Life Technologies INC., Carlsbad, CA, USA) and anti-digoxigenin rhodamine (Roche Diagnostics GmbH), respectively. The chromosomes were counterstained with ProLong Gold antifade reagent containing DAPI (Invitrogen, Life Technologies) under a cover glass. Chromosomes were studied under a Nikon Eclipse 400 microscope.

RESULTS

All the species analysed (see Table 1) had the same diploid chromosome number with 2n = 29 chromosomes and sex determination system X0 (male). We classified autosomes according to size as one long (L) metacentric pair, five medium-sized (M) and eight short (S) acrocentric pairs. The X chromosome was bi-armed and slightly smaller than the long pair of autosomes (Fig. 2a–k). So, the number of chromosome arms including the X chromosome (FN) is 32. All individuals had paracentromeric C-bands and in most cases, these heterochromatin blocks were thin and only on the first pair of metacentric chromosomes of *Psalmatophanes barettoi* and *Calliphona koenigi* were they thick. In *P. barettoi*, distally located thick C-bands were located on pair M₂, whereas the distal band in other species/specimens on the same bivalent was thin (Fig. 2a, b, e, f, h).

In order to analyse the distribution of ribosomal and telomeric DNA on the chromosomes in the karyotypes of all taxa, two-colour FISH was used with two differently labelled probes. A similar pattern of chromosomal localization of 18S rDNA probes was observed in all taxa, excluding *Tettigonia balcanica*. Generally, FISH revealed the presence of rDNA only near the distal region of the second pair of chromosomes (Fig. 2d, g, j). Only in *T. balcanica* was an additional heteromorphic cluster (in term of present/absent) of 18S rDNA detected on the distally located region of another medium-sized bivalent (M_5 or M_6) (Fig. 2k). The rDNA cluster sometimes varied in size between homologous chromosomes (Fig. 2d, g, j, k). In the taxa studied one (per haploid genome) large cluster of 18S rDNA coinciding with a single active NOR was visualized by staining with Ag-NOR and a thin C-band/CMA$_3$ positive region was observed (Fig. 2c, i). The telomeric DNA hybridized at the distal ends of all chromosomes. In some bivalents hybridization signals differed in size and intensity between chromosomes of the same taxa, but there was no significant variation between taxa.

DISCUSSION

Up to now, cytotaxonomic studies on the Glyphonotini have included two species of *Glyphonotus*, *G. thoracicus* (Fischer von Waldheim, 1846) and *G. coniciplicus* Uvarov, 1914 from Kazakhstan (2n = 21; Bugrov, 1990; unpubl. data) and 15 Australian species of the genera *Chlorodectes* Rentz, 1985; *Ectopistidectes* Rentz, 1985 and *Metaballus* Herman, 1974 (2n = 23–27; Ueshima & Rentz, 1990). In these species, diploid chromosome numbers ranged from 21 to 27 in males and their karyotypes were characterized by the distinct morphology of the chromosomes, which consist of only acrocentric or one to three bi-armed autosomes/sex chromosome. Furthermore, the North American and Australian Tettigoniini genera *Capnobotes* Scudder, 1897 and *Zacycloptera* Caudell, 1907 and *Nanodectes* Rentz, 1985 have 15 to 23 chromosomes of a different morphology (Ueshima & Rentz, 1979, 1990, 1991). Thus, these species/genera have an evolutionary more advanced karyotype with centric fusions and/or various numbers of tandem fusions and pericentric inversions. However, males of all the species of the genus *Tettigonia* (i.e. *T. cantans*, *T. caudata*, *T. viridissima*, *T. ussuriana* Uvarov, 1939 and *T. uvarovi* Ebner, 1946) throughout the Palaearctic region have a karyotype with 29 chromosomes (Warchałowska-Śliwa, 1984, 1998; Warchałowska-Śliwa & Maryańska-Nadachowska, 1995a; Warchałowska-Śliwa et al., 2002).

Representatives of the genera examined in this study are included in two tribes based on differences in the shape of their cerci and teeth. On the other hand, these genera have the same male karyotype with 2n = 29, chromosome morphology and the ancestral X0 sex chromosome determination system. If we assume that most species of the Palaearctic Tettigoniinae are characterized by a basic/ancestral karyotype with 2n = 31 and FN = 31 in males (for review see Warchałowska-Śliwa, 1998), the chromosome number of species included in this study could be

the result of one Robertsonian translocation (the first autosome pair becomes bi-armed) and a pericentric inversion that modified the ancestral acrocentric X chromosome into the metacentric X (FN = 32). A similar type of translocation is reported in some other species of Tettigoniinae (e.g. Grzywacz et al., 2017a; Warchałowska-Śliwa et al., 2005, 2017). In addition, the present and previous studies indicate that the karyotypes of *Tettigonia* are stable and there are no changes in the chromosome number and general morphology of the X chromosome in this genus. Currently, an acrocentric X chromosome is only reported in *T. uvarovi* (Warchałowska-Śliwa et al., 2002).

Recently the location of ribosomal genes were studied in order to understand genomic differentiation and distinguish between species and evolutionary lines in some bush-crickets of the subfamily Tettigoniinae (Grzywacz et al., 2017a; Warchałowska-Śliwa et al., 2017). One rDNA/NOR locus (per haploid genome) is coincident with a single active NOR and GC-rich heterochromatin region located near the distal part of the second pair of chromosomes. This appears to be a feature of the typical karyotype of species of *Psalmatophanes*, *Calliphona* and *Tettigonia*. A single bivalent carrying the 18S rDNA cluster, but in the interstitial or paracentromeric regions, is reported in other tettigoniids, e.g. in European Platycleidini (Grzywacz et al., 2017a) and Pholidopterini (Warchałowska-Śliwa et al., 2017). Furthermore, one active NOR appears to be a typical feature of karyotypes of European Tettigoniinae (Warchałowska-Śliwa et al., 2005). Only in *Tettigonia balcanica* (one male analysed, present paper) was there an additional heteromorphic cluster, of 18S rDNA detected on a distally located region of another medium-sized bivalent. In addition, some individuals exhibit different intensities of rDNA hybridization on homologous pairs of autosomes. Similar heteromorphism is reported in other bush-crickets and attributed to amplification or loss due to either unequal crossing-over, translocation rearrangements or tandem duplications of ribosomal genes (e.g. Warchałowska-Śliwa et al., 2013). The terminal locations of hybridization signals in *Psalmatophanes, Calliphona* and *Tettigonia* indicate that the telomeres are composed of (TTAGG)$_n$ repeats as in other Orthoptera (e.g. Warchałowska-Śliwa et al., 2011, 2013, 2017; Grzywacz et al., 2014). The number and morphology of chromosomes and marker differentiation did not differ in *Psalmatophanes barettoi*, *Calliphona koenigi* and *Tettigonia*.

The data on morphological characters (Krauss, 1892; Chopard, 1938; Bolívar, 1940, 1991; Holzapfel & Cantrall, 1972), results of molecular phylogenetic studies (Arnedo et al., 2008) and biogeography/distribution [both genera allo- respectively parapatric to *Tettigonia*; candidates for an assumed peripatric speciation (see maps for *Tettigonia* in Grzywacz et al., 2017b; Cigliano et al., 2020)] indicate that *Calliphona* and *Psalmatophanes* are probably closely related to *Tettigonia*. In terms of the number and structure of their chromosomes both genera are similar cytogenetically to *Tettigonia*, but differ distinctly from *Glyphonotus*. Based on the cytogenetic information presented and pre-

vious morphological and molecular evidence, we strongly recommend that *Psalmatophanes* and *Calliphona* be transferred from the tribe Glyphonotini to the tribe Tettigoniini.

It should be mentioned that all the species of *Glyphonotus* (see Miram, 1925) and *Calliphona koenigi* have infuscated hind wings, which are displayed during defence (Miram, 1925; Pfau & Pfau, 2002). We consider patterned or coloured hind wings as an analogous trait, also seen e.g., in *Capnobotes fuliginosus* (Thomas, 1972) (Tettigoniinae) (but not in other species of *Capnobotes* Scudder, 1897; see Rentz & Birchim, 1968) and in species of *Clonia* Stål, 1855 (Saginae) (Kaltenbach, 1990). All these species are large and orthopterans capable of inflicting a severe bite.

The information on the relationships of the tribe Glyphonotini is contradictory. Gorochov (1988) re-established the subfamily Glyphonotinae Tarbinsky, 1932 as a monogeneric group outside Tettigoniinae and close to Conocephalinae. On the other hand, molecular genetic data suggest a close relationship between *Glyphonotus* (*sinensis*) and holarctic tettigoniines (Liu Chunxiang, unpubl.). Rentz & Colless (1990) accepted Gorochov's argument and established the tribe Glyphonotini, but within Tettigoniinae. However, there are technical errors in their disjunctive tribal diagnosis. Rentz & Colless (1990) give a list of 16 characters of which all Glyphonotini must have at least eight. The first character, number 5 / state 2, is not found in any Glyphonotini according to their data matrix. Character 32/6 is not on the list of the characters (only up to 32/3). Based on the characters in the list, the genera under Glyphonotini, *Apote* Scudder, 1897 and *Metaballus* Herman, 1884, have only seven and are thus not qualified as Glyphonotini. On the other hand, *Tettigonia* has also only seven. If after correction this genus also has eight characters, its inclusion would automatically synonymize the tribe in its present definition. The similarity of both genera is underlined by the fact that the type species of *Glyphonotus* [*G. thoracicus* (Fischer von Waldheim, 1846)] is described under *Locusta*, a former name for *Tettigonia*.

In the first printed version of the *Orthoptera Species File* (OSF), Otte (1997) adds further species with additional problems: (1) genus *Hyphinomos* Uvarov, 1921 belongs to the Tettigoniini according to Rentz & Colless (1990; as *Hyphonomos*) and Onconotinae according to Sergeev (1995). Otte (1997), however, places it in Glyphonotini without comment. From the characters listed in Rentz & Colless (1990), *Hyphinomos* is far from Glyphonotini (has only three of the 16 characters mentioned above). (2) genus *Evergoderes* Bolivar, 1936 was not studied by Rentz & Colless (1990), but Otte (1997) lists it in Glyphonotini without comment.

Mugleston et al. (2018) indicate there are a large number of unsolved problems with the phylogeny of Tettigonioidea. These authors also mention serious problems within the Tettigoniinae, but have neither studied or even mention Glyphonotini. We follow their advice "Tettigoniinae should only include the Holarctic shield back tribes", which indicates that the Holarctic genera may be more closely related to each other than the remaining genera and

that currently Glyphonotini should comprise only *Glyphonotus*, with the remaining four Holarctic genera (*Apote*, *Cyrtophyllicus* Hebard, 1908, *Evergoderes* and *Hyphinomos*) in Tettigoniini sensu lato. The three Australian genera are currently placed in Tettigoniinae without tribal affiliation.

In conclusion, the present study has focused on the cytogenetic mapping of rRNA gene and telomeric sequences, active NOR and general distribution of heterochromatin in different genera/tribes, which is new data on the karyology of the Tettigoniinae. A chromosomal characterization of several species, karyotypic evidence for their relationships and additional support for the identification of species are some of the major contributions that cytogenetics can make to tettigoniid taxonomy. The results of chromosome analyses of Tettigoniinae clearly show the need for a taxonomic revision of this subfamily, whose karyotypic characteristics provide useful and interesting data. Thus, we believe that cytogenetics, morphology and molecular evidence together may resolve the problems with the classification of the groups of Tettigoniinae.

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