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Cytotaxonomy and karyology of the tribe Otiorhynchini (Coleoptera: Curculionidae)

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Abstract. A cytogenetic study of bisexual species belonging to the genera *Cirrorhynchus*, *Dodecastichus* and *Otiorhynchus* is presented in order to confirm their taxonomic position. The karyotype characterization was accomplished by an analysis of mitotic and meiotic chromosomes after differential staining, namely by C-banding, silver impregnation, DAPI and CMA_3. A review of the cytogenetic data for the tribe Otiorhynchini contributed to knowledge of chromosomal evolution in this group. An investigation of five of the species studied showed some similarities such as a sex chromosome system of "parachute type" (Xy_p), the presence of 10 autosomal bivalents (2n = 22) and heterochromatin localized around centromeres. These observations are similar to those already described for Otiorhynchini species, and confirm the karyological conservatism of this weevil group. In contrast, another species *Cirrorhynchus kelecsenyi* has an additional four autosomal bivalents ($n♂ = 14 + Xy_p$, 2n = 30), which differs considerably from the chromosomal homogeneity of the other genera. Karyotypic evolution in this species was achieved most probably by increasing the number of chromosomes by centric fissions, resulting in variation in the number of acrocentric chromosomes. DAPI-positive and CMA_3-negative reactions of heterochromatic DNA in all the species studied suggest that it has an AT-rich composition. Impregnating chromosomes with silver nitrate reveals NORs on one pair of autosomes, and probably argentophilic material in the interspace between the X and y sex chromosomes. The karyological findings support the taxonomical revision of Otiorhynchini based on morphological characters.

INTRODUCTION

The Curculionidae is one of the largest beetle families with some 50,000 described species (Lawrence & Newton, 1995). So far, the karyology of about 600 species of Curculionidae has been investigated, although the great majority of the cytogenetic findings reported for weevils only refer to male chromosome numbers and sex determination system at meiotic metaphase I. Much less research has focused on the banded karyotypes of curculionids (Hsiao & Hsiao, 1984; Holecová et al., 2002; Rożek et al., 2004; Lachowska et al., 2004, 2005, 2006a, b). Data on the karyology of Curculionidae varies greatly from genus to genus and from subfamily to subfamily. There are many species-rich genera in which chromosomal composition has not yet been determined.

The tribe Otiorhynchini comprises ten genera (*Cirrorhynchus*, *Dodecastichus*, *Limatogaster*, *Otiorhynchus*, *Neotournieria*, *Parameira*, *Parotiorhynchus*, *Rhynchotious*, *Solariola*, and *Tylotus*) autochthonous exclusively to the Palaearctic region (Magnano, 1998). Only ten species of *Otiorhynchus* have been introduced into North America. Adults are generalists, feeding on various plants, whereas larvae are root-eating. Numerous species are apterous, characterized by nocturnal activity (Arnoldi, 1975; Dieckmann, 1980; Smreczyński, 1966). This group of weevils is well known for having a large number of parthenogenetic lineages, which have a much broader distribution than their sexual counterparts (Suomalainen et al., 1987). The *Otiorhynchus*-complex comprises about 1,500 species and is not only the largest and most speciose group within the Curculionidae, but also among taxons of higher rank (e.g. subgenera and species groups). The systematics of the *Otiorhynchus*-complex has been controversial for a long time. According to systems proposed by Stierlin (1883), Reitter (1916), Penecke (1935) and Arnoldi (1975), the genus *Otiorhynchus* should be divided into several subgenera and groups of species. The latest system of Magnano (1998) is the result of more than twenty years of studying *Otiorhynchus* and related genera. According to this author the *Otiorhynchus*-complex comprises eight separate genera: *Dodecastichus* Stierlin, 1861; *Cirrorhynchus* Apfelbeck, 1898; *Limatogaster* Apfelbeck, 1899; *Otiorhynchus* Germar, 1824; *Neotournieria* Apfelbeck, 1832; *Parotiorhynchus* Magnano, 1998; *Rhynchotious* Magnano, 1998; *Tylotus* Schönherr, 1823. The largest genus *Otiorhynchus* is divided into 105 subgenera (Magnano, 1998). At present, studies on the systematics and phylogeny of beetles are based on morphological, as well as on genetic and cytogenetic data (Angus et al., 2000; Gomez-Zurita et al., 2004; Petitpierre et al., 2004; Dutton & Angus, 2007). So far 34 bisexual species and 18 parthenogenetic species or races of the tribe Otiorhynchini from central and northern Europe, the Balkan Peninsula, and Sicily have been karyologically studied (Suomalainen, 1947; Smith & Virkki, 1978; Mikulska, 1951, 1960; Tucić & Mesaroš, 1992; Holecová et al., 1997a, b; Lachowska et al., 1998; Lachowska & Holecová, 2000; Holecová et al., 2002).

TABLE 1. Species of weevils whose karyotype was determined.

Species	Geographic source and date of collection
Cirrorhynchus kelecsenyi Frivaldszky, 1892	C Slovakia, Strážovské vrchy Mts., Zliechov (48°56′N, 18°26′E), June 9, 2006
Dodecastichus inflatus (Gyllenhal, 1834)	SW Slovakia, Malé Karpaty Mts, Pezinská Baba hill (48°21′N, 17°11′E), May 19, 2006
Otiorhynchus (s. str.) *coecus* Germar, 1824 = *niger* (Fabricius, 1775)	C Slovakia, Strážovské vrchy Mts, Strážov Nature Reserve (48°57′N, 18°28′E), June 9, 2006
Otiorhynchus (s. str.) *cornicinus* Stierlin, 1861 = *laevigatus* (Fabricius, 1792)	C Slovakia, Zvolenská kotlina basin, Jakub-Roháčovo (48°46′N, 19°08′E), May 26, 2006
Otiorhynchus (s. str.) *multipunctatus* (Fabricius, 1792)	C Slovakia, Strážovské vrchy Mts, Zliechov (48°56′N, 18°26' E), June 9, 2006
Otiorhynchus (*Phalantorrhynchus*) *morio* (Fabricius, 1781)	C Slovakia, Strážovské vrchy Mts, Strážov Nature Reserve (48°57′N, 18°28′E), June 9, 2006

The present paper is a continuation of research on the karyology of Palaearctic weevils. The aim of this study is: (1) to provide more information on the chromosomes of bisexual species of Otiorhynchini- and determine whether an ancestral karyotype ($n♂ = 10 + Xy_p$), characteristic for most weevils, predominates in the analysed group; (2) to characterize karyotypic diversity in six species belonging to three genera (*Cirrorhynchus*, *Dodecastichus* and *Otiorhynchus*) using differential chromosome banding techniques; (3) to compare the chromosomal results and the taxonomical position of *Cirrorhynchus kelecsenyi.*

MATERIAL AND METHODS

Adults of both sexes were collected in Slovakia in May and June 2006 (Table 1). Voucher specimens are deposited in the Institute of Systematics and Evolution of Animals PAS, Kraków, Poland. Gonads (9–10 from each species) were dissected under a stereomicroscope in several drops of hypotonic 0.9% sodium citrate solution containing 0.005% colchicine. The

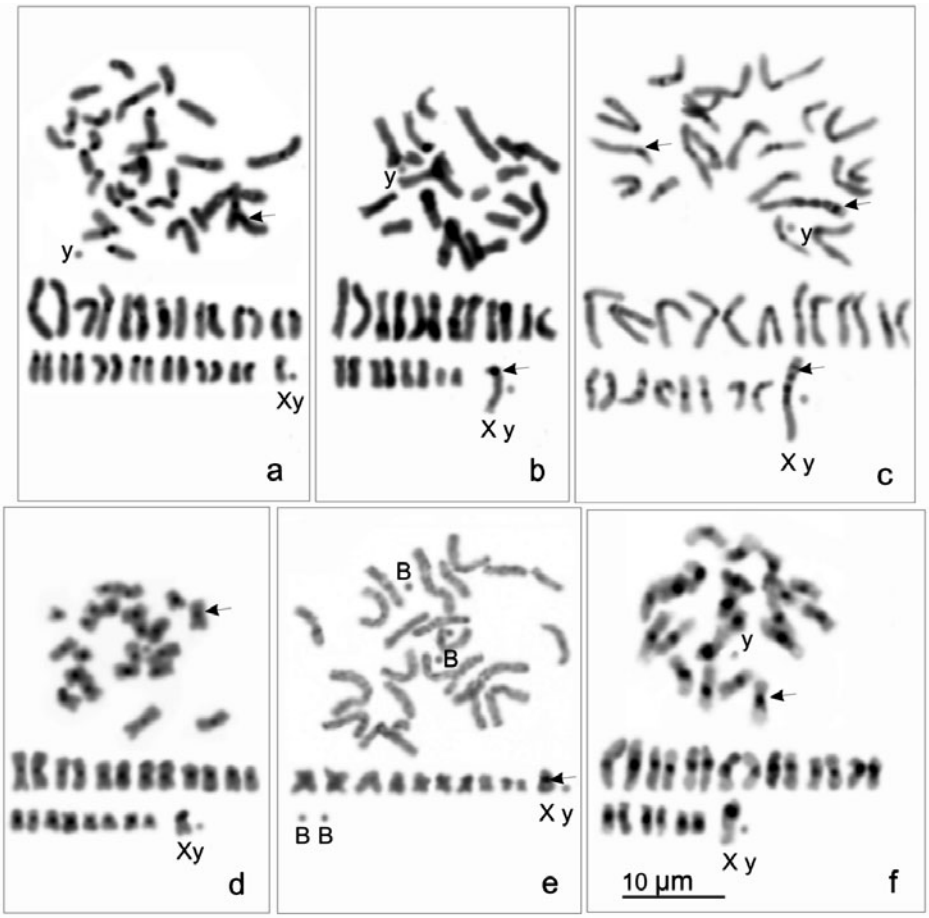

Fig. 1. C-band staining of the chromosomes at mitotic metaphase in: a – *Cirrorhynchus kelecsenyi*; b – *Dodecastichus inflatus*; c – *Otiorhynchus coecus*; d – *Otiorhynchus cornicinus*; e – *Otiorhynchus multipunctatus*; f – *Otiorhynchus morio.* Arrows indicate C-bands; X, y – sex chromosomes; B – supernumerary chromosome.

TABLE 2. Chromosome numbers of the species studied and their relative lengths (% TCL), and the centromeric index (AR) of particular chromosome pairs.

Pair no.	*Cirrorhynchus kelecsenyi* 2n = 30, n♂ = 14 + Xy_p mitotic metaphase		*Dodecastichus inflatus* 2n = 22, n♂ = 10 + Xy_p mitotic metaphase		*Otiorhynchus coecus* 2n = 22, n♂ = 10 + Xy_p mitotic metaphase	
	%TCL	AR	%TCL	AR	%TCL	AR
1	12.30	1.87	13.43	1.01	12.52	1.40
2	11.03	1.29	11.22	1.27	11.74	1.14
3	8.48	1.01	11.99	1.42	10.02	1.27
4	7.40	4.91	10.12	1.44	9.61	1.40
5	6.58	1.82	8.71	1.12	8.41	3.10
6	6.46	3.32	8.68	5.05	7.55	1.37
7	6.22	3.27	6.86	1.66	7.23	1.21
8	6.05	3.23	6.44	1.30	6.85	1.61
9	5.68	–	6.40	1.32	6.79	1.08
10	5.47	1.47	4.16	–	6.10	1.27
11	4.98	3.63	–	–	–	–
12	4.72	–	–	–	–	–
13	4.32	3.06	–	–	–	–
14	4.18	3.01	–	–	–	–
X	5.02	1.51	10.67	4.17	11.95	1.24
y	1.11	–	1.42	–	1.24	–

Pair no.	*Otiorhynchus cornicinus* 2n = 22, n♂ = 10 + Xy_p mitotic metaphase		*Otiorhynchus multipunctatus* 2n = 22 + 1–2, n♂ = 10 + Xy_p + 1–2B metaphase II		*Otiorhynchus morio* 2n = 22, n♂ = 10 + Xy_p mitotic metaphase	
	%TCL	AR	%TCL	AR	%TCL	AR
1	12.97	1.14	11.55	1.26	13.50	1.15
2	11.16	1.10	10.61	1.05	11.27	1.21
3	10.13	1.02	10.52	1.35	10.80	1.52
4	9.81	1.18	8.82	1.80	9.68	1.26
5	9.38	1.35	8.42	1.15	9.31	1.18
6	9.10	1.40	8.40	1.16	7.87	1.08
7	8.25	1.20	7.38	1.10	7.45	1.17
8	6.94	1.21	7.32	1.12	6.93	1.42
9	5.87	1.51	6.31	1.07	6.80	1.20
10	5.63	1.06	5.08	1.11	4.85	1.14
X	8.69	1.45	10.61	1.78	9.76	1.16
y	2.06	–	1.67	–	1.72	–
2B	–	–	3.04	–	–	–

gonads were transferred into a small volume of the same solution and incubated for 30–45 min at room temperature. Then the gonads were fixed according to the method described by Rożek (1994) with a minor modification (Rożek & Lachowska, 2001). C-banding was performed using the procedure described by Sumner (1972) with some modifications (Lachowska et al., 2006a). The slides were stained with 4% Giemsa in phosphate buffer (pH 6.8) for 10 to 20 min. For NOR silver staining the method described by Howell & Black (1980) was used with some modifications (Lachowska et al., 2005). The DNA binding fluorochromes, GC-specific chromomycin A_3 (CMA_3) and AT-specific 4'-6-diamidino-2-phenylindole (DAPI), were used according to the methods described by Schweizer (1976) and Donlon & Magenis (1983), with minor modifications. The slides were first subjected to the C-banding procedure and, to improve the fluorochrome staining, 0.5% methanol was included in the fluorescent dye. After staining, the slides were mounted in antifade medium consisting of 1% n-propylgallate in a 10 M phosphate buffer solution with 70% glycerol at pH 7.0. Evaluation of chromosome morphology was based on ten mitotic metaphases. In order to facilitate the arrangements of karyograms, the chromosome lengths were calculated as percentages of the total chromosome length of the haploid set (% TCL), which also includes the sex chromosomes. Chromosomes were classified according to Levan et al. (1964). Spermatogonial metaphase, meiotic stages, and interphase nuclei were analyzed and photographed using a Nikon Eclipse 400 light microscope and CCD DS-U1 camera (Nikon, Tokyo, Japan) and the software Lucia Image, version 5.0 (Laboratory Imaging, Prague, Czech Republic).

RESULTS

Herein, we report chromosomal findings for six bisexual species of which two are new records for the cytogenetic knowledge of the tribe, with the aim to discuss the trends in chromosome evolution in Otiorhynchini. Five of the species show some similarities such as a sex chromosome system of achiasmatic parachute type (Xy_p) and presence of 10 autosomal bivalents

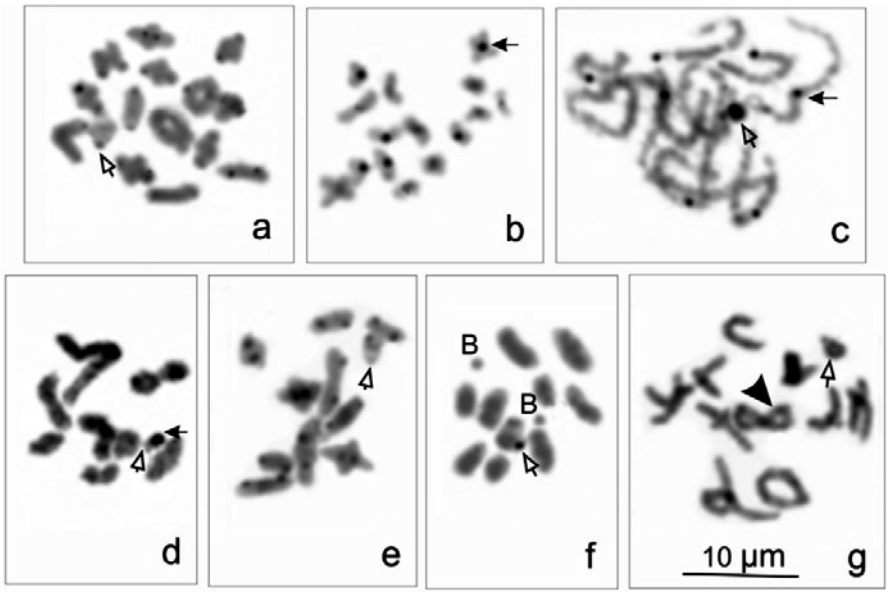

Fig. 2. Meiotic chromosomes after C-band staining. a – diakinesis in *Cirrorhynchus kelecsenyi*; b – metaphase II in *Cirrorhynchus kelecsenyi*; c – pachytene in *Dodecastichus inflatus*; d – metaphase I in *Dodecastichus inflatus*; e – diakinesis in *Otiorhynchus coecus*; f – metaphase I in *Otiorhynchus multipunctatus*; g – diakinesis in *Otiorhynchus morio.* Solid arrows indicate C-band blocks, open arrows Xy_p associations, solid arrowhead the longest bivalent with three chiasmata.

(Figs 2d–g, 3a–c, e–f). One species possesses 14 autosomal bivalents and Xy_p (Fig. 2a). Examination of diakinesis shows that long autosomal bivalents have either two terminal chiasmata, one terminal and one interstitial chiasma, or only one interstitial or terminal chiasma. Therefore, they form rod-shape figures, crosses and rings (Figs 2a, e–g, 3a–b). An exception is *Otiorhynchus morio*, with the longest autosomes often forming trichiasmate (two terminal and one interstitial) bivalents (Fig. 2g). Short bivalents of rod morphology are connected by one terminal chiasma. The numbers of bivalents of different shape are not stable, i.e. the same bivalents are sometimes connected by one chiasma and at other times by two chiasmata. The results show that the pattern of meiotic behaviour of the chromosomes is similar for all the beetles examined here.

Cirrorhynchus kelecsenyi ($2n = 30$, $n♂ = 14 + Xy_p$) – the karyotype consists of 30 chromosomes of different

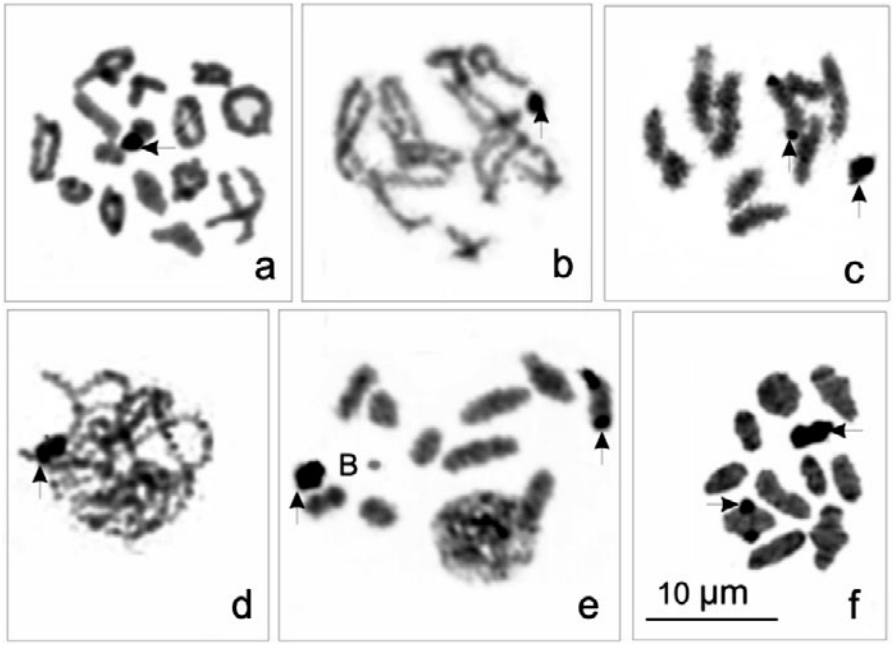

Fig. 3. Spermatocyte nuclei after silver staining. a – diakinesis in *Cirrorhynchus kelecsenyi*; b – early diakinesis in *Dodecastichus inflatus*; c – metaphase I in *Otiorhynchus coecus*; d – pachytene in *Otiorhynchus cornicinus*; e – metaphase I in *Otiorhynchus multipunctatus*; f – metaphase I in *Otiorhynchus morio.* Arrows show silver impregnation.

morphology. Three pairs of autosomes are metacentric, two are submetacentric, seven are subtelocentric and two acrocentric. The X chromosome is metacentric and short (relative length of 5.02%) while the y chromosome is dot-like (1.11%) (Fig. 1a). The two first pairs are the longest with relative lengths of 12.30% and 11.03%, the remaining autosomes are shorter with 8.48%–4.18% relative length (Table 2). C-positive segments are visible around centromeres in the majority of chromosomes, with the exceptions of the 6th, 11th, and 14th pairs with short, heterochromatic arms. Only the 4th pair is euchromatic, the y chromosome is negatively heteropycnotic (Fig. 2a, b). The argentophilic site is localized on sex chromosomes and is detectable only during meiotic prophase and metaphase I (Fig. 3a). After DAPI staining, bright signals were observed in the centromeric regions (Fig. 4a).

Dodecastichus inflatus (2n = 22, n♂ = 10 + Xy_p) is defined by a symmetric karyotype with a prevalence of metacentric chromosomes and a y chromosome of dot-like shape. The 6th autosomal pair and X chromosome with a secondary constriction have a subtelocentric morphology, the 10th pair is acrocentric (Fig. 1b). The longest chromosomes include the 1st–4th pairs of 13.43%–10.12% relative length, whereas the 5th–10th pairs account for 8.71%–4.16% of the total complement length. The X chromosome is one of the longest elements with a relative length of 10.67%, while the y chromosome is the smallest, presenting 1.42% of the relative length of the entire karyotype (Table 2). In pachytene, short segments of heterochromatin on autosomes are visible but undetectable during mitotic metaphase. Centromeric C-band is distinguishable on the X chromosome in mitotic stages and also metaphase I. The dot-shaped y chromosome is C-negative (Figs 1b, 2c–d). An Ag NO_3-positive cluster is situated on the sex chromosomes stained during meiotic prophase and metaphase I (Fig. 3b). CMA_3/DAPI produces homogeneous staining with no bright regions (not shown).

Otiorhynchus coecus (2n = 22, n♂ = 10 + Xy_p), karyotype is composed mainly of metacentric chromosomes with the exception of two subtelocentric autosomes (5th pair) and a dot-like y chromosome (Fig. 1c). The relative length of autosomes is 12.52%–6.10%, the X chromosome comprises 11.95%, whereas the y chromosome only 1.24% (Table 2). Constitutive heterochromatin appears in centromeric regions on all autosomes and the X chromosome, moreover the latter has two intercalary bands, and autosomes from the 8th pair have one intercalary band (Fig. 1c). Argentophilic blocks are situated on the sex chromosomes and the 4th pair of autosomes, visible from leptotene to metaphase I (Fig. 3c). Only DAPI positive pericentromeric regions were observed (Fig. 4b).

Otiorhynchus cornicinus (2n = 22, n♂ = 10 + Xy_p) has a symmetric karyotype with uniform chromosome mor-

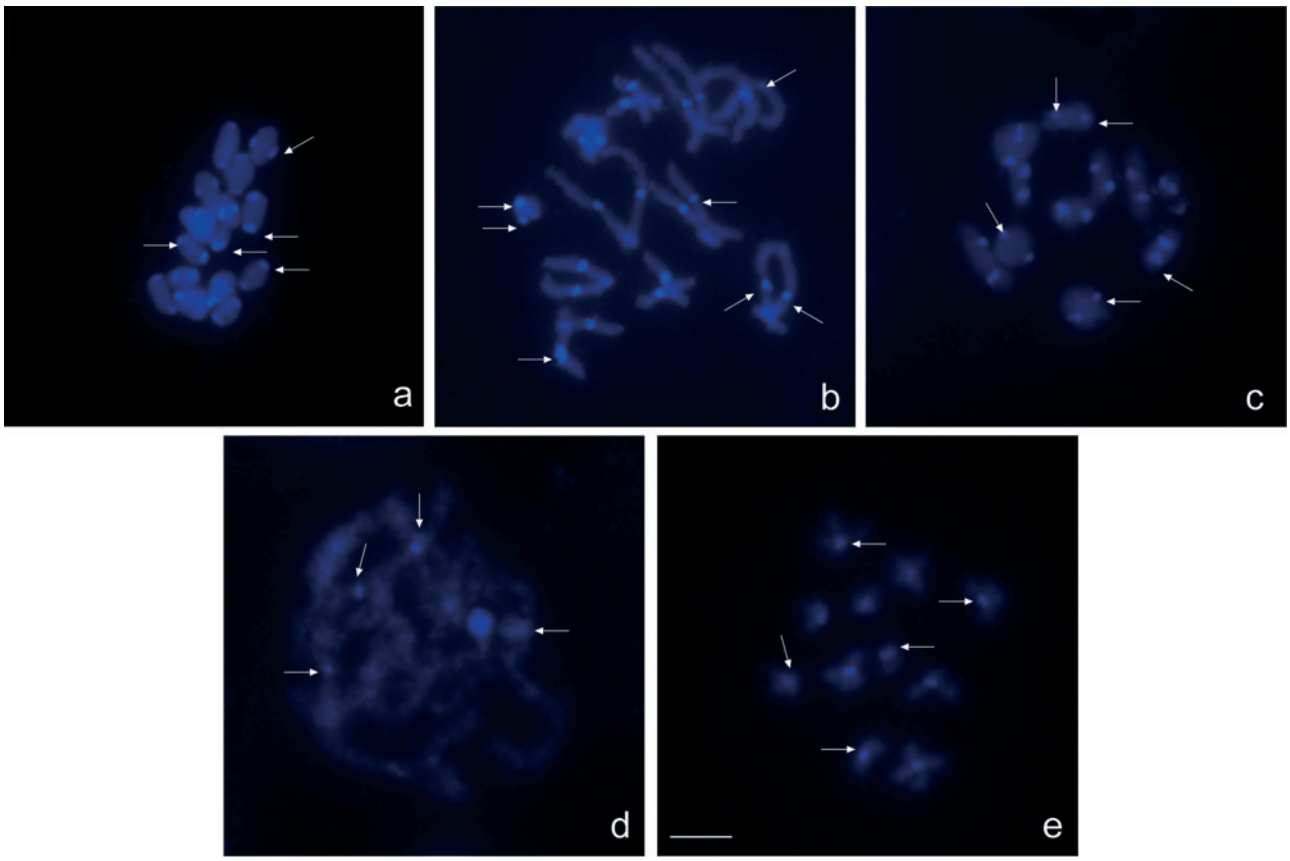

Fig. 4. Chromosomes after DAPI staining. a – metaphase I in *Cirrorhynchus kelecsenyi*; b – diakinesis in *Otiorhynchus coecus*; c – metaphase I in *Otiorhynchus cornicinus*; d – pachytene in *Otiorhynchus multipunctatus*; e – metaphase II in *Otiorhynchus morio.* Arrows point to heterochromatic blocks. Scale bar = 5 μm.

phology. All autosomes and the X chromosome have a median centromere and the y chromosome is dot-like (Fig. 1d). The relative length of the longest chromosomes varies between 12.7%–10.13% (pairs 1–3), the shorter chromosomes are pairs 4–10 with relative lengths of 9.81%–5.63%, the X chromosome – 8.69% and y – 2.06% (Table 2). The heterochromatin visualized by C-banding is limited to the pericentromeric region of all autosomes and one arm of the X chromosome (Fig. 1d). From meiotic prophase to metaphase I the sex bivalent was strongly silver stained (Fig. 3d). Only DAPI-positive blocks were detected (Fig. 4c).

Otiorhynchus multipunctatus (2n = 22 + 1–2 B, n♂ = 10 + Xy_p + 1–2 B). Because there were no good quality plates with mitotic metaphases, its karyotype is described on the basis of metaphase II. The symmetric karyotype contains metacentric autosomes, submetacentric 4th pair- and the X chromosome, the y chromosome is dot-like. On mitotic and meiotic plates 1–2 small additional elements were also visible, probably representing B-chromosomes (Fig. 1e). The sizes of the B-chromosomes are similar to the size of the y chromosome. The relative length of autosomes varies between 11.55%–5.08%, the X chromosome makes up 10.61% of the karyotype, while the y chromosome – 1.67%, and 2B – 3.04% (Table 2). Observation during metaphase I revealed the presence of heterochromatin only around the centromere of the X chromosome (Fig. 2f). During meiotic prophase and metaphase I, silver positive regions were located on the sex chromosomes and one pair of autosomes (Fig. 3e). DAPI positive signals were visible only during pachytene (Fig. 4d)

The karyotype of *Otiorhynchus morio* (2n = 22, n♂ = 10 + Xy_p) consists of chromosomes of similar length and morphology with median centromeres, only the y chromosome is dot-like (Fig. 1f). Autosome pairs 1–10 make up 13.50%–4.85% of the relative length, the X chromosome – 9.76% and the y chromosome – 1.72% (Table 2). All autosomes possess centromeric C-bands of different sizes, whereas a large heterochromatic block occurs on one arm of the X chromosome in an intercalary position (Fig. 2g). There is argentophilic material on the sex chromosomes and on one pair of autosomes (Fig. 3f). DAPI signals were observed in the centromeric position (Fig. 4e). Weak CMA_3 staining labeled one autosomal bivalent (not shown).

DISCUSSION

Below the chromosomal results for the six species are assessed in order to see whether there is an agreement with the proposed taxonomy. Based on taxonomical characters, the genus *Dodecastichus* is characterized by the presence of elytra with 12 or 13 striae, and occurs only in Italy, Central and Eastern Europe, and the Balkans. The genus *Otiorhynchus* is morphologically heterogeneous, has elytra with 10 striae, ventrites without longitudinal furrows, fore and middle tibia not flattened, and femora untoothed or (in some subgenera) the hind femora toothed. Despite the restricted distributions of many endemic species, the genus as a whole is widely distributed throughout the Palaearctic region (Magnano, 1998). The bisexual species from *Dodecastichus, Otiorhynchus* and *Tylotus* examined have an identical diploid chromosome number, 2n = 22, and meioformula n♂ = 10 + Xy_p (for some species the Xy sex determination system has been described but probably it is mistake because sometimes the "parachute" system is hardly visible on squash preparations) (Table 3). This confirms the karyological conservatism in this weevil group because it is the most characteristic chromosome number of weevils, and probably represents the ancestral state in the Curculionidae family as a whole. All results show that most of the chromosomes are meta- or submetacentric, a condition which is almost the rule in the karyotypic architecture in Otiorhynchini. The karyotype of *Otiorhynchus multipunctatus* is of some interest because it shows the presence of B chromosomes, clearly distinguishable from the regular members of the complement. The size of these additional chromosomes is approximately the same as that of the y heterochromosome. Because of the poor knowledge of B-chromosomes in weevils, it is difficult to comment on their genesis. Of the up to 600 species of Curculionidae examined karyologically, only four species have supernumerary chromosomes (Ennis, 1972; Smith & Brower, 1974; Dey, 1989; Holecová et al., 2005).

The application of the C-banding technique reveals a clear band pattern. C-banded karyotyping is occasionally used for the identification of closely related species in some coleopteran groups, e.g. Carabidae, Aphodiidae, Hydrophilidae, etc., where conventional staining techniques often give insufficient information (Angus et al., 2000; Wilson & Angus, 2004). In the species examined, the chromosomes resemble one another in having the C-bands restricted mostly to the area around the centromere, which is characteristic of the majority of insects (Juan & Petitpierre, 1989; Imai, 1991; Rożek, 1998; Almeida et al., 2000; Proença et al., 2002; Zacaro et al., 2004). An intercalary C-band was detected only on two chromosomes of *O. coecus.* On the X chromosome, the constitutive heterochromatin is located in the centromeric region in all species and also in an intercalary position in *O. morio* and *O. coecus*. In all species examined, the y chromosome does not possess a particular heterochromatic marking, although the C-banding technique does not stain all types of heterochromatin (Sumner, 1990). In Curculionidae, heterochromatin occurs mainly in small proportions and very often when the chromosomes become more condensed during the mitotic metaphase, diakinesis, metaphase I and II, these short segments are weak or are not visible at all (Rożek et al., 2004; Lachowska et al., 2005).

$AgNO_3$ chromosome staining is very useful for the analysis of nucleolar organizer regions (NORs), although this technique mainly reveals transcriptionally active NORs (Sumner, 1990). In beetles, NORs can be located on the autosomal pairs and/or sex chromosomes, although most data show that the nucleolus organizer is widely distributed on one autosomal pair (Moura et al., 2003; Bione et al., 2005). In three of the six species examined two Ag-

TABLE 3. Overview of the karyotypic data of the bisexual species of Otiorhynchini.

Species	Chromosomal formula of males	Chromosomal morphology	References
Cirrorhynchus kelecsenyi (Frivaldsky, 1892)	2n = 30, n♂ = 14+Xy$_p$	metacentric, submetacentric, subtelocentric, acrocentric, y-dot	Present paper
Dodecastichus atripes (Apfelbeck, 1918)	2n = 22, n♂ = 10+Xy$_p$	metacentric, y-dot	Tucić & Mesaros, 1992
Dodecastichus aurosignatus vlasuljensis Apfelbeck, 1894	2n = 22, n♂ = 10+Xy$_p$	metacentric, y-dot	Tucić & Mesaros, 1992
Dodecastichus dolomitae dryadis (Apfelbeck, 1895)	2n = 22, n♂ = 10+Xy$_p$	metacentric, y-dot	Tucić & Mesaros, 1992
Dodecastichus geniculatus (Germar, 1817)	2n = 22, n♂ = 10+Xy$_p$	–	Smith & Virkki, 1978
Dodecastichus inflatus (Gyllenhal, 1834)	2n = 22, n♂ = 10+Xy$_p$	metacentric, subtelocentric, acrocentric, y-dot	Present paper
Dodecastichus obsoletus (Stierlin, 1861) as *D. speiseri* Apfelbeck, 1894	2n = 22, n♂ = 10+Xy$_p$	metacentric, y-dot	Tucić & Mesaros, 1992
Otiorhynchus alpicola Boheman, 1843	2n = 22, n♂ = 10+Xy$_p$	metacentric, y-dot	Tucić & Mesaros, 1992
Otiorhynchus alpicola atterimus Boheman, 1843	2n = 22, n♂ = 10+Xy$_p$	metacentric, y-dot	Tucić & Mesaros, 1992
Otiorhynchus apenninus Stierlin, 1883 as *O. salicicola* Boheman, 1843	2n = 22, n♂ = 10+Xy$_p$	–	Smith & Virkki, 1978
Otiorhynchus arcticus (Fabricius, 1780)	2n = 22, n♂ = 10+Xy$_p$	–	Smith & Virkki, 1978
Otiorhynchus armadillo (Rossi, 1792)	2n = 22, n♂ = 10+Xy$_p$	–	Smith & Virkki, 1978
Otiorhynchus austriacus (Fabricius, 1801)	2n = 22, n♂ = 10+Xy$_p$	–	Smith & Virkki, 1978
Otiorhynchus bisulcatus (Fabricius, 1781)	2n = 22, n♂ = 10+Xy$_p$	–	Smith & Virkki, 1978
Otiorhynchus carmagnolae (Villa & Villa, 1835)	2n = 22, n♂ = 10+Xy	–	Smith & Virkki, 1978
Otiorhynchus coecus Germar, 1824 as *O. niger* (Fabricius, 1775)	2n = 22, n♂ = 10+Xy$_p$	metacentric, subtelocentric, y-dot	Lachowska & Holecová, 2000; Holecová et al., 2002; Present paper
Otiorhynchus cornicinus Stierlin, 1861	2n = 22, n♂ = 10+Xy$_p$	metacentric, y-dot	Present paper
Otiorhynchus corvus Boheman, 1843	2n = 22, n♂ = 10+Xy	metacentric, y-dot	Smith & Virkki, 1978; Holecová et al., 1997a
Otiorhynchus croaticus Stierlin, 1861	2n = 22, n♂ = 10+Xy$_p$	metacentric, y-dot	Tucić & Mesaros, 1992
Otiorhynchus equestris (Richter, 1820)	2n = 22, n♂ = 10+Xy$_p$	–	Smith & Virkki, 1978
Otiorhynchus gemmatus (Scopoli, 1763)	2n = 22, n♂ = 10+Xy$_p$	–	Smith & Virkki, 1978
Otiorhynchus kollari Gyllenhal, 1834	2n = 22, n♂ = 10+Xy	–	Smith & Virkki, 1978
Otiorhynchus koritnicensis Apfelbeck, 1918	2n = 22, n♂ = 10+Xy$_p$	metacentric, y-dot	Tucić & Mesaros, 1992
Otiorhynchus meridionalis Gyllenhal, 1834	2n = 22, n♂ = 10+Xy	–	Smith & Virkki, 1978
Otiorhynchus minutesquamosus Solari & Solari, 1908	2n = 22, n♂ = 10+Xy$_p$	metacentric, submetacentric, y-dot	Holecová et al., 1997b
Otiorhynchus morio (Fabricius, 1781)	2n = 22, n♂ = 10+Xy$_p$	metacentric, submetacentric, y-dot	Mikulska, 1960; Holecová et al., 2002; Present paper
Otiorhynchus multipunctatus (Fabricius, 1792)	2n = 22+1–2B, n♂ = 10+Xy$_p$+1–2B	metacentric, submetacentric, y-dot	Smith & Virkki, 1978 Present paper
Otiorhynchus obsidianus Boheman, 1843	2n = 22, n♂ = 10+Xy	–	Smith & Virkki, 1978
Otiorhynchus obtusus Boheman, 1843	2n = 22, n♂ = 10+Xy	–	Smith & Virkki, 1978
Otiorhynchus opulentus Germar, 1834	2n = 22, n♂ = 10+Xy$_p$	metacentric, submetacentric, y-dot	Lachowska et al., 1998
Otiorhynchus praecellens bosnarum Stierlin, 1886	2n = 22, n♂ = 10+Xy$_p$	metacentric, y-dot	Tucić & Mesaros, 1992
Otiorhynchus repletus Boheman, 1843	2n = 22, n♂ = 10+Xy	–	Smith & Virkki, 1978
Otiorhynchus rotifer Apfelbeck, 1828	2n = 22, n♂ = 10+Xy$_p$	metacentric, y-dot	Tucić & Mesaros, 1992
Otiorhynchus sensitivus (Scopoli, 1763)	2n = 22, n♂ = 10+Xy$_p$	–	Smith & Virkki, 1978
Otiorhynchus strumosus Heller, 1897	2n = 22, n♂ = 10+Xy$_p$	metacentric, y-dot	Tucić & Mesaros, 1992
Otiorhynchus tenebricosus (Herbst, 1784) as *O. fuscipes* Olivier, 1807	2n = 22, n♂ = 10+Xy	–	Smith & Virkki, 1978
Tylotus chrysops (Herbst, 1797) as *Otiorhynchus chrysops* Herbst, 1797	2n = 22	–	Smith & Virkki, 1978

stained spots occur, one is situated on the sex chromosomes, the second on one pair of autosomes. In meiotic cells of Coleoptera, NOR activity commences at the beginning of the meiotic prophase and disappears in the middle of the diplotene phase. The nucleolar masses produced can persist for a longer time in species with a prolonged diplotene (Virkki et al., 1991; Bione et al., 2005). This phenomenon was observed in all species studied here, but the presence of argentophilic masses on the sex chromosomes up until the late phase of meiosis I may indicate that Xy_p association is not necessarily due to an NOR. Studies on the segregation of sex chromosomes in curculionids showed that, even when the NORs are autosomal, the lumen of the sex bivalent is filled with a proteinaceous substance with an affinity for silver from diakinesis to anaphase I. It is suggested that this substance may play an adhesive role, controlling the correct separation (Virkki et al., 1991; Moura et al., 2003; Bione et al., 2005). Because in the other three species only one NOR occurs on the autosomes, our data appears in accordance with the hypothesis that an autosome pair functions as a nucleolus organizer, and the presence of non-nucleolar argyrophilous substances in the Xy_p bivalents contributes to regular association and segregation during meiosis. However, only the employment of fluorescent in situ hybridization with an rDNA probe would precisely identify the NORs in these species.

Chromosome staining by DNA base specific fluorochromes is little used in cytogenetic studies of Coleoptera (Vitturi et al., 1999; Colomba et al., 2006; Moura et al., 2003; Schneider et al., 2006) and has never hitherto been applied to Curculionidae. The use of fluorescent DNA-banding dyes with different specificities gives a better characterization of heterochromatic regions in terms of their relative enrichment with A–T or G–C base pairs. In the species of *Otiorhynchus* studied C-bands fluoresced brightly after DAPI staining suggesting the occurrence of a high amount of A–T base pairs in the DNA sequences making up the heterochromatic C-bands. Some differences in fluorescent intensity could be explained by the degree of condensation, i.e. the more the chromosomes are elongated, the weaker the visible signals. The sequential CMA_3 staining of chromosomes of the *Otiorhynchus* species studied showed that heterochromatin is negatively stained by chromomycine, which supports the hypothesis that there is an abundance of A–T in heterochromatin. The fluorochrome CMA_3 staining labels NORs independently of their activity, and the fluorescence is associated with G–C content typical of genes coding for ribosomal RNA (rDNA) (Anokhin & Nokkala, 2004). The correlation between CMA_3 bands and NORs is quite common in insects (Brito et al., 2003). However, a weak fluorescence after CMA_3 application, possibly coincident with NORs, was visible only in *O. morio*. The lack of positive signals in other species may suggest a small number of rDNA genes; alternatively, the absence of CMA_3 bands may be due to technical reasons because sometimes this band disappears when C banding is applied before sequential staining with chromomycine (Brito et al., 2003).

The taxonomic position of *Cirrorhynchus kelecsenyi* has changed. Previously it was included within the genus *Otiorhynchus* (Stierlin, 1883; Reitter, 1916; Winkler, 1932), but later it was put in a separate genus, *Cirrorhynchus* because it has elythra with 10 striae, ventrites without longitudinal furrows, and male tibia hollowed, the hind one with a long fringe of hair along the inner edge. The male ventrite 5 is characterized by two long tufts of hairs pointing forward on hind edge. The distribution of the genus is very limited (Italy, Slovakia, Hungary and Balkans) (Magnano, 1998). The karyotype of *C. kelecsenyi* consists of 30 chromosomes and differs strikingly from that in all other Otiorhynchini species examined, not only because of its higher chromosome number but also due to its asymmetry of chromosome sizes and existence of acrocentric chromosomes with short heterochromatic arms (Table 3). The increased number of small acrocentric chromosomes support the suggestion that the karyotypic evolution in this species was achieved by centric fissions of the ancestral metacentric chromosomes. Also the sex bivalent is smaller than in the species of the two other genera. The present karyological study supports the results of the taxonomical revision of this group made by Magnano (1998). According to the karyological data *Otiorhynchus* and *Dodecastichus* are closely related genera, whereas *Cirrorhynchus* is a distinct taxon.

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