PL-ISSN 0015-5497 (print), ISSN 1734-9168 (online)
Folia Biologica (Kraków), vol. 61 (2013), No 3-4
doi: 10.3409/fb61_3-4.177

Cytogenetic Analysis of *Otiorhynchus bisulcatus* (Fabricius, 1781) and *O.(Zadrehus) atroapterus* (De Geer, 1775) (Coleoptera, Curculionidae, Entiminae) using C Bands, NORs, and DAPI/CMA_3 Staining*

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Accepted May 15, 2013

HOLECOVÁ M., MARYAŃSKA-NADACHOWSKA A., ROŻEK M. 2013. Cytogenetic analysis of *Otiorhynchus bisulcatus* (Fabricius, 1781) and *O.* (*Zadrehus*) *atroapterus* (De Geer, 1775) (Coleoptera, Curculionidae, Entiminae) using C bands, NORs, and DAPI/CMA_3 staining. Folia Biologica (Kraków) **61**: 177-183.

The structure of the karyotypes of two *Otiorhynchus* species belonging to separate subgenera, viz. *Otiorhynchus* s.str. *bisulcatus* and *O.* (*Zadrehus*) *atroapterus*, is compared and described for the first time. Both species have the same chromosome number (2n=22), sex chromosome system of an achiasmate parachute type (Xy_p), symmetric karyotype with the prevalence of metacentrics, similar meiotic behaviour, localization of NORs and positive DAPI signals. The main differences involve the morphology of autosomes and the X chromosome in the C-banding pattern and DAPI/ CMA_3 signals as well as in the presence of additional B chromosomes.

Key words: Coleoptera, Curculionidae, karyotypes, C-banding, Ag-banding, DAPI/CMA_3 – staining.

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The tribe Otiorhynchini comprises eight genera of weevils autochthonous exclusively to the Palaeartic region. Only ten species of the genus *Otiorhynchus* Germar, 1822 have been introduced into the Nearctic region. All species of *Otiorhynchus* are apterous, foliophagous as adults, whereas their larvae develop in soil, feeding on roots. They are well known as inhabitants not only of woodland, forest patches and bushes, but also gardens, hedgerows, and other types of urban green areas. The *Otiorhynchus*-complex comprises about 1,500 species and is one of the largest and most speciose within the curculionids (KOCH 1992; MAGNANO 1998; WANAT *et al*. 2011).

The systematics of the genus *Otiorhynchus* is complicated and the taxonomic status of individual subgenera and/or species groups has been changed several times. The genus *Otiorhynchus* is morphologically very heterogenous. It is characterized by having elytra with 10 striae, ventrites without longitudinal furrows, fore and middle tibiae not flattened, and femora untoothed or (in some subgenera) the hind femora toothed. Despite the restricted distribution of many endemic species, the genus as a whole is widely distributed throughout the Palaearctic region (MAGNANO 1998). The subgenus *Otiorhynchus* s. str. Germar, 1822 is very diversified with regard to distribution and species richness. The taxon *Zadrehus* Reitter, 1912 was originally described as a species group of the subgenus *Aramichnus* Gozis, 1822 (REITTER 1912). According to the latest revision of MAGNANO (1998) the taxon *Zadrehus* was reclassified as a separate subgenus of the genus *Otiorhynchus*. Both subgenera (*Otiorhynchus* s. str. and *Zadrehus*) differ in morphology of the rostral dorsum, antennal fossae, pronotum, tibae, femora, mesocoxae, elytral striae, intervals and male genitalia as well.

So far only 34 bisexual species and 18 parthenogenetic species or races of the tribe Otiorhynchini from central and northern Europe, the Balkan Pen-

*Partially supported by VEGA, grant number 2/0035/13 and 1/0066/13 to Milada HOLECOVÁ.

insula and Sicily have been karyologically examined. Karyotype characterization was accomplished through analysis of mitotic and meiotic chromosomes after conventional or differentiation staining (Giemsa staining, C-banding, silver impregnation, DAPI and CMA_3). All described parthenogenetic species are examples of geographic parthenogenesis and they have a polyploid chromosome number (3n) but the haploid set consists of 11 chromosomes. The karyotype of bisexual species consists of 2n = 22 and a meioformula n♂ = 10 +Xy_p which seems ancestral for all curculionids (SUOMALAINEN 1947; SMITH & VIRKKI 1978; MIKULSKA 1951, 1960; TUCIĆ & MESAROŠ 1992; HOLECOVÁ *et al.* 1997 a, b, 2002; LACHOWSKA *et al.* 1998, 2008a, b; LACHOWSKA & HOLECOVÁ 2000; ROŻEK *et al.* 2009).

The present paper examines the karyotypes of two species from central Europe – *Otiorhynchus* s.str. *bisulcatus* and *O.* (*Zadrehus*) *atroapterus* (=*rotundatus*) using differential staining techniques. $AgNO_3$-banding was used to reveal the nucleolus organizing chromosomes and to locate the NORs. C-banding was used to study the distribution of heterochromatin. To characterize the molecular composition of the heterochromatin the preparations were stained with DNA-specific fluorochromes DAPI and CMA_3 which selectively stain AT-rich and GC-rich DNA regions, respectively. The aim of the study was to compare chromosome morphology of both examined species belonging to separate subgenera and to describe similarities and/or differences in karyotype structure.

Material and Methods

For the cytogenetic study, adults of both species were collected in Slovakia and Poland in May 2011 and August 2010-2012 (Table 1). Voucher specimens are deposited in the Institute of Systematics and Evolution of Animals, Polish Academy of Sciences, Kraków. Mitotic and meiotic chromosomes were obtained from testicular cells according to the method described by ROŻEK *et al.* (2009). C-banding was performed using the procedure described by SUMNER (1972) with minor modifications (LACHOWSKA *et al.* 2006b). The slides were stained with 4% Giemsa phosphate buffer (pH 6.8) for 10 to 20 min. For the 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 to reveal the molecular composition of C-heterochromatin according to the methods described by SCHWEITZER (1976) and DONLON & MAGENIS (1983). Chromosomes were classified according to LEVAN *et al.* (1964). Evaluation of chromosome morphology was based on ten mitotic metaphases. Chromosome lengths were calculated as percentages of the total chromosome lenght of the haploid set (% TCL), which also includes the sex chromosomes. Spermatogonial metaphases, meiotic stages, and interphase nuclei were analyzed and photographed with a Nikon Eclipse 400 light and Nikon Eclipse E400 fluorescence microscope, at 1000x. Microphotographs were made with a Nikon DS-U1 camera (Nikon, Tokyo, Japan), using the software Lucia Image version 5.0 (Laboratory Imaging, Prague, Czech Republic). The images were optimized for best contrast and brightness by means of the Corel Photo-Paint 11 image-processing software.

Results

The same chromosome number 2n=22 was observed at spermatogonial metaphases (Figs 1,8) in both examined species. The meioformula n = 10+Xy_p was identical in all male metaphase I plates.

Otiorhynchus s. str. *bisulcatus* (2n♂ = 20 +Xy_p). The male diploid complement consists of 20 autosomes and X and y sex chromosomes. The karyotype is symmetric with chromosomes forming a series descreasing in size. Metacentric structure is evident in eight pairs of autosomes as well as in the X chromosome, whereas two pairs of submetacentrics (5th and 7th) are also recorded (Table 2, Fig. 1). The relative length of autosomes varies between 13.50%-5.09%, the long X chromosome makes up 14.13% of the karyotype, while the dot-

Table 1

Origin of weevil species used for karyological analysis

Species	Geographic source and date of collection	Host plant and habitat
Otiorhynchus bisulcatus	SW Slovakia, Malé Karpaty Mts., Naháč – Katarínska Nature Reserve (48°33'05'' N, 17°32'32'' E), 340 m a.s.l., May and June, 2011	*Fraxinus excelsior* the forest margin
Otiorhynchus (*Zadrehus*) *atroapterus*	S Poland, Kraków (50°03'22'' N, 19°56'24'' E), 182 m a.s.l., August, 2010, 2011, 2012	*Ligustrum vulgare* the hedgerow

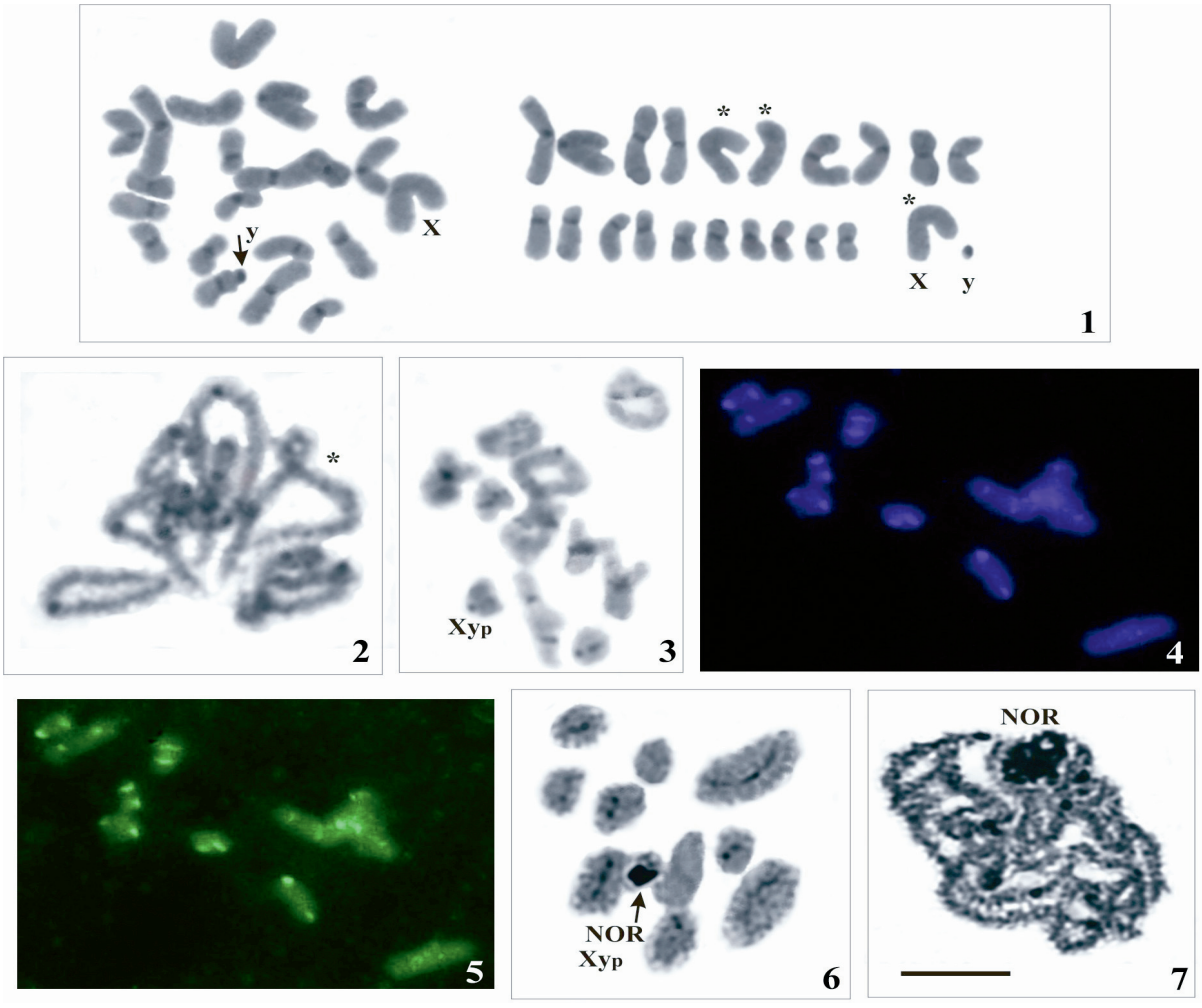
Table 2

Relative length (% TCL) and centromeric index (AR) of particular chromosome pairs

Pair No.	*Otiorhynchus bisulcatus*		*Otiorhynchus* (*Zadrehus*) *atroapterus*	
	%TCL	AR	%TCL	AR
1	13.50	1.38	14.17	1.30
2	13.14	1.31	12.35	1.46
3	12.19	1.30	10.42	1.02
4	9.54	1.21	10.20	1.78
5	7.88	2.56	9.93	1.03
6	7.14	1.20	8.95	1.27
7	6.64	2.03	8.40	1.51
8	6.08	1.06	7.45	1.04
9	5.09	1.04	5.60	1.95
10	4.66	1.03	5.07	1.24
X	14.13	1.18	6.72	6.62
y	2.37	-	2.20	-

shaped y chromosome – only 2.37% (Table 2). The heterochromatin visualized by C-banding is limited to short blocks in centromeric regions of autosomes except for the C-negative 3rd pair. The X chromosome is euchromatic, while the dot-like y chromosome is heterochromatic (Figs 1, 3). Chromosome staining with DAPI and CMA_3 shows pericentric bright signals DAPI+/CMA_3+ at pachytene/diplotene and metaphase I on ten of eleven bivalents, therefore the obtained results indicate that heterochromatin consists of repeats rich in A-T and C-G nucleotides, respectively (Figs 4, 5).

O. (*Zadrehus*) *atroapterus* ($2n♂ = 20 + Xy_p + 2B$). The symmetric karyotype with uniform chromosome morphology contains eight pairs of metacentric autosomes, two submetacentrics (the 4th and 9th pair), the subtelocentric X chromosome and the dot-like y (Table 2, Fig. 8). Often in mitotic and meiotic plates, 2 small additional elements were also recognizable representing supernumerary B-chromosomes (Figs 8, 10). The relative length of autosomes is 14.17%-5.07%. The X chromosome comprises 6.70%, whereas the y only 2.20%. The



Figs 1-7. Chromosomes of *Otiorhynchus bisulcatus*. Fig. 1. C-banded mitotic metaphase and karyogram, the asterisks indicate the third pair of autosomes and X chromosome without C-bands. Fig. 2. Diplotene, the arrow indicates the 3rd pair of autosomes without C-bands. Fig. 3. Diakinesis. Fig. 4. DAPI staining – metaphase I. Fig. 5. CMA_3 staining – metaphase I. Fig. 6. Metaphase I. Fig. 7. Pachytene bouquet. Bar = 10 μm.

dot-like B chromosomes are slightly longer in comparison with the y (Table 1, Figs 8-10). All autosomes possess short pericentromeric C-bands of similar sizes, whereas a slightly longer heterochromatic block occurs in the centromeric region of the X. The dot-shaped y is totally heterochromatic (Figs 8, 10).

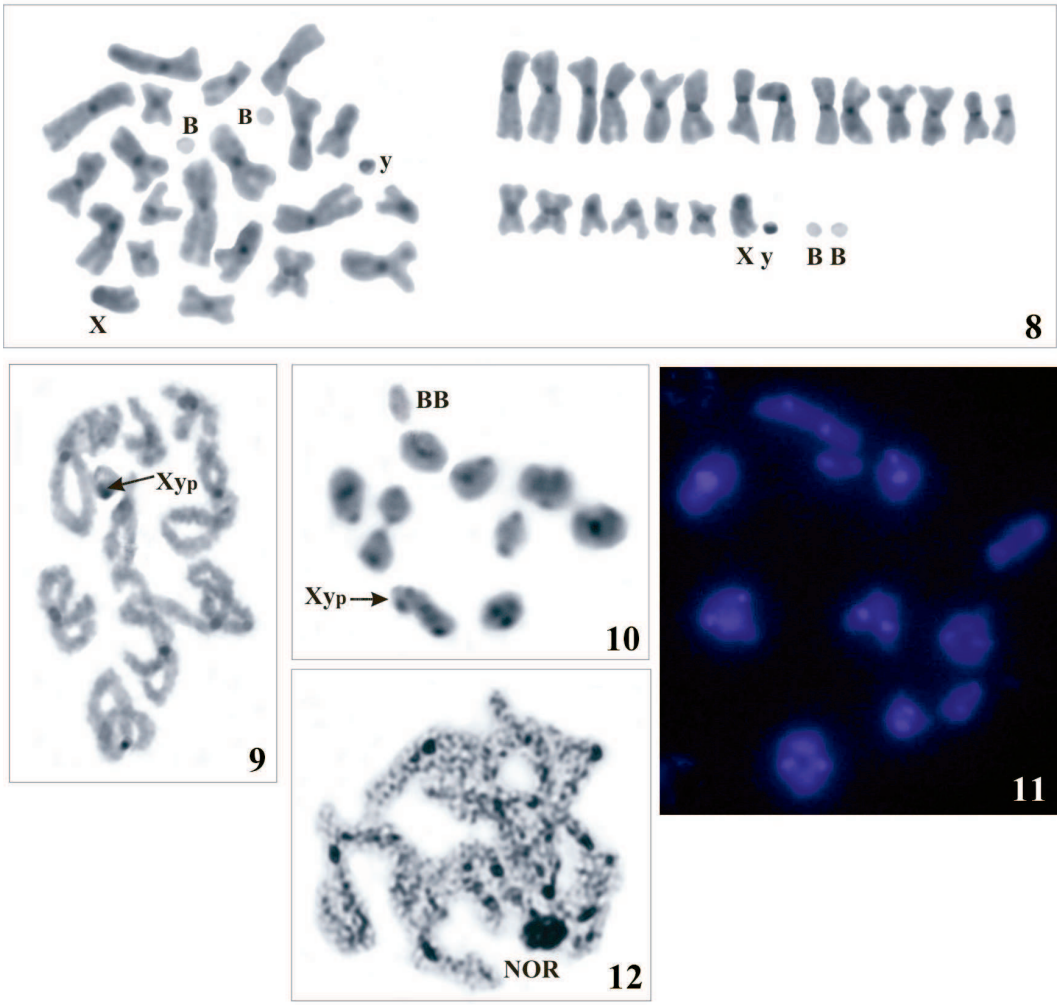
After DAPI staining at pachytene/diplotene/diakinesis, bright signals were observed in the pericentromeric regions of all pairs of chromosomes. After CMA_3 staining signals on chromosomes were not visible (Fig. 11).

Examination of spermatocyte I cell divisions shows significant similarity of individual meiotic stages in both *Otiorhynchus* species. As a consequence of weaker chromosome spiralisation, larger blocks of constitutive heterochromatin are clearly visible during leptotene, zygotene, pachytene, diplotene and diakinesis in comparison with mitotic metaphase (Figs 2, 3 and 9). During early diakinesis the longest autosomal pairs possess 3-4 chiasmata each (terminal and intercalar) whereas the shorter pairs are connected by 1-2 chiasmata. Later after terminalisation of chiasmata the bivalents form rings, crosses and rods (Figs 3, 9). Ten autosomal bivalents and one achiasmate sex heterovalent Xy_p are clearly seen in metaphase I plates (Fig. 10). An additional euchromatic element in *Otiorhynchus atroapterus* is observed in the majority of metaphases I. It probably originated from two B chromosomes, often occurring at mitotic metaphase (Fig. 10).

In both examined species argentophilic blocks are localized in sex chromosomes and are detectable only during all stages of the meiotic prophase and metaphase I, whereas they did not stain and remained undetected in the mitotic metaphase plates (Figs 6, 7, 12).

Discussion

Both examined species of *Otiorhynchus* show some similariries such as a sex chromosome sys-



Figs 8-12. Chromosomes of *Otiorhynchus atroapterus*. Fig. 8. C-banded mitotic metaphase and karyogram. Fig. 9. Early diakinesis. Fig. 10. Metaphase I. Fig. 11. DAPI staining – metaphase I. Fig. 12. Diplotene.

tem of the achiasmate parachute type (Xy_p) and the presence of 10 autosome pairs. This confirms the karyological conservatism in this genus because it is the most characteristic chromosome number and seems to be the ancestral state in the family Curculionidae (LACHOWSKA *et al.* 1998). The examined karyotypes are characterized by the prevalence of metacentric chromosomes, a condition which is almost the rule in the karyotypic architecture not only in Otiorhynchini but also in the majority of broad-nosed weevils (Entiminae) (SMITH & VIRKKI 1978; LACHOWSKA *et al.* 1998, 2006a, 2008a).

The karyotype of *Otiorhynchus atroapterus* is of interest because it shows the presence of euchromatic B-chromosomes, clearly distinguishable from the regular members of the complement. The size of these additional elements is slightly larger than that of the y heterochromosome. The present state of knowledge on B-chromosomes in weevils does not offer a clear explanation of their genesis. It is possible that they originated by fragmentation of one arm of the X chromosome of *Otiorhynchus atroapterus*. The subtelocentric X chromosome seems to be indirect evidence. In curculionid species supernumerary chromosomes are rare. From among 600 curculionid species examined karyologically, they were found only in seven species (ENNIS 1972; SMITH & BROWER 1974; DEY 1989; HOLECOVÁ *et al.* 2005; LACHOWSKA *et al*. 2008a, and the present study).

Up to now C-banding was occasionally used for identification of closely related species in some coleopteran groups, e.g. Carabidae, Aphodiidae, Hydrophilidae etc. in which conventional staining techniques often provide insufficient information (ANGUS *et al.* 2000; WILSON & ANGUS 2004; LACHOWSKA *et al.* 2009). In the examined species the chromosomes resemble one another in having C-bands restricted mostly to the area around the centromere which is characteristic of the majority of insects (JUAN & PETITPIERRE 1989; IMAI 1991; ALMEIDA *et al.* 2000; PROENÇA *et al.* 2002; ZACARO *et al.* 2004). Pericentromeric blocks are present in the majority of autosomes and in the X chromosome in *Otiorhynchus atroapterus*, while the y chromosome is heterochromatic in both examined species. In addition to the pericentromeric C-bands in *Otiorhynchus*, an intercalary position of the constitutive heterochromatin was also detected in chromosomes of *O.* (*Otiorhynchus*) *coecus* and *O.* (*Phalantorrhynchus*) *morio* (LACHOWSKA *et al*. 2008a). In the X chromosome the constitutive heterochromatin, if present, is located either in the centromeric region or in an intercalary position. In both examined species, the y chromosome is heterochromatic. In Otiorhynchini (in genera *Cirrorhynchus, Dodecastichus, Otiorhynchus*) analyzed in a previous study (LACHOWSKA *et al*. 2008a) the dot-shaped y was C-negative although the C-banding did not show all types of heterochromatin (SUMNER 1990). It is common knowledge that in Curculionidae, heterochromatin occurs mainly in a small proportion. Usually these short segments are very weak or invisible when the chromosomes become more condensed during the mitotic metaphase and meiotic diakinesis, metaphase I and II (HOLECOVÁ *et al.* 2002; LACHOWSKA *et al.* 2005, 2006a, b).

In Coleoptera $AgNO_3$ staining has been useful for the analysis of nucleolus organizer regions (NORs) (VIRKKI *et al.* 1991; BIONE *et al.* 2005a, b). In many species of beetles from different families, NORs may be located in the autosome pairs and/or sex chromosomes, although most data show that the nucleolus organizer is most often distributed in one autosome pair (VIRKKI 1983; VIRKKI *et al.* 1984; VITTURI *et al.* 1999; COLOMBA *et al.* 2000; MOURA *et al*. 2003; BIONE *et al.* 2005a; ALMEIDA *et al.* 2006; SCHNEIDER *et al.* 2007; LACHOWSKA *et al.* 2008a; MENDES-NETO *et al.* 2010). In both examined species the Ag-stained NOR is situated in the sex chromosomes. The presence of argentophilic masses in the sex chromosomes up until the late phase of meiosis I may indicate that the Xy_p association is not necessarily due to a NOR. Studies on segregation of sex chromosomes in weevils showed that even when the NORs are autosomally located, the lumen of the sex bivalent is filled with a proteinaceous substance with an affinity for silver from diakinesis to anaphase I. This substance may probably play an adhesive role, controlling the correct separation at anaphase I (VIRKKI *et al.* 1990,1991; JUAN *et al.* 1991, 1993; PETITPIERRE 1996; MOURA *et al.* 2003; BIONE *et al.* 2005a, b; SCHNEIDER *et al.* 2007; LACHOWSKA *et al.* 2008a; MENDES-NETO *et al.* 2010). Because in other species of Otiorhynchini only one NOR is located in the autosomes (LACHOWSKA *et al.* 2008a), our data are in accordance with the hypothesis that an autosome pair functions as a nucleolus organizer, and the presence of non-nucleolus argentophilous substances in the Xy_p bivalents contributes to regular association and segregation during meiosis. However, only the application of fluorescence *in situ* hybridization (FISH) with an rDNA probe would precisely identify the NORs in these species.

Chromosome staining by DNA base specific fluorochromes is seldom used in cytogenetic studies of Coleoptera (JUAN 1989; VITTURI *et al.* 1999; COLOMBA *et al.* 2000; MOURA *et al.* 2003; SCHNEIDER *et al.* 2007; LACHOWSKA *et al.* 2008a; MENDES-NETO *et al.* 2010; KARAGYAN & LACHOWSKA 2012). The use of fluorescent DNA-banding dyes with different specificities provides a better characterization of heterochromatic regions in terms of their relative enrichment with A-T or G-C

base pairs. C-bands fluoresced brightly after DAPI staining in both examined *Otiorhynchus* species. This suggests the occurrence of a high amount of A-T base pairs in the DNA sequences making up the heterochromatic C-bands. Positive DAPI signals were found in the majority of weevils previously studied confirming that A-T pairs often make up the main part of heterochromatin (LACHOWSKA 2008; LACHOWSKA *et al.* 2008). However, after CMA_3 staining, we found positive signals in the majority of chromosomes of *O. bisulcatus* showing that heterochromatin in this species consists of A-T and C-G pairs.

CMA_3 staining labeled NORs independently of their activity, and the fluorescence is associated with G-C content typical for genes encoding ribosomal RNA (rDNA) (ANOKHIN & NOKKALA 2004). The correlation between NORs and CMA_3 bands is quite common in some insects, including beetles (BRITO *et al.* 2003), but it is not always possible to identify rDNA. Silver nitrate stains argentophilic acid protein involved in rDNA activity but can also stain other proteins. CMA_3 stains C-G rich parts of chromosomes including NORs. Numerous positive CMA_3 signals may cause difficulties in identification of NORs and/or make it impossible as in *O. bisulcatus*.

In this study, the fluorescent signals after CMA_3 were positive in *O. bisulcatus* but negative in *O.* (*Zadrehus*) *atroapterus*. This observation constitutes another difference between the karyotypes of both species besides differences in the morphology of X chromosomes and C-banding pattern in autosomes.

In general, the distribution of AT- or GC-rich clusters of constitutive heterochromatin among beetles studied by fluorochrome staining is variable. For instance, positive CMA_3 and negative DAPI signals were found in some Elateridae (SCHNEIDER *et al.* 2007), Buprestidae (KARAGYAN & LACHOWSKA 2012) and most Scarabaeoidea (VITTURI *et al.* 1999; MOURA *et al.* 2003; BIONE *et al.* 2005b; CABRAL-DE-MELLO *et al.* 2010). Positive DAPI signals were found in the majority of the Curculionidae (LACHOWSKA 2008; LACHOWSKA *et al.* 2008a and the present paper). Rarely, positive signals in both DAPI and CMA_3 were revealed in Scarabaeidae (BIONE *et al.* 2005b) in Curculionidae (LACHOWSKA 2008, the present study) and Chrysomelidae (ALMEIDA *et al.* 2006).

In conclusion, data from our previous (LACHOWSKA *et al.* 2008a) and present study offer important insights into the karyotype characteristics of the genus *Otiorhynchus* which may be useful in elucidation of relationships among the species of the tribe Otiorhynchini as well as between weevils and representatives of other coleopteran families.

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