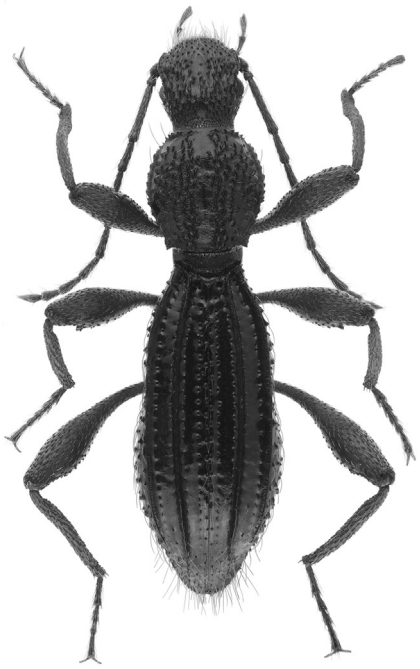
РОССИЙСКАЯ АКАДЕМИЯ НАУК
Южный научный центр

RUSSIAN ACADEMY OF SCIENCES
Southern Scientific Centre

Кавказский Энтомологический Бюллетень

CAUCASIAN ENTOMOLOGICAL BULLETIN

Том 19. Вып. 1
Vol. 19. Iss. 1



Ростов-на-Дону
2023

Cytogenetic analysis on *Turkonalassus quercanus* Keskin, Nabozhenko et Alpagut-Keskin, 2017 (Coleoptera: Tenebrionidae: Helopini)

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Faculty of Science, Department of Zoology, Section of Biology, Ege University, İzmir 35100 Turkey. E-mail: utkucalisan@gmail.com, nursen.alpagut@ege.edu.tr

Abstract. Cytogenetic features of the endemic Western Anatolian tenebrionid species *Turkonalassus quercanus* Keskin, Nabozhenko et Alpagut-Keskin, 2017 were analyzed using conventional and differential staining. Chromosome preparations were obtained from the gonads of both males and females. The karyotype of *T. quercanus* was found to be $2n = 20 (9 + Xy_p)$, which is considered the modal number for Tenebrionidae. The heteromorphic sex chromosomes of *T. quercanus* form a parachute like bivalent at metaphase I (MI) of male meiosis. Both conventional and differential staining have shown that predominantly metacentric chromosomes of *T. quercanus* exhibit a typical pericentromeric heterochromatin pattern. As per results of the silver staining, the existence of a prominent nucleolus at prophase I and a highly impregnated area associated with Xy_p at MI are indicated the sex chromosomal location of NOR. In comparison with previously published cytogenetic data on other species of the tribe Helopini which are presenting the same karyotype formula, our results suggest that a series of chromosomal rearrangements may have been involved in their karyotype evolution.

Key words: cytogenetics, Tenebrionidae, Helopini, *Turkonalassus*, *Nalassus*, sex chromosomes, NOR, heterochromatin.

Цитогенетический анализ *Turkonalassus quercanus* Keskin, Nabozhenko et Alpagut-Keskin, 2017 (Coleoptera: Tenebrionidae: Helopini)

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Факультет наук, отделение зоологии, секция биологии, Эгейский университет, Измир 35100 Турция. E-mail: utkucalisan@gmail.com, nursen.alpagut@ege.edu.tr

Резюме. Цитогенетические признаки эндемичного западноанатолийского жука-чернотелки *Turkonalassus quercanus* Keskin, Nabozhenko et Alpagut-Keskin, 2017 были проанализированы с использованием обычного и дифференциального окрашивания. Хромосомные препараты получали из гонад как самцов, так и самок. Кариотип *T. quercanus* равен $2n = 20 (9 + Xy_p)$, что считается модальным числом для Tenebrionidae. Гетероморфные половые хромосомы *T. quercanus* формируют ассоциацию «парашют» в мейотической метафазе I (MI) у самца. Как обычное, так и дифференциальное окрашивание показало, что преимущественно метацентрические хромосомы демонстрируют типичный паттерн перицентромерного гетерохроматина. По результатам окрашивания серебром наличие заметного ядрышка в профазе I и сильно импрегнированного участка, связанного с Xy_p в MI, указывает на локализацию ядрышковых организаторов в половых хромосомах. По сравнению с ранее опубликованными цитогенетическими данными по другим видам трибы Helopini, имеющим ту же формулу кариотипа, наши результаты позволяют предположить, что в эволюции их кариотипа могла быть задействована серия хромосомных перестроек.

Ключевые слова: цитогенетика, Tenebrionidae, Helopini, *Turkonalassus*, *Nalassus*, половые хромосомы, ядрышковые организаторы, гетерохроматин.

Introduction

The genus *Turkonalassus* Keskin, Nabozhenko et Alpagut-Keskin, 2017 comprises cold adapted, lichen-feeding tenebrionid beetles. Most of the *Turkonalassus* species have been described from subalpine or alpine habitats throughout Anatolian high-mountain ranges [Keskin et al., 2017; Nabozhenko et al., 2021]. All *Turkonalassus* species, except *Turkonalassus macedonicus* Keskin, Nabozhenko et Alpagut-Keskin, 2017 found in Greece and Bulgaria, are endemics of Turkey [Keskin, Nabozhenko, 2010; Keskin et al., 2017; Nabozhenko et al., 2021]. All species of this genus are allopatric and strongly isolated geographically from each other. One of the interesting features is the obligatory presence of creeping shrubs of Juniperus communis L., 1753 in the habitats of *Turkonalassus* species [Nabozhenko et al., 2021]. Only one Anatolian species, *T. quercanus*, is associated with oak forests (Quercus cerris L., 1753) without juniper shrub [Keskin et al., 2017].

The genus *Turkonalassus*, while possessing certain *Nalassus* Mulsant, 1854 (Coleoptera: Tenebrionidae) characters like structure of epipleura, aedeagus and female genital tubes, differentiates from it by the ventral side of head structures, and absence of the hairbrush on abdominal ventrites which are typical for many *Nalassus* species [Keskin et al., 2017]. These two genera of the tribe Helopini were also determined as two separate lineages based on phylogenetic analyses of MP20 and COI sequences, which are consistent with patterns of their morphological differentiation and geographic distributions (B. Keskin et al., unpublished data). In most cases, Anatolian *Turkonalassus*

Research Article / Научная статья
DOI: 10.23885/181433262023191-1521

and *Nalassus* members appear as high-altitude species, found in rocky and forested environments, with strong endemism [Keskin, Nabozhenko, 2010; Keskin et al., 2017].

Turkonalassus quercanus, endemic to relatively small Western Anatolian Sultan Mountain Range, indicates strong relations with the genus *Nalassus*. This species is differentially diagnosed by partly (but better, than in other species) developed hind wings, the structure of the aedeagus and the pronotum, otherwise morphologically similar to *T. adimonius* (Allard, 1876) and *T. pineus* Keskin, Nabozhenko et Alpagut-Keskin, 2017. In phylogenetic analysis, MP20 and COI trees revealed that *T. quercanus* is close to *T. petrophilus* Keskin, Nabozhenko et Alpagut-Keskin, 2017 (B. Keskin et al., unpublished data).

Considering Tenebrionidae is one of the larger families of Coleoptera, the group is severely understudied cytogenetically, and little is known about their karyotype evolution. Karyotypes of about 250 tenebrionid species are determined so far in the subfamilies Alleculinae, Diaperinae, Lagriinae, Pimelinae and Tenebrioninae [e.g., Holecová et al., 2008; Juan, Petitpierre, 1991a; Blackmon, Demuth, 2015; Gregory, 2023]. These studies cover a small portion of the family. Diploid number within Tenebrionidae is mostly $2n = 20$, but it varies greatly; changing from $2n = 14$ to $2n = 38$ [Juan, Petitpierre, 1991a; Pons, 2004; Holecová et al., 2008; Lira-Neto et al., 2012]. Genera *Nalassus* and *Turkonalassus* are not exempt from the said great research gap. The only chromosome study of these two genera has been performed with *Turkonalassus bozdagus* (Keskin et Nabozhenko, 2010) (originally described in the genus *Nalassus*) and *N. plebejus* (Küster, 1850) [Şendoğan, Alpagut-Keskin, 2016]. Major karyological differences concerning centromere positions, heterochromatin distribution, NOR localization and the properties of the X chromosomes were revealed between these two species [Şendoğan, Alpagut-Keskin, 2016].

The aim of this study is to obtain the first cytogenetic data on the endemic Western Anatolian species *Turkonalassus quercanus* using both female and male specimens. To investigate the extent of cytogenetic variations in the tribe Helopini, the specific patterns obtained for *T. quercanus* karyotype were also compared with previously published cytogenetic data on the tribe in general.

Material and methods

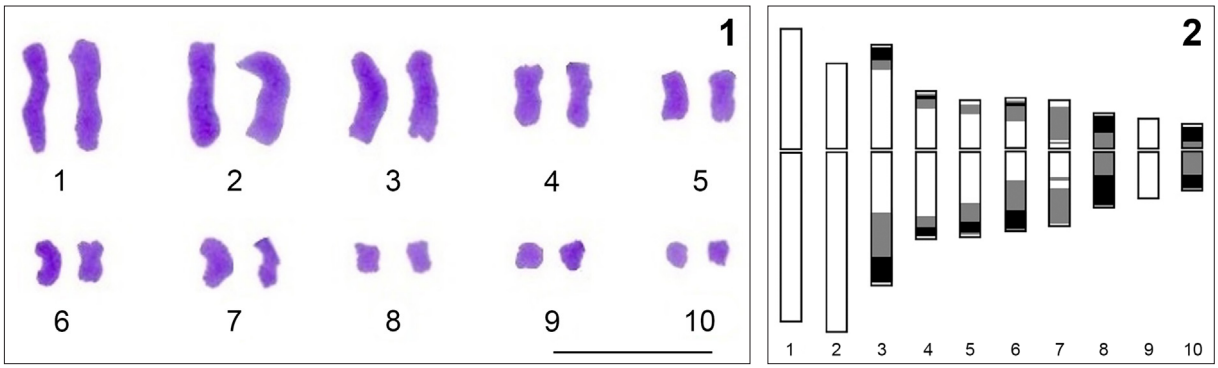
The specimens of *Turkonalassus quercanus* were retrieved from Sultandağı, Afyonkarahisar (38°27′41″N / 31°15′36″E, 1510 m; 20 males and 17 females) and Akşehir, Konya (38°20′39″N / 31°22′58″E, 1545 m; 11 males and 4 females), on Quercus cerris trunks. Adult beetles were collected after the dusk, during their active early-night period.

Two methods were applied for the gonad preparation: microspreading method [Chandley et al., 1994] and splashing method [Murakami, Imai, 1974], with some modifications. Dissected gonads were treated with Stenman's hypotonic solution [Stenman et al., 1975] (5 min for males, 10 min for females) and fixed in 3 : 1 ethanol: acetic acid for at least 30 min in –20 °C. Gonads were macerated with sterilized needles before the application of the methods.

The slides were stained with 4% Romanowsky – Giemsa diluted with Gibco Gurr's phosphate buffer pH 6.8, for 20 min. For the determination of the NORs, silver impregnation method was applied [Patkin, Sorokin, 1983]. Dehydrated slides were counterstained with 4% Romanowsky – Giemsa after the silver impregnation. Vectashield Antifade Medium with DAPI (H-1200) was used to determine AT rich heterochromatic regions. DAPI stained slides were examined with Olympus BX53 fluorescent microscope. Other slides were analyzed and photographed with Zeiss Axioscope light microscope using ZEN software. The chromosomal measurements were carried out with the LEVAN plugin [Sakamoto, Zacaro, 2009] of the Image J software [Schneider et al., 2012]. CHIAS plugin [Kato et al., 2011] was used for the creation of the karyotype and the idiogram.

Results

Diploid chromosome number was revealed as $2n = 20$ while the chromosomal formula $9 + Xy_p$ was determined in the analysis of the spermatogonial and oogonial cells. Karyotype and idiogram were constructed using female cells (Figs 1, 2). Oogonial chromosome morphology



Figs 1–2. *Turkonalassus quercanus* mitotic chromosomes, female.
1 – karyotype; 2 – ideogram. Scale bar 5 μm.
Рис. 1–2. Митотические хромосомы *Turkonalassus quercanus*, самка.
1 – кариотип; 2 – идеограмма. Масштабная линейка 5 μm.

Table 1. Chromosome morphologies and measurements of *Turkonalassus quercanus* (female).
Таблица 1. Морфология хромосом и измерения кариотипа *Turkonalassus quercanus* (самка).

Chromosome Хромосома	Length (μm) Длина (μm)	CI	%RL	AR	Morphology Морфология
1	3.390	42	18.2	1.38	m
2	3.043	39	16.4	1.56	sm
3	2.804	43	15.1	1.44	m
4	1.934	43	10.4	1.34	m
5	1.586	45	8.5	1.25	m
6	1.478	42	7.9	1.41	m
7	1.444	47	7.7	1.11	m
8	1.075	42	5.7	1.46	m
9	0.989	41	5.3	1.46	m
10	0.804	46	4.3	1.17	m

Note. CI – centromere index; RL – relative length; AR – arm ratio; m – metacentric; sm – submetacentric.
Примечание. CI – центромерный индекс; RL – относительная длина; AR – соотношение плеч; m – метацентрический; sm – субметацентрический.

predominantly exhibited metacentric character (Table 1). The 2nd chromosomal pair was submetacentric, while the rest were metacentric. The largest chromosome was measured as 3.390 μm, while the shortest one was 0.804 μm in length (Table 1). The chromosome set appeared suitable to be handled in three main length groups including three relatively large pairs, four middle-length pairs, and three small pairs.

Female and male prophase I pachytene nuclei appeared to have heterochromatin blocks in all chromosomes (Figs 3–6) whereas no specific heterochromatin area was observed in the female mitotic metaphase chromosomes (Fig. 1). Females, having homomorphic sex chromosomes, showed large pericentromeric heterochromatin blocks on some pairs, and smaller heterochromatin areas on the rest (Fig. 3). Male prophase exhibited supporting patterns (Fig. 4). A medium-sized chromosome in the male metaphase that cannot be paired was considered as possible X chromosome (Fig. 5). The parachute like bivalent formation of X and y chromosomes was observed in male MI (Fig. 6).

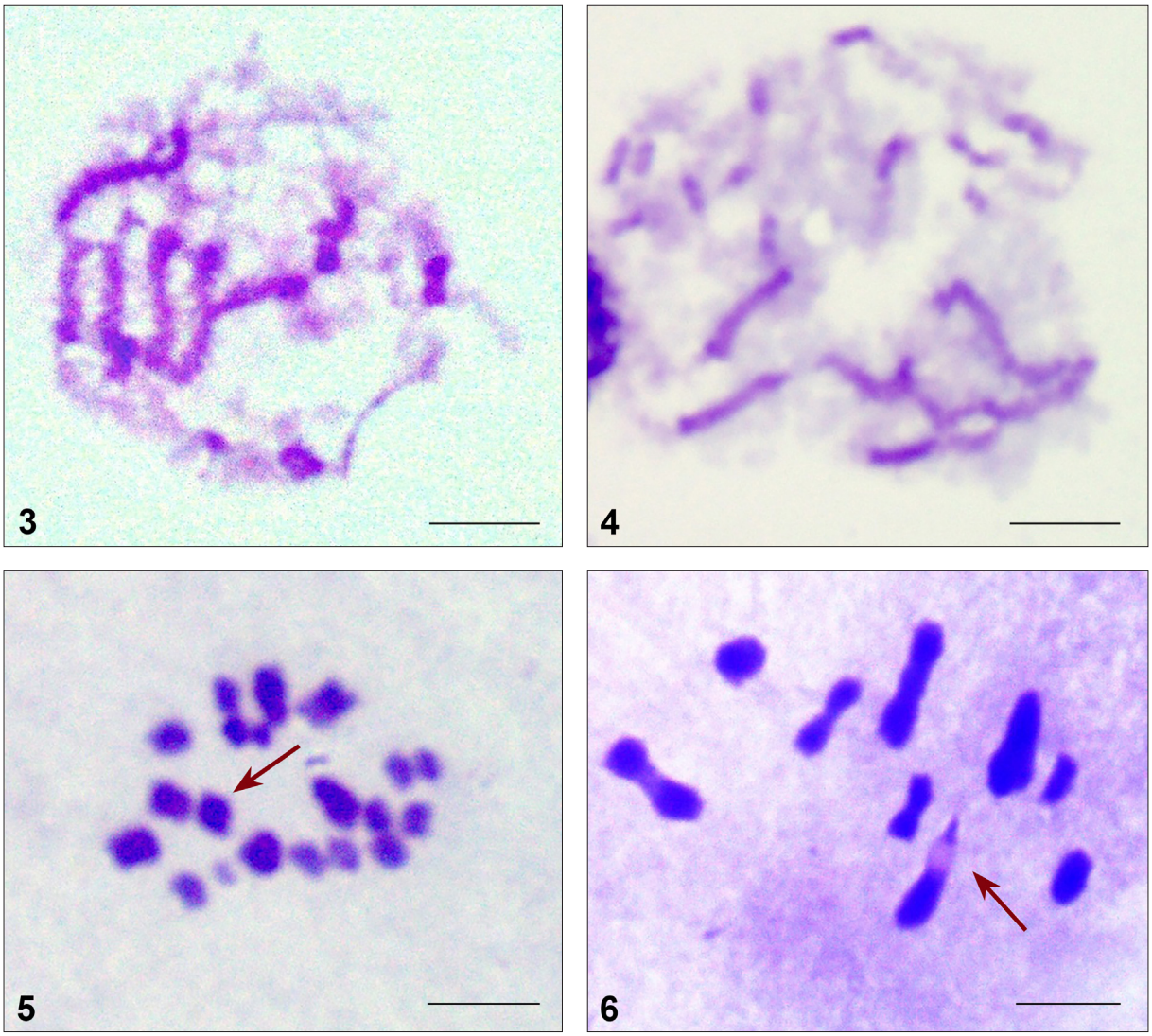
With silver nitrate staining, highly impregnated pericentromeric heterochromatin regions and prominent nucleolus were observed in prophase I plates (Fig. 8). At the male MI, NORs related to the Xy_p sex bivalent are presented (Fig. 7). Pericentromeric location of AT rich heterochromatic regions of both pachytene and metaphase chromosomes were observed with fluorescent DAPI staining (Figs 9, 10).

Discussion

Tenebrionid species with a diploid chromosome number of $2n = 20$ and Xy_p sex determining system are very frequent [Juan, Petitpierre, 1991a; Palmer, Petitpierre, 1997; Pons, 2004]. However, their karyotypes can show great differences in diploid number, chromosome morphology, heterochromatin distribution, and sex determining systems [Juan, Petitpierre, 1990, 1991a, b; Petitpierre et al., 1991; Juan et al., 1993; Bruvo-Mađarić et al., 2007].

Cytogenetic data on the tribe Helopini are only known for some *Nesotes* Allard, 1876 [Juan, Petitpierre, 1986, 1989, 1991a, b], *Euboeus* Boieldieu, 1865 [Palmer, Petitpierre, 1997], *Turkonalassus*, *Nalassus* [Şendoğan, Alpagut-Keskin, 2016], *Accanthopus* [Şendoğan et al., 2019] and *Helops* Fabricius, 1775 [Öğren, 2018] species. However, male MI plates reported for the genera *Nesotes* ($2n = 20$, Xy_p) and *Euboeus* ($2n = 20$, XY) [Juan, Petitpierre, 1986, 1989, 1991a, b] do not allow detailed comparison of chromosome features. Measurements in *T. quercanus* demonstrate a chromosome set ranging between 0.804 and 3.390 μm. In comparison with existing mitotic chromosome measurements of other Helopini species, such as *Turkonalassus bozdagus* (1.097–4.315 μm), *Nalassus plebejus* (1.010–4.442 μm), *Helops glabriventris* Reitter, 1885 (0.78–4.57 μm) and *Accanthopus velikensis* Piller et Mitterpacher, 1783 (0.759–4.999 μm), *Turkonalassus quercanus* chromosomes are revealed to be quite short. While all chromosomes except one submetacentric pair are metacentric in *T. quercanus* (Table 1), previous studies have shown that Helopini karyotypes may have a variable number of metacentric, submetacentric, and subtelocentric elements. A variability of chromosome morphology has already been reported for several Coleopteran families such as Cicindelidae, Chrysomelidae, Meloidae, Scarabaeidae and Tenebrionidae [Serrano, 1981; Petitpierre, 1983; Juan et al., 1990; DeAlmeida et al., 2000; Petitpierre, Garnería, 2003; Wilson, Angus, 2005; de Julio et al., 2010; Petitpierre, 2011].

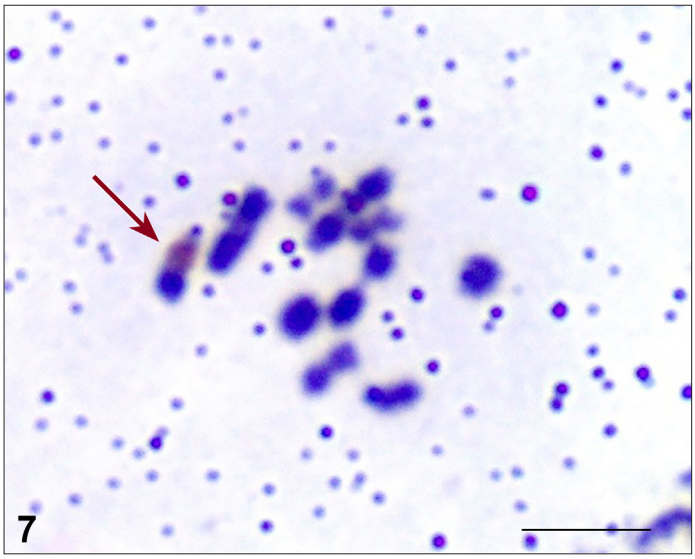
In the majority of the tenebrionid species, the pericentromeric regions of the chromosomes typically have distinctive dark blocks [Juan, Petitpierre, 1989; Juan et al., 1990; DeAlmeida et al., 2000; Moura et al., 2003; Pons, 2004; Goll et al., 2013; Şendoğan, Alpagut-Keskin, 2016]. However, more complex patterns are also known [Juan, Petitpierre, 1989; Dutrillaux et al., 2006]. These heterochromatic regions, which have mostly AT rich sequences, may differ both in size and sequence composition among tenebrionid karyotypes [Juan et al., 1993; Plohl et al., 1993; Ugarković et al., 1994; Pons et al., 2002, Goll et al., 2013]. The presence of large pericentromeric heterochromatin blocks on all chromosomes of *T. quercanus* has been confirmed using conventional (Figs 3, 4) and differential staining (Figs 8, 10). Although, a similar pericentromeric heterochromatin pattern is reported for *T. bozdagus* [Şendoğan, Alpagut-

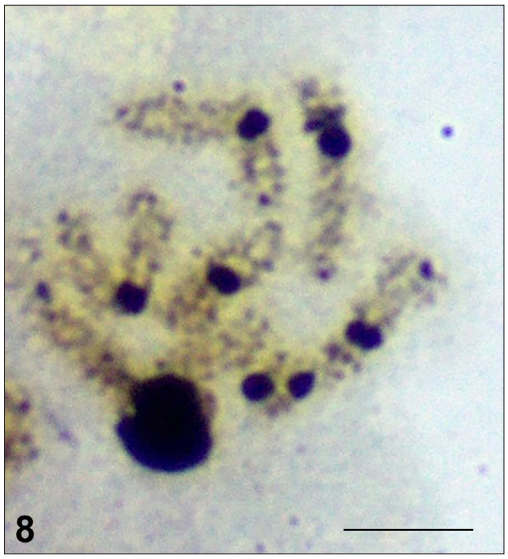


Figs 3–6. Romanowsky–Giemsa-stained meiotic chromosomes of *T. quercanus*.
3 – pachytene chromosomes, female; 4 – the same, male; 5 – male metaphase to anaphase (arrow indicate X chromosome) 6 – male MI chromosomes (arrow indicate Xy_p heteromorphic pair). Scale bars 5 μm.
Рис. 3–6. Мейотические хромосомы *Turkonalassus quercanus*, окрашенные по Романовскому – Гимзе.
3 – хромосомы пахитены, самка; 4 – то же, самец; 5 – от метафазы к анафазе, самец (стрелка указывает на Х-хромосому); 6 – MI хромосомы, самец (стрелка указывает на Xy_p гетерохроматическую пару).

Keskin, 2016] and *Accanthopus velikensis* [Şendoğan et al., 2019], variations in the sizes of the heterochromatin blocks, and presence of additional telomeric blocks on *A. velikensis* chromosomes differentiate karyotypes of these Helopini species (Figs 3, 4) [Şendoğan, Alpagut-Keskin, 2016: figs 2a, b]. However, some other Helopini species have a very different heterochromatin distribution pattern [Şendoğan, Alpagut-Keskin, 2016; Şendoğan et al., 2019; Öğren, 2018]. The presence of relatively small amounts of heterochromatin dispersed throughout the whole length of *Helops glabriventris* and *Nalassus plebejus* chromosomes implies that the changes in the heterochromatin amount and distribution may have played important roles in the chromosomal evolution of Helopini.

Silver impregnation method is used for the detection of the nucleolus organizer regions [Howell, Black, 1980; Patkin, Sorokin, 1983]. With this method, proteins present in the area where transcriptionally active ribosomal DNA exists can be detected [Goodpasture, Bloom, 1975]. NORs can be located in various places on chromosomes, in different species [Juan et al., 1993; Vitturi et al., 1999; Colomba et al., 2000; Bione et al., 2005a, b; Pons, 2004; Rożek et al., 2004; Schneider et al., 2007; Holecová et al., 2008; Karagyan et al., 2012; Lira-Neto et al., 2012; Goll et al., 2013; Öğren, 2018; Şendoğan et al., 2019]. The connection of NORs with sex bivalents has been shown in other tenebrionid studies [Juan et al., 1993; Wolf, 1997; Vitturi et al., 1999; DeAlmeida et al., 2000; Şendoğan, Alpagut-Keskin, 2016]. *Turkonalassus quercanus* MI plates impregnated with silver nitrate showed NORs connected to sex chromosomes (Figs 9, 10). Sex bivalent related NORs are also known in *T. bozdagus* [Şendoğan, Alpagut-Keskin,

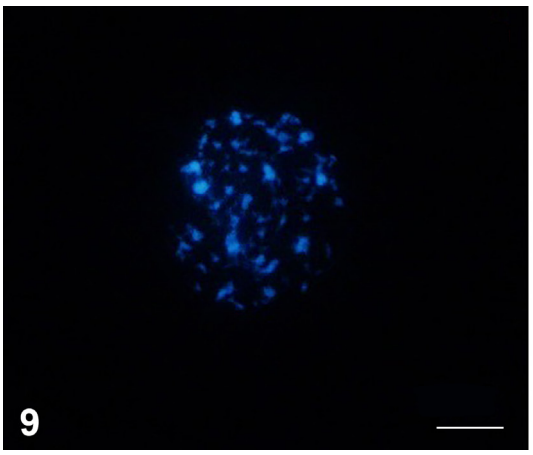


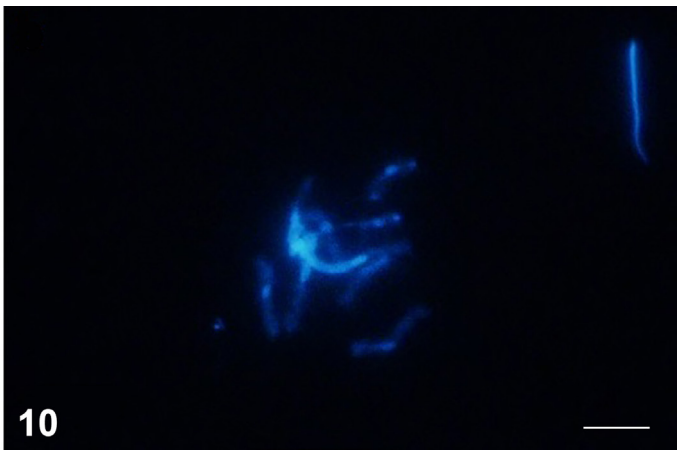


Figs 7–8. Silver nitrate-stained *Turkonalassus quercanus* meiotic chromosomes.
7 – Xy_p sex bivalent in male MI plate (arrow indicates argyrophilic sex bivalent); 8 – a prominent nucleolus associated with one of the medium sized chromosomes, and pericentromeric heterochromatin regions seen as smaller dots in prophase I chromosomes. Scale bars 5 μm.
Рис. 7–8. Мейотические хромосомы *Turkonalassus quercanus*, окашенные нитратом серебра.
7 – половой бивалент Xy_p на пластинке MI самца (стрелка указывает на аргирофильный половой бивалент); 8 – заметное ядрышко, связанное с одной из хромосом среднего размера, и перицентромерные области гетерохроматина в виде более мелких точек в хромосомах профазы I. Масштабные линейки 5 μm.

2016]. On the other hand, while autosomal localization of NORs was reported for *Helops glabriventris*, the potential NORs of *Nalassus plebejus* and *Accanthopus velikensis* were observed only in prophase I nuclei.

A brief comparison of karyotypes of *T. quercanus* and *T. bozdagus* allow us to identify major cytogenetic differences that may provide valuable information about their divergence process. These two congeneric species exhibit differences in chromosome lengths, number of metacentric/submetacentric chromosomes and sex bivalents (Table 2). The differences found in chromosome lengths and morphology between these two *Turkonalassus* species are thought to be related to pericentromeric inversions that resulted in centromeric shift. This type of pericentromeric rearrangements were already reported for several Tenebrionid species [Juan et al., 1990; DeAlmeida et al., 2000; Şendoğan, Alpagut-Keskin, 2016]. Although there is no direct measurement of *T. quercanus* X chromosome due to missing male set suitable to karyotype construction, *T. quercanus* X chromosome clearly differs from *T. bozdagus* X which is the largest element of the karyotype (Fig. 5). Additionally, appearance of a prominent secondary constriction on the long arm of the giant X chromosome in *T. bozdagus*, is not evident for *T. quercanus* metaphase chromosomes. The differences in relative length of X chromosomes between closely related species generally





Figs 9–10. AT rich heterochromatin regions in *T. quercanus*, male.
9 – mitotic metaphase; 10 – pachytene. Scale bars 10 μm.
Рис. 9–10. Богатые AT гетерохроматиновые области у *T. quercanus*, самец.
9 – митотическая метафаза; 10 – пахитена. Масштабные линейки 10 μm.

Table 2. Cytogenetic properties of two species of *Turkonalassus*.
Таблица 2. Цитогенетические характеристики двух видов *Turkonalassus*.

Parameter Параметр	*T. quercanus*	*T. bozdagus**
Chromosome length Длина хромосом	0.804–3.390 μm 3 large, 4 medium and 3 small 3 крупных, 4 средних, 3 маленьких	1.097–4.315 μm gradually decreasing постепенно уменьшающиеся
Chromosome morphology Морфология хромосом	9 metacentric, 1 submetacentric / 9 метацинтрических, 1 субметацентрическая	7 large metacentric, 3 submetacentric / 7 крупных метацентрических, 3 субметацентрических
Sex bivalents Половые биваленты	Xy_p	giant Xy_p гигантский Xy_p
NOR localization Локализация ядрышковых организаторов	sex bivalent половой бивалент	sex bivalent половой бивалент
Heterochromatin Гетерохроматин	centromeric or pericentromeric / центромерный или перицентромерный	centromeric or pericentromeric центромерный или перицентромерный
Secondary constriction Вторичное сужение	non apparent or existent не выражено или представлено	on the long arm of the X chromosome на длинном плече Х-хромосомы

Note. * – data from Şendoğan and Alpagut-Keskin [2016].
Примечание. * – данные по [Şendoğan, Alpagut-Keskin, 2016].

thought to be derived from either heterochromatin amplification or translocation [Juan, Petitpierre, 1989; Dutrillaux, Dutrillaux, 2009].

In conclusion, cytogenetic data of *T. quercanus* presented here revealed that its karyotype shows the similar pattern observed in most of the Tenebrionid species, with slight differences. The differences in the chromosome lengths and morphology between helopine species presenting an identical formula suggest that a series of chromosomal rearrangements such as pericentromeric inversions, unequal reciprocal translocations, chromosomal shifts, and changes in the amount of constitutive heterochromatin were involved in their karyotype evolution. Therefore, in further studies, the identification and chromosomal mapping of genes or specific sequences that may have played important roles in the tenebrionid speciation are needed. To better understand the tenebrionid karyotype evolution, comparative molecular cytogenetic analysis should be conducted on closely related species groups in major tenebrionid lineages.

Acknowledgements

We feel deeply grateful to Dr Maxim Nabozhenko and Dr Bekir Keskin as we are able to benefit from their knowledge on Helopini. We are thanking to Dirim Şendoğan and Coşku Öğren for their help in both laboratory and the field work. We also would like to express our gratitude towards Ege University Institute of Nuclear Sciences Cell Culture and Cell Imaging Laboratory.

This study was supported by Ege University Scientific Research Projects (17-FEN-033).

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Received / Поступила: 29.09.2022
Accepted / Принята: 9.01.2023
Published online / Опубликована онлайн: 25.01.2023