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Cytogenetics of *Accanthopus velikensis* (Piller et Mitterpacher, 1783) (Tenebrionidae: Helopini)

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Abstract. The karyotype and cytogenetic features of darkling beetle *Accanthopus velikensis* were analysed using conventional and differential staining. The diploid number was determined as 2n = 20 and the presence of Xy_p sex determination system was observed with DAPI and silver staining as well as conventional staining. Although a single nucleolar material was observed in prophase I nuclei, multiple argyrophilic signals in diakinesis-metaphase I plates makes it difficult to determine the exact NOR location. Both conventionally and differentially stained plates showed that heterochromatin is mostly concentrated on centromeric regions of *A. velikensis* chromosomes. Obvious telomeric signals on some rod shaped bivalents as well as the X chromosome were also detected with $AgNO_3$ and DAPI staining. Although presented karyotype of *A. velikensis* resemble to those of other Helopini members and follows the common patterns of Tenebrionid karyotypes, slight differences in chromosome morphologies, NORs and the heterochromatin distribution were detected. Our specimens also showed a unique haplotype for COI sequences with an 84-83% sequence similarity to database sequences for Tenebrionidae.

Keywords. Karyotype, NOR, COI, DNA barcoding, Helopini, Tenebrionidae.

INTRODUCTION

Accanthopus Dejean, 1821 (= *Enoplopus* Solier, 1848) is a small tenebrionid genus with two lichen-feeding species, *A. velikensis* and *A. reitteri* (Brenske, 1884) distributed in Southern and partly Central Europe and occurring in *Fagus*, *Abies* and *Quercus* forests. Although the genus is considered to be included in the tribe Helopini since Lacordaire (1859), several additional taxonomic placements have been also proposed. Historically, the genus has been placed in either a separate tribe (*Enoplopites* – Solier 1848; Reitter 1917) or different subtribes in Helopini (i.e. Enoplopina – Reitter 1922; Nabozhenko 2018; Cylindrinotina – Ardoin 1958; Helopina – Nabozhenko 2008; Nabozhenko and Löbl 2008). Ardoin (1958) also suggested erecting a separate tribe within the subfamily Tenebrioninae for this genus.

The genus *Accanthopus* has unusual external and internal structures, some of which support its position in the tribe Helopini. Several structures including the inner prothoracic skeleton, ovipositor, defensive glands female genital tubes are typical for Helopini (Tschinkel & Doyen 1980, Nabozhenko, 2005). On the other hand, the genus possess numerous non-helopine characters, such as very wide and spherical body, mentum with sexual dimorphism, profemora with strong and large acute tooth dorsally, strongly widened epipleura, very short and wide metaventrite, structures of mesonotum, metendosternite, aedeagus (Ardoin 1958), male inner sternite VIII and lobes of gastral spicula. Therefore, the position of *Accanthopus* in relation to other Helopini and Tenebrioninae lineages needs to be tested with additional data sets and integrated with molecular phylogenetic analyses.

The cytogenetic data among Tenebrionids have covered only about 1% of the species diversity (Guenin 1950, 1951a,b; Smith 1952; Smith and Virkki 1978; Yadav et al. 1980; Petitpierre et al. 1991; Juan and Petitpierre 1991a; Holecová et al. 2008; Blackmon and Jeffery 2015; Gregory 2016). In general, most of the species present a karyotype with 2n = 20, but the diploid number ranges from 2n = 14 to 2n = 38 in Tenebrionidae (Juan and Petitpierre 1991a; Pons 2004; Holecová et al. 2008; Lira-Neto et al. 2012). Based on available data, main karyological patterns in tenebrionid beetles were noticed in chromosome morphology, sex determining systems and distribution of heterochromatin (Juan and Petitpierre 1990; Petitpierre et al. 1991; Juan and Petitpierre 1991a, 1991b; Juan et al. 1993; Bruvo-Madaric et al. 2007; Şendoğan and Alpagut Keskin 2016).

Although chromosomal data are available for several representatives of subfamilies Allecullinae, Diaperinae, Lagriinae, Pimelinae, and Tenebrioninae, even basic information is scarce or totally lacking for other subfamilies (Juan and Petitpierre 1991a; Blackmon and Jeffery 2015). The chromosomes of *Accanthopus* have not yet been studied. Furthermore, cytogenetic data concerning the tribe Helopini are only known for some *Nesotes* Allard, 1876, *Euboeus* Boieldieu, 1865 (=*Probaticus* Seidlitz, 1896), *Nalassus* Mulsant, 1854 and *Turkonalassus* Keskin et al., 2017 species (Juan and Petitpierre 1986, 1989, 1991a, 1991b; Palmer and Petitpierre 1997; Şendoğan and Alpagut Keskin 2016).

Considering the limited cytogenetics information, the increase of chromosomal data may provide valuable phylogenetic signals about tenebrionid diversity. In this study, the mitotic and meiotic chromosomes of both sexes of *A. velikensis* were analysed using conventional and differential staining methods, with the aim of providing new data that will improve the knowledge on Tenebrionidae cytogenetics. We also sequenced the mt COI gene, for genetic identification of our *A. velikensis* specimens and barcoding of presented karyotype for further phylogenetic analysis.

MATERIALS AND METHODS

Specimens

Accanthopus velikensis specimens were collected from Pınarhisar, Kırklareli (41º46’02” N/27º37’51” E, 835 m). Adult beetles were collected on the trunks of trees at night when they are active.

Chromosome Analysis

Mitotic and meiotic chromosomes of 9 male and one female specimens were analysed using conventional and differential staining. Chromosome spreads were prepared from male and female gonads following the microspreading (Chandley et al. 1994) or splashing (Murakami and Imai 1974) methods with some modifications (Şendoğan and Alpagut Keskin 2016). The slides were stained with 4% Giemsa for 20 minutes for conventional staining. Silver impregnation technique of Patkin and Sorokin (1983) was performed to figure out the position of NOR regions. Chromosome spreads were examined and photographed with Zeiss Axio Scope A1 light microscope using ZEN software. The chromosomal measurements were obtained using the Levan plugin (Sakamoto and Zacaro 2009) and the karyotype was created with the CHIAS plugin (Kato et al. 2011) of the program Image J (Rasband 1997-2015). Heterochromatin distribution patterns were visualized with fluoroshield-DAPI (Sigma) specific to AT-rich chromosomal regions under Olympus BX50 fluorescent microscope.

mt COI barcoding

Genomic DNA was obtained from the thorax of the specimens using the Promega 96-well plate kit according to the manufacturer’s instructions. The mitochondrial cytochrome oxidase I (COI) gene was amplified using the primers JerryTen and PatTen (Papadopoulou et al. 2009) for genetic identification of *A. velikensis* specimens and barcoding of the karyotype. PCR products were purified and then sequenced in both directions. Sequencher 5.0 software was used to assemble and edit sequence chromatograms (Gene Codes, Ann Arbor, MI) and the COI sequences were submitted to GenBank for accession numbers. We performed a haplotype analysis using DnaSP v.5.10.1 (Rozas et al. 2017) and a BLAST search for all our sequences, in order to compare them with sequences deposited in GenBank.

RESULTS

We amplified the partial 829 bp sequences of cytochrome oxidase gene. Our specimens showed a unique haplotype for COI sequences with an 84-83% sequence similarity to database sequences for Tenebrionidae.

The cytogenetic analyses of spermatogonial and oogonial metaphase plates of *Accanthopus velikensis* revealed the diploid number to be 2n = 20, consisting of 2 pairs of metacentric and 7 pairs of submetacentric chromosomes (Figure 1-2, Table 1). While in male metaphase plates a minute subtelocentric *y* and a small submetacentric X chromosome appear as a heteromorphic pair (Figure 2b), no heteromorphism was observed among female metaphase plates (Figure 1a). X and *y* chromosomes are the smallest elements of the *A. velikensis* karyotype with the lengths of 2.434 µm and 0.759 µm, respectively (Table 1).

The observation of male metaphase I plates determined meioformula as 9 + Xy_p. The heteromorphic pair that composed the Xy_p was clearly observed in both conventionally and differentially stained male metaphase I plates (Figure 3a-c).

In diplotene/diakinesis of *A. velikensis*, 7 rod-shaped (terminal chiasma), and 3 ring-shaped (two terminal chiasmata) bivalents were observed (Figure 3d, 4d). In diakinesis/metaphase I; most of the homologous chromosomes

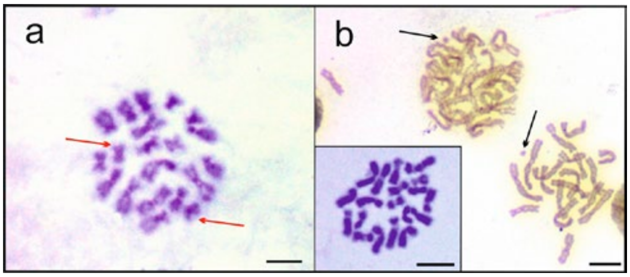

Fig. 1. (a) Oogonial metaphase (b) Spermatogonial metaphase of *Accanthopus velikensis*. Red and black arrows show X and minute y chromosomes respectively. Bars = 5µm.

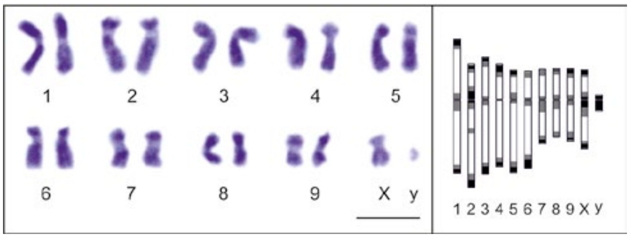

Fig. 2. Male karyotype and idiogram of *A. velikensis* 2n = 20. Bar = 5µm.

Table 1. Chromosome morphologies and measurements of *Accanthopus velikensis*. CI: centromere index, RL: relative length. (Centromere positions were determined according to Levan et al. 1964).

Chromosome	Length (µm)	%RL	CI	Morphology
1	4.999	13.7	45	M
2	4.466	12.2	28	SM
3	4.336	11.9	38	SM
4	3.771	10.3	28	SM
5	3.771	10.4	36	SM
6	3.553	9.8	30	SM
7	2.955	8.1	39	SM
8	2.782	7.6	44	SM
9	2.608	7.2	45	M
X	2.434	6.7	39	SM
y	0.759	2.1	23	ST

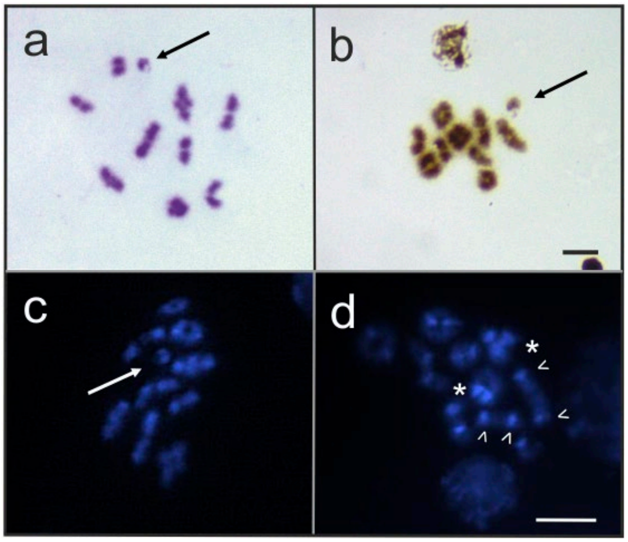

Fig. 3. Xy_p sex bivalents and heterochromatin in (a-c) Metaphase I, (a) Giemsa (b) Silver nitrate (c) DAPI staining (d) Diplotene-diakinesis (DAPI staining) Arrows show Xy_p sex bivalents, asterisk show heterochromatin. Arrowheads indicate telomeric signals on some of the rod shaped bivalents Bars = 5µm.

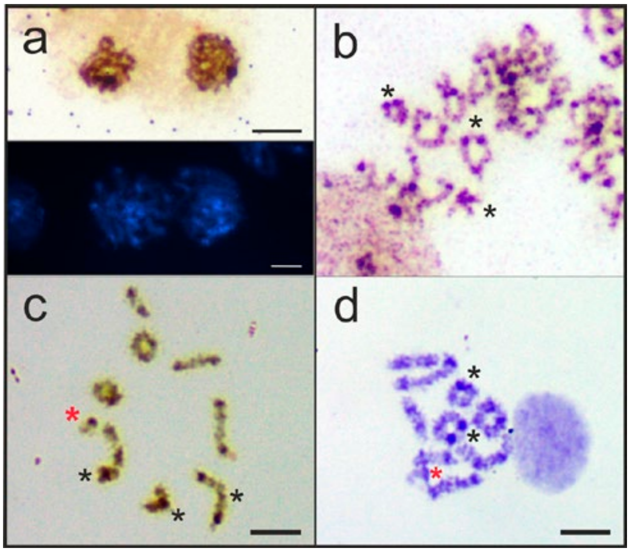

Fig. 4. NORs and heterochromatin in (a) Prophase I nuclei (silver and DAPI staining), (b) Diplotene-diakinesis, (c-d) pachytene after Silver (b-c) and Giemsa staining (d). Red asterisk indicate Xy_p and the presence of obvious signal in the long arm telomeric region of submetacentric X, black asterisks show differentially stained chromosome regions. Bars = 5µm (b-c possess the same scaling).

formed rod shaped bivalents and 1-2 cross-bivalents were also observed due to interstitial chiasma (Figure 3a–c).

In prophase nuclei, silver nitrate staining revealed the existence of a single impregnated mass of nucleolar material (Fig 4a). Additionally, obvious signals in the telomeric and pericentromeric regions of some autosomal pairs as well as Xy_p, were also observed in both silver nitrate and Giemsa stained diakinesis-metaphase I plates (Figure 4b-d). Giemsa staining of prophase nuclei indicated that all chromosomes of *A. velikensis* showed dark heterochromatic blocks mainly located in centromeric and pericentromeric regions (Figure 4d). Also with silver nitrate (Figure 4b-c) and DAPI staining (Figure 3d, 4a) rich telomeric and interstitial signals were observed in the large arms of most of the chromosomes.

In metaphase II plates, while some haploid sets seemed to be n = 9 due to minute *y* chromosome not being detectable (Figure 4b) the plates with the X chromosome showed the normal haploid number 10 (Figure 4a).

DISCUSSION

Due to predominant occurrence of the diploid number 2n=20 and parachute configuration of sex bivalents in the studied species, tenebrionid beetles considered as karyologically conservative group (Juan and Petitpierre 1988; 1991a; Juan et al., 1989; Palmer and Petitpierre 1997; Pons 2004). On the other hand, variations in sex chromosomes, NORs and heterochromatin distribution in spite of the shared modal number reveal that intrachromosomal rearrangements have played a major role in tenebrionid karyotype divergence (Juan et al. 1990; Almeida et al. 2000). The extent of diploid numbers between 14-38 within the family suggests that interchromosomal rearrangements such as Robertsonian processes or polyploidy could have also involved in karyotype evolution (Juan and Petitpierre 1991a; Petitpierre et al., 1991; Almeida et al. 2000; Pons 2004; Holecová et al. 2008; Lira-Neto et al. 2012; Goll et al. 2013).

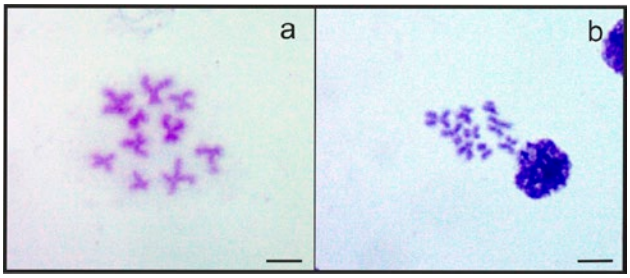

Fig. 5. (a.-b) Metaphase II plates (a) Haploid set with X chromosome (b) Haploid set with minute y chromosome which cannot be seen Bar = 5µm.

We showed that the karyotype of *A. velikensis* consists of 10 pairs of chromosomes (2n=20, Xy_p) which are mostly submetacentric (Figure 1, 2, 3 a-c). This formula (n=10, Xy_p) was reported for other Helopini species as well i.e. *Nesotes* (Juan and Petitpierre 1986, 1989, 1991a), *Nalassus* and *Turkonalassus* (Şendoğan and Alpagut Keskin 2016). Despite this general resemblance, presence of mostly submetacentric chromosomes slightly differentiate *A. velikensis* karyotype from other Helopini possessing predominantly metacentric chromosomes. Furthermore, relative lengths of sex bivalents are obviously different in present karyotypes of Helopini. While X chromosomes of *A. velikensis* and *N. plebejus* (Küster, 1850) show similar relative lengths (6.9 % and 6.55 % respectively), *T. bozdagus* (Keskin et Nabozhenko, 2010) have clearly larger X (13.74 % of total complement) which has a conspicuous secondary constriction on the long arm. However, diploid numbers reported for the other helopine genera *Nesotes* (2n=20, Xy_p) and *Euboeus* (2n=20, XY) are based only on male metaphase I plates (Juan and Petitpierre 1986, 1989, 1991a, 1991b), and do not allow detailed comparison of chromosome morphologies.

Studies on differential patterns of karyotypes in Tenebrionidae and some other coleopteran families revealed the occurrence of heterochromatic blocks in mainly pericentromeric regions and autosomal or sex chromosomal location of NORs (Juan and Petitpierre 1989; Juan et al. 1993; Pons 2004; Rozek et al. 2004; Schneider et al. 2007; Holecová et al. 2008; Karagyan et al. 2012; Goll et al 2013; Şendoğan and Alpagut Keskin 2016). The presence of heterochromatin blocks on pericentromeric regions of *A. velikensis* chromosomes was demonstrated with both $AgNO_3$ and DAPI staining (Figure 3d, 4). Additionally, telomeric signals on some rod shaped bivalents as well as the X chromosome (Fig 3d, 4 b-d) were detected. Our results showed that even a single NOR site was present in prophase I nuclei (Figure 4a), chromosomes in diakinesis-metaphase I plates gave multiple signals (Figure 4b-d). Therefore, further testing of exact NOR locations with rDNA-FISH probes is required to determine whether these signals are directly associated with NORs or a result of heterochromatin condensation.

In conclusion, karyotype of *A. velikensis* resemble those of other Helopini members and follows the common patterns of tenebrionid karyotypes with slight differences in chromosome morphologies, NORs and heterochromatin distribution. To truly understand these specific patterns of *A. velikensis* karyotype, comparative molecular cytogenetic studies with related taxa is required. In order to broaden the knowledge on the chromosomal evolution of tribe Helopini and assess the situation/position of *A. velikensis* within the tribe, cytogenetic studies should be combined with molecular phylogenetic analyses as well.

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