Eur. J. Entomol. **108**: 509–517, 2011
http://www.eje.cz/scripts/viewabstract.php?abstract=1647
ISSN 1210-5759 (print), 1802-8829 (online)

Comparative analysis of the location of rDNA in the Palaearctic bushcricket genus *Isophya* (Orthoptera: Tettigoniidae: Phaneropterinae)

BEATA GRZYWACZ[1], ANNA MARYAŃSKA-NADACHOWSKA[1], DRAGAN P. CHOBANOV[2], TATJANA KARAMYSHEVA[3] and ELŻBIETA WARCHAŁOWSKA-ŚLIWA[1]

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

[2]Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, Sofia, Bulgaria

[3]Institute of Cytology and Genetics, Siberian Branch of the Russian Academy of Sciences, Novosibirsk, Russia

Key words. Orthoptera, FISH, Ag-NOR, rDNA, telomeric repeats

Abstract. The present study focused on the evolution of the karyotype in 21 taxa of the genus *Isophya*, which was done by mapping the location on the chromosomes of ribosomal RNA (rRNA) coding genes using fluorescence in situ hybridization (FISH) with an 18S rDNA probe and using silver staining ($AgNO_3$) to evaluate the activity of major rDNA clusters. Since the chromosome number and sex determination do not vary in this genus, the above markers were used in a detailed comparison of the cytogenetic features of species of *Isophya*. The species analyzed were placed into three groups based on the location of rDNA on their chromosomes: (1) rDNA-FISH signals only on the two long pairs of autosomes, (2) rDNA-FISH signals on one long and one short pair of autosomes, and (3) rDNA-FISH signals on three to five different sized pairs of autosomes. These groupings partly correspond to the morphological groupings proposed in earlier studies. One long pair of autosomes frequently carried rDNA in all the *Isophya* species and probably is a plesiomorphic character for these taxa. The cytogenetic mapping revealed great variability among *Isophya* species in the chromosomal location of major rDNA clusters. Our results suggest that the observed variation in the number of rDNA clusters can be an important species-group specific phylogenetic marker. Analysis of 18S rDNA hybridization signals showed that the evolutionary dynamics of rDNA in this genus is remarkably high and accompanied by changes in the structure of chromosomes bearing rDNA at an inter- and intra-specific level. The telomeric sequence $(TTAGG)_n$ hybridized with the termini of most of chromosomes, however, some chromosome ends lacked signals probably due to a low copy number of telomeric repeats.

INTRODUCTION

The genus *Isophya* Brunner von Wattenwyl (1878) is one of the largest bushcricket genera in including 89 species (Eades et al., 2011) (*I. medimontana* Nedelkov, 1907 is actually regarded as a subspecies by Harz, 1969). It occurs in Central Europe, the Carpathian Basin, the Balkans, southern Ukraine, Asia Minor and the Caucasus region, eastwards reaching north western Iran and Iraq and an isolated area of the Altai Mountains (Bey-Bienko, 1954). Most species of *Isophya* have restricted ranges and thus the genus includes a high number of endemic taxa (Sevgili, 2003; Sevgili & Heller, 2003). The status of many species remains unresolved because *Isophya* contains several morphologically very similar species (Heller, 1988, 2006; Warchałowska-Śliwa et al., 2008; Chobanov, 2009a, b). In spite of the existing comprehensive bioacoustic and morphological (e.g. Heller et al., 2004; Sevgili et al., 2006), classical cytogenetic (e.g. Warchałowska-Śliwa et al., 2008) and molecular studies (Grzywacz & Warchałowska-Śliwa, 2008; Grzywacz-Gibała et al., 2010), the relationships within the group are still poorly known. The development of new cytogenetic-molecular markers is important for a better understanding of the genome organization and phylogeny of this genus.

Over 25 species and subspecies of *Isophya* have been studied cytotaxonomically (Warchałowska-Śliwa et al., 2008). The majority of these species have a karyotype consisting of 2n = 31 (male) / 32 (female) acrocentric chromosomes with an X0/XX mechanism of sex determination. This karyotype is suggested as ancestral for most species of tettigoniids (e.g. White, 1973; Warchałowska-Śliwa, 1998). Only one species, *I. hemiptera* (from the northern Caucasus), has a neo-X/neo-Y sex determination system (Warchałowska-Śliwa & Bugrov, 1998). Thus, the species of this genus have the same number of chromosomes and sex determination system. In the sex chromosome (X), the most remarkable changes include a pericentric inversion that converted the ancestral acrocentric X into a subacro/submetacentric X. Inter-specific autosomal differentiation involved the distribution and quantity of C-heterochromatin and the number of nucleolar organizer regions (NORs), thus revealing discrete changes that reflect the level of genomic organization (Warchałowska-Śliwa et al., 2008).

Recently, fluorescence in situ hybridization (FISH) techniques were extensively used for mapping DNA sequences directly on chromosomes, which has significantly increased our understanding of karyotype structure and evolution. In particular, major ribosomal RNA (rRNA) genes, the so-called rDNA, clustered in the nucleolar organizer regions (NORs) have been found useful as markers for karyotype comparisons in many insect species at the genus level, e.g. tiger beetles (Zacaro

et al., 2004), grasshoppers (e.g. Loreto et al., 2008) and bushcrickets (Warchałowska-Śliwa et al., 2009). Other repetitive sequences, the so-called telomeric DNA, are located mainly at chromosome termini. Telomeres were used as markers for identifying the ends of chromosomes and in the majority of insect orders, including Orthoptera, are composed of multiple copies of short, tandemly arranged TTAGG sequences. Clusters of telomeric repeats are thought to indicate chromosomal rearrangements related to changes in chromosome number and evolution in insects (López-Fernández et al., 2004, Warchałowska-Śliwa et al., 2009).

The stable karyotype of *Isophya* species and other short-winged species belonging to different genera of the tribe Barbitistini, namely *Ancistrura*, *Andreiniimon*, *Barbitistes*, *Metaplastes*, *Poecilimon*, *Polysarcus*, etc. (see review Warchałowska-Śliwa, 1998), is a simple model the study of which could contribute greatly to understanding the evolutionary dynamics of rDNA. The location of rRNA genes in Phaneropterinae by FISH has so far only been done for a single species (Hemp et al., 2010). Recently, silver impregnation was used to identify NORs in 15 species of the genus *Isophya* (Warchałowska-Śliwa et al., 2008).

The present study reports the results of follow-up research on the reconstruction of the ancestral karyotype based on the number and distribution of rDNA clusters in 21 species/subspecies of the genus *Isophya* using FISH with 18S rDNA probe as a potentially useful marker. In addition, NOR activity was verified by silver impregnation for 15 species previously analysed and 6 more species added for which this information was not available. A comparison of rDNA-FISH results with those after silver impregnation enabled us to identify non-active NOR clusters and precisely locate the active NORs. In all rDNA-FISH experiments, we used simultaneously a $(TTAGG)_n$ probe for a better identification of chromosome ends, particularly at diplotene/diakinesis. This study is the first on the chromosome evolution and phylogeny of Phaneropterinae bushcrickets.

MATERIAL AND METHODS

The 55 specimens analyzed represent 21 taxa of the genus *Isophya.* Adults or nymphs were collected during June/July from 2006 to 2010 in Bulgaria, Poland, Germany, Serbia and Russia (for localities see Table 1).

Gonads of the specimens were dissected out, treated with a hypotonic solution (0.9% sodium citrate) and then fixed in Carnoy (3 : 1 ethanol : acetic acid). Chromosome preparations were made by macerating cellular suspensions in drops of 45% acetic acid on slides, which were then squashed under cover slips. Then the cover slips were removed by the dry ice procedure and the slides air dried. The C-banding was carried out according to Sumner (1972) with a slight modification. The silver staining method ($AgNO_3$) for visualising NORs was applied as previously reported (Warchałowska-Śliwa & Maryańska-Nadachowska, 1992).

Fluorescence in situ hybridization with 18S rDNA (rDNA-FISH) and $(TTAGG)_n$ telomeric probes

For rDNA-FISH, a 1.8 kb fragment of 18S rDNA was generated by PCR using genomic DNA isolated from *Isophya rammei* (Orthoptera) as a template. The amplification was performed in a final volume of 25 µl containing 1.5 mM $MgCl_2$, 2.5 mM dNTP, 10 µM of each of the two 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), 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. The probe was labelled by nick translation with biotin-11-dUTP (Invitrogen, Tokyo, Japan).

The $(TTAGG)_n$ telomeric probe was generated by PCR using a modified version of the López-Fernández et al. (2004) technique. PCR was performed 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.

For FISH using both the rDNA and telomeric probes, we used the procedure described in Lichter et al. (1988). Chromosome preparations were treated with 100 µg/ml RNase A. After denaturation the chromosomes were hybridized with a probe mixture containing 20 ng of 18S rDNA probe, the $(TTAGG)_n$ telomeric probe and 10 µg of sonicated salmon sperm DNA (Invitrogen). Hybridization signals were detected with avidin-Alexa 488 (Invitrogen) and mouse anti-digoxigenin antibodies conjugated to Cy3 (Sigma-Aldrich, Tokyo, Japan). The preparations were counterstained with 20 µl DAPI (Sigma-Aldrich, Tokyo, Japan) for 15 min and then mounted in anti-fade based on DABCO (1-4-diazabicyclo[2.2.2]octane; Sigma-Aldrich) (see Sahara et al., 1999).

At least 15 meiotic divisions (diplotene/diakinesis) per male and two (rarely 3) males per species were analyzed using FISH and $AgNO_3$ techniques. In addition, spermatogonial or oogonial metaphases for some species were examined. FISH and silver staining techniques were used on the same species to determine whether these markers gave the same results when used on specimens collected from different geographical locations (Table 1). Photographs were taken with a Nikon Eclipse 400 light microscope fitted with a CCD DS-U1 camera using Chroma filter sets (for FISH). The software Lucia Image 5.0 was applied and images were mounted in Adobe Photoshop.

RESULTS

As described elsewhere (e.g. Warchałowska-Śliwa et al., 2008 except for *I. hemiptera*), the karyotypes of all the species analyzed consist of 31 chromosomes in the male and 32 in the female with an X0/XX sex determination system. Fifteen pairs of acrocentric autosomes can be arranged into two size groups: four long (L) and eleven medium and short (M/S) pairs. The pairs gradually decrease in size resulting in some problems with the precise identification of chromosome pairs (especially among the long and short ones). The acrocentric, subacrocentric, or submetacentric X chromosome is the longest element in the set (Fig. 1a).

The localization of rDNA in *Isophya*, resolved by FISH and its activity analyzed by silver impregnation, are shown Figs 1–5 and summarized in Table 1. FISH revealed two (rarely one) to five clusters of rDNA (Fig.

TABLE 1. Species of *Isophya*: Data on where the species used in this study were collected, comparison of the distribution of rDNA on chromosomes and variation in the size of hybridization signal between homologous chromosomes.

rDNA group	Species and morphological species group	Localities where collected	Geographical coordinates	rDNA-FISH signal
I	**#*I. pavelii*** Brunner von Wattenwyl, 1882 (= *I. rammei* Peshev, 1981 – synonym after Chobanov 2009a; as *I. rammei* in Warchałowska-Śliwa et al., 2008) *rectipennis* group (= *pavelii* Warchałowska-Śliwa et al., 2008)	Bulgaria, Strandzha Mts, 29.v.2006	41°57′ 27°33′	2, 3/4*, X
	I. rectipennis Brunner von Wattenwyl, 1882 *rectipennis* group (= *pavelii* in Warchałowska-Śliwa et al., 2008)	Bulgaria, East Stara Planina Mts, 17.vi.2006	42°43′ 26°22′	3/4, 5
	I. speciosa (Frivaldsky, 1865) *schneideri* group	Bulgaria I. Rhodope Mts, 23.vi.2008	41°44′ 25°58′	1/2, 3/4
		II. Foothills of Stara Planina Mts (Predbalkan Range), 29.v.2006	43°05′ 25°40′	1/2, 3/4
	I. modestior Brunner von Wattenwyl, 1882 *costata* group	Serbia, Novi Sad vicinity, 10.vi.2007	45°11′ 19°49′	1/2, 3/4*
	I. pienensis Maran, 1954 *kraussii* group	Poland, Bieszczady Mts, 10.vi.2007	49°06′ 22°39′	1/2 low activity, 3/4
	I. kraussii Brunner von Wattenwyl, 1878 *kraussii* group	Germany, Bavaria, vi.2009	49°30′ 11°31′	2, 3/4
II	***I. gulae*** Peshev, 1981 *pyrenaea* group	Bulgaria, Tundzha Range, Elhovo, 23.vi.2008	42°11′ 26°33′	2/3, 12/13
	I. obtusa Brunner von Wattenwyl, 1882 *pyrenaea* group	Bulgaria, Central Stara Planina Mts, vi.2007	42°45′ 24°54′	2/3, 12/13
	I. altaica Bey-Bienko, 1926 *pyrenaea* group	Russia, Altai Mts, 6.vii.2007	50°37′ 56°62′	3, 12/13
	I. camptoxypha (Fieber, 1854) or ***I. posthumoidalis*** Bazyluk, 1971 *pyrenaea* group	Poland, Tatra Mts, 5.vii.2007	49°18′ 19°57′	3/4, 11/12
	#I. bureschi Peshev, 1981 *modesta* group	Bulgaria, #I. Rila Mts, Jundula, 9.vi.2008	42°03′ 23°51′	3/4, 11/12
		II. Sashtinska Sredna Gora Mt, 25.vi.2008	42°32′ 24°07′	11/12
		III. North Pirin Mts, Gotse Delchev lodge, 14.vi.2006	41°47′ 23°33′	3/4**, 11/12
		IV. Plana Mt., 18.vii.2010	42°29′ 23°23′	11/12 or 3/4, 11/12
	I. andreevae Peshev, 1981 *modesta* group	Bulgaria, Struma valley, 20.iv.2006	41°47′ 23°09′	3/4, 10/11
	I. miksici Peshev, 1985 (as *I. plevnensis* in Warchałowska-Śliwa et al., 2008) *modesta* group	Bulgaria I. West Stara Planina Mts, 24.vi.2006	43°10′ 23°29′	2/3, 12/13
		II. Danubian Plane, 24.vi.2006	43°27′ 24°14′	2/3, 12/13
	I. plevnensis Peshev, 1985 (as *I. pravdini pravdini*; in Warchałowska-Śliwa et al., 2008) *modesta* group	Bulgaria, Central Stara Planina Mts, 12.vi.2007	42°47' 24°55'	2/3, 12/13
	#I. longicaudata adamovici Peshev, 1985 (as *I. pravdini adamovici* in Warchałowska-Śliwa et al., 2008) *modesta* group	Bulgaria, East Stara Planina Mts, 17.vi.2006	42°43′ 26°22′	3/4, 12/13*
	I. longicaudata longicaudata Ramme 1951 (as *I. modesta longicaudata* in Warchałowska-Śliwa et al., 2008) *modesta* group	Bulgaria, North Black Sea coast, 21.vi.2006 Bulgaria, Dobrudzha, 16.vi.2010	43°23′ 28°28′ 43°47′ 27°35′	3/4, 12/13 3/4, 12/13
	#I. rhodopensis / I. leonorae *modesta* group	Bulgaria #I. West Rhodope Mts, near lake Shiroka Polana, 19.vi.2008	41°44′ 24°09′	3/4, 12/13
III	***I. tosevski*** Pavicevic, 1983 *modesta* group	Macedonia, near Doiran lake, 5.–6.v.2005	41°15′ 22°43′	3/4, 5, 6, 7/8, 12/13
	I. leonorae Kaltenbach, 1965 (as *I. kisi* in Warchałowska-Śliwa et al., 2008) *modesta* group	Bulgaria, Alibotush Mt, 9.viii.2006	41°23′ 23°36′	2/3, 3/4, 6/7, 8, 12/13
	I. petkovi Peshev, 1959 *modesta* group	Bulgaria I. East Rhodope Mts, Murgovo, 15.vi.2006	41°43′ 25°28′	3/4, 5/6, 7/8, 12/13; 1 medium-sized with low activity
		II. East Rhodope Mts, Gluhite Kamani, 30.v.2006, 23.viii.2008	41°44′ 25°58′	3/4, 5/6, 7/8, 12/13; 1 medium-sized with low activity
	#I. rhodopensis Ramme 1951 *modesta* group	Bulgaria I. Rhodope Mts, Trigrad, 14.vi.2006	41°37′ 24°22′	3/4, 5/6, 12/13**
		#II. Rodopi Mts, Smolyan, 15.vi.2006, 21.vi.2008	41°36′ 24°40′	3/4, 5, 6**, 8/9, 12/13 or 3/4, 5, 8/9, 12/13
	#I. rhodopensis / I. leonorae *modesta* group	II. West Rhodope Mts, Trigrad-Zhrebovo, 20.vi.2008 III. Rodopi Mts, Stoykite village, 20.vi.2008	41°34′ 24°24′ 41°39′ 24°38′	3/4*, 5*, 6/7*, 12/13* 3/4, 5/6, 12/13*

* high or low activity and ** present or absent; # one or two females were examined; a slash between two numbers indicates imprecise identification of the pair of chromosomes (bivalent).

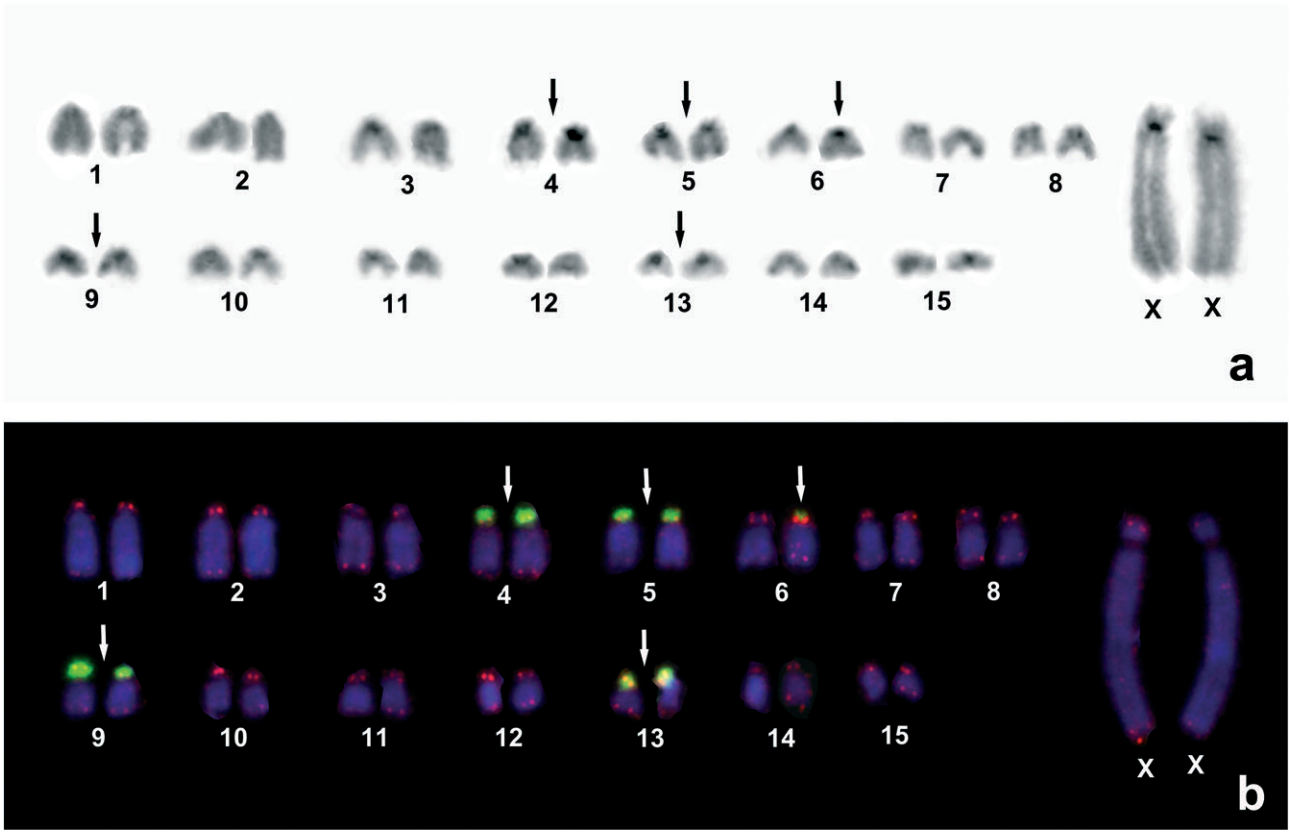

Fig. 1. C-banding staining (a) and FISH (b), using both 18S rDNA (green) and telomeric DNA (red) probes, of the karyotype of *Isophya rhodopensis* (Smoljan population). Arrows indicate (a) thick C-bands and (b) rDNA sites near centromeric regions of the acrocentric chromosomes. C-heterochromatin (a) and both the TTAGG and rDNA signals (b) on M_6 varied between two homologous chromosomes. X – sex chromosome. Bar = 10 μm.

1b), located on the autosomes and sporadically on the X chromosome. The clusters were observed at meiosis from diakinesis to metaphase I on bivalents and at mitosis on the spermatogonial or oogonial metaphase chromosomes. Hybridization with rDNA probes produced an identically positioned signal at the oogonial mitotic metaphase and on the spermatogonial mitotic and meiotic chromosomes. According to the differences in number and location of 18S rDNA signals, three groups are specified (Table 1) as follows.

Group I. In six species the rDNA clusters were evident on two long bivalents at diplotene/diakinesis and four autosomes at mitotic metaphase (Fig. 2a–d). They are located near the centromeric regions of the autosomes (probably 1st or 2nd – 1/2 and 3rd or 4th – 3/4) of *I. pavelii* (Fig. 2a), *I. speciosa* (both populations), *I. modestior* (Fig. 2b), *I. pienensis* and *I. kraussii*, and distal to the centromere on 3/4 and 5th in *I. rectipennis* (Fig. 2c, d). Additionally, a low intensity rDNA-FISH signal near the centromeric region of the sex chromosome was observed in *I. pavelii* (Fig. 2a).

Group II. Eleven species carried rDNA on a pair of long (probably 3/4) and a pair of short chromosomes (probably 11, 12 or 13) always near the centromeric regions (Fig. 3a, b). Intra-specific variation in this pattern was found in *I. bureschi*. Individuals collected from two populations (Rila Mts and Pirin Mts) revealed signals on two autosomal pairs – long and short (L and S) autosomes, whereas one population (Sastinska Sredna Gora Mt.) showed a hybridization signal only on one pair of short chromosomes (Fig. 3d, e). In addition, only one out of four individuals collected on Plana Mt. (between the two mentioned regions) possessed extra rDNA sequences on the long bivalent.

Group III. In this group FISH revealed rDNA only near the centromeric region on three or four bivalents, differing in size, of both populations of *I. petkovi* (Fig. 4a) and on five bivalents of *I. leonorae* and *I. tosevski* (Fig. 4b). Analysis of *I. rhodopensis* and individuals determined as *I. rhodopensis/leonorae* collected from different populations showed variation in the position of 18S rDNA on three to five pairs of chromosomes (Fig. 1b).

In some species comparisons, the rDNA-carrying chromosomes differed in the size of the hybridization signal on different autosomes. For example, in *I. camptoxypha* the rDNA hybridization signal varied between autosomes, the long pair had two discrete clusters whereas the short pair only one signal (Fig. 5a). These differences suggest chromosomal rearrangements. Sometimes the rDNA cluster was on the shortest bivalent (Fig. 4a). The rDNA signal on the long chromosome of *I. pavelii* and *I. modestior*, or short one of *I. longicaudata adamovici*, also varied in size (Table 1 – see the chromosome number marked with an asterisk). In *I. rhodopensis* (some popula-

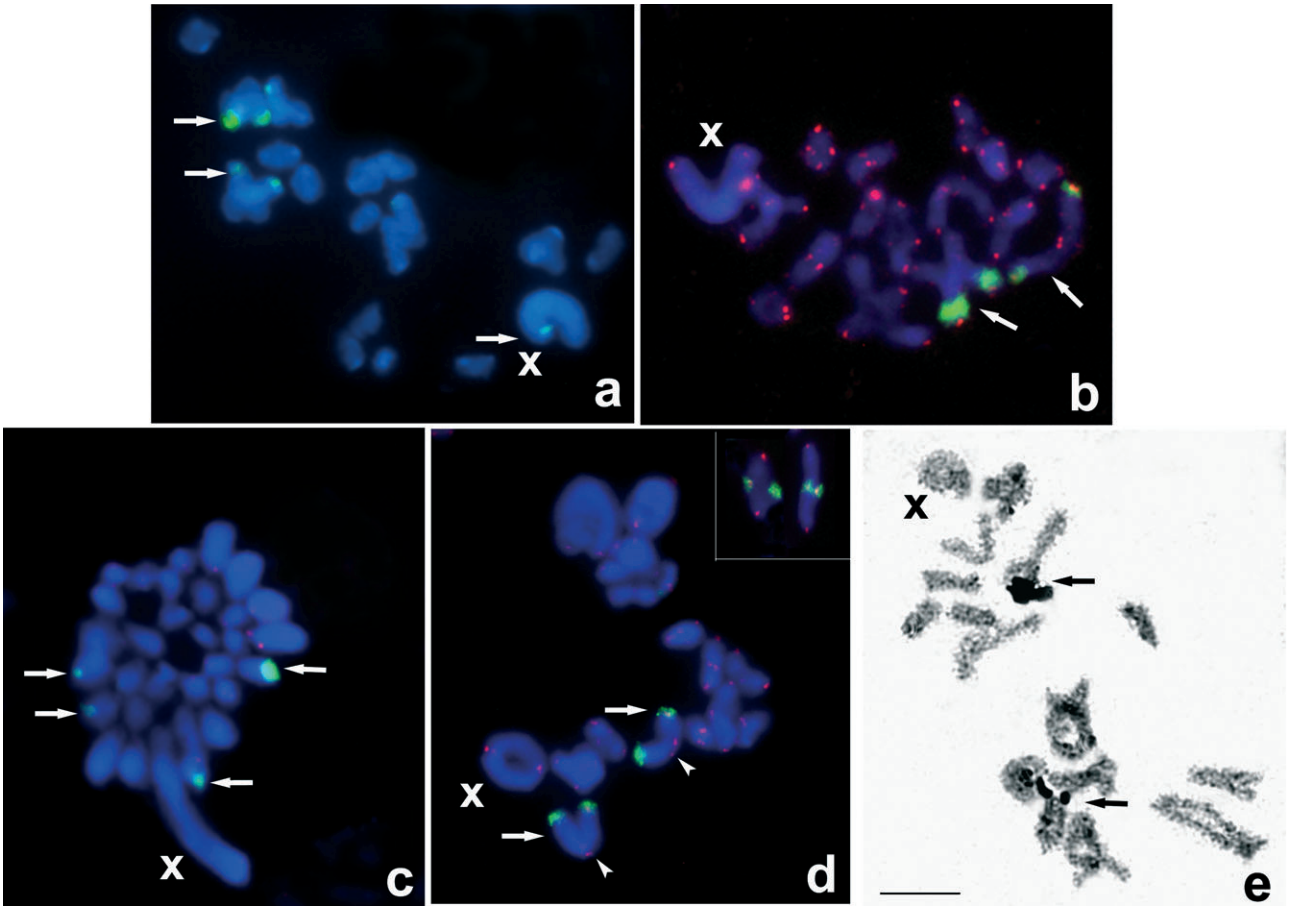

Fig. 2. Diplotene (a) in *I. pavelii* with rDNA signals and (b) in *I. modestior* FISH, using both the 18S rDNA (green) and telomeric DNA (red) probes. Arrows indicate (a, b) rDNA sites in centromeric regions on two long bivalents (probably $L_{1/2}$, $L_{3/4}$) and (a) a low intensity site on the X chromosome. Spermatogonial mitotic metaphase (c) and (d) diplotene in *I. rectipennis* from the Sliven population using both the 18S rDNA and telomeric probes; arrows indicate rDNA clusters on probably L_4 and M_5 bivalents distal to the centromere (arrowheads), with (d) terminal or (in the right corner) interstitial chiasmata; (e) silver-stained diplotene with two active NORs (arrows). TTAGG signals in (c) and (d) are weak. Bar = 10 µm.

tions) and *I. bureschi*, the rDNA-FISH signal was not observed on one of the homologous chromosomes (Figs 1b, 5b) (Table 1 – marked with two asterisks).

The present analysis of the locations of active NORs was not always congruent with the results of Warchałowska-Śliwa et al. (2008). These differences probably result from the lower number of cells analyzed by Warchałowska-Śliwa et al. (2008), leading to an imprecise determination of the size of the NOR bearing chromosome and/or inability to detect the very small Ag-NORs on some chromosomes. In the present study, both the NOR activity and rDNA-FISH pattern were always recorded on meiotic bivalents at prophase I of the same individuals of each species. In most of the species analyzed, FISH signals were coincident with the active NORs visualized by $AgNO_3$ staining (Table 1 and Figs 2e, 3c, 4c). However, a lack of full congruency between the location of rDNA and NOR activity was recorded for the X chromosome of *I. pavelii*, the 1/2 autosome of *I. pienensis*, the 12/13 of *I. tosevski* and in some individuals of *I. rhodopensis/leonorae* (the population from Rodopi Mts, Trigrad-Zhrebevo).

FISH with the $(TTAGG)_n$ probe (tDNA-FISH) was used on the spermatogonial/oogonial mitoses and spermatocyte nuclei at different stages of meiosis. In all *Isophya* species the FISH signals were detected at the distal ends of most autosomes. Differences in the intensity of hybridization signals were detected among most of the autosomes of each species and on the X chromosome of some species (Fig. 5c–f). Generally, the tDNA-FISH signals on chromosomes of *I. altaica, I. plevnensis* or *I. rectipennis* (Fig. 2c, d) were weaker than on those of *I. gulae*, *I. leonorae*, *I. modestior* and *I. camptoxypha* (Figs 2b, 3b). tDNA-FISH signals were recorded on the centromeric regions of subacro/submetacentric X chromosomes. The telomeric FISH signals on this chromosome differed in most of the species.

DISCUSSION

The present study focused on the evolution of the karyotype in *Isophya* by mapping the location of rRNA coding genes on chromosomes using FISH and using silver staining ($AgNO_3$) to evaluate the activity of rDNA clusters. These markers were used in an attempt to

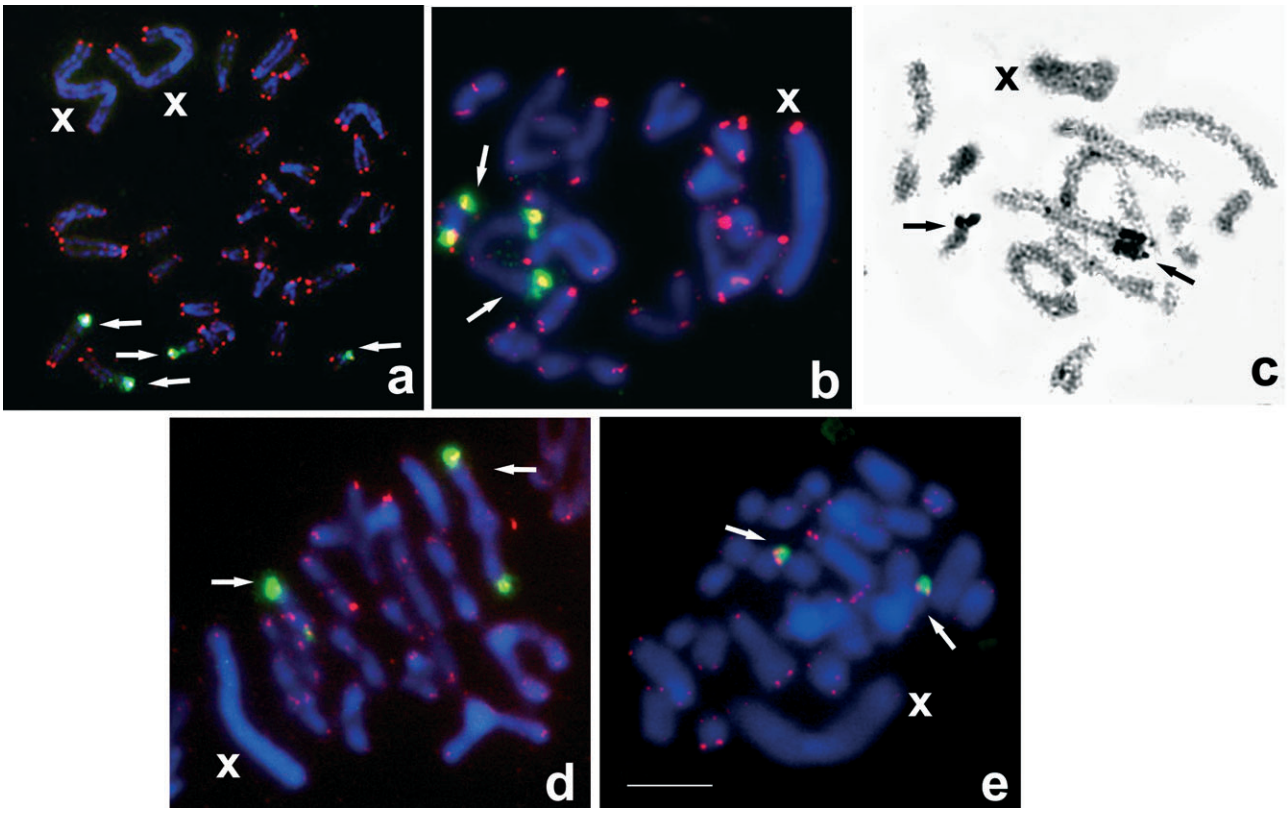

Fig. 3. FISH using both the 18S rDNA (green) and telomeric DNA (red) probes (a, b, d, e). (a) Mitotic metaphase of a female of *I. altaica* and (b) diakinesis in *I. camptoxypha* with rDNA signals near the centromeric regions (arrows) on one long (3/4) and one short pair of chromosomes (11/13); (c) silver-stained diplotene in *I. camptoxypha* with two active NORs (arrows). (d) Diplotene and (e) spermatogonial mitotic metaphase in *I. bureschi* with intra-specific polymorphism; rDNA cluster on (d) two bivalents (population Rila Mts) and (e) only on one short chromosome (population from Sashtinska Sredna Gora Mt.). TTAGG signals in (e) are weak. Bar = 10 μm.

improve the characterisation of the chromosomes and compare the cytogenetic features of this genus.

rDNA-FISH patterns vary among the *Isophya* species in terms of the number of signals and sizes of chromosomes bearing the 18S rDNA cluster. Quantitative analysis of data (Table 1) showed that the number of autosomes (within the haploid genome) bearing rDNA clusters were two (in 15 species), exceptionally one (only in two populations of *I. bureschi*) and rarely three to five (6 species). Comparative analysis of the location of rDNA among individuals from two or more geographical populations of the same species demonstrated the same FISH signals in *I. speciosa*, *I. miksici* and *I. petkovi*. On the other hand, the number of 18S rDNA sites detected in populations and/or individuals of *I. bureschi*, *I. rhodopensis* and *I. rhodopensis/leonorae* varied from one to two in *I. bureschi* or three to five and two to five in the Rhodopean specimens of *I. rhodopensis* and *I. rhodopensis/leonorae*, respectively.

With respect to other Tettigoniidae so far analyzed, the chromosome complement of *Isophya* shows an unusually high number of active NORs. For example in most Tettigoniinae (see Warchałowska-Śliwa et al., 2005) and Saginae (Warchałowska-Śliwa et al., 2009) the active NOR with rDNA loci is located only on a single bivalent. In the African species *Lunidia viridis* (Phaneropteridae) a cluster of rDNA repeats corresponding to an active NOR occur on a single large bivalent (Hemp et al., 2010). The increased number of autosomes bearing NORs and/or the varied location of these regions on the chromosomes of some *Isophya* species should be considered as a derived state caused by unknown chromosome rearrangements. The inter-specific and intra-specific variation in the chromosomal location of rDNA (analyzed using FISH) and NOR activity (using Ag-NOR staining) could be explained by different mechanisms: either structural chromosomal rearrangements such as translocations or inversions, ectopic re-combinations (e.g. Nguyen et al., 2010), or transposition of rDNA repeats to new locations (see Cabrero & Camacho, 2008). In most species of *Isophya* the rDNA loci were located near the centromeric region, with the exception of *I. rectipennis* in which 18S rDNA sites were distally located. Most probably, this species exemplifies a small rearrangement in two long chromosomes resulting from a paracentric inversion that changed the position of the rDNA loci. In some species with rDNA loci situated on long or short autosomes, the size of the positive FISH fluorescent signals varied between homologous arms (e.g. Figs 3d, 5b). The latter suggests the existence of polymorphism in the number of rDNA transcriptional units. Rearrangements such as translocations between homologous and non-homologous chromo-

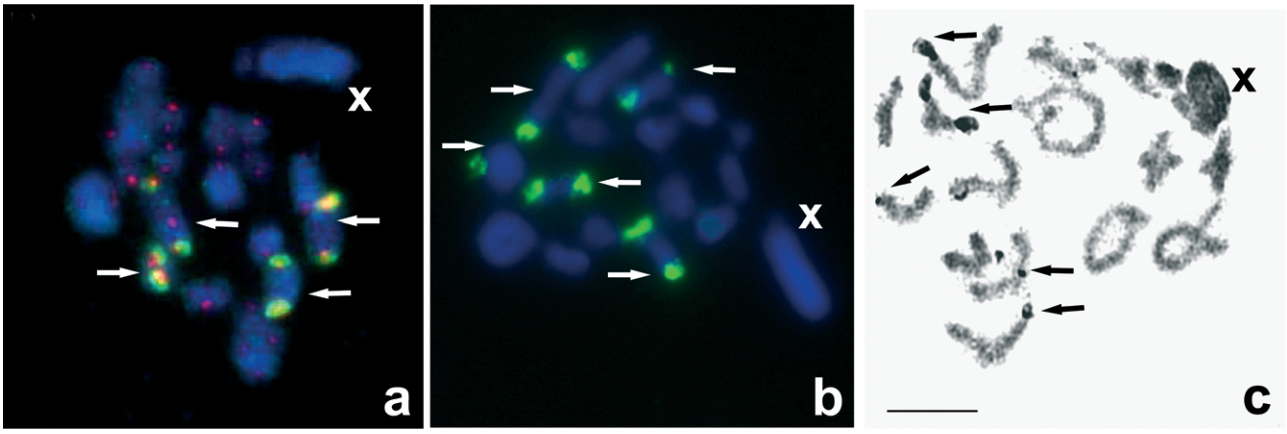

Fig. 4. rDNA-FISH signals (green; arrows) at diakinesis on (a) four bivalents of *I. petkovi* that differ in size and (b) five bivalents of *I. leonorae* near the centromeric region; (c) silver-stained diplotene in *I. leonorae* with five active NORs (arrows). Bar = 10 μm.

somes or duplication of loci by unequal crossing-over probably resulting in different numbers of copies of homologous chromosome, could be implicated by the heteromorphic FISH signal (e.g. Boroń et al., 2006).

The rDNA was frequently on a long chromosome (3/4) in all *Isophya* species and this is probably a plesiomorphic state for these taxa. On the other hand, a short chromosome (probably 11/13) was usually the characteristic location in species belonging to the second and third groups (Table 1). Only *I. pavelii* showed an extra inactive cluster of rDNA near the centromeric region of the X chromosome. It is more likely that this cluster is a result of rDNA spread than a remnant of a NOR lost in other species due to fast divergence of the X chromosome. Although, there are differences in the rates of differentiation between sex chromosomes and the autosomes after hybridization between closely related grasshoppers (Gosálvez et al., 1997; Bridle et al., 2002).

In most orthopterans studied so far, the active NORs are usually located in the constitutive heterochromatin (e.g. Cabrero & Camacho, 1986; Marchi & Pili, 1994; López-León et al., 1999; Warchałowska-Śliwa et al., 2005). In accordance with this pattern, the rDNA clusters in *Isophya* are located in the same chromosome regions in which mostly thick, heterochromatic C-positive bands are present (Fig. 1a, b; see also Warchałowska-Śliwa et al., 2008), probably composed of multiple repetitive DNA sequences. Silver staining was used to evaluate the activity of rDNA clusters. However, it is known, that the conventional silver staining method is sometimes unspecific. It is generally used to reveal only active major ribosomal genes, but in some case, it also detects genomic features other than NORs, thus producing false positive results (Dobigny et al., 2004). The pattern of rDNA hybridization has advantages over classical cytogenetic methods; it is highly specific and moreover, may contribute valuable additional information on homologies between chromosomal segments.

In *Isophya* the number and chromosomal locations of NORs detected in the first meiocytes indicate substantial, although incomplete coincidence between FISH and $AgNO_3$ results. Thus, not all rDNA clusters detected by

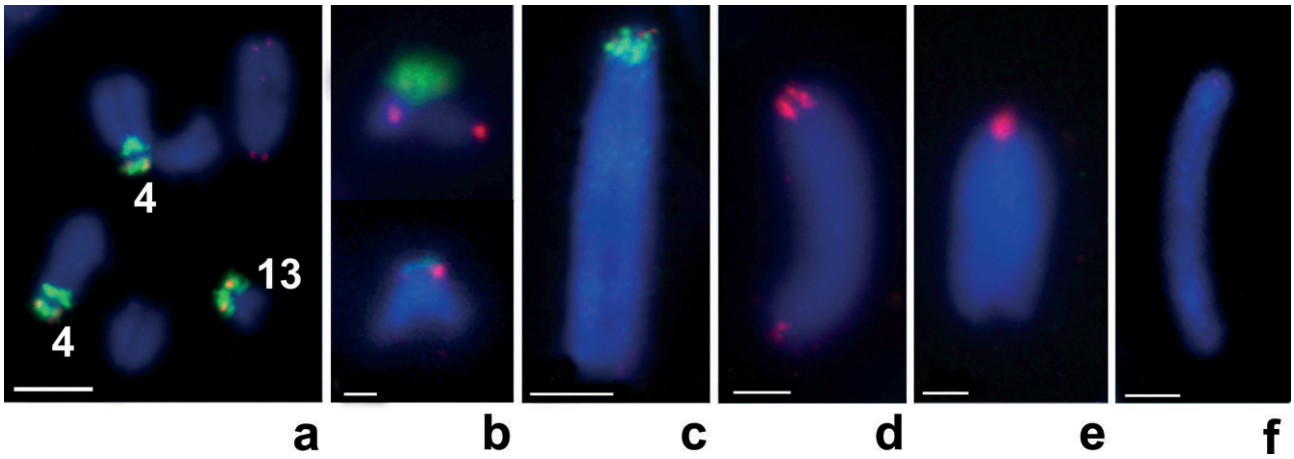

Fig. 5. Differences in intensity of the hybridization signals on *Isophya* chromosomes after FISH with 18S rDNA (green) and telomeric DNA probes (red). Hybridization areas varied in size (a) between autosomes in *I. camptoxypha* and (b) homologous chromosomes in *I. bureschi*; c–f – the X chromosome of the male; (c) FISH using both the 18S rDNA (green) and telomeric DNA (red) probes in *I. pavelii*; differences in the intensity of the tDNA signals (d) in *I. camptoxypha*, (e) *I. andreevae* and (f) *I. toshevski*. Bars = 1 μm (a–d, f) and 2 μm (e).

FISH are active during meiosis (i.e. form nucleoli). This is probably connected with inactive rDNA loci on the X chromosome of *I. pavelii*, on one of the long autosomes of *I. pienensis* and in one *I. bureschi* individual. Similar to our results, the combined use of FISH and silver impregnation methods reveals the existence of inactive rRNA genes in some species of grasshoppers (e.g. López-León et al., 1999; Cabrero & Camacho, 2008). So, as shown by silver impregnation in *Isophya* species not all rDNA clusters detected by FISH are active during meiosis and the present analysis of the location of active NORs is not completely congruent with the results of Warchałowska-Śliwa et al. (2008). Also the fact that the individual NOR-bearing chromosomes could not be identified weakens the usage of rDNA/NOR as a marker for determining phylogenetic relationships between *Isophya* species. The significance of rDNA clusters when chromosome homologies are not identified in evolutionary studies are critically discussed by Dobigny et al. (2004).

The terminal locations of hybridization signals in *Isophya* showed that the telomeres are composed of $(TTAGG)_n$ repeats, as in other orthopterans (e.g. López-Fernández et al., 2004; Warchałowska-Śliwa et al., 2009; Hemp et al., 2010). Differences in the intensity of the hybridization signal detected among some autosomes and X chromosomes between species may be related to the presence of a different number of repeats of ribosomal sequences. However, for a precise comparison, an analysis with measurement of signal intensity is necessary. On the other hand, the lack of tDNA-FISH signals could probably be due to a low copy of telomeric repeats, insufficient for detection by the standard FISH technique (Cabrero et al., 2007; Warchałowska-Śliwa et al., 2009).

A high level of genetic polymorphism was detected within the genus *Isophya* by using RAPD (Random Amplification of Polymorphism DNA) markers (Grzywacz & Warchałowska-Śliwa, 2008) and four DNA fragments – two mitochondrial (cytochrome oxidase II – COII, cytochrome b – cytb) and two nuclear sequences (internal transcribed spacer I and II – ITS1, ITS2) (Grzywacz-Gibała et al., 2010). These results show some conflict with the systematics of the group based on morphological and bioacoustic data and represent only the first step in the reconstruction of the phylogeny of *Isophya*.

The first mapping of the chromosomal location of 18S ribosomal genes revealed variability among the *Isophya* species and partly corresponded to the morphological groupings proposed in earlier studies (e.g. Warchałowska-Śliwa et al., 2008; Chobanov 2009a, b). Six species from the first group (Table 1) based on the localization of the 18S rDNA (Table 1) showed a FISH signal only on two long autosomes (1/2, 3/4). These species belong to four morphological groups (after Warchałowska-Śliwa et al., 2008): (1) the most primitive ones belong to the *Isophya rectipennis* group (*I. rectipennis*, *I. pavelii*, etc.), (2) the highly morpho-acoustically specialized *schneideri* (*I. speciosa*) group, and (3/4) the *costata* / *kraussii* relatives. Species with rDNA loci on one long (3/4) and one short (11–13) chromosome belong to the *pyrenaea* group (*I. gulae*, *I. obtusa*, *I. altaica* and *I. camptoxypha*) and partly to the *modesta* group (after Warchałowska-Śliwa et al., 2008). Furthermore, in the remaining species of the *modesta* group, the number of autosomes bearing rDNA loci ranged from two to five. The variation observed between and within taxa of the *modesta* group at the genetic level, including the number of rDNA sites (present paper) and variation in DNA fragments (COII, cytb, ITS1, ITS2) (Grzywacz-Gibała et al., 2010), is higher than the morpho-behavioural diversity known in this group. The great similarities between taxa of this group and observed intra-specific variation suggest the recent evolution of the *I. modesta* relatives (Grzywacz-Gibała et al., 2010). The genetic differences (based both on rDNA markers and DNA fragments) between populations of *I. bureschi*, *I. rhodopensis* and *I. rhodopensis/leonorae* suggest ongoing divergence in isolated or remote populations and probable hybridization in the zones of secondary contact.

In conclusion, the present study has focused on the cytogenetic mapping of rRNA coding genes and telomeric sequences in the genus *Isophya*. The variation in the number and situation of 18S rDNA loci and/or Ag-NORs in the stable karyotype of this genus are probably important species-group specific phylogenetic markers. Analysis of these markers reveals that the evolutionary dynamics of rDNA in this genus is remarkably high, which is confirmed by changes in the chromosomes bearing signals at inter- and intra-specific levels. Future karyotype analyses using FISH with rRNA gene sequences and telomeric sequences should be carried out on other related genera of Barbitistini to gain a more comprehensive view of chromosome evolution in the Phaneropterinae.

ACKNOWLEDGEMENTS. This research was supported by the Ministry of Science and Higher Education of Poland, grant N N303 611738 to E. Warchałowska-Śliwa & B. Grzywacz. T. Karamysheva was partially supported by the Mianowski Fund – Foundation for the Promotion of Science (2009), Poland, and by the Russian Foundation for Basic Research, grant 08-04-00167. D.P. Chobanov was supported by grants DO 02-259/08 of the Ministry of Education, Youth and Science of Bulgaria, BG051PO001-3.3.04/41 (European Social Fund through the Ministry of Education, Youth and Science of Bulgaria) and the Orthopterists' Society Small Grants Program.

REFERENCES

Bey-Bienko G.Ya. 1954: *Phaneropterinae. Fauna of the USSR. Orthoptera 2(2)*. Zoological Institute of the Academy of Sciences, Moscow-Leningrad, 385 pp.

Boroń A., Porycka K., Ito D., Abe S. & Kirtiklis L. 2009: Comparative molecular cytogenetic analysis of three Leuciscus species (Pisces, Cyprinidae) using chromosome banding and FISH with rDNA. *Genetica* **135**: 199–207.

Bridle J.R., De la Torre J., Bella J.L., Butlin R.K. & Gósalvez J. 2002: Low levels of chromosomal differentiation between the grasshoppers Chorthippus brunneus and Chorthippus jacobsi (Orthoptera; Acrididae) in Northern Spain. *Genetica* **114**: 121–127.

CABRERO J. & CAMACHO J.P.M. 1986: Cytogenetic studies in gomphocerinae grasshoppers. II. Chromosomal location of active Nucleolar Organizing Region. *Can. J. Genet. Cytol*. **28**: 540–544.

CABRERO J. & CAMACHO J.P.M. 2008: Location and expression of ribosomal RNA genes in grasshoppers: Abundance of silent and cryptic loci. *Chromosome Res.* **16**: 595–607.

CABRERO J., GÓMEZ R. & CAMACHO J.P.M. 2007: No evidence of the presence of Wolbachia in the parthenogenetic insect Saga pedo. *Proc. XI. Eur. Soc. Evol. Biol. Congress, Uppsala, Sweden, 20–25 August 2007*. Uppsala, p. 277.

CHOBANOV D.P. 2009a: New records and a new synonym of Orthoptera from Bulgaria. *Articulata* **24**: 79–108.

CHOBANOV D.P. 2009b: Phylogeny and systematics of the Isophya modesta group (Phaneropteridae) based on morphology and bioacoustics. *Metaleptea* **28**: 20–27.

DOBIGNY G., DUCROZ J.-F., ROBINSON T.J & VOLOBOUEV V. 2004: Cytogenetic and cladistics. *Syst. Biol*. **53**: 470–484.

EADES D.C., OTTE D., CIGLIANO M.M. & BRAUN H. 2011: *Orthoptera Species File Online. Version 2.0/4.0.* [09.ii.2011]. http://Orthoptera.SpeciesFile.org

GOSÁLVEZ J., MASON P.L. & LÓPEZ-FERNÁNDEZ C. 1997: Differentiation of individuals, populations and species of Orthoptera: the past, present and future of chromosome markers. In Gangwere S.J., Muralirangan M.C. & Mularirangan M. (eds): *The Bionomics of Grasshoppers, Katydids and their Kin*. CAB International, Wallingford, pp. 355–383.

GRZYWACZ B. & WARCHAŁOWSKA-ŚLIWA E. 2008: Polymorphism of the genus Isophya (Orthoptera, Phaneropteridae, Barbitistinae) revealed by RAPD. *Folia Biol. (Kraków)* **56**: 153–157.

GRZYWACZ-GIBAŁA B., CHOBANOV D.P. & WARCHAŁOWSKA-ŚLIWA E. 2010: Preliminary phylogenetic analysis of the genus Isophya (Orthoptera, Phaneropteridae) based on molecular data. *Zootaxa* **2621**: 27–44.

HARZ K. 1969: *Die Orthopteran Europas I. Series Entomologica*. W. Junk, The Hague, 750 pp.

HEMP C., HELLER K.-G., WARCHAŁOWSKA-ŚLIWA E. & HEMP A. 2010: Ecology, bioacoustics and chromosome analysis of a new genus and species of African Phaneropterinae (Orthoptera: Tettigoniidae). *Org. Divers. Evol.* **10**: 215–226.

HELLER K.-G. 1988: *Bioakustik der europäischen Laubheuschrecken*. Margraf, Weikersheim, 360 pp.

HELLER K.-G. 2006: Song evolution and speciation in bushcrickets. In Drosopoulos S. & Claridge M.F. (eds): *Insect Sounds and Communication*. CRC Press, Boca Raton, pp. 137–152.

HELLER K.-G., ORCI K.M., GREIN G. & INGRISCH S. 2004: The Isophya species of Central and Western Europe (Orthoptera: Tettigonioidea: Phaneropteridae). *Tijdsch. Entomol*. **147**: 237–258.

LICHTER P., CREMER T., TANG C.J., WATKINS P.C., MANUELIDIS L.& WARD D.C. 1988: Rapid detection of human chromosome 21 aberration by in situ hybridisation. *Proc. Natl. Acad. Sci. USA* **85**: 9664–9668.

LÓPEZ-FERNÁNDEZ C., PRADILLO E., ZABAL-AGUIRRE M., FERNÁNDEZ J.L., GARCIA DE LA VEGA C. & GISÁLVEZ J. 2004: Telomeric and interstitial telomeric-like DNA sequence in Orthoptera genomes. *Genome* **47**: 757–763.

LÓPEZ-LEÓN M.D., CABRERO J. & CAMACHO J.P.M. 1999: Usually high amount of inactive ribosomal DNA in the grasshopper Stauroderus scalaris. *Chromosome Res*. **7**: 83–88.

LORETO V., CABRERO J., CAMACHO J.P.M. & SOUZA M.J. 2008: Comparative analysis of rDNA location in five Neotropical gomphocerine grasshopper species. *Genetica* **132**: 95–101.

MARCHI A. & PILI E. 1994: Ribosomal RNA genes in mosquitoes: localization by fluorescence in situ hybridization (FISH). *Heredity* **72**: 599–605.

NGUYEN P., SAHARA K., YOSHIDO A. & MAREC F. 2010: Evolutionary dynamics of rDNA clusters on chromosomes of moths and butterflies (Lepidoptera). *Genetica* **138**: 343–354.

SAHARA K., MAREC F. & TRAUT W. 1999: TTAGG telomeric repeats in chromosomes of some insects and other arthropods. *Chromosome Res.* **7**: 449–460.

SEVGILI H. 2003: A new species of bushcricket (Orthoptera: Tettigoniidae) of the Palearctic genus Isophya (Phaneropterinae) from Turkey. *Entomol. News* **114**: 129–137.

SEVGILI H. & HELLER K.-G. 2003: A new species of the genus Isophya Brunner von Wattenwyl (Orthoptera: Tetigoniidae: Phaneropterinae). *Tijdsch. Entomol*. **146**: 39–44.

SEVGILI H., ÇIPLAK B., HELLER K.-G. & DEMISROY A. 2006: Morphology, bioacoustics and phylogeography of the Isophya major group (Orthoptera: Tettigoniidae: Phaneropterinae): A species complex occurring in Anatolia and Cyprus. *Eur. J. Entomol.* **103**: 657–671.

SUMNER S.G. 1972: A simple technique for demonstrating centromere heterochromatin. *Exp. Cell Res.* **75**: 304–306.

WARCHAŁOWSKA-ŚLIWA E. 1998: Karyotype characteristics of katydid orthopterans (Ensifera, Tettigoniidae) and remarks on their evolution at different taxonomic levels. *Folia Biol. (Kraków)* **46**: 143–176.

WARCHAŁOWSKA-ŚLIWA E. & BUGROV A.G. 1998: Karyotypes and C-banding patterns of some Phaneropterinae katydids (Orthoptera, Tettigonioidea) with special attention to a post-reductional division of the neo-X and the neo-Y sex chromosomes in Isophya hemiptera. *Folia Biol. (Kraków)* **46**: 47–54.

WARCHAŁOWSKA-ŚLIWA E. & MARYAŃSKA-NADACHOWSKA A. 1992: Karyotypes, C-bands, NORs location in spermatogenesis of Isophya brevipennis Brunner (Orthoptera: Phaneropteridae). *Caryologia* **45**: 83–89.

WARCHAŁOWSKA-ŚLIWA E., HELLER K.-G. & MARYAŃSKA-NADACHOWSKA A. 2005: Cytogenetic variability of European Tettigoniinae (Orthoptera, Tettigoniidae): Karyotypes, C-and Ag-NOR-banding. *Folia Biol. (Kraków)* **53**: 161–171.

WARCHAŁOWSKA-ŚLIWA E., CHOBANOV D.P., GRZYWACZ B. & MARYAŃSKA-NADACHOWSKA A. 2008: Taxonomy of the Genus Isophya (Orthoptera, Phaneropteridae, Barbitistinae): Comparison of karyological and morphological data. *Folia Biol. (Kraków)* **56**: 227–241.

WARCHAŁOWSKA-ŚLIWA E., GRZYWACZ B., MARYAŃSKA-NADACHOWSKA A., KARAMYSHEVA T.V., RUBTSOV N.B. & CHOBANOV D.P. 2009: Chromosomal differentiation among bisexual European species of Saga Charp. (Orthoptera, Tettigoniidae, Saginae) detected by both classical and molecular methods. *Eur. J. Entomol*. **106**: 1–9.

WHITE M.J.D. 1973: *Animal Cytology and Evolution, 3rd ed.* Cambridge Univ. Press, London, 961 pp.

WHITING M.F., CARPENTER J.C., WHEELER Q.D. & WHEELER W. C. 1997: The Strepsiptera problem: phylogeny of the holometabolous insect orders inferred from 18S and 28S ribosomal DNA sequences and morphology. *Syst. Biol.* **46**: 1–68.

ZACARO A.A., PROENÇA S.J.R, LOPEZ-ANDRADE C. & SERRANO A.R.M. 2004: Cytogenetic analysis of Ctenostomini by C-banding and rDNA localization and its relevance to the knowledge of the evolution of tiger beetles (Coleoptera: Cicindelidae). *Genetica* **122**: 261–268.

Received March 15, 2011; revised and accepted June 3, 2011