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ORIGINAL ARTICLE

Cytogenetic studies on three tenebrionid beetles, *Tenebrio molitor*, *Alphitobius diaperinus* and *Zophobas morio* (Coleoptera: Tenebrionidae): An overview and new data

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Abstract. Here, a comprehensive cytogenetic analysis of three species of tenebrionid beetles: *Alphitobius diaperinus*, *Tenebrio molitor* and *Zophobas morio*, is presented. This paper also contains a review of the cytogenetic information for each species and adds new data. The male karyotype of both *T. molitor* and *Z. morio* is 2n = 18 + Xy_p, with large heterochromatic blocks in the pericentromeric regions of all chromosomes. The male *A. diaperinus* karyotype is 2n = 18 + X0, also with heterochromatic pericentromeric regions in all chromosomes. The location of the nucleolar organizer regions (NORs) differs in each species: in *A. diaperinus*, it is located on the smallest pair of autosomes, while in *T. molitor,* it is on two pairs of autosomes and both sex chromosomes. In contrast, it is exclusively located on the X chromosome in *Z. morio*. Telomere analysis revealed that all species have TCAGG repeats in their telomeres, but lack the canonical TTAGG insect telomeric motif. In addition, a study of the distribution of satellite DNA and composition revealed that each species has a main satellite DNA family forming the pericentromeric heterochromatin. Fluorescence in situ hybridization of each of these satellites did not produce hybridization signal in the other two species, indicating a divergence in repetitive DNA composition among them. This study adds to the understanding of chromosomal organization, heterochromatin distribution and repetitive DNA dynamics in tenebrionid beetles and sheds light on their cytogenetic diversity and evolutionary significance.

INTRODUCTION

The species *Tenebrio molitor*, Linnaeus, 1758, *Alphitobius diaperinus*, Panzer 1797 and *Zophobas morio*, Fabricius, 1776 belong to the family Tenebrionidae (Coleoptera, Polyphaga), one of the largest beetle families with probably more than 30,000 described species (Bouchard et al., 2021). *T. molitor*, commonly known as the mealworm beetle, is widely recognized and extensively studied due to its significant ecological roles and as a model organism in various research fields (Matyja et al., 2020; Rankic et al., 2021; Czarniewska et al., 2023). Moreover, these three species are of economic importance as they are frequently used as food for pets, such as reptiles, birds and other insectivorous animals. Recently their potential as food for both humans and animals has been studied (Rumbos & Athanassiou, 2021; Errico et al., 2022; Rigopoulou et al., 2023). The use of insects as a source of food is increasing, according to the FAO (2021), due to increasing costs and environmental effect associated with the production of animal protein, along with concerns over food insecurity in specific regions and global population growth. Thus, it is crucial to explore alternatives to traditional livestock and commonly consumed meat products. Currently, the European Food Safety Authority (EFSA) has approved four insect species, including two tenebrionids, *T. molitor* and *A. diaperinus*, for human consumption (European Commission, 2023). This endorsement highlights the potential of insects as a sustainable and nutritious food source for humans.

Of the described tenebrionids, the chromosome number is only known for approximately 250 species (Blackmon & Demuth, 2015). The diploid chromosome numbers range from 2n = 14 reported in four species: *Arthromacra aenea* Say, 1824 and *Isomira variabilis* Horn, 1875 (Smith & Virkki, 1978), *Diaperis boleti* Linnaeus, 1758 (Juan & Petitpierre, 1986) and *Scotobius miliaris* Billberg, 1815

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(Vidal, 1984) to 2n = 38, reported in *Blaps gibba* Laporte, 1840 (Vitturi et al., 1996), with 2n = 20 being the most common in the species studied and the modal karyotype in Coleoptera**.** Within the Tenebrionidae, several genera in the subfamilies Tenebrioninae and Pimelinae have been the subject of numerous studies on the composition, location and evolution of satDNA (satDNA) (Petitpierre et al., 1995; Ugarković et al., 1995; Meštrović et al., 1998; Pons et al., 2004; Mravinac & Plohl, 2010; Pavlek et al., 2015; Gržan et al., 2023; among others). These species are characterized by the presence of a high amount of satellite DNA mainly located in the pericentromeric regions of the chromosomes, which in some species can reach up to 50% of the genome (Juan & Petitpierre, 1989).

The aim of this paper was to review and add new data on karyotypes, organization of the heterochromatin, telomeres and arrangement of rDNA genes in three species in the Tenebrioninae subfamily (*T. molitor*, *A. diaperinus* and *Z. morio*) and contributing to the understanding of their cytogenetic diversity, chromosomal organization and evolutionary importance.

MATERIAL AND METHODS

Material collection and chromosome preparations

Specimens of both pupae and adult males of *A. diaperinus*, *T. molitor* and *Z. morio* were utilized in this study. Living specimens were acquired from ARTROPOSFERA (https://artroposfera.es/).

Chromosome preparations were obtained from the males. Testes from both the pupal and adult stages were carefully extracted, immersed in distilled water for 45 min to induce an osmotic shock and then preserved in absolute ethanol : glacial acetic acid solution (3 : 1). For chromosome preparations, testes were macerated in 50% glacial acetic acid. The resulting mixture was spread in droplets on a glass slide and placed on a hot plate at 42°C until the slides were dry. Following this, the slides were dehydrated using an ethanol series (70%, 80% and 100%, each for 30 s) and were subsequently stored at –20°C until utilized. Chromosome spreads were stained with Giemsa or mounted with VECTASHIELD containing DAPI (4'-6-diamino-2-phenylindole) fluorochrome (Vector Labs, Burlingame, CA, USA) before examining under an Olympus (Hamburg, Germany) BX51 fluorescence microscope equipped with an Olympus DP70 camera. Photographs were taken and processed using the DP Manager and Adobe Photoshop CS4 software (Adobe Systems, San Jose, CA, USA).

C-banding

The heterochromatin blocks were visualized using C-banding, following the protocol described by Sumner (1972) with some modifications. The slides were initially treated with a 0.2 M hydrochloric acid solution for 10 min at 25°C. Then incubated in a 5% barium hydroxide solution at 60°C for 1 min and 50 s, then washed with water, followed by a brief rinse in the initial hydrochloric acid solution and a final wash in 2 × SSC at 60°C for 2 min. Finally, the slides were stained with DAPI at a concentration of 0.75 µg/mL and mounted with VECTASHIELD.

Extraction of genomic DNA and isolation of satellite DNA using restriction endonucleases

Main satDNAs from *T. molitor* and *A. diaperinus* were previously isolated (Petitpierre et al., 1988; Plohl & Ugarković, 1994a). Previously no satDNA families were reported for *Z. morio*. Therefore, in this study, digestion with restriction endonucleases was used to characterize the satDNAs of *Z. morio.*

Z. morio total genomic DNA (gDNA) was extracted from the heads and legs of males using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA), according to the manufacturer's protocol. DNA was digested overnight using several restriction endonucleases using 4 U/µg DNA. The restriction endonucleases used were those present at the multiple cloning site of the pUC19 plasmid (*Hind*III, *Sph*I, *Pst*I, *Sal*I, *Xba*I and *Bam*HI). Fragments of about 300 and 600 bp, generated by digestion with *Sph*I (Thermo Fisher Scientific, Waltham, MA, USA), were eluted from the agarose gel and ligated into the pUC19 vector that was linearized by digestion with *Sph*I. Ligation reactions were used to transform competent *Escherichia coli* Escherich, 1885 DH5α bacteria (Zymo Research, Orange, CA, USA). Recombinant cells were selected from colonies grown on LB/ampicillin/IPTG/X-Gal plates. Both strands of the recombinant plasmids were sequenced using the universal primers SP6 and T7. The identified satDNA family was called Zmorio-S. Multiple-sequence alignments of the identified repeats were performed using the BioEdit Sequence Alignment Editor (Hall, 1999). The sequence data were analysed and compared with the DNA databases using NCBI's BLAST.

Probes and fluorescence in situ hybridization (FISH)

The location of nucleolar organizer regions (NORs) on chromosomes was mapped using the *Drosophila melanogaster* Meigen, 1830 plasmid pDmra.51#1 as a probe. This plasmid contains an 11.5 kb rDNA unit that encodes both the 18S and 28S genes (Endow, 1982). Plasmid DNA (1 µg) was labelled with biotin-16-dUTP (Roche Diagnostics GmbH, Mannheim, Germany) using the Biotin Nick Translation Mix (Roche), precipitated and dissolved in the hybridization solution (50% v/v deionized formamide, 10% v/v dextran sulfate, 2 × SSC) with a final concentration of 15 ng/µL.

Two telomeric probes were used, one with the ancestral insect telomeric motif, the TTAGG repeat, and another with the alternative telomere TCAGG repeat, reported in several tenebrionids (Prušáková et al., 2021). The telomeric repeats were generated by PCR without template using the primers $(TTAGG)_6$ and $(TAACC)_6$, or $(TCAGG)_6$ and $(TGACC)_6$, following a similar procedure to that described by Ijdo et al. (1991). The PCR reaction was carried out in a total volume of 100 µL, using 100 pmol of each primer and 2.5 U of *Taq* polymerase. The PCR cycling program comprised 30 cycles, with an initial step of 1 min at 95°C, followed by 1 min at 50°C, 1 min at 72°C and a final extension of 10 min at 72°C. PCR generated fragments were purified and labelled with biotin-16-dUTP or digoxigenin-11-dUTP (Roche) by nick translation using the DNA Polymerase I/DNase

Table 1. Oligonucleotides designed for the main satellite DNA families.

Species	Primer	Primer sequence
Tenebrio molitor	T_molit_sat142bp	GTTCTTGCGTCGTTTTACTTCGAAATGTACAAG
Alphitobius diaperinus	Tdiap-128	TGTGTTCCGGCGAAACCTTTAGAGCTACGACGG
Zophobas morio	Zmorio-SphI-F	TTTCCGAGCCCGTAGGGTAT
	Zmorio-SphI-R	TCTGAGCCACGAAACACACT

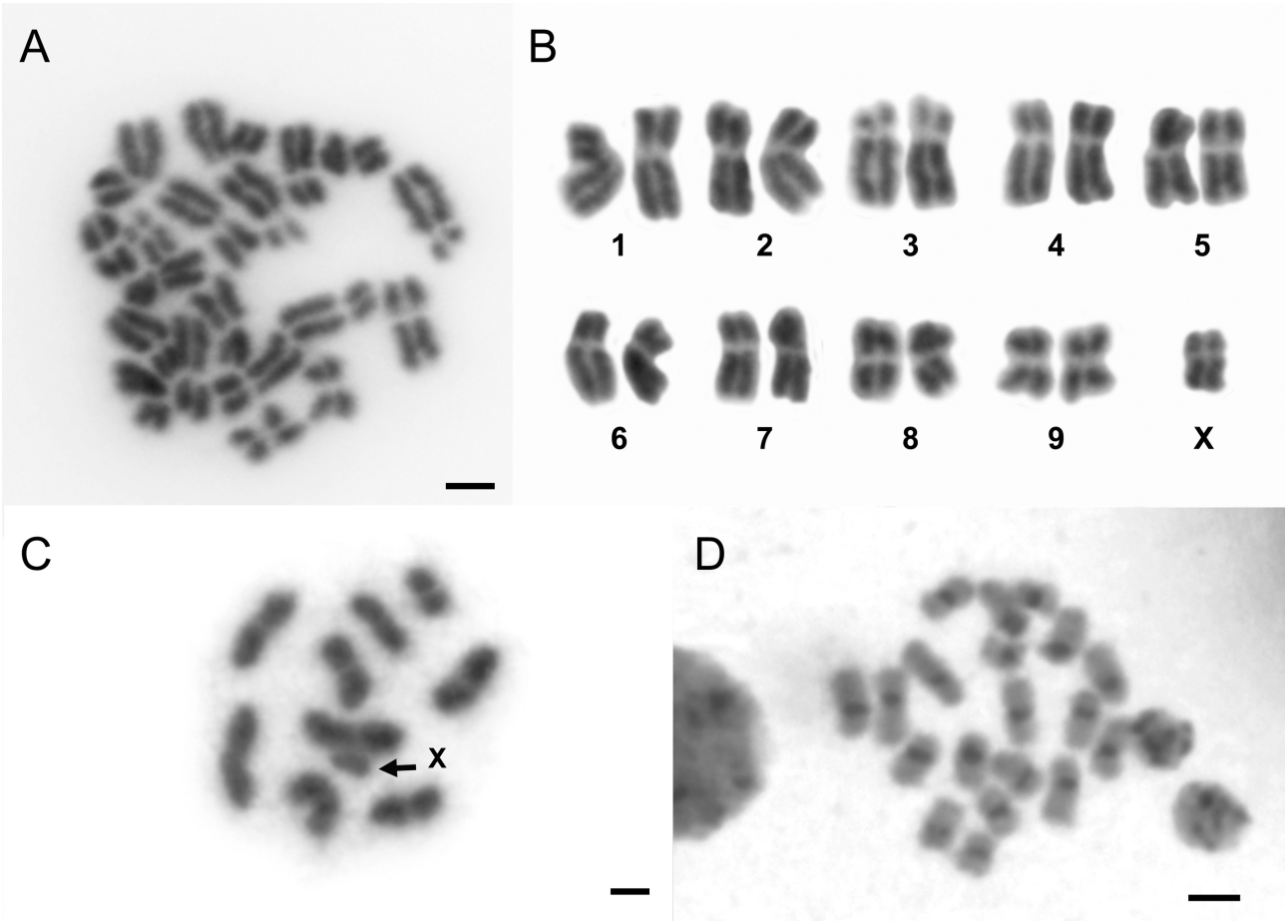

Fig. 1. *Alphitobius diaperinus*. Mitotic metaphase plate (A) and karyotype of a male (B). (C) Meiotic metaphase plate of a male showing the X chromosome as a univalent (arrow). (D) C-banding showing the presence of heterochromatic blocks in the pericentromeric region of all chromosomes. Bar = 5 µm.

I mix (Invitrogen, San Diego, CA). The probe was diluted in hybridization solution (10 ng/µL).

The location of the satDNA on chromosomes was done using specific oligonucleotides designed using the consensus sequences of the corresponding repeat (Table 1, Supplementary material). Oligonucleotides (150 pmol) were labelled with digoxigenin-11-dUTP (Roche) or biotin-16-dUTP (Roche) using 200 U of terminal transferase (Roche) for 20 min at 37°C. Hybridization solutions were prepared to a final concentration of 3 pmol/µL of probes in 50% formamide.

FISH was done using the protocol outlined by Cabral-de-Mello & Marec (2021). Before hybridization, slides were treated with RNase A (100 µg/mL in 2 × SSC) for 60 min at 37°C and then washed in 2 × SSC. Then the slides were incubated in a solution of formaldehyde (3.7% formaldehyde in 4 × SSC, 0.1% v/v Tween-20, 1% w/v skimmed milk) and dehydrated for 5 min each in 70%, 90% and 100% ethanol.

Hybridization solutions were incubated for 10 min at 95°C to denature the probes and placed on ice for 3–5 min. This step was not used when oligonucleotide probes were used. Hybridization was done by applying 25 µL of probe solution to each slide that was then covered with a glass coverslip. Slides were heated for 3 min at 70°C and immediately chilled on ice for 3 min. The slides were transferred to a moist chamber and incubated overnight at 37°C. After hybridization, coverslips were removed, and the slides were washed three times in 2 × SSC at room temperature. Before immunological detection, the slides were incubated 15 min in the Washing Blocking Buffer (WBB, 4 × SSC, 0.1% v/v Tween-20, 1% w/v skimmed milk).

Biotin-labelled probes were detected using Alexa Fluor 488-conjugated streptavidin (Invitrogen) at a concentration of 10 µg/mL, while digoxigenin-labelled probes were detected using anti-digoxigenin-rhodamine (Roche) at a concentration of 1 µg/mL, both diluted with WBB. After incubation for 60 min at 37°C the slides were washed three times in WBB at room temperature, air-dried and mounted with VECTASHIELD with DAPI.

RESULTS AND DISCUSSION

Karyotype and C-banding

The karyotypes and sex chromosomes in *A. diaperinus* are 2n = 19, X0 in males and 2n = 20, XX in females (Sharma et al., 1973). According to these authors, the male karyotype consists of 17 metacentric and 2 submetacentric chromosomes. Based on the current study, the submetacentric chromosomes are the third largest autosome pair (Fig. 1A, B). The X chromosome is metacentric and smaller than the autosomes. At meiosis I in males the X chromosome appears as a univalent (Fig. 1C). C-banding revealed large heterochromatic blocks in the pericentromeric regions of all chromosomes (Fig. 1D), which is consistent with previous reports (Plohl & Ugarković, 1994a; Bruvo et al., 1995).

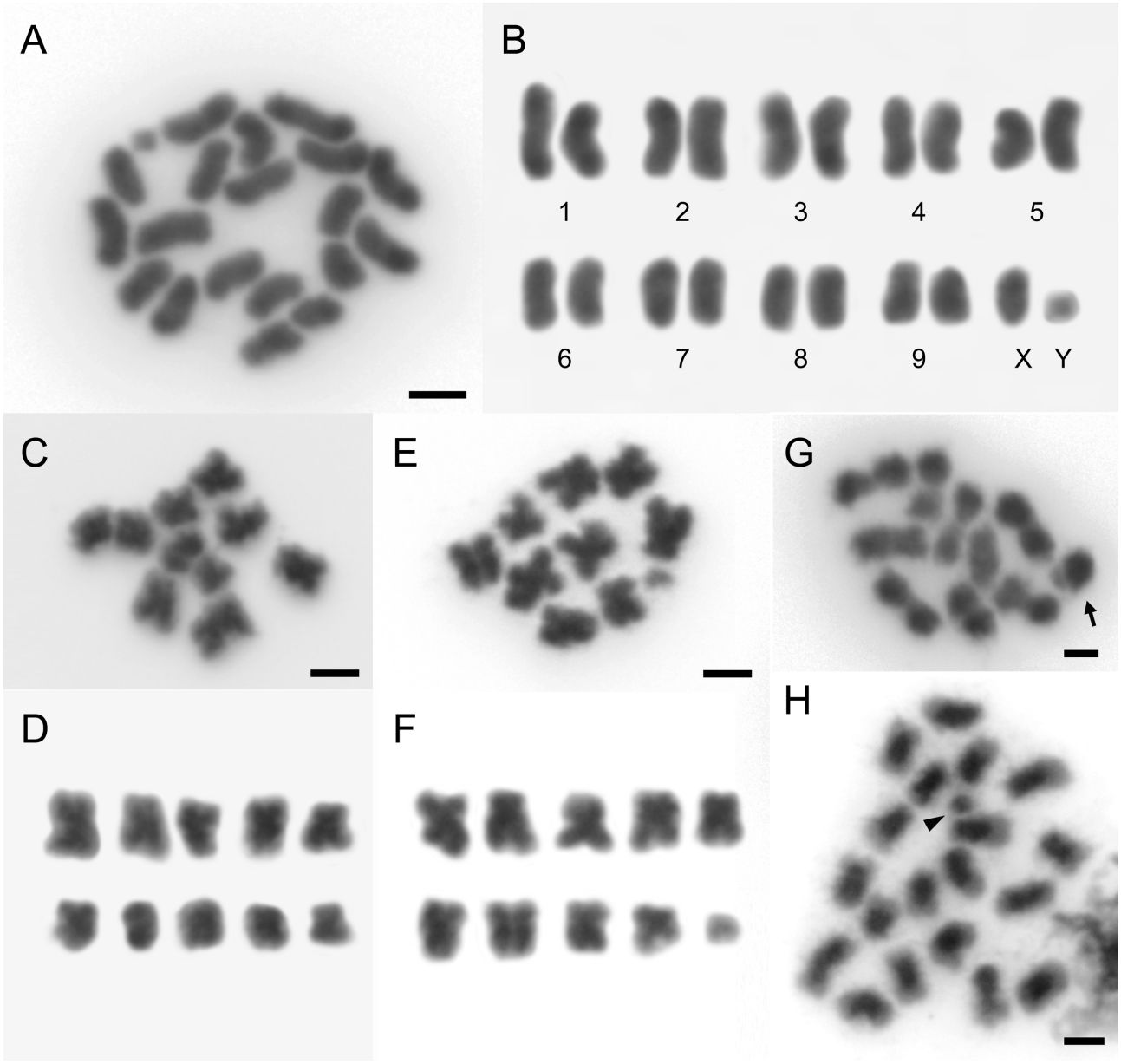

Fig. 2. *Tenebrio molitor*. (A, B) Mitotic metaphase plate and karyotype of a male. Male meiotic metaphase II plates and karyotypes, one with the X chromosome (C, D) and the other with the small Y chromosome (E, F). (G) Meiotic metaphase I showing the typical Xy_p parachute configuration of the sex chromosomes (arrow). (H) C-banding showing the presence of heterochromatic blocks in the pericentromeric region of all chromosomes. Arrowhead indicates the Y chromosome. Bar = 5 μm.

T. molitor was one of the first beetles to be cytogenetically analysed (Smith, 1953). The study by Stevens (1905) is of great relevance within cytogenetics as it was the first one to establish the existence of sex chromosomes, revealing an unequal pair in males and two equal-sized chromosomes in females. Stevens (1905) reports that the chromosome number for *T. molitor* is 2n = 20, with 19 large chromosomes of similar size and a minute chromosome present only in male cells, corresponding to the Y chromosome, as observed here (Fig. 2A, B). Guenin (1951) studied several tenebrionids, including *T. molitor*, confirming the chromosome number in this species and revealing that most mitotic chromosomes are metacentric, but some appear submetacentric. Although the morphology of chromosomes in mitotic metaphases is not clear, in metaphase II of meiosis, when chromatids separate, it is clear that the autosomes and X chromosomes are biarmed, but some of them seem to be submetacentric rather than metacentric (Fig. 2C–F). In metaphase I, the X and the Y chromosomes are associated in the typical Xy_p parachute configuration (Fig. 2G), a non-chiasmata association between a biarmed X chromosome and the minute Y chromosome. Guenin (1951) also analysed the chromosomes of the sister species *Tenebrio obscurus* Fabricius, 1792 and reports a karyotype almost identical to that recorded for *T. molitor*. In addition, Smith (1952) reports that the karyotype of *Tenebrio picipes* Herbst, 1797 is very similar to that of *T. molitor*, with the only difference being that the X chromosome is much smaller than the autosomes, but larger than the Y chromosome. Currently, North American *T. picipes* is now assigned to

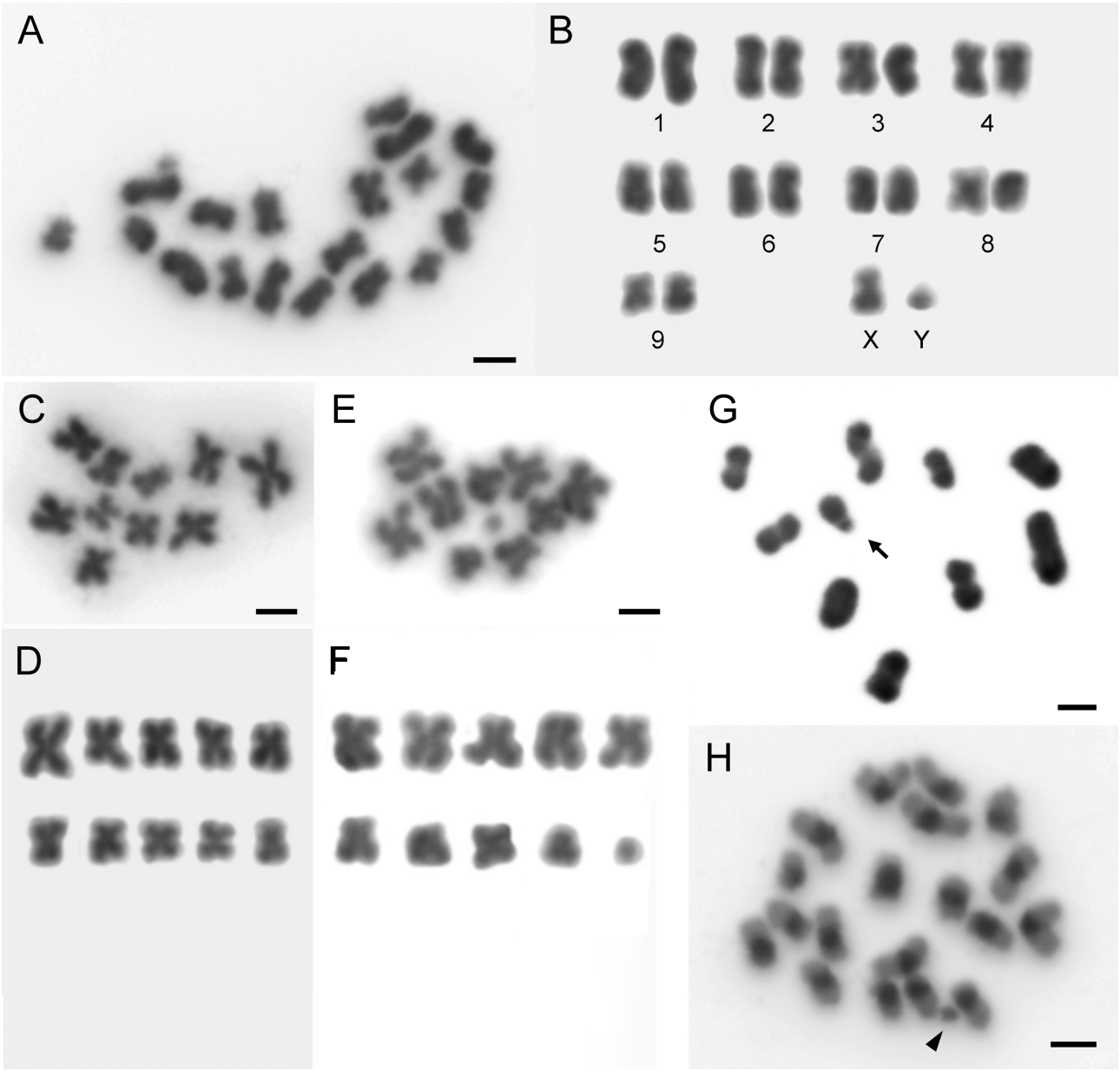

Fig. 3. *Zophobas morio*. (A, B) Mitotic metaphase plate and karyotype of a male. Male meiotic metaphase II plates and karyotypes, one with the X chromosome (C, D) and the other with the small Y chromosome (E, F). (G) Meiotic metaphase I showing the typical Xy_p parachute configuration of the sex chromosomes (arrow). (H) C-banding showing the presence of heterochromatic blocks in the pericentromeric region of all chromosomes. Arrowhead indicates the Y chromosome. Bar = 5 µm.

the genus *Neatus* LeConte as *Neatus tenebrioides* Palisot de Beauvois 1812 (Bousquet et al., 2018). The *T. molitor* autosomes and the X chromosome are characterized by the presence of prominent heterochromatic blocks (Weith, 1985; Juan & Petitpierre, 1991) that make up more than half of the length of the mitotic chromosomes (Fig. 2H).

The karyotype of *Z. morio* (= *Z. atratus*, *Z. opacus*) was recently determined by Cabral-de-Mello & Marec (2021) as 2n = 20, Xy_p. All of its autosomes are clearly metacentric, while the X chromosome is submetacentric (Fig. 3A, B). As in *T. molitor*, the chromosome morphology is most clearly visible at metaphase II (Fig. 3C–3F). The Y chromosome is dot-like and associates with the X chromosome at meiosis I, forming the typical Xy_p (Fig. 3G). C-banding, done for the first-time on this species, revealed pericentromeric heterochromatic blocks on all chromosomes (Fig. 3H). For the genus *Zophobas* there is only cytogenetic data for *Zophobas* aff. *confusus*, which has a very similar karyotype to *Z. morio* (Lira-Neto et al., 2012).

The Xy_p sex chromosome system, reported in *T. molitor* and *Z. morio*, is the predominant sex chromosome system within Tenebrionidae and suborder Polyphaga (Juan & Petitpierre, 1991; Blackmon & Demuth, 2015). The XX/X0 system in *A. diaperinus* is less frequently recoded in tenebrionids and is reported in fewer than ten species in different subfamilies (Blackmon & Demuth, 2015). Similarly, within the suborder Polyphaga, the X0 sex chromosome system is generally less prevalent and complex sex chromosome systems are reported in several families

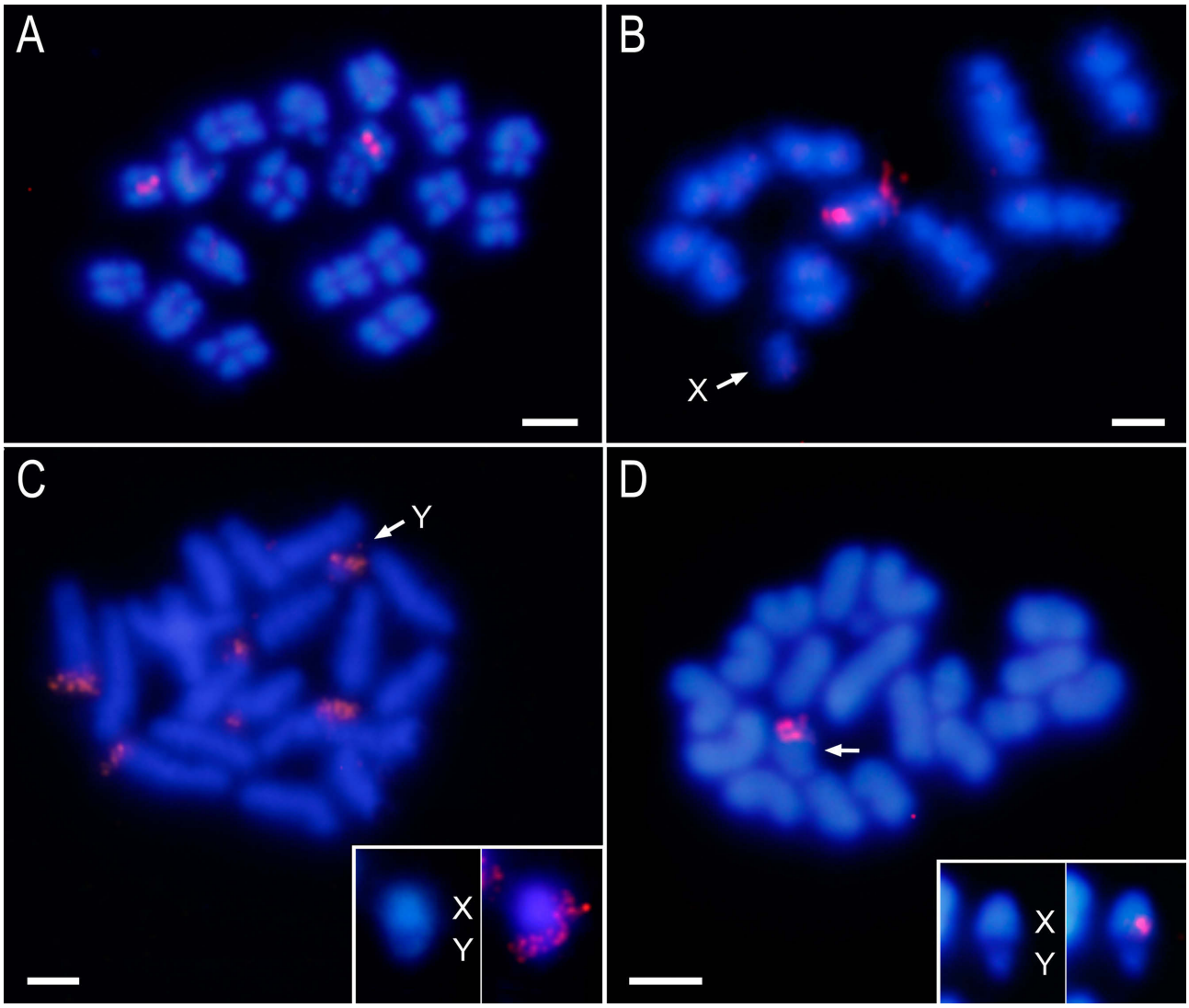

Fig. 4. FISH showing the location of the NORs on chromosomes. (A, B) *Alphitobius diaperinus*; mitotic (A) and meiotic (B) metaphase plates showing the NORs located in the smallest pair of autosomes. (C) Mitotic metaphase plate of *Tenebrio molitor* showing the location of the NORs on six chromosomes. Insert shows the Xy_p bivalent showing hybridization signals on both sex chromosomes. (D) Mitotic metaphase of *Zophobas morio* showing the location of the NOR on the X chromosome. Insert shows the Xy_p bivalent with hybridization signals on the X chromosome. Bar = 5 μm.

(Blackmon et al., 2017; Bracewell et al., 2023; Dutrillaux & Dutrillaux, 2023).

Chromosome location of the rDNA genes

Eukaryotic genomes contain a large number of tandem ribosomal DNA (rDNA) repeats, which provide an ample supply of ribosomal RNAs for the formation of ribosomes. These repetitive arrays are referred to as nucleolar organizing regions (NORs). Eukaryotic rDNA consists of tandem repeats that include the large (45S) and small (5S) transcriptional units. The large unit encodes the 18S, 5.8S and 28S rRNAs, separated by internal transcribed spacers I and II (Long & Dawid, 1980). Traditionally, NORs are localized using silver staining, which has limitations in staining only active NORs and potentially staining heterochromatic regions (Dobigny et al., 2002; Caperta et al., 2007). Currently, FISH is considered to be the most effective tool for mapping the rDNA genes, regardless of whether they are expressed. Comparative analysis of rDNA sequences and gene organization in species aids evolutionary and phylogenetic studies, which offer insights into species relationships and diversification events (Menezes et al., 2021; Pita et al., 2022; Wang et al., 2023).

The location of NORs on chromosomes of the species studied was determined using FISH, which revealed three distinct patterns. In *A. diaperinus* NORs were identified on the smallest pair of autosomes (Fig. 4A, B). In *T. molitor,* NORs are located on two pairs of autosomes and both sex chromosomes (Fig. 4C), which is consistent with previous observations of Juan et al. (1993). In *Z. morio*, NORs are exclusively located on the X chromosome (Fig. 4D) and in *Z.* aff. *confusus* on the Xy_p sex chromosomes, based on silver nitrate impregnation (Lira-Neto et al., 2012). It is noteworthy that silver nitrate staining may be associated with proteins linked to the sex chromosomes (Goll et al., 2013). Interestingly, in some species of Coleoptera the results are different when silver impregnation and FISH are

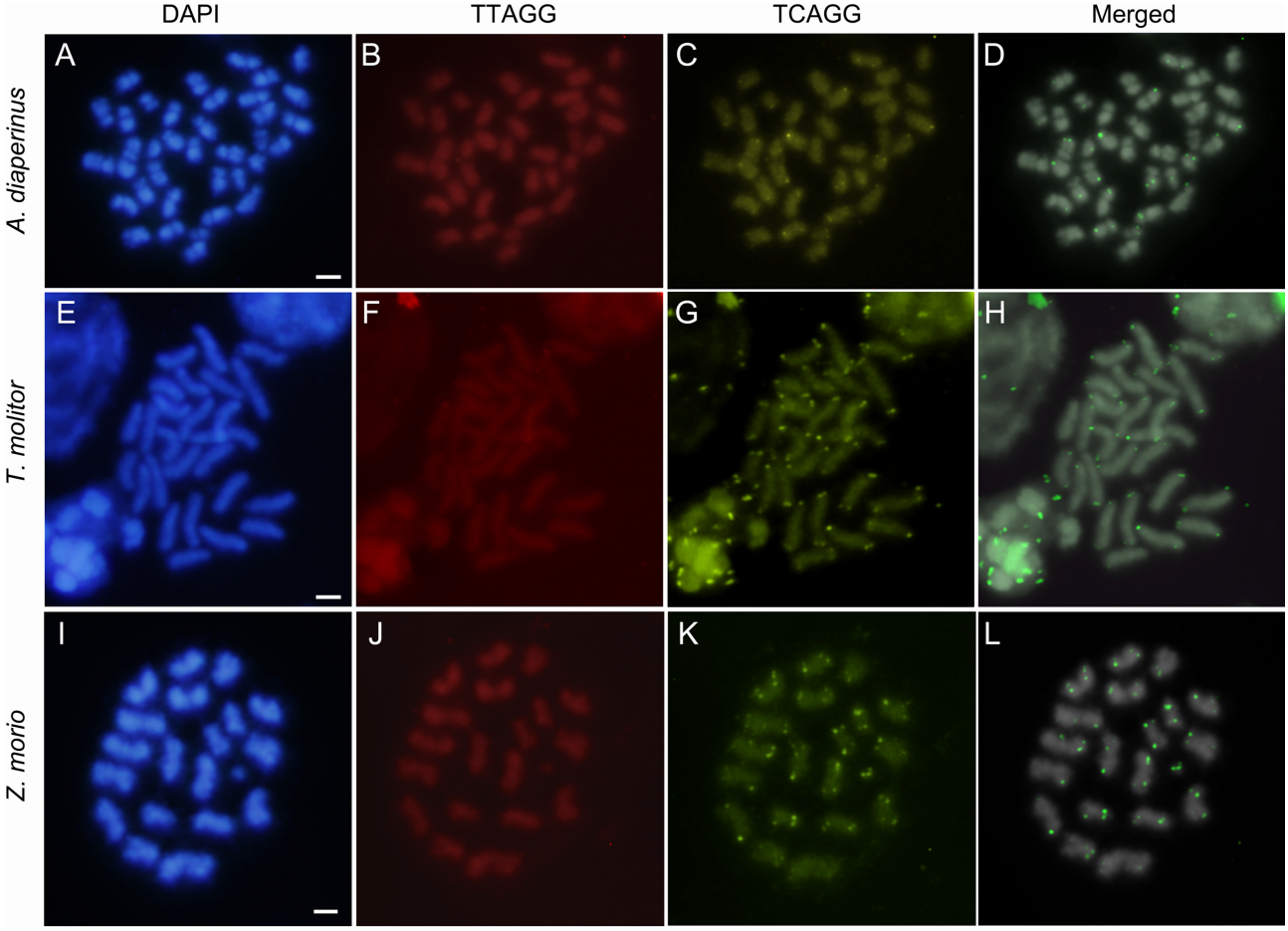

Fig. 5. FISH with telomeric DNA probes in mitotic metaphases of *A. diaperinus*, *T. molitor* and *Z. morio*. For each species the photographs show staining with DAPI, hybridization with a probe containing the TTAGG repeat, hybridization with a probe containing the TCAGG repeat, and the merged image. Bar = 5 μm.

used to locate NORs (Dutrillaux & Dutrillaux, 2012; Goll et al., 2015).

In the families of Coleoptera, there is no well-defined pattern in the distribution of NORs (Ruiz-Torres et al., 2021). Generally, the majority of Coleoptera have two rDNA clusters located on a single pair of autosomes. This pattern is reported in approximately 80% and 65% of the species of Adephaga and Polyphaga, respectively (Lopes et al., 2017, and references therein). However, in Polyphaga the ribosomal genes are present on the autosomes, the sex chromosomes or both, according to the data reported for species belonging to the families Scarabaeidae, Chrysomelidae, Tenebrionidae, Coccinelidae and Geotrupidae (Goll et al., 2015). This variable distribution of NORs is similar to that recorded in the species studied here. An unusually high number of chromosomes with rDNA is reported in two species of Coleoptera, both with 2n = 20, a chromosome number very common in Coleoptera. Specifically, *Coprophanaeus ensifer* Germar, 1821 (Scarabaeidae) has rDNA clusters on seven autosomal bivalents and the Y chromosome (Oliveira et al., 2010) and *Hycleus scutellatus* Rosenhauer, 1856 (Meloidae) has rDNA clusters on 12 different mitotic chromosomes (Ruiz-Torres et al., 2021). Although the causes of the variability in rDNA number and location should be better studied in Coleoptera, it is suggested that in Scarabaeidae it is related to the occurrence of heterochromatin, rather than to macro-chromosomal rearrangements (Cabral-de-Mello et al., 2011).

Telomeres

Telomeres consist of repetitive DNA sequences and specialized proteins that prevent the degradation and fusion of the ends of chromosomes, thus maintaining the structural integrity of chromosomes. In general, telomeres consist of a tandem repetition of short repeats that are synthetized by a specific ribonucleoprotein, telomerase (Zakian, 2012). The telomere motif $(TTAGG)_n$ is considered ancestral for the class Insecta, although this motif has been lost in several insect orders (Frydrychová et al., 2004; Kuznetsova et al., 2020). In many insects that lack the TTAGG repeat in their telomeres, the organization of these structures is unknown. Recently, the use of chromosome-level genome assemblies has allowed the determination of the organisation in some of them, in which telomeric long and short T-containing motifs are present (Lukhtanov & Pazhenkova, 2023). Coleoptera is one of the most heterogeneous groups in terms of telomere composition, with species harbouring TTAGG telomeric repeats and many species lacking this sequence (Prušáková et al., 2021). One of the families of beetles in which the telomeric sequence TTAGG is currently not re-

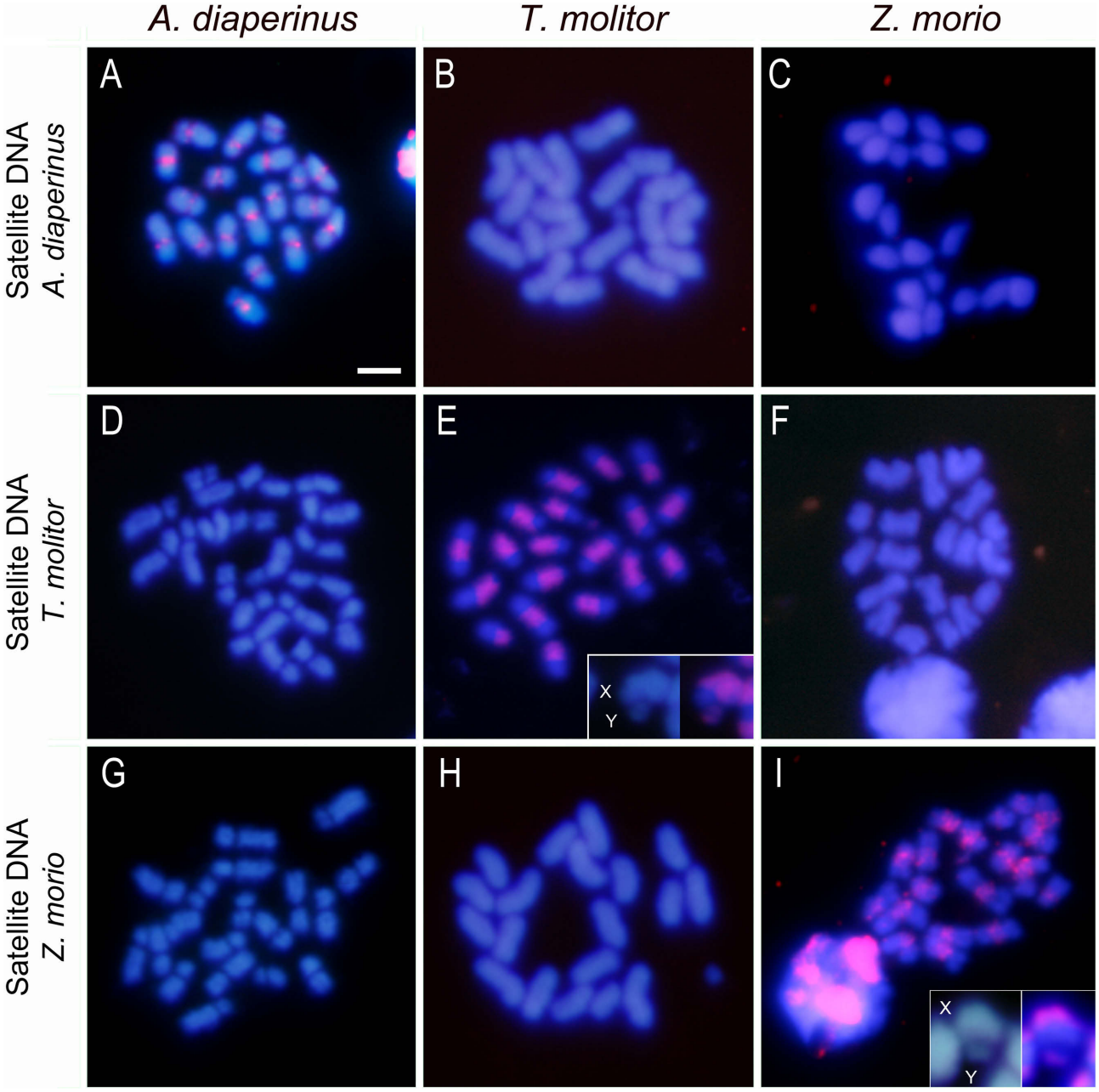

Fig. 6. Chromosome location of the satDNA on mitotic metaphases. Each photograph shows the results of FISH in metaphases plates of each of the three tenebrionid species using the main satDNA of one of them as a probe. The inset in (E) shows the Xy_p bivalent of *Tenebrio molitor* showing hybridization signals on both sex chromosomes. The inset in the last photograph (I) shows the Xy_p bivalent of *Zophobas morio* with hybridization signals on both sex chromosomes. Bar = 5 µm.

corded is the Tenebrionidae, in which this sequence is replaced by TCAGG, as in *T. molitor* (Fig. 5E–H) and several species in the genera *Tribolium* and *Pimelia* (Mravinac et al., 2011; Prušáková et al., 2021). Similarly, FISH carried out using the TTAGG repeat did not generate hybridization signals in either *A. diaperinus* or *Z. morio*, whereas terminal hybridization signals were recorded when the TCAGG repeat was used as a probe (Fig. 5).

These results for two previously unstudied genera of Tenebrionidae, indicate that the replacement of the TTAGG repeat by TCAGG appears to be a common feature in the family Tenebrionidae. The presence of the telomeric TCAGG repeat in non-tenebrionid species such as *Malachius bipustulatus* Linnaeus, 1758 (Melyridae) and *Pyrochroa serraticornis* Scopoli, 1763 (Pyrochroidae) (Lukhtanov & Pazhenkova, 2023) indicates that the shift from TTAGG to TCAGG has also occurred in other families of Coleoptera (Prušáková et al., 2021; Lukhtanov & Pazhenkova, 2023). Prušáková et al. (2021), in a comprehensive study of telomeric DNA sequences in beetles, consider that the diversity of telomeric motifs is positively related to the species richness of a particular taxon regardless of its age. These authors also point out that telomere dysfunction can initiate rapid genomic changes leading to reproductive isolation and speciation.

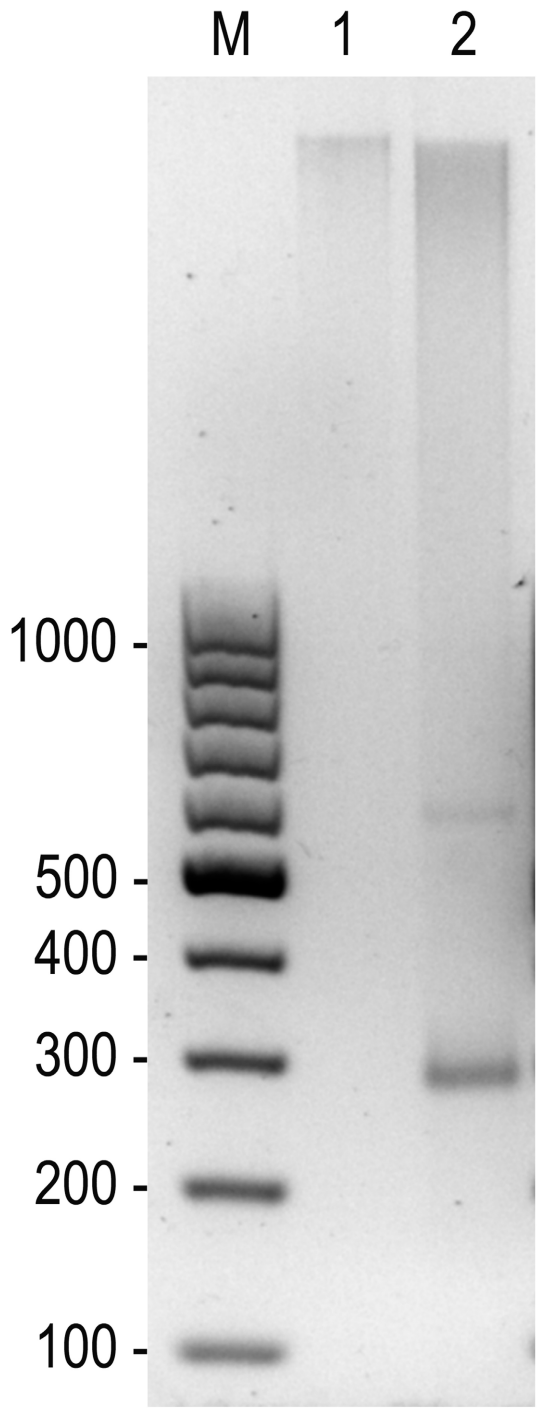

Fig. 7. Electrophoresis in a 2% agarose gel of *Zophobas morio* gDNA after digestion with *Sph*I (lane 2). Lane 1, undigested DNA. Digestion with *Sph*I reveals a band of repetitive DNA of about 300 bp and another of about 600 bp. The numbers on the left indicate the size in bp of the molecular size marker DNA (M).

Heterochromatin and satellite DNA

Satellite DNA is the primary component of heterochromatin and consists of repetitive DNA sequences that are tandemly repeated and arranged in a head-to-tail configuration. Recent molecular studies reveal the presence of many families of satDNA in eukaryotic genomes, which influence their architecture and functioning (Garrido-Ramos, 2017). Collectively, these families are referred to as the "satellitome" (Ruiz-Ruano et al., 2016).

As commented on above, in the three species of tenebrionid studied, C-banding revealed the presence of large heterochromatic blocks in the pericentromeric regions of all chromosomes. In Tenebrionidae, numerous studies have focused on the analysis of satDNA using classical methods (reviewed in Petitpierre et al., 1995; Ugarković et al., 1995; Palomeque & Lorite, 2008), whereas very few have used genomic data. In *A. diaperinus*, Plohl & Ugarković (1994a) report a satDNA that makes up 25% of the total genomic DNA and consists of three monomers (tH1, tH2 and tH3) organized in higher order repeating structures, specifically a dimer, composed of tH1 and tH3, and a trimer containing tH1, tH2 and tH3 in series. FISH revealed that these variants are uniformly distributed throughout the pericentromeric heterochromatic regions of all chromosomes (Bruvo et al., 1995). The FISH used in the present study used an oligonucleotide probe based on the satDNA of *A. diaperinus*, designed in a conserved region among the three monomers described by Plohl & Ugarković (1994a) (Supplementary material). A hybridization pattern similar to that described by Bruvo et al. (1995) was recorded for chromosomes of *A. diaperinus* (Fig. 6A). No hybridization signals were recorded for the chromosomes of *T. molitor* (Fig. 6B) or those of *Z. morio* (Fig. 6C) when this probe was used. No similar sequences were found in the sequences of *T. molitor* and *Z. morio* deposited in the NCBI GenBank database based on a BLAST analysis.

The satDNA of *T. molitor* was among the first to be characterized in insects based on the digestion of genomic DNA using restriction endonucleases (Petitpierre et al., 1988). It consists of the repetition of 142-bp monomeric units and is present in all chromosomes, including both sex chromosomes (Ugarković et al., 1989; Juan et al., 1990, 1993). Recently, Oppert et al. (2023) report a new sequencing and assembly of the *T. molitor* genome and a comparison of this data with other published long-read assemblies (Elefttheriou et al., 2022; Kaur et al., 2023). Oppert et al. (2023) report that mainly 11 satDNAs constitute the satellitome of this species and that they make up 28% of the genome. One of these satellites, TmSat1, is the most abundant, making up 26.5% of the *T. molitor* genome and corresponds to the 142-bp satDNA previously described by Petitpierre et al. (1988). This value is very different from previous estimates, which indicate that the amount of this satDNA is around 50% (Petitpierre et al., 1988; Davis & Wyatt, 1989; Plohl et al., 1990). These authors point out that 9 of the 11 satDNAs are also found in the genomes of other insects based on the results of the BLAST analysis. Specifically, the sequences TmSat1 are very similar to those in the genome of *T. obscurus*, *Z. morio* and other species of insects. The similarity of TmSat1 in the genomes of *T. molitor* and *T. obscurus* was previously reported by Plohl & Ugarković (1994b). The FISH in this study used an oligonucleotide probe based on the 142-bp monomeric repeat sequence. Hybridization signals were recorded for all chromosomes of *T. molitor* (Fig. 6E), which is consistent with the reports

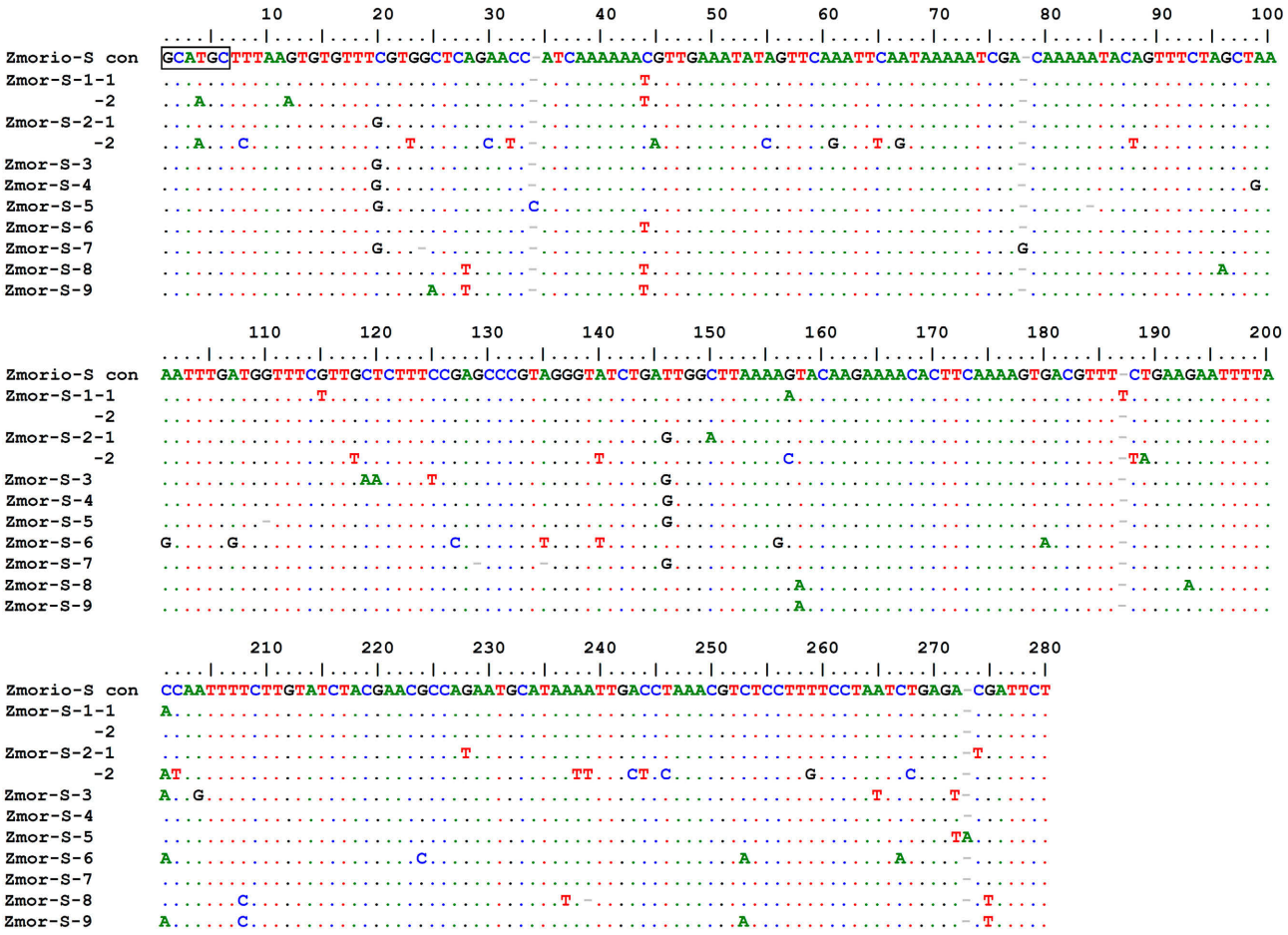

Fig. 8. Multiple sequence alignment of all sequenced monomers from the Zmorio-S satDNA family (NCBI accession numbers PP502217–PP502225) and the consensus sequence derived from them. The sequences designated 1 and 2 are monomeric units from the same dimer clone. The target for the *Sph*I restriction endonuclease is boxed.

of other authors (Ugarković et al., 1989; Juan et al., 1990, 1993). No hybridization signals were recorded for the chromosomes of *A. diaperinus* (Fig. 6D) and *Z. morio* (Fig. 6F) using this probe. A BLAST analysis, however, confirmed the presence of TmSat1 in the *Z. morio* genome (NCBI RefSeq assembly GCA_027724725), which includes 4178 scaffolds and no assembled chromosomes. The search in this genome reported only a scaffold with similarity with the TmSat1 of *T. molitor* (QUZm001 scaffold_1065, GenBank accession number JALNTZ010001065). This scaffold is 28,315 bp in length and only contains tandem repetitions of a 142-bp sequence that is very similar to TmSat1 (some sequences in the scaffold have a 99% similarity to the TmSat1 consensus sequence). Dot plot analysis of the scaffold sequence reveals that the 200 copies of the 142-bp sequence are organized into six different arrays in which the orientation of the 142-bp repeated sequences changes (Supplementary material). The low proportion of TmSat1 in the *Z. morio* genome most likely accounts for the absence of clear hybridization signals obtained using the particular FISH technique used.

Z. morio genome was recently sequenced (Kaur et al., 2023), however, there is no comprehensive profile of its entire satDNA collection. The genomic DNA of this species was digested with several restriction endonucleases to isolate the satDNA. Electrophoresis of genomic DNA digested using *Sph*I produces a band of approximately 300 bp and other of about 600 bp that were cloned and sequenced (Fig. 7). The analysis and alignments of these sequences indicate that it is a satDNA with a monomeric repetition length of 276 bp (Fig. 8). BLAST analysis of the *Z. morio* genome revealed the presence of this satDNA in many scaffolds, each with multiple matches. The analysis also indicated a tandem arrangement of the monomers. Overall, data indicate that this satDNA is likely to be the most abundant satDNAs in the *Z. morio* genome. FISH using oligonucleotides based on a sequence of this satDNA (Supplementary material) results in positive hybridization signals for all chromosomes, including the sex chromosomes, of *Z. morio* (Fig. 6I). No hybridization signals were recorded using this probe on the chromosomes of *A. diaperinus* (Fig. 6G) or *T. molitor* (Fig. 6H). In the three tenebrionid species analysed, the use of specific probes for the major satDNA of each species resulted in positive hybridization signals in the pericentromeric regions of all chromosomes, which coincide with the heterochromatin revealed by C banding.

Traditionally, it has been believed that insect genomes contain a main satDNA family, which usually constitutes the primary component of heterochromatin located in the pericentromeric regions (reviewed by Palomeque & Lorite,

2008; Garrido-Ramos, 2017). Recent molecular studies have revealed the presence of multiple satDNA families in insect genomes. Generally, the most abundant satDNA families in the genome tend to be concentrated in heterochromatic regions, which are densely packed and often transcriptionally inactive areas of chromatin. In contrast, less abundant families are distributed more widely and may be organized as short, dispersed arrays within the euchromatic regions. The presence of satDNAs in both heterochromatin and euchromatin is reported for insects in several orders, such Coleoptera (Pavlek et al., 2015; Montiel et al., 2022; Rico-Porras et al., 2024), Orthoptera (Palacios-Gimenez et al., 2020), Hemiptera (Pita et al., 2017; Bardella et al., 2020; Cabral-de-Mello et al., 2023; Mora et al., 2023), Lepidoptera (Cabral-de-Mello et al., 2021) and Diptera (Sproul et al., 2020). Recent studies on satDNA indicate their potential and significant functions in processes like chromosomal evolution, speciation, and even genetic regulation through chromatin modulation, not only in insects but also in eukaryotes in general (reviewed by Flynn et al., 2024; Di Tommaso & Giunta, 2024).

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Unpaginated Supplementary material follows on next page.

Satellite DNAs specific oligonucleotides design

Alphitobius diaperinus

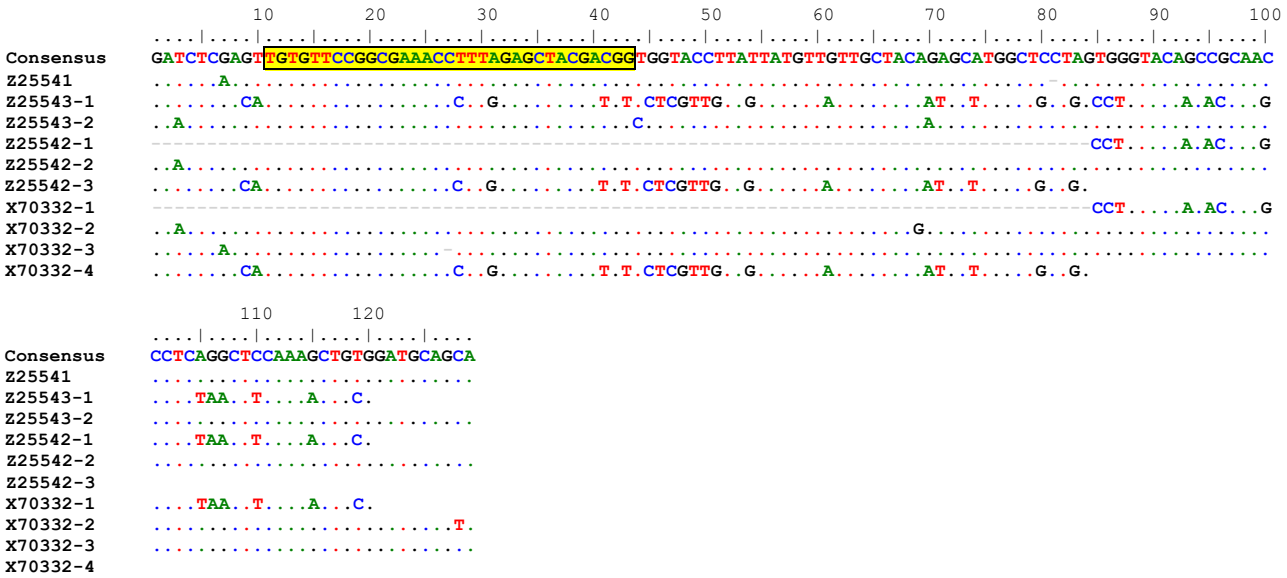
Sequences for the satellite DNA of *Alphitobius diaperinus* has been recovered from Plohl and Ugarković (1994) Mol. Gen. Genet. 242 (3), 297-304

>Z25541.1 A.diaperinus satellite DNA 128 bp
GATCTCAAGTTGTGTTCCGGCGAAACCTTTAGAGCTACGACGGTGGTACCTTATTATGTTGTTGCTACAGAGCATGGCTCTAGTGGGTACAGC
CGCAACCCTCAGGCTCCAAAGCTGTGGATGCAGCA

>Z25543.1 A.diaperinus satellite DNA 249 bp Sau3a
GATCTCGACATGTGTTCCGGCGAAACCCTTGGAGCTACGATGTTCTCGTTGTAGTATGTTATTGCTACAATGCTTGGCTGCTGGCCTGTACAA
CACCAAGCCTCTAACTTCAAAACTGCGGAACTCGAGTTGTGTTCCGGCGAAACCTTTAGAGCTACGACGGCGGTACCTTATTATGTTGTTGCT
ACAAAGCATGGCTCCTAGTGGGTACAGCCGCAACCCTCAGGCTCCAAAGCTGTGGATGCAGCA

>Z25542.1 A.diaperinus satellite DNA 249 bp HaeIII
CCTGTACAACACCAAGCCTCTAACTTCAAAACTGCGGAACTCGAGTTGTGTTCCGGCGAAACCTTTAGAGCTACGACGGTGGTACCTTATTAT
GTTGTTGCTACAGAGCATGGCTCCTAGTGGGTACAGCCGCAACCCTCAGGCTCCAAAGCTGTGGATGCAGCAGATCTCGACATGTGTTCCGGC
GAAACCCTTGGAGCTACGATGTTCTCGTTGTAGTATGTTATTGCTACAATGCTTGGCTGCTGG

>X70332.1 A.diaperinus satellite DNA 277 bp
CCTGTACAACACCAAGCCTCTAACTTCAAAACTGCGGAACTCGAGTTGTGTTCCGGCGAAACCTTTAGAGCTACGACGGTGGTACCTTATTAT
GTTGTTGCTACGGAGCATGGCTCCTAGTGGGTACAGCCGCAACCCTCAGGCTCCAAAGCTGTGGATGCAGTAGATCTCAAGTTGTGTTCCGGC
GAAACTTTAGAGCTACGACGGTGGTACCTTATTATGTTGTTGCTACAGAGCATGGCTCCTAGTGGGTACAGCCGCAACCCTCAGGCTCCAAAG
CTGTGGATGCAGCAGATCTCGACATGTGTTCCGGCGAAACCCTTGGAGCTACGATGTTCTCGTTGTAGTATGTTATTGCTACAATGCTTGGCT
GCTGG



Box indicates the sequence used for the oligonucleotide:

Tdiap-128: 5´-TGTGTTCCGGCGAAACCTTTAGAGCTACGACGG

Tenebrio molitor

Sequence of the satellite DNA of *Tenebrio molitor* has been recovered from Plohl et al. (1990). Mol. Gen. Genet. 242 (3), 297-304

>L19313.1 Tenebrio molitor satellite repeat sequence 142 bp
GAATTCTGTAGTTCTTGCGTCGTTTTACTTCGAAATGTACAAGTTCCACGACGAAATTCC

GATTCGCACTTAGTTTTTCGTGATCCTACAAAGTTGCGAGCGAAAAAACGTATTTAGAGGA

AAGTTAGCGCCTTGGAACCTG

Box indicates the sequence used for the oligonucleotide:

T_molit_sat142bp: 5´-GTTCTTGCGTCGTTTTACTTCGAAATGTACAAG

Zophobas morio

Sequence of the satellite DNA of *Zophobas morio* has been obtained in this study

>Zmorio-S consensus sequence 276 bp
GCATGCTTTAAGTGTGTTTCGTGGCTCAGAACCATCAAAAAACGTTGAAATATAGTTCAAATTCAATAAAAATCGACAAAAATACAGTTTCTA
GCTAAAATTTGATGGTTTCGTTGCTCTTTCCGAGCCCGTAGGGTATCTGATTGGCTTAAAAGTACAAGAAAACACTTCAAAAGTGACGTTTCT
GAAGAATTTTACCAATTTTCTTGTATCTACGAACGCCAGAATGCATAAAATTGACCTAAACGTCTCCTTTTCCTAATCTGAGACGATTCT

Zmorio-SAT GCATGCTTTAAGTGTGTTTCGTGGCTCAGAACCATCAAAAAACGTTGAAATATAGTTCAAATTCAATAAAAATCGACAAA 80

Zmorio-SAT AATACAGTTTCTAGCTAAAATTTGATGGTTTCGTTGCTCTTTCCGAGCCCGTAGGGTATCTGATTGGCTTAAAAGTACAA 160

Zmorio-SAT GAAAACACTTCAAAAGTGACGTTTCTGAAGAATTTTACCAATTTTCTTGTATCTACGAACGCCAGAATGCATAAAATTGA 240

Zmorio-SAT CCTAAACGTCTCCTTTTCCTAATCTGAGACGATTCT 276

Boxes indicate the sequence used for the oligonucleotides:

Zmorio-SphI-F: 5´-TTTCCGAGCCCGTAGGGTAT
Zmorio-SphI-R: 5´-TCTGAGCCACGAAACACACT

Tenebrio molitor satDNA in *Zophobas morio*

QUZm001 scaffold_1065 (GenBank accession number JALNTZ010001065) of the *Z. morio* genome. Dot-plot analysis shows that this scaffold of 28,315 bp in length contains six arrays of a 142-bp sequence in which the orientation of the 142-bp repeated sequences changes.