

## Taxonomy of the Genus *Isophya* (Orthoptera, Phaneropteridae, Barbitistinae): Comparison of Karyological and Morphological Data

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The genus *Isophya* represents one of the largest orthopteran genera with about 45 species occurring in Europe. All over its range the genus includes groups of sibling species, the recognition of which is hampered by their morphological similarity. In part, some species were grouped according to their morphology: the *Isophya straubei*-group, the *I. amplipennis*-group and the *I. major*-group. Nevertheless, many species have not been studied using new methods. This is particularly true for 15 taxa described from the Balkans, more or less resembling *I. modesta*, known mostly from old, unsatisfactorily informative descriptions. Chromosomal analysis of 25 species/subspecies of the genus *Isophya* showed the karyotypic evolution among species. The karyotype of 18 species/subspecies was investigated for the first time. The most remarkable changes in the sex chromosomes were the ancient acrocentric X chromosome inversions in 19 species and an X chromosome/autosome mutual tandem translocation, from which the sex determination system neo-XY originated in *I. hemiptera*. Karyotype differentiation has been less rapid in autosomes than in sex chromosomes, in this case interspecific autosomal differentiation has involved the distribution and quantity of C-heterochromatin as well as the number of NORs. Cytogenetic analysis revealed some changes reflecting the level of genomic organization, and the results suggest some association of related taxa from biogeographic regions.

Key words: Orthoptera, katydids, chromosomes, cytotaxonomy, taxonomy, C-banding, NORs.

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The tettigonioid family Phaneropteridae, sometimes considered as the subfamily Phaneropterinae, includes about 2200 species occurring throughout the world. The subfamily Barbitistinae (HELLER *et al.* 2006 = Odonturinae after GOROCHOV 1995) includes short-winged species of the genera *Poecilimon*, *Isophya*, *Barbitistes*, *Ancistrura*, *Leptophyes*, etc. distributed throughout the West-Palaearctic region.

The genus *Isophya* Brunner von Wattenwyl (1878) represents one of the largest orthopteran genera, comprising about 82 species (91 after EADES *et al.* 2007 <http://Orthoptera.SpeciesFile.org>, but includes some previously synonymized and some Neotropical forms, representing different genera and, most likely, a different subfamily (see e.g. KARABAG 1960; unpublished data), which apparently should be excluded from this classification).

Of the latter, approximately 45 occur in Europe (HELLER *et al.* 1998). The distributional range of *Isophya* covers Central Europe (as far west as the Pyrenees and as far north as Southern Germany and Poland), the Carpathian Basin, the Balkans (as far south as Northeastern Greece), Southern Ukraine, Asia Minor (following the Eastern Mediterranean coast southwards to Israel) and the Caucasus region eastwards to Northwestern Iran and Iraq. Further east one species (*I. altaica* Bey-Bienko) occurs in an isolated area of Northeastern Kazakhstan and the Altai (BEY-BIENKO 1954). The centre of diversity includes the Eastern Balkans, the Caucasus and Asia Minor, the latter is considered to be the generic radiation centre (LA GRECA 1999). Most species of *Isophya* show restricted ranges, often confined to a particular to-

pography (SEVGILI *et al.* 2006), thus the genus exhibits a high rate of endemism (SEVGILI 2003; SEVGILI & HELLER 2003).

Throughout its range the genus includes groups of sibling species, the recognition of which is hampered by their morphological similarity, lack of sclerotized genitalia and simple cercus shape, thus their description and recognition were based mainly on subtle differences (BEY-BIENKO 1954; HELLER *et al.* 2004; SEVGILI *et al.* 2006). Only after application of the bioacoustical approach in characterizing different species (ZHANTIEV & DUBROVIN 1977; HELLER 1988; INGRISCH 1991; etc.) some revisions of local fauna or species groups have been done and particular distinctive features in morphology were found (HELLER *et al.* 2004, SEVGILI *et al.* 2006). Nonetheless, the status of many species remains unresolved, moreover the recognition of females is most often possible only after relating to a conspecific male. In addition to the bioacoustics and external morphology, cytogenetic analysis can be helpful in revealing species-specific features in *Isophya* and can provide for the refinement of the systematics of this genus and for studies on speciation in this group.

Over 60 species and subspecies of nine genera of Barbitistinae have been studied cytotaxonomically (MESSINA *et al.* 1975; WARCHAŁOWSKA-ŚLIWA 1984, 1998; WARCHAŁOWSKA-ŚLIWA & BUGROV 1998; WARCHAŁOWSKA-ŚLIWA & HELLER 1998; WARCHAŁOWSKA-ŚLIWA & MARYAŃSKA-NADACHOWSKA 1992; WARCHAŁOWSKA-ŚLIWA *et al.* 1987, 1992, 1995, 1996, 2000). The majority of these species have a karyotype consisting of  $2n=31$  acrocentric chromosomes in the male with the X0/XX mechanism of sex determination. In four species of Phaneropteridae from the Palearctic region, the neo-XY/neo-XX system of sex determination has been described (ALICATA *et al.* 1974; MESSINA 1981; MESSINA *et al.* 1975; WARCHAŁOWSKA-ŚLIWA & BUGROV 1998). The karyotype of the genus *Isophya* has been studied to date only in five species from Central Europe, the Caucasus and the Altai (WARCHAŁOWSKA-ŚLIWA & BUGROV 1998; WARCHAŁOWSKA-ŚLIWA & HELLER 1998; WARCHAŁOWSKA-ŚLIWA & MARYAŃSKA-NADACHOWSKA 1992; WARCHAŁOWSKA-ŚLIWA *et al.* 1996). In these species, stability of the chromosome number ( $2n=31$  in the male) and sex determination was observed. Only one species, *Isophya hemiptera*, from the Northern Caucasus shows a neo-X/neo-Y system, which has been demonstrated to have originated from X-autosome fusion through meiotic and mitotic analysis (WARCHAŁOWSKA-ŚLIWA & BUGROV 1998).

In an attempt to contribute to a better understanding of the taxonomy of the genus *Isophya*, this paper provides additional information on the

genome organization including karyotype/cytotaxonomy structure. The goal of this work is to characterize karyotype diversity in 25 species/subspecies, using two differential staining methods: revealing constitutive heterochromatin (C-banding patterns) and nucleolar organizer regions (NORs) to determine mechanisms of chromosomal differentiation during the evolution of these species.

## Material and Methods

Adult males, females and nymphs were collected in Bulgaria, Russia, Poland, Macedonia, and Serbia (see Table 1 for taxonomic data and specific localities). Voucher specimens are deposited in the Chobanov's collection in the Institute of Zoology, Bulgarian Academy of Sciences (Sofia) and in the Institute of Systematics and Evolution of Animals, Polish Academy of Sciences (Kraków).

For morphological comparison only adult specimens were examined, which, if nymphs were collected, were obtained by breeding in captivity until imaginal moulting.

Testes and ovaries were excised, incubated in a hypotonic solution (0.9% sodium citrate), and then fixed in ethanol:acetic acid (3:1). The fixed material was squashed in 45% acetic acid. Coverslips were removed by the dry ice procedure and then preparations were air-dried. The C-banding examination was carried out according to SUMNER (1972) with slight modification. The silver staining method for NORs was performed as previously reported (WARCHAŁOWSKA-ŚLIWA & MARYAŃSKA-NADACHOWSKA 1992). Chromosomal morphology was determined according to the nomenclature proposed by LEVAN *et al.* 1964. The fixed material is deposited in the Institute of Systematics and Evolution of Animals, Polish Academy of Sciences (Kraków).

## Results

### Cytogenetics

A comparison of chromosomes of 25 species determined the similarities and differences between some karyotypic elements. Karyotypes of 18 species/subspecies were investigated for the first time. The karyotype analyses were derived from mitotic spermatogonial/oogonial metaphases and meiotic bivalents. The chromosome complements ( $2n$ ) of all species (excluding *I. hemiptera* with  $2n=30$  and a neo-XY sex mechanism; WARCHAŁOWSKA-ŚLIWA & BUGROV 1998) consist of 31 chromosomes in the male and 32 in the female with the X0/XX sex determination system. All autosomes were acrocentric, consisting of four

Table 1

C-heterochromatin, NORs and B chromosomes in species of the genus *Isophya*; X chromosome: telocentric with thin paracentromeric C-band (TYPE 1A); acrocentric with thick C-band (TYPE 1B); subacrocentric with thin C-bands (TYPE 2A); subacrocentric with thick double C-band (TYPE 2B); submetacentric with thin C-band (TYPE 3), (e.g. 10/11 impossible precision identification pair of chromosomes and bivalent numbers)

No	Species and species group	Collection localities	Numbers of individuals	X type (in mitosis) information additional to X type is included in this column	C-bands in autosome 1, 2, ... numbers of chromosomes ; * intraspecific variation of C-heterochromatin (thick/thin or present/absent)	Position of NOR 1, 2, ... numbers of bivalents	B chromosome	References
1	<i>I. modestior</i> Brunner von Wattenwyl, 1882 <i>I. costata</i> group	Serbia, Novi Sad vicinity, Frushka Gora Mt, ~300 m, 10.06.2007	2 males	1A	1, 3, 9 thick paracentromeric, the remaining autosomes thin, 4* and 10/11* paracentromeric, 9 thick telomeric	3/4, one small bivalent	no	
2	<i>I. rammei</i> Peshev, 1981 <i>I. pavelii</i> group	Bulgaria, 1. Strandzha Mts, Tsarnogorovo locality S of Malko Turnovo, 350 m, 29.05.2006 (coll. as nymphs, moulted in captivity) 2. Strandzha Mts, Tsarnogorovo locality S of Malko Turnovo, 350 m, 29.06.2007	2 males, 3 females  3 males	1B interstitial C-band near telomeric region	Thick paracentromeric in most chromosomes, without telomeric	3/4, one small bivalent  no	no	
3	<i>I. rectipennis</i> Brunner von Wattenwyl, 1882 <i>I. pavelii</i> group	Bulgaria, 1. Strandzha Range near Veselie village, 06. 1994 2. E Stara Planina, Karandila locality above Sliven, 1000 m, 17.06.2006 (coll. as nymphs, moulted in captivity) 3. N Black Sea coast, Sofia University Botanical Garden, 20.06.2006	2 males 4 males  1 male, 1 female	1A interstitial and telomeric C-bands	All paracentromeric thin, 2/3, 4-6*, 1S telomeric 3/4 and two small autosomes* with thick paracentromeric *, the remaining thin, 2/3, 4-6, 1S telomeric	no  Two medium sized bivalents  no	no	WARCHAŁOWSKA-SŁIWA <i>et al.</i> 1996 and present paper
4	<i>I. kalishevskii</i> Adelung, 1907 <i>I. schneideri</i> group	Russia, Caucasus, Krasnodar Kray, Chugush Mts, 07. 1997	4 males	1B interstitial C-band near paracentromeric region	3, 6, 10* thick paracentromeric, the remaining autosomes thin, 2, 3, 4, 6, 7/8 telomeric, 4 * interstitial (only in one male)	no		

Table 1 cont.

5	<b><i>I. schneideri</i></b> Brunner von Wattenwyl, 1882 <i>I. schneideri</i> group	Dagestan, near Guniv village, 08.1987	3 males	1B thick paracentromeric, interstitial C-band	Thick paracentromeric in all chromosomes	Two small sized bivalents	no	WARCHALOWSKA-ŚLIWA <i>et al.</i> 1995
6	<b><i>I. speciosa</i></b> (Frivaldsky, 1865) <i>I. schneideri</i> group	Bulgaria, 1. C Stara Planina Mts, Pleven Lodge, 23.05.2005 (coll. as nymphs, moulted in captivity) 2. Predbalkan Range, Veliko Turnovo, 300-350 m, 29.05.2006	1 male  2 females	2A telomeric C-bands in both arms	1, 3, 12 thick paracentromeric, the remaining autosomes thin, without telomeric	12	no	
7	<b><i>I. hospodar</i></b> (Saussure, 1898) <i>I. straubei</i> group	Bulgaria, E Rodopi Mts, Dolni Glavanak village, 350 m, 25.04.2005 (coll. as nymphs, moulted in captivity)	1 male	2A very short euchromatic arm	3 pairs of autosomes thick paracentromeric, the remaining autosomes thin	no	no	
8	<b><i>I. tosevski</i></b> Pavicevic, 1983 <i>I. modesta</i> group	Macedonia, Doiran Lake, 5-6.05.2005 (coll. as nymphs, moulted in captivity)	3 males	2A telomeric C-bands in both arms	2/3 (centromeric+interstitial), 15 thick paracentromeric, the remaining thin	2/3, 6/7, one small bivalent	no	
9	<b><i>I. andreevae</i></b> Peshev, 1981 <i>I. modesta</i> group	Bulgaria, 1. Rila Mts, Eleshnitsa Lodge, 850 m, 13.06.06 (coll. as nymphs, moulted in captivity) 2. Strouma valley, Kresna Gorge, 350-450 m, 19-20.04.2006 (coll. as nymphs, moulted in captivity)	4 males, 2 females  3 males, 1 female  1 male 1 female	2B telomeric C-band in short arm	5*, 9* thick paracentromeric, the remaining thin, 3 interstitial near paracentromeric, 6, 7, 9, 10, 12, 13, 14 telomeric	9/10  9/10	Type 1	
10	<b><i>I. miksici</i></b> Peshev, 1985 <i>I. modesta</i> group	Bulgaria, W Stara Planina Mts, Vracanska Mt., near Pushevitsa Lodge, 1000-1200 m, 24.06.2006	4 males, 1 female	2A	13 thick paracentromeric and 3* (one male no. 2), 7/8* (one male no. 3), the remaining thin without telomeric	3/4, 13	Type 2	
11	<b><i>I. plevnensis</i></b> Peshev, 1985 <i>I. modesta</i> group	Bulgaria, C Danubian Plane, Iskar, 24.06.2006	2 males	2B	3/4, 13/14 thick paracentromeric, the remaining thin, without telomeric	3/4, 4/5	no	
12	<b><i>I. pravdini pravdini</i></b> Peshev, 1985 <i>I. modesta</i> group	Bulgaria, 1. C Stara Planina Mts, Apriltsi-Vidima quarter, 1100 m, 23.05.2005 (coll. as nymphs, moulted in captivity) 2. C Stara Planina Mts, Apriltsi-Ostrets quarter, 1000-1100 m, 12.06.2007 (coll. as nymphs, moulted in captivity).	3 males  1 male	2A distal C-band in short arm	3/4, 6/7, 13/14 thick paracentromeric, the remaining thin, 5/6, 7-10 telomeric  3/4, in three small pairs thick paracentromeric	3/4, 13/14  3/4, 13/14	no	

Table 1 cont.

13	<b><i>I. pravdini adamovici</i></b> Peshev, 1985 <i>I. modesta</i> group	Bulgaria, E Stara Planina Mts, Karandila locality above Sliven, 1000 m, 17.06.2006 (coll. and fixed as nymphs)	2 males, 1 female	2A/B interstitial C-band near paracentro- meric region	4, 5, 10, 13 thick paracentromeric, the remaining thin, 4 telomeric	3/4, 13/14	Type 2	
14	<b><i>I. modesta longicaudata</i></b> Ramme, 1951 <i>I. modesta</i> group	Bulgaria, 1. N Black Sea coast, Sofia University Bot- anical Garden, 20.06.2006 2. N Black Sea coast, Bolata locality N of Kaliakra cape, 21.06.2006	1 male,  3 males	2B interstitial C-band near paracentro- meric region	3/4, 5, 8/9, 13 thick paracentromeric, the remaining thin, 3/4 telomeric	one small bivalent  one small bivalent	no	
15	<b><i>I. rhodopensis</i></b> Ramme, 1951 <i>I. modesta</i> group; <i>rhodopensis</i>	Bulgaria, 1. Trigrad 14.06.06  2. Smoljan 15.06.06	1 male  4 males, 1 female	2A interstitial C-band near paracentro- meric region and telomeric C-band in long arm	3/4, 13/14 and 13/15* thick paracentromeric  3/4* thick, 4/3*, 13/15* paracentromeric. Telomeric in all autosomes excluding 1 in both localities	3-4 NORs probably 3/4, 6/7, two small bivalents	no	
16	<b><i>I. petkovi</i></b> Peshev, 1959 <i>I. modesta</i> group; <i>rhodopensis</i>	Bulgaria, 1. W Rodopi Mts, Asenovgrad - Bachkovo village, 350-400 m, 9-10.06.2001 2. W Rodopi Mts, Asenovgrad - Bachkovo village, 350-400 m, 9-10.06.2001 3. E Rodopi Mts, Gorata Ridge, Gluhite Kamuni locality N of Dubovets village, ~500 m, 30.05.2006 4. E Rodopi Mts, Perpericon historic site near Murgovo village, 15.06.06	2 males, 1 female no.7,11  1 male no.10  1 male no.10  1 male	2B very short euchroma- tic arm  2A  2B	3/4, 8,9, 12, 13 thick paracentromeric, the remaining thin, 5, 6, 7, 8, and two small pairs telomeric 3/4 thick paracentromeric, the remaining thin, 5, 6, 8-13, 15 telomeric  All paracentromeric thick, without telomeric	3/4, 9/10, 13/15   3/4, 9/10, 13/15  3/4, 9/10, 13/15	no	
17	<b><i>I. kisi</i></b> Peshev, 1981 <i>I. modesta</i> group; <i>rhodopensis</i>	Bulgaria, 1. Alibotush Mt., Livade locality, 1700 m, 9.08.2006  2. N Pirin Mts., Ban- sko - Gotse Delchev Lodge, 1000-1300 m, 14.06.2006	4 males  1 male	2B very short euchroma- tic arm	3/4, 6, and three small pairs * (thin/thick) paracentromeric, the remaining thin, 5, 9, 10 telomeric  3, 4, 6 –13 thick paracentromeric, the remaining thin, 5, 9, 10 telomeric	3/4, 5/6, 12/13	Type 2	

Table 1 cont.

18	<i>I. bureschi</i> Peshev, 1959 <i>I. modesta</i> group	Bulgaria, 1. N Pirin Mts., Bansko - Gotse Delchev Lodge, 1000-1300 m, 14.06.2006 (coll. and fixed as nymphs) 2. N Pirin Mts, above Bansko, 1150 m, 8.08.2006	1 male, 1 female  2 males	2A	9/10 thick paracentromeric, the remaining thin, 3* thick paracentromeric (2 males from Piryn and 1 male, 1 female from Goce) and 8/9* paracentromeric (in 1 male from Goce), 3 telomeric	9/11	no	
19	<i>I. gulae</i> Peshev, 1981 <i>I. pyrenaee</i> group	Bulgaria, Tundzha Range, Dolna Topchiya reserve near Elhovo, 13.06.2005	1 male	2A very short euchromatic arm	2/3 thick paracentromeric, the remaining thin	no	no	
20	<i>I. obtusa</i> Brunner von Wattenwyl, 1882 <i>I. pyrenaee</i> group	Bulgaria, 1. C Stara Planina Mts, Pleven Lodge, 1600 m, 23.-24.05.2005 (coll. as nymphs, moulted in captivity) 2. same locality, 25.05.2007 (coll. as nymphs, moulted in captivity, fixed 2.-12.06.2007)	2 males  4 males and 1 male, 1 female	2A very thin interstitial C-band near telomeric region	2/3 thick (interst/subacro-thick) paracentromeric, the remaining thin, 11/12* paracentromeric (in two males), 13/14 telomeric 3* paracentromeric (in two males), 10/12* (in three males)	no	3/4, one small bivalent	Type 2
21	<i>I. altaica</i> Bey-Bienko, 1926 <i>I. pyrenaee</i> group	Russia, Altai Mts, Cherge, 06/07 1990, 1995	3 males	3	3 thick paracentromeric, the remaining thin	3, one small bivalent	no	WARCHAŁOWSKA-ŚLIWA <i>et al.</i> 1996
22	<i>I. camptoxypha</i> (Fieber, 1854) (syn. <i>I. brevipennis</i> Brunner von Wattenwyl, 1878) <sup>1</sup>	Poland, Ojców National Park, 1987-1989	25 males and 5 females	2A	3 thick paracentromeric, the remaining thin, telomeric in most of autosomes 1, 2, 4 5, 6, 7, 9, 10, 11, 12	3, one small bivalent	no	WARCHAŁOWSKA-ŚLIWA & MARYAŃSKA-NADACHOWSKA, 1992
	<i>I. camptoxypha</i> (Fieber, 1854) or <i>I. posthumoidalis</i> Bazyluk, 1971 <sup>2</sup>	Poland Tatra Mt., Jaworzynka Valley, 07.2007	1 male and 2 females	3 telomeric C-band in short arm	3, 10/11 thick paracentromeric, the remaining thin	3, one small bivalent		present paper
23	<i>I. kraussii</i> Brunner von Wattenwyl, 1878 <i>I. kraussii</i> group	Germany, Bavaria, near Erlangen, 1997	11 males	3 subtelo/ /submeta- centric	All paracentromeric thin, 10-13 telomeric	no	no	WARCHAŁOWSKA-ŚLIWA & HELLER 1998
24	<i>I. pienensis</i> Maran, 1954 <i>I. kraussii</i> group	Poland, Bieszczady, Połonina Caryńska, 24.07.2007	5 males	2A telomeric C-bands in both arms	3 thick paracentromeric, the remaining thin, 1 telomeric	3/4	no	
25	<i>I. hemiptera</i> Bey-Bienko, 1954 <i>I. hemiptera</i> group	Russia, Caucasus, Krasnodar Kray, Chugush Mts, 07. 1997	3 males	Neo-XY	1-6, 9, 11, 13, 14, neoY, thick, the rest thin paracentromeric, telomeric neoY	no	no	WARCHAŁOWSKA-ŚLIWA & BUGROV 1998

<sup>1</sup> Probably this species erroneously determined;<sup>2</sup> Only one male is determined.

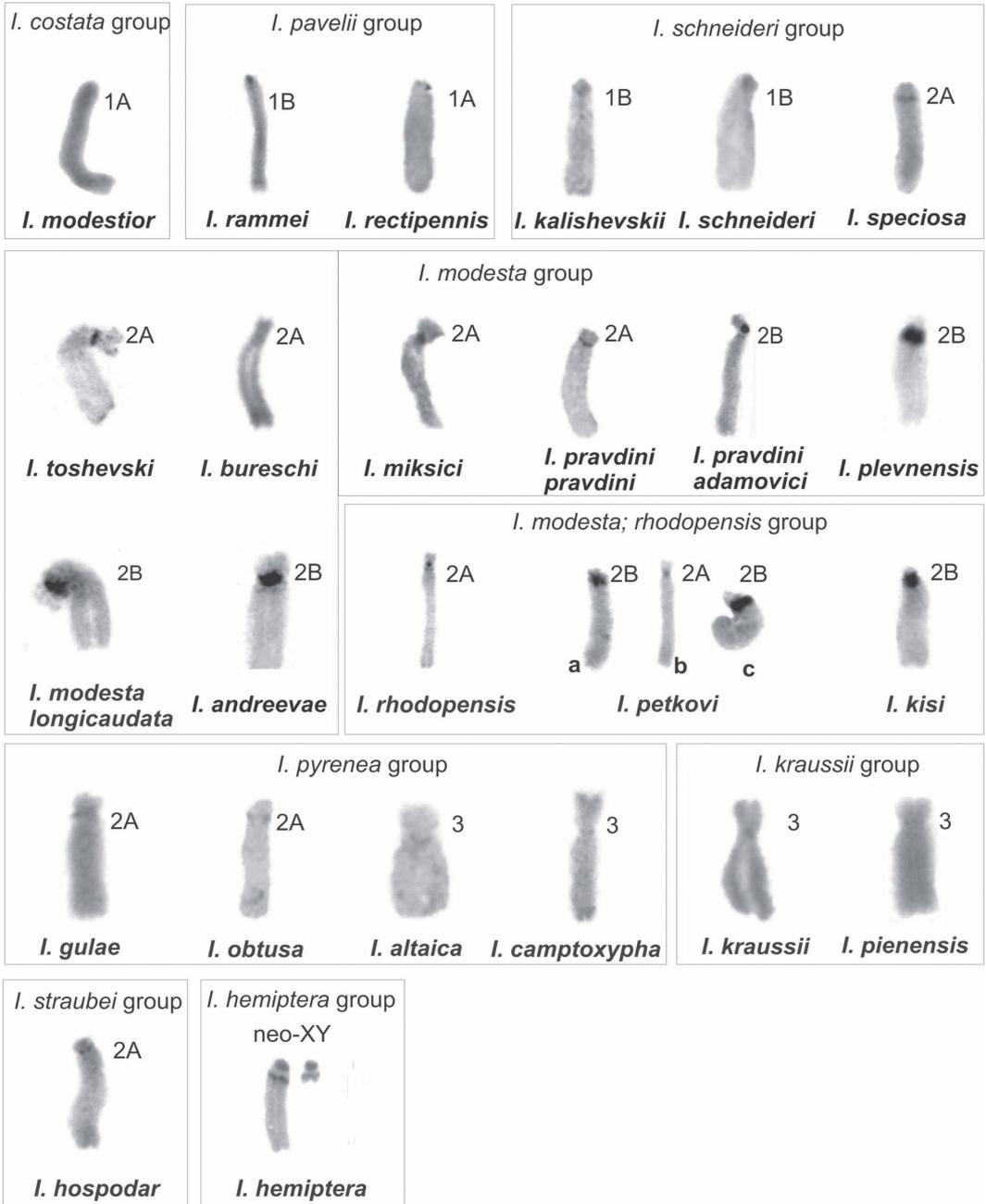


Fig. 1. Morphotypes of the X chromosome in species groups of *Isophya*; according to differences in the amount of heterochromatin, two subtypes of X chromosomes - with thin (Type 1A and 2A and 3) or thick (Type 1B, 2B) paracentromeric C-bands are observed. Three populations of *I. petkovi* (a, b, c) differ in the proportion of short in relation to long arms and in the amount heterochromatin of paracentromeric region of the submetacentric X. *Isophya hemiptera* from Northern Caucasus shows a neo-X and neo-Y system.

long and eleven medium and short pairs gradually decreasing in size, the X chromosome was the largest element. However, in some species, beside an acrocentric, a subacrocentric or submetacentric X chromosome occurs, changing the number of chromosomal arms (Fundamental numbers = FN) in some species from 31 to 32 in the male. The morphologies of the X chromosome (type 1 – acrocentric; type 2 – subacrocentric and type 3 – submetacentric), C-banding patterns and NOR locations in all chromosomes are reported in Table

1, including karyotypical features described earlier. Figures 1-4 give some examples of the results. In some cases, the paracentromeric C-bands are restricted to the centromere region (thin C-bands), whereas in other cases, C-bands occupy the regions next to the centromeres (thick=double C-bands).

A comparison of the morphology and constitutive heterochromatin of the X chromosome in the studied species is shown in Figure 1. All morphotypes of X are divided into three types differing in

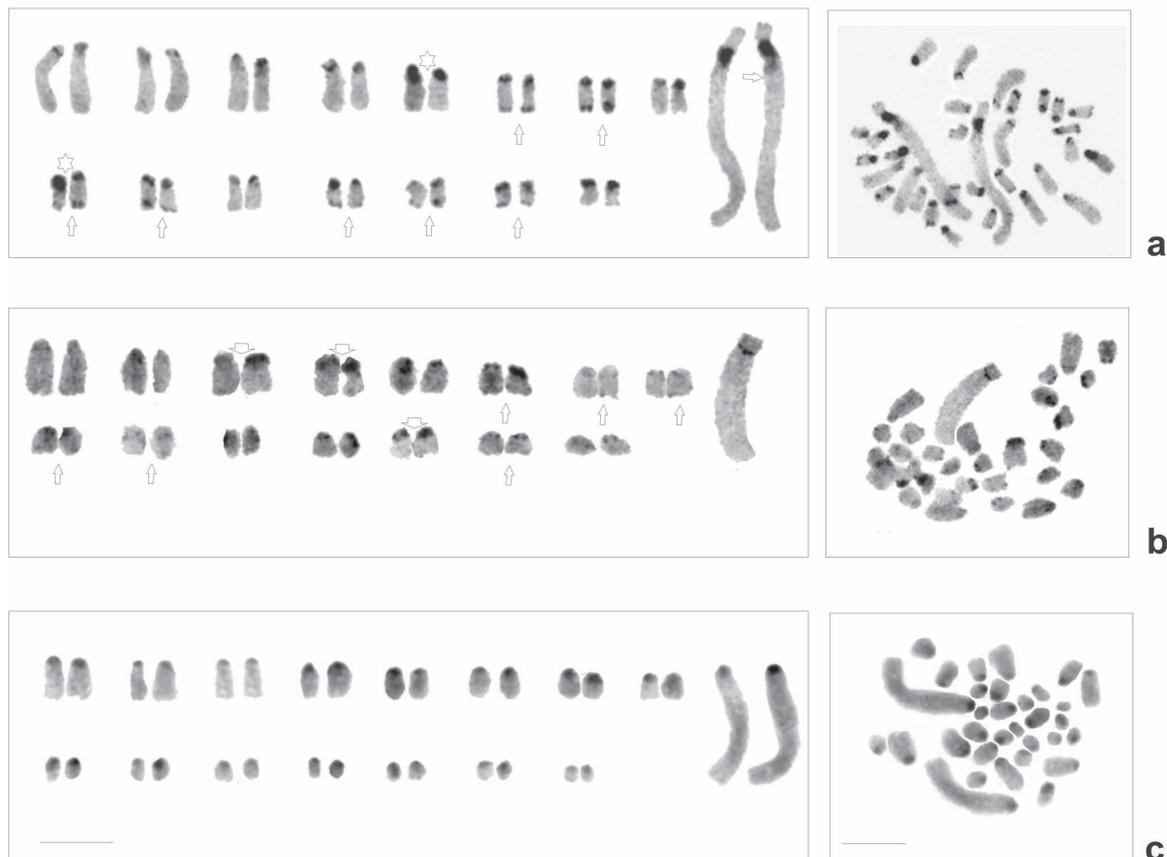


Fig. 2. C-banded karyotypes and mitotic metaphase: *I. andreevae* (a), *I. pravdini pravdini* (b) and *I. rammei* (c); arrows indicate telomeric C-bands in autosomes and an interstitial one located near the paracentromeric region of the X chromosome in *I. andreevae*; stars indicate variation in two pairs of homologous chromosomes (presence/absence or more/less heterochromatin); indicate thick bands in some pairs of chromosomes. Bars = 10  $\mu\text{m}$ .

morphology. According to differences in the amount of heterochromatin, two subtypes of X chromosomes – with thin (Type 1A, 2A and 3) or thick (Type 1B, 2B) paracentromeric C-bands, were specified. It is worth mentioning that the three populations of *I. petkovi* differ in the proportion of short in relation to long arms and in the amount of heterochromatin of the paracentromeric regions of the submetacentric X chromosomes (Fig. 1). A thin interstitial C-band is located near the paracentromeric region of the X chromosome in *I. kalishevski*, *I. rhodopensis*, and *I. andreevae* (Fig. 1), in the middle part of X in *I. rectipennis* and near the telomeric region in *I. rammei* and *I. bureschi*. If a thin telomeric C-band was present, it was located in one arm in Type 1 of the X chromosome in *I. rectipennis*, in Type 2 in *I. speciosa*, *I. rhodopensis*, *I. toshevski* and *I. pravdini adamovici*, as well as in Type 3 in both *I. camptoxypha* (or *posthumoidals*) and *I. pienensis*. Thin telomeric C-bands in both arms were observed only in *I. speciosa*, *I. toshevski* and *I. pienensis*.

Little regularity was observed in C-heterochromatin content and distribution in autosomes (Table 1). Thin paracentromeric C-bands

appeared in most autosomes, however, in some species there were thick bands in one, two, three, and very rarely in four pairs of chromosomes of different sizes (Fig. 2b). Nonetheless, thick C-bands occur in most autosomes in *I. rammei* (Fig. 2c), *I. schneideri* and *I. hemiptera*. It is worth mentioning that the C-heterochromatin in some species shows polymorphism in quantity. In *I. modestior*, *I. kalishevski*, *I. kisi*, *I. bureschi*, *I. obtusa*, *I. miksici*, *I. rhodopensis*, and *I. andreevae* (Fig. 2a), variation in one, two or three pairs of homologous chromosomes occurs (presence/absence or more/less heterochromatin (indicated in Table 1 by asterisks). Telomeric C-bands were present in different pairs of autosomes. However, in three species, *I. camptoxypha*, *I. andreevae* (Fig. 2a) and *I. rhodopensis*, these bands were detected in most autosomes.

The total numbers of active NORs bearing bivalents among species are indicated in Table 1. For other species NORs are not shown owing to the absence of diplotenes in the studied cells. Chromosomes of different species may differ in number of NORs. All the NORs were localized in paracentromeric regions, probably in the secondary constriction regions under thick C-banding. In *I. speciosa*,

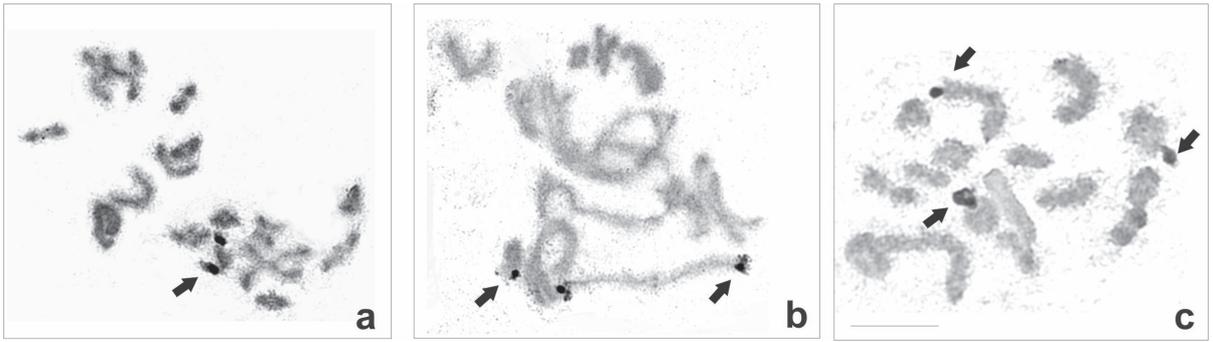


Fig. 3. NORs are localized in paracentromeric regions during diplotene to diakinesis (arrows); in *I. andreevae*, single active NOR located on the small bivalent (a); in *I. pravdini* two NORs detected on the M3/4 and one small bivalent (b); three active NORs are located in *I. petkovi* (c). Bar = 10  $\mu\text{m}$ .

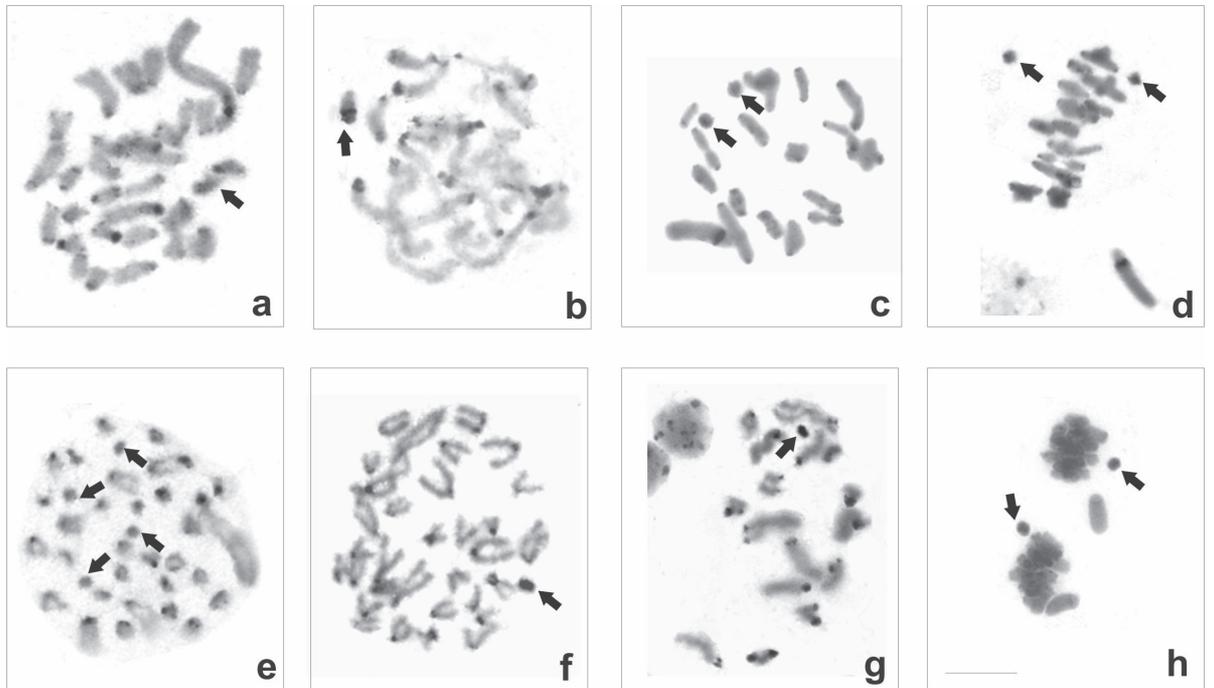


Fig. 4. Two morphotypes of B chromosomes: B1 (a-d) and B2 (e-h). Figs a-d; B1 type in *I. andreevae* shows a large C-band near the centromere, a very short euchromatic region in the short arms and an euchromatic part in the distal region; mitosis (a), diplotene (b), diakinesis (c) and metaphase I/anaphase I (d). B2 is smaller than all autosomes, heterochromatic; (e) B chromosomes in *I. kisi* (mitosis); (f-h) *I. miksici*, one B chromosome in mitosis (f), diakinesis (g) and metaphase I/anaphase I (h). Arrows indicate B chromosomes. Bar = 10  $\mu\text{m}$ .

*I. bureschi*, *I. longicaudata* and *I. andreevae*, a single active NOR located on the small bivalent was observed (Fig. 3a). Eleven species (see Table 1) possess two NORs detected on the M3/4 and one small bivalent (Fig. 3b). Three active NORs were located in *I. kisi*, *I. petkovi*, *I. plevnensis* and *I. toshevski*, whereas *I. rhodopensis* probably possesses four NORs connected with the M3/4, M6/7 and two small bivalents (Fig. 3c).

B chromosomes, which are supernumeraries to the standard chromosome complement, were found in five species of the genus *Isophya* (Table 1). B chromosome variants were divided into two categories, according to their size and C-band content

(B1 and B2). In *I. andreevae* (one male) and *I. obtusa* (three males), the size of the B1 type was similar to that of the small pair of autosomes, was of the acrocentric/subacrocentric type and mitotically and meiotically unstable. This type shows a large C-band near the centromere, a very short euchromatic region in the short arms and an euchromatic part in the distal region (Fig. 4 a-d). The B2 chromosome occurred in *I. kisi* (two males), *I. miksici* (two males) and *I. pravdini adamovici* (three males). It was smaller than all autosomes, heterochromatic, and unstable in both mitotic and meiotic divisions in male (Fig. 4e-h).

## Morphology

Many species of the genus *Isophya* were known only from their descriptions and just recently new data greatly helped in understanding the taxonomic system of this group (ZHANTIEV & DUBROVIN 1977; HELLER 1988; INGRISCH 1991; HELLER *et al.* 2004; SEVGILI *et al.* 2006). In part, some species were grouped according to their morphology (e.g. HELLER 1988: *Isophya straubei*-group, SEVGILI 2003: *Isophya amplipennis*-group; SEVGILI *et al.* 2006: *Isophya major*-group). Nevertheless, many species have not been studied using new methods. This is particularly true for 15 taxa described from the Balkans and more or less resembling *I. modesta*, known mostly from old, uninformative descriptions (BRUNNER VON WATTENWYL 1882; PESHEV 1959a, 1959b; KALTENBACH 1965; PESHEV 1981, 1985; PAVIČEVIĆ 1983).

In the present paper we use the morphology of the studied taxa to outline tentative groups of allied species as a basis for karyological characterization. The morphological characteristics of the species studied is taken from BEY-BIENKO (1954), HELLER *et al.* (2004), etc., but mostly from our own, unpublished data. In some cases earlier data on karyotypes, as well as published or unpublished male calling song structure, were involved in resolving particular problems. We do not concentrate here on species characterization by using morphology or bioacoustics, which will be a part of future studies. The distribution of every taxon we deal with is presented.

### *Isophya pavelii* group

A morphologically primitive group of species with some transition to *Isophya straubei* group.

It includes taxa of middle to large size, with long tegmina. Fastigium verticis slightly wider or narrower than scapus. Pronotal disc not saddle-shaped, ventral edge of hind femora not spinulate or with one spine. Stridulatory file from well developed to partially reduced (in *I. rectipennis*), bearing 50-100 teeth in studied species. Apical part of male cerci gradually curved, attenuated, with long slender spine. Ovipositor short to moderately long. The group includes a few species from the Eastern Balkan Peninsula and Asia Minor. Bioacoustic comparisons point to further divisions in this group.

*I. rammei* Peshev, 1981 – known only from its type locality near Malko Turnovo, Strandzha Mountains in southeastern Bulgaria. New data support its synonymy with *I. pavelii* Brunner von Wattenwyl, 1878.

*I. rectipennis* Brunner von Wattenwyl, 1878 – a narrow distribution from southeastern Romania, through Eastern Bulgaria and European Turkey to northwestern Turkey.

### *Isophya straubei* group

A morphologically primitive group of species of middle to large size with long tegmina. Fastigium verticis wider than scapus. Pronotal disc not or weakly saddle-shaped, ventral edge of hind femora not spinulate. Stridulatory file usually reduced in its apical part (bearing smaller and narrow teeth) or well developed (in *I. straubei straubei*) bearing 60-125 teeth in the studied species. Male cerci stout, gradually curved, apically pointed to a small spine. Ovipositor short to moderately long. Includes a few species from the eastern Balkan Peninsula and Asia Minor.

*I. hospodar* (Saussure, 1898) – distributed in the easternmost part of the Balkan Peninsula, similar to *I. rectipennis* but without penetrating into Asia Minor.

### *Isophya modesta* group

Includes the largest species of the genus. Fastigium verticis wide but narrower than scapus. Pronotal disc long, not saddle-shaped, ventral edge of hind femora spinulate. Tegmina long and wide, stridulatory file well developed with stable width but variable in the number of teeth, counted from 55 to 180 in the studied species. Male cerci stout, with a straight apical part, apically obtuse, bearing a strong spine the length and width of which varies. The ovipositor is moderately to very long for the genus. Includes a large number of taxa described from the Balkans to Central Europe.

*I. modesta longicaudata* Ramme, 1951 – the subspecies has been reported from Dobrudzha (Romania and Bulgaria) and the Bulgarian Black Sea coast. New unpublished data support its specific status (K.-G. Heller, personal communication; own data).

*I. pravdini adamovici* Peshev, 1985 – this taxon is known only from its type locality in eastern Stara Planina Mts (E Bulgaria) above Sliven. New data (unpublished) put it in close relation with *I. modesta longicaudata* Ramme.

*I. pravdini pravdini* Peshev, 1985 – this taxon was known only from its type locality at the northern slope of central Stara Planina Mts above Troyan. We found it in the surroundings of Apriltsi in the same region.

*I. plevnensis* Peshev, 1985 – known from central North Bulgaria (the Central Danubian plane and

the neighboring foothills). Some specimens show similarity to *I. pravdini pravdini*, some – to *I. miksici*.

*I. miksici* Peshev, 1985 – known from the western Stara Planina Mountains in Bulgaria.

*I. andreevae* Peshev, 1981 – the species has a restricted area, confined to the central part of the Strouma Valley (SW Bulgaria) and the neighboring mountain slopes (e.g. Rila, Pirin, Maleshvska, Belassitsa).

*I. tosevski* Pavicevic, 1983 – closely related to *I. andreevae*, it occurs in the neighboring lowland territories of southeastern Macedonia and north-central Greece.

Superspecies *I. rhodopensis*. Includes the next three sibling taxa which show great similarity in morphology, stridulatory file structure and song (unpublished data).

*I. rhodopensis* Ramme, 1951 – the species range is confined to the Rhodope Mountains in Bulgaria (and probably Greece).

*I. petkovi* Peshev, 1959 – new data on the morphology and bioacoustics (unpublished) support the synonymy of this taxon with *I. rhodopensis*.

*I. kisi* Peshev, 1981 – described from Pirin and Alibotush Mts in Southwestern Bulgaria. New data (unpublished) support its synonymy with *I. leonora* Kaltenbach, 1965, known from the mountains of northeastern Greece.

*Isophya bureschi* Peshev, 1959 – known from northwestern Pirin and southern Rila Mountains in southwestern Bulgaria. Morphologically the species is somewhat aside the other species mentioned here. Similarities with *I. stysi* from Central Europe are observed.

#### *Isophya pyrenaea* group

The species of the group are moderately large to large in size. Fastigium verticis is narrower than scapus. Pronotal disc is typically widened in metazona with sinuate side edges. The ventral edge of hind femora is weakly or not spinulate. The male tegmina are slightly to distinctly bulged and darkly coloured. Colouration in males is frequently variegated, females are normally uniformly green. Stridulatory file has 50 – 130 teeth in the studied species. Male cerci stout, gradually curved, apically obtuse (less conspicuous in *I. altaica*, *I. pyrenaea* and some specimens of *I. camptoxypha*), bearing short spine (longer in *I. altaica*). The ovipositor is moderately long. Includes a few species known from high altitudes in the Central Balkans (*I. obtusa*), Western (*I. pyrenaea* (Serville, 1839)) and Central Europe (*I. camptoxypha* (Fieber, 1853), Altai (*I. altaica*) and the lowlands of south-

eastern Bulgaria (*I. gulae*). After careful study this group can be additionally divided.

*I. altaica* Bey-Bienko, 1926 – the range of this species is separated by a huge gap of 2500-3000 km (BEY-BIENKO 1954) from the main range of the genus and even more from the range of its relatives. Thus, the species is a relict from a formerly wider area of distribution.

*I. camptoxypha* (Fieber, 1853) (syn. *I. brevipennis* Brunner von Wattenwyl, 1878 – syn. after Heller et al. 2004) – Carpathian Mountains of Slovakia, Poland, Ukraine and Romania, as well as in isolated mountainous areas of western and southern Hungary, reaching Austria.

*I. obtusa* Brunner von Wattenwyl, 1882 – the species was described from the high parts of the western Stara Planina Mts in Serbia. Since that time it has been found only in the central parts of Stara Planina in Bulgaria.

*I. gulae* Peshev, 1981 – up to the present, known only from its type locality (the region of Elhovo, Tundzha valley) in southeastern Bulgaria.

#### *Isophya costata* group

The species are moderately large to large in size. Fastigium verticis is narrower than scapus and only in *I. costata* is as wide as or slightly wider than the scapus. Pronotal disc long, widened in metazona. The ventral edge of the hind femora may or may not be spinulate. The male tegmina are distinctly widened and short. The stridulatory file in these species usually bears a very high number (up to 280) of teeth, but variable only in *I. modestior* (from 95 to 250) (HELLER et al. 2004). The shape of male cerci varies. Ovipositor is moderately short to long. Includes possibly a large number of species known from the northwestern Balkans and Central and Eastern Europe (territories north of the Black Sea). There seems to be a transition to the next group.

*I. modestior* Brunner von Wattenwyl, 1882 – the range of the species covers the Carpathian Mountains (Southeastern Poland, Slovakia, Eastern Hungary, Romania, Ukraine), western Stara Planina Mountains and the mountainous Kraishite region (southeastern Serbia and northwestern Bulgaria), reaching the Alps (Austria and Italy) and the Dinaric Mountains (southern Serbia and northern Macedonia).

#### *Isophya kraussii* group

Species of moderate size. Fastigium verticis narrower than scapus. Pronotal disc long, widened in metazona. Ventral edge of hind femora not spinu-

late. Male tegmina distinctly shortened. Stridulatory file with a very high number (usually 200-300) of fine teeth. Male cerci moderately stout to moderately slender, apical part gradually curved, more or less pointed. Ovipositor moderately short. A few species from Central Europe and the northwestern – most part of the Balkans are designated to this group but some other eastwardly distributed species (e.g. *I. zubovskii* Bey-Bienko, 1954) need to be studied in order to outline more correctly the two groups, *costata* and *kraussii*.

*I. kraussii* Brunner von Wattenwyl, 1878 – the species has a wide range, covering Central Europe – Germany, Poland, Czech Republic, Slovakia, Hungary, Austria.

*I. pienensis* Maran, 1954 – the species was found in Slovakia, Poland, Romania, and Ukraine.

*Isophya schneideri* (*amplipennis*) group [SEVGILI (2003) included some species into the *Isophya amplipennis*- group, omitting *I. schneideri* and some other species, which we consider as related. Thus, here we use the name *I. schneideri*.]

This is the most morphologically and bioacoustically advanced group. Includes species of small to moderate size. Fastigium verticis much narrower than scapus. Pronotal disc shortened, saddle shaped. Ventral edge of hind femora not spinulate. Male tegmina are distinctly bulged. The stridulatory file is of similar shape and width but with a variable number of teeth between species (70-180) (HELLER 1988; SEVGILI 2003). The male cerci are fine, gradually curved, pointed to a small or bigger spine. Ovipositor is short. A phenomenon typical for the group is melanism. This is the most numerous group with many species described from Asia Minor and the Caucasus region. Two species penetrates into the Balkan region.

*I. schneideri* Brunner von Wattenwyl, 1878 – occurs in the Caucasus region, eastern Turkey and northwestern Iran.

*I. kalishevskii* Adelung, 1907 – western Georgia (Abkhazia) and south-eastern Russia in the territory of the Western Caucasus.

*I. speciosa* (Frivaldszky, 1867) – one of the widely distributed species, found in the central and eastern part of the Balkan peninsula (Bosnia and Herzegovina, Montenegro, Macedonia, Northern Greece, Bulgaria, Eastern Serbia, European Turkey), southern Romania and the western part of Asia Minor.

#### *Isophya hemiptera* group

Species of small to moderate size. Fastigium verticis much narrower than scapus. Pronotal disc very short, saddle shaped. Ventral edge of hind

femora not spinulate. Male tegmina very long for the genus, bulged. Stridulatory file structure is not known. Male cerci stout, straight, at apex curved and attenuated to a pointed tooth. Ovipositor is short. Only one species – *I. hemiptera* Bey-Bienko, 1954, known from the Caucasus region, unique not only in morphology but also in karyotype characteristics (see Results and Discussion).

## Discussion

The aim of this study was to provide an initial step in understanding the evolution of the Palearctic genus *Isophya* on the basis of karyotype with comparison to morphological data.

Comparative cytogenetic studies of more than 60 species and subspecies of Palearctic genera of Barbitistinae showed that the karyotype of the majority of these species consists of  $2n=31$  in the male (all chromosomes are acrocentric) (see review WARCHAŁOWSKA-ŚLIWA 1998). This number is considered as basic/ancestral for tettigoniids. Most of these taxa did not undergo extensive karyotypic changes during speciation and tend to be karyologically conservative at the generic level. In *Isophya*, karyotype evolution has been achieved by reduction of chromosomal number through a change of the X0 sex determination mechanism to neo-XY, involving tandem fusion between autosomes and sex chromosomes only in *I. hemiptera*. The absence of heterochromatinization in the neo-XY sex bivalent suggests a relatively recent origin for the neo-XY system in this species (WARCHAŁOWSKA-ŚLIWA & BUGROV, 1988). Though it has been put in the *I. amplipennis* group by SEVGILI (2003), we separate this species in a monotypic species group – the *I. hemiptera* group, following the unique sex-determination system and some morphological features (cercal structure, long tegmina).

Variation in the number of acrocentric chromosomes among species of *Isophya* involved only the sex chromosome (Table 1). This could be due to the occurrence of a pericentric inversion that converted the original/ancestral acrocentric X chromosome to subacro/submetacentric status. These karyotype characteristics, found in 19 species/subspecies of *Isophya*, are more frequent than those observed in other closely related genera. Changes in morphology of the X chromosome in Barbitistinae were only found in *Poecilimon laevisissimus* (MESSINA *et al.* 1975) and *Leptophyes punctatissima* (WARCHAŁOWSKA-ŚLIWA & HELLER 1998). This character could probably help resolve the taxonomy if it is used in defining the morphological differences between groups of the genus *Isophya*. Of the five species showing acrocentric X chromo-

somes, two (*I. kalishevski* and *I. schneideri*, *I. schneideri* group) are distributed in the Caucasus region, while *I. modestior* (*I. costata* group) occurs in the southeastern part of Central Europe and the northwestern Balkans and *I. rectipennis* and *I. rammei* (both *I. pavelii* group) occur along the southwestern coast of the Black Sea and the neighboring continental regions. On the other hand, all the Balkan members of the *I. modesta* group are characterized by a subacrocentric X chromosome, however, there was a considerable discrepancy between species in relation to the constitutive heterochromatin content. Differences in the amount of heterochromatin mainly in the pericentromeric region of the X chromosome and the autosomes, as well as additional C-bands in the interstitial and/or telomeric region, could have originated from multiple duplications of small repetitive DNA segments as well as/or from transfer of heterochromatic material among equidistant sites of non-homologous chromosomes (SCHWEIZER & LOIDL 1987).

The C-banding technique, which allows for the identification of constitutive heterochromatin, has been extensively used in analyses of orthopteroid chromosomes and differences between species belonging to the same genus and between species of different genera are a result of its amount and pattern. A comparison of C-banding patterns in autosomes of the *Isophya* species is not straightforward. While some species, e.g. *I. hemiptera*, *I. rammei*, and *I. schneideri*, possess thick paracentromeric C-bands in the majority of chromosomes, others (see Table 1) show thin paracentromeric C-bands in all or most chromosomes. Interstitial C-bands in the autosomes occurred only in one long-sized pair in *I. toshevski*, whereas telomeric C-bands were observed in most chromosomes of *I. rhodopenis*, *I. andreevae* and *I. camtoxypha*.

Heterochromatin polymorphism is common in a wide variety of eukaryote species (e.g. SUMNER 1990, 2003). Interspecific intraspecific polymorphism, mainly associated with different numbers of additional heterochromatin blocks (indicated with star in Table 1), was present in some species of *Isophya* and also found in the closely related genus *Poecilimon* (WARCHAŁOWSKA-ŚLIWA *et al.* 2000). The heteromorphism of C-band distribution among the *Isophya* species shown in this paper may be a result of heterochromatin addition or deletion (thin/thick). Three possible hypotheses are proposed to explain this polymorphism: 1) displacement by translocation/inversion, 2) the process of euchromatin transformation, 3) amplification of an existing DNA sequence (SUMNER 1990). Of these three, the last one seems to be the most plausible hypothesis explaining the origin of a thick extra-segment observed in most

chromosomes. The heteromorphism in some autosomal pairs suggests that these chromosomes are more or less tolerant to losses or gains in that material (JOHN 1988).

Interspecific variation in the number of NORs (Table 1) was generally not dependent on the species groups. However, three NORs were detected on some bivalents of the closely related *I. kisi*, *I. rhodopenis* and in all analyzed populations of *I. petkovi*. NORs were located in autosomes and occurred predominantly on the 3<sup>rd</sup>/4<sup>th</sup> and one of the small bivalents. The variation of bivalents numbers bearing NORs may have arisen from the Ag-staining, which reveals only the NORs that are active during meiosis (from leptotene to diplotene). Therefore, NORs can be treated as additional markers for analyses of relationships between species of this group.

A comparative analysis of karyotype between populations of *I. petkovi* shows polymorphism in morphology of the X chromosomes as well as in quantity and distribution of the C-banding-pattern both in X and the autosomes and in NOR location. Different specimens show karyotypic similarity either to *I. kisi* or *I. rhodopenis*. Since these three forms are quite similar morphologically and, moreover, show a smooth transition in their song structure (CHOBANOV, unpublished data), their specific status does not seem well defined and thus they may represent subspecies or only transitional forms of a species. This data suggests strong karyotypic variability or provides the hypothesis that these taxa form a hybrid zone.

The occurrence of supernumerary chromosomes (B) has been previously noted especially in grasshoppers and a few species of tettigoniids (e.g. BATTAGLIA 1964; JOHN & LEVIS 1968; WHITE 1973; HEWITT 1979; CAMACHO *et al.* 1981; WARCHAŁOWSKA-ŚLIWA & HELLER 1998; WARCHAŁOWSKA-ŚLIWA *et al.* 1992, 2007). Additionally, we found small mitotically and meiotically unstable B chromosomes in five species of the genus *Isophya*. The traditional hypothesis on the origin of B chromosomes is that they are derived from the autosomes (JONES & REES 1982). However, recent cytological and molecular studies indicate that this element is partially due to intraspecific from autosomal complement or interspecific hybridization from closely related species (CAMACHO *et al.* 2000). In the case of *Isophya* the second explanation is more probable, but this phenomenon requires further detailed cytogenetic and molecular studies. Comparing sequences of shared DNA by both autosomes and Bs may shed light onto the karyological processes by which the B chromosomes arise.

In conclusion, chromosomal analysis of 25 species/subspecies of the genus *Isophya* suggests karyotypic evolution among species. In the sex chromosome, the most remarkable changes were the ancient acrocentric X chromosome inversion in 19 species and the X chromosome/autosome mutual tandem translocation, from which the sex determination system of the neo-XY originated in *I. hemiptera*. Karyotype differentiation has been less rapid in autosomes than in sex chromosomes, in this case interspecific autosomal differentiation has involved the distribution and quantity of C-heterochromatin as well as the number of NORs. Methods used in this study may reveal part of the evolutionary history of *Isophya*, and are broadening the perspectives of studies of their phylogenetic relationships. For a better understanding the generic karyotype evolution, further studies employing molecular techniques (e.g. RAPD, FISH as well as mtDNA and rDNA sequencing) are necessary. Finally, the cytogenetic analysis reveals some changes, reflecting the level of genomic organization, and the results suggest some association of related taxa from biogeographic regions. This leads to the conclusion that the present geographical arrangement of many species greatly reflects their natural phylogenetic relationships. Nonetheless, some isolated populations of species with similar morphology show differences in karyotype, e.g. *I. obtusa* and *I. altaica*. New data is needed for completing the natural grouping of these taxa.

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