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ORIGINAL ARTICLE

Comparative cytogenetics of *Lachnaia* species (Coleoptera: Chrysomelidae) reveals a novel telomeric motif (TTTGG) in insects

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Abstract

Cytogenetic analyses of *Lachnaia hirta*, *Lachnaia tristigma*, and *Lachnaia vicina* have revealed that all species show a similar karyotype with $2n = 24$ and a meioformula of $11 + Xy +$. Despite this, their sex chromosomes show certain differences in morphology. All species exhibit pericentromeric heterochromatin on all autosomes and X chromosomes, with the Y chromosome being heterochromatic. The X and Y chromosomes carry the nucleolar organizer regions in the three species, although *L. vicina* also has them in one autosomal pair. No clear hybridization signals were obtained using a probe with the TTAGG repeat, which is the ancestral DNA motif of telomeres in insects. The genomes of the three species have been sequenced. The obtained data have been used for the identification of telomeric motifs through bioinformatics analyses, including the Telomeric Repeats Identification Pipeline, a bioinformatics tool for identifying telomeric repeat motifs. According to the data, the TTTGG sequence is suggested as the telomeric repeat in these species, a finding confirmed by fluorescence in situ hybridization. The TTTGG motif constitutes a new telomeric repeat motif in Coleoptera. This new motif has been found at the chromosome ends of another beetle species, the oak borer *Platypus cylindrus* (Coleoptera, Curculionidae), and in a wasp species, *Oxytorus armatus* (Hymenoptera, Ichneumonoidea), whose genomes are assembled at the chromosome level. The detection of this telomeric repeat in two families of beetles and in a hymenopteran species suggests that this new telomeric motif has independently emerged in evolutionarily distant groups of insects.

Keywords Coleoptera · Chrysomelidae · Cryptocephalinae · C-banding · rDNA · Telomeric repeat motif

Introduction

Chrysomelidae belongs to the order Polyphaga and is considered one of the most diverse families of beetles (Bouchard et al., 2011, 2017). Leaf beetles form a species-rich beetle group known by their often very colorful. Their dietary focus primarily consists of plant materials, establishing them as crucial herbivores within their respective ecosystems (Jolivet & Hawkeswood, 1995).

The subfamily Cryptocephalinae (Chrysomelidae) comprises approximately 5300 species worldwide, exhibiting remarkable diversity, particularly in tropical and subtropical areas. Cryptocephalinae is currently subdivided into five tribes: Clytrini, Cryptocephalini, Fulcidacini, Pachybrachini, and Mylassini (Gómez-Zurita & Cardoso, 2021). Some representatives of the subfamily Cryptocephalinae constitute potential pests of crops such as pistachios or *Quercus* species, including species of the genera *Lachnaia* and *Labidostomis* (Gómez et al., 2022; Martín-Santafé et al., 2014).

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Numerous investigations have been focused on analyzing the karyotypic characteristics of Chrysomelidae. Within this taxonomic group, the chromosome number and chromosome morphology vary widely, ranging from $2n = 8$ to $2n = 72$ (Lorite et al., 2002a; Petitpierre et al., 1988), although the most common chromosome number is $2n = 24$ (Blackmon & Demuth, 2015). Most chrysomelid males exhibit the typical Xyp sex chromosome system, considered ancestral in Polyphaga beetles (Smith & Virkki, 1978). In male metaphase I, the sex chromosomes form the characteristic "parachute-like" structure (Xyp), an achiasmatic association of one large or medium-sized X chromosome and one small or even tiny Y chromosome. However, other sex chromosome systems are present in Coleoptera, although with much less frequency, such as the presence of two sex chromosomes of similar size (Petitpierre, 2023). In some species belonging to other Chrysomelidae subfamilies, such as Megalopodinae and Cryptocephalinae, other systems with asynaptic sex chromosomes have been described (Petitpierre et al., 1988).

C-banding techniques are used to identify constitutive heterochromatin, typically enriched in highly repetitive DNA such as satellite DNA (satDNA) (Sumner, 1972). In Coleoptera, the analyzed patterns of C-bands are highly variable, but in general, pericentromeric heterochromatin has been described on most or nearly all autosomes and on the X chromosome, while the heterochromatin distribution on the Y chromosome is highly variable (reviewed by Dutrillaux & Dutrillaux, 2019). In chrysomelids, despite scarce and fragmentary information on C-banding, it is considered that most species exhibit pericentromeric heterochromatin on autosomes and intercalary heterochromatin of variable amounts and locations on sex chromosomes, especially on the Y chromosome (reviewed by Petitpierre, 2023; Rozek et al., 2004).

Eukaryotic genomes contain tandem repeat clusters of ribosomal DNA (rDNA), known as nucleolar organizer regions (NORs). The localization and chromosomal distribution of the NORs have been studied in numerous organisms and data included in the Animal rDNA Database (Sochorová et al., 2018). As the NORs are important components of the eukaryotic genome, they are widely used as a chromosomal marker. Despite this, there is no well-defined pattern of NOR distribution in coleopterans. Generally, it is considered that most species have two rDNA clusters per diploid genome, typically located on a pair of autosomes. This pattern occurs in approximately 80% and 65% of Adephaga and Polyphaga species, respectively, including those with highly divergent diploid numbers and/or sex chromosome systems (Lopes et al., 2017; Schneider et al., 2007). In the family Chrysomelidae, NOR distribution has only been studied in the subfamilies Alticinae, Cassidinae, and Chrysomelinae, although data regarding only the first two subfamilies are included in the Animal rDNA Database (https://www.animalrdnadatabase.com/, accessed on 22 July 2024). In Chrysomelidae, most studied species present a single NOR on an autosome, less frequently two NORs with autosomal localization, and much less frequently, NORs on the X chromosome (Almeida et al., 2010; Gómez-Zurita et al., 2004; Lopes et al., 2017; Petitpierre, 2023, among others).

Telomeres are protein-DNA complexes located at the ends of eukaryotic chromosomes (Blackburn, 1991; reviewed in Jenner et al., 2022). Telomeres protect the ends of chromosomes, and their loss causes instability and usually cell death (reviewed in Kalmykova, 2023). They exhibit a conserved simple telomeric tandem repeat motif consisting predominantly of $(TTAGGG)_n$ in vertebrates (reviewed in Aksenova & Mirkin, 2019) and $(TTAGG)_n$ in arthropods (reviewed in Kuznetsova et al., 2020). Zhou et al. (2022) have developed a bioinformatic pipeline named Telomeric Repeats Identification Pipeline (TRIP), which confirmed that $(TTAGG)_n$ is the ancestral telomeric repeat in 19 orders of insects. Prušáková et al. (2021) reported that this ancestral telomeric repeat motif has been repeatedly or completely lost in over half of the studied beetle superfamilies, being replaced in numerous species by two alternative telomeric motifs, $(TCAGG)_n$ and $(TTAGGG)_n$, in at least three beetle superfamilies' (Curculionoidea, Chrysomeloidea, and Staphylinoidea). However, in many species where this motif has been lost, the structure of their chromosomal end remains unknown, indicating newly accumulated data point to a great diversity in terms of telomeric structure in insects (Lukhtanov & Pazhenkova, 2023). Additionally, the $(TTAGG)_n$ motif appears to have been irreversibly lost in the Diptera order (reviewed in Kuznetsova et al., 2020). Recently, TELOBASE (https://bio.tools/telobase, accessed on 22 July 2024) has been developed as a database to compile telomeric sequences across the tree of life (Lyčka et al., 2024).

Given the complex chromosomal variability and the importance of numerous Chrysomelidae beetles, including several genera from Cryptocephalinae, as pests in different crops, we have selected three species of the genus *Lachnaia* to study chromosomal evolution patterns at the intrageneric level. The genus *Lachnaia* belongs to the tribe Clytrini (Cryptocephalinae) (Gómez-Zurita & Cardoso, 2021; Zhang et al., 2022). We used classical and molecular cytogenetic analyses, which allowed us to provide a detailed description of the karyotypes followed by a comparative analysis. Additionally, we advanced the study of the complex organization of telomeres in beetles, using the three species as a model.

Material and methods

Insect collection and chromosome preparations

Male specimens of the *Lachnaia hirta* Fabricius, 1801, *L. tristigma* Lacordaire, 1848, and *L. vicina* Lacordaire, 1848

species were used in the study. The specimens were collected from the "Marroquíes Norte" park located on the periphery of Jaén city (Andalucía, Spain) as well as at the University of Jaén Garden of Native Flora. Since these species are not endangered, no special permissions were required. The species differentiation was established by applying the dichotomous key described by Petitpierre (2016), complemented by examining the morphology of the aedeagus.

Testes of the three species were extracted, immersed in distilled water for 45 min for hypotonic shock, fixed in modified Carnoy solution (ethanol and glacial acetic acid, 3:1), and stored at − 20 °C until further use. For chromosome preparations, the testes were macerated by pipetting in 50% glacial acetic acid inside a microtube. The resulting mixture was spread onto a glass slide, which was subsequently placed on a heating plate set at 42 °C. Following this, the prepared slides were dehydrated using an ethanol series (70%, 80%, and 100%, 1 min each step) and stored at − 20 °C until use. The chromosome spreads were stained with either Giemsa or mounted using VECTASHIELD with DAPI (4′−6-diamino-2-phenylindole) (Vector Labs, Burlingame, CA, USA). Slides were examined using a BX51 Olympus® fluorescence microscope (Olympus, Hamburg, Germany) equipped with a CCD camera (Olympus® DP70). Image acquisition and processing were performed using DP Manager software v1.1.1.71 and Adobe Photoshop CS4 software (Adobe Systems, San Jose, CA, USA).

C-banding

The heterochromatin distribution was revealed using C-banding, following the procedure outlined by Sumner (1972) with some modifications. Initially, the slides were treated with a 0.2 M hydrochloric acid solution for 10 min at 25 °C. Subsequently, the slides were incubated in a 5% barium hydroxide solution at 60 °C for 1 min and 50 s, followed by rinsing with water. After a brief rinse in the initial hydrochloric acid solution and a final wash in 2 × SSC at 60 °C for 2 min, the slides were ultimately stained with DAPI at a concentration of 0.75 µg/mL and then mounted with VECTASHIELD.

Probes and fluorescence in situ hybridization (FISH)

The location of the NORs was obtained by FISH. A fragment of the 18S rDNA was amplified using the primers 18S-965 and 18S-1573R (Mullin et al., 2005). The PCR product was labeled with biotin-16-dUTP or digoxigenin-11-dUTP (Roche Diagnostics GmbH, Mannheim, Germany) via nick translation using the DNA Polymerase I/DNase I mix (Invitrogen, San Diego, CA), precipitated, and dissolved in the hybridization solution (50% v/v deionized formamide, 10% v/v dextran sulfate, 2 × SSC) to a final concentration of 15 ng /µL.

Two probes with the repeats TTAGG and TTTGG were generated by PCR. The $(TTAGG)_n$ sequence is considered to be an ancestral DNA motif of telomeres in insects, and the $(TTTGG)_n$ sequence is the new telomeric motif found here (see results). The PCR reactions were conducted in the absence of a template, employing the self-annealing primer pairs $(TTAGG)_6$-$(TAACC)_6$ or $(TTTGG)_6$-$(CCAAA)_6$, respectively, following a similar procedure to that described by Ijdo et al. (1991). Each PCR reaction was carried out in a total volume of 100 µL, utilizing 100 pmol of each primer and 2.5 U of *Taq* polymerase. The PCR program comprised 30 cycles, starting with a denaturation step of 1 min at 95 °C, followed by annealing at 50 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR-generated fragments were purified and labeled with biotin-16-dUTP or digoxigenin-11-dUTP (Roche) using the DNA Polymerase I/DNase I mix. The resulting probes were diluted in the hybridization solution to a final concentration of 10 ng/µL.

FISH and immunological detection were performed following the protocol described by Cabral-de-Mello and Marec (2021) with some modifications (Rico-Porras et al., 2024). Biotin-labeled probes were detected using Streptavidin Alexa Fluor 488-conjugated (Invitrogen, CA, USA), while digoxigenin-labeled probes were detected using anti-digoxigenin-rhodamine (Roche). The chromosomes were counterstained with DAPI, and the slides were mounted with VECTASHIELD. Images were acquired and processed as above described.

Genome sequencing and searching telomeric motif by bioinformatics analysis

Lachnaia hirta, *L. tristigma*, and *L. vicina* total genomic DNA (gDNA) was extracted from males using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA), following the manufacturer's protocol. Three micrograms of gDNA were submitted to the company Novogene for sequencing on the Illumina HiSeq 2000 platform (San Diego, CA, USA). A 350-bp fragment library and 150-bp paired-end reads were obtained, providing a total of 2.8 Gb of sequencing data for *L. hirta*, 3.4 Gb for *L. tristigma*, and 3.0 Gb for *L. vicina*. The quality of the raw reads was assessed using the FastQC program (Andrews, 2010). Reads with a quality score below 20 and those containing adapters were filtered out using Trimmomatic v0.39 (Bolger et al., 2014).

After filtering, the two FASTQ files obtained for each of the three species were processed utilizing the Telomeric Repeats Identification Pipeline (TRIP), a bioinformatics tool recognized for its speed and accuracy in identifying

telomeric-repeat motifs (TRMs) candidates, using default parameters (Zhou et al., 2022). Specifically, RepeatDetector was used to extract tandemly repeated sequences ranging from 2 to 25 base pairs from sequencing reads, allowing zero-mismatch tolerance for discrepancies. Subsequently, RepeatSummary alphabetically ordered the repeated monomers, determining the total number of reads containing each repetition and calculating the count and total length of each repeated unit. The C value (0.25) of *L. vicina* (Animal Genome Size Database, https://www.genomesize.com/search.php, accessed on 22 July 2024) was applied to the program for accurate analysis. TRIP generates 19 summary statistics and identifies candidate TRMs based on metrics such as the number of repetitions within reads, total length of tandem repeats, and the percentage representation of each repeat in sequencing reads.

To confirm the presence or absence of the ancestral insect telomeric repeat (TTAGG) and the TRM candidate identified by TRIP analysis (TTTGG), their abundance and divergence were estimated using RepeatMasker v4.1.4 (Smit et al., 2013–2015) with the "-a" option to keep the alignment and the RMBlast search engine. For this analysis, one million raw reads were randomly selected and aligned against a concatenated sequence of the TTAGG and TTTGG repeats. Average divergence was computed by considering sequence distances using the Kimura 2-parameter model, facilitated by the Perl script calcDivergenceFromAlign.pl from the RepeatMasker suite.

Additionally, Bowtie2 alignment analysis (Langmead & Salzberg, 2012) was performed to mask occurrences of the TTAGG and TTTGG repeats within the reads. Default parameters, including the "-p" option for paired-end reads analysis, were applied. Subsequently, the Bowtie2 results were filtered to determine the quantity of paired-end reads that were masked due to the presence of these telomeric repeats. Finally, the CompuTEL v1.2 program (Nersisyan & Arakelyan, 2015) was used to analyze the mean telomere length in all three species. Both the TTAGG and TTTGG telomeric motifs were examined. Default parameters were used, with the exception of the number of chromosomes ($2n = 24$), the genome length (C value = 0.25, as estimated for *L. vicina*), and the analyzed motifs (TTAGG or TTTGG).

To test the presence of this new telomeric motif in insect genome assemblies, a general BLASTn search was performed on the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 15 July 2024). The search used a sequence consisting of ten concatenated repetitions of the TTTGG repeat. This repetitive sequence served as a probe to scan the genome assemblies and detect potential matches that would indicate the presence of the telomeric motif in the insect genomes included in the database.

Results and discussion

Karyotype analysis and C-banding

The karyotypic analysis shows that the three analyzed *Lachnaia* species have very similar karyotypes, with the same chromosome number ($2n = 24$) and acrocentric autosomes (Fig. 1). The main differences among the karyotypes were found for the sex chromosomes. In *L. hirta* (Fig. 1a), the X chromosome is submetacentric whereas the Y chromosome, the smallest chromosome, appears to be also acrocentric. In *L. tristigma* (Fig. 1b), the X chromosome is acrocentric, and the small Y chromosome seems to be also acrocentric. In *L. vicina* (Fig. 1c), the X chromosome is submetacentric, and, unlike the two previous species, the Y chromosome is larger than several autosome pairs.

At meiosis, the three species show asynaptic sex chromosomes (Fig. 2), with the meioformula (11 + Xy +). The meioformula nomenclature follows Petitpierre et al. (1988), although modified according to Blackmon and Demuth (2014), who indicate that "distant-pairing sex bivalents are denoted by Xy +," terminology also used in the Coleoptera Karyotype Database (Blackmon & Demuth, 2015). The chromosomal number and meioformula are similar to those described for *Lachnaia pubescens* Dufour, 1820 (Petitpierre, 1988). The chromosome number of *L. vicina* aligns with previous reports for this species (Petitpierre et al., 1993), although its sex chromosome system had not been previously described, and the chromosome number is not included in the Coleoptera Karyotype Database (https://evobir.shinyapps.io/ColeopteraDB/, accessed on 22 July 2024) (Blackmon & Demuth, 2015). Despite the limited number of species examined, most species within the tribe Clytrini have a low chromosome number, $2n = 22$–24 (Blackmon & Demuth, 2015; Petitpierre et al., 1988), which is consistent with the species studied in this work.

C-banding in the three species revealed heterochromatin blocks located at the pericentromeric regions of all autosomes and the X chromosomes, as well as almost fully heterochromatic Y chromosomes (Fig. 3). In *L. hirta*, however, the X chromosome also exhibits significant portion of heterochromatin on its short arm (Fig. 3a). In metaphase I, the heterochromatic blocks are visible as they stain intensely with DAPI (Fig. 2).

Although the three analyzed *Lachnaia* species have similar karyotypes, several characteristics differentiate them, mainly by differences in the sex chromosomes. In all cases, changes in chromosomal morphology appear to be related to variations in the heterochromatin, as has been considered in other insects (Hirai et al., 2004; Imai

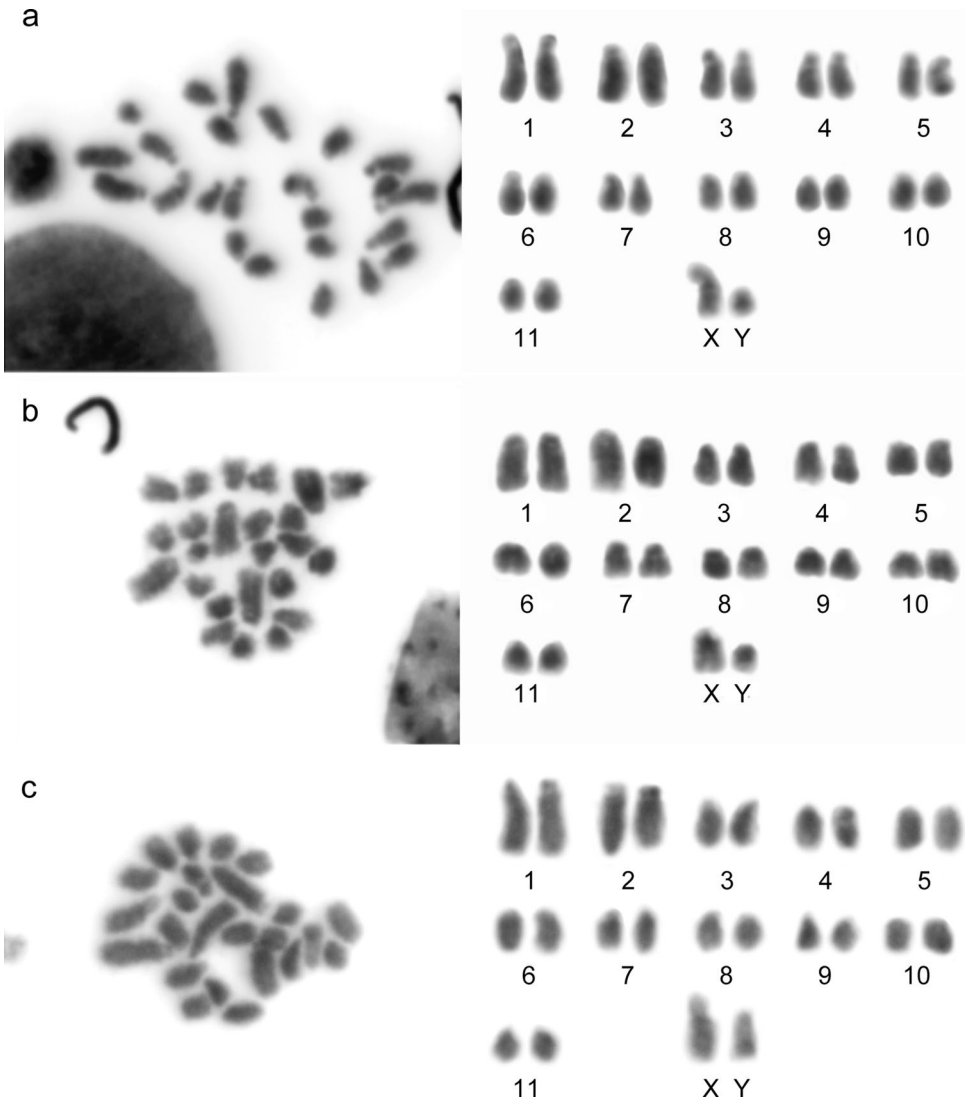

Fig. 1 **a** *Lachnaia hirta*, mitotic metaphase and karyotype of an adult male stained with DAPI (inverted grayscale image). **b** *Lachnaia tristigma*, mitotic metaphase and karyotype of an adult male stained with DAPI. **c** *Lachnaia vicina*, mitotic metaphase and karyotype of an adult male stained with DAPI

et al., 1988). In the family Cryptocephalinae, C-banding has only been applied in *Cryptocephalus globicollis* Suffrian, 1847 (Dutrillaux et al., 2023), revealing a banding pattern quite similar to that observed in these species, particularly *L. tristigma*. Likewise, pericentromeric heterochromatin on all chromosomes, including the X and Y, has been observed in other leaf beetles, such as *Chrysolina carnifex* Fabricius, 1792 (Palomeque et al., 2005).

Chromosome location of the nucleolus organizer regions

The localization of the NORs has been performed in the three species. In *L. hirta*, hybridization with the rDNA probe revealed positive signals on the short arm of the X chromosome and on the Y chromosome (Fig. 4a–d). The hybridization signal on the Y chromosome appears slightly smaller than that observed on the X chromosome. Similarly, in *L. tristigma*, both the X and Y chromosomes exhibited positive hybridization signals (Fig. 4e–h). However, in contrast to *L. hirta*, the hybridization signal on the Y chromosome appears to be larger than that observed on the X chromosome. In *L. vicina*, positive hybridization signals were observed on the X and Y chromosomes, similar to the two previous species (Fig. 4i–l). In this case, the signal from the X chromosome appears to be greater than that on the Y chromosome, as in *L. hirta*. Additionally, hybridization signals were detected on the short arm of an autosome pair.

Until now, the localization of NORs has not been performed in any species of the Cryptocephalinae subfamily. Our results reveal a different NOR distribution pattern in the three studied species. Specifically, *L. vicina* shows NORs on the sex chromosomes and, unlike the other two species, also in one autosomal pair. This variation in the chromosomal distribution of NORs has also been reported in leaf beetles of the subfamily Alticinae (Azambuja et al., 2020),

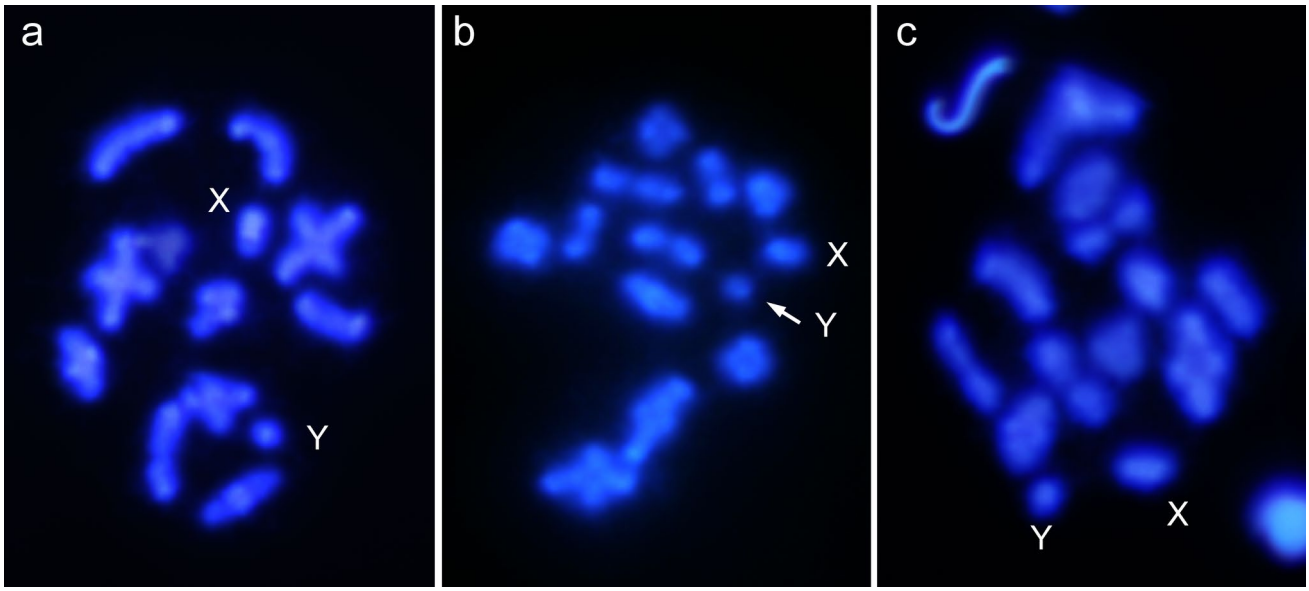

Fig. 2 Male meiotic metaphases stained with DAPI showing the Xy + sex-determining system (asynaptic sex-chromosomes) in **a** *Lachnaia hirta*, **b** *Lachnaia tristigma*, and **c** *Lachnaia vicina*

as well as in other beetles (Cabral-de-Mello et al., 2011) and in other groups of insects (Pita et al., 2022; Provazníková et al., 2021). We also observed differences in the size of the hybridization signal on the X and Y chromosomes of the three studied species, indicating differences in the number of ribosomal cluster copies. This phenomenon has also been observed in other beetle insects. Cabral-de-Mello et al. (2011) suggest that such variation could be attributed to ectopic recombination and transposition, as well as to inversions and translocations within the genome. The X chromosomes in *L. hirta* and *L. vicina* are submetacentric, unlike in *L. tristigma*, which has an acrocentric X chromosome. FISH with rDNA shows a much more intense signal in the first two species compared to *L. tristigma*. This suggests that in *L. hirta* and *L. vicina*, there has been an rDNA amplification, which in *L. hirta* has been accompanied by a heterochromatin amplification, as its short arm is heterochromatic. This dual amplification of heterochromatin and rDNA has been described in other beetle groups, such as those in the family Scarabaeidae (Cabral-de-Mello et al., 2011).

Study of telomeric sequences

To detect the telomeric sequences in these species, first, we performed FISH using the ancestral insect telomeric motif, TTAGG, as a probe. The FISH results indicated the presence of this telomeric repeat only in *L. hirta,* with hybridization signals observed on a limited number of chromosomes (Fig. 5a). No hybridization signals were detected with this probe in *L. vicina* or *L. tristigma* (results not shown). We selected the TTAGG probe based on its prevalence in beetles, as reported by Prušáková et al. (2021) and data available in TELOBASE (http://cfb.ceitec.muni.cz/telobase/, accessed on 15 July 2024). Specifically, TTAGG has been documented in beetles within the subfamily Cryptocephalinae, including species such as *Cryptocephalus trimaculatus* Rossi, 1790 and *C. sericeus* Linnaeus, 1758, which harbor the ancestral insect telomeric sequence (Prušáková et al., 2021). The genus *Cryptocephalus* belongs to the tribe Cryptocephalini within the Cryptocephalinae subfamily (Gómez-Zurita & Cardoso, 2021). On the other hand, the species *Clytra laeviscula* Ratzeburg, 1837, which belongs to the tribe Clytrini, like the genus *Lachnaia* (Gómez-Zurita & Cardoso, 2021), did not show positive results for the TTAGG telomeric motif (Prušáková et al., 2021).

Since the telomeric motif TTAGG were not consistently observed in *Lachnaia* species, their genomes were sequenced to determine their telomeric sequences. The FASTQ files for each species were processed using the Telomeric Repeats Identification Pipeline (TRIP), a bioinformatics tool designed to identify telomeric repeat motifs (TRMs). Although a definitive TRM candidate was not identified in all three species, likely due to the low sequencing coverage used in the analyses, the TRIP analysis successfully identified a series of candidate TRMs across all species. Specifically, a detailed examination of the 19 statistical outputs from the TRIP pipeline identified the sequence AAACC (or TTTGG) as a plausible TRM candidate in all three species (Fig. 6). Further analysis of potential TRMs in *L. hirta* also revealed the sequence AACCT (Fig. 6a), corresponding to the ancestral telomeric repeat TTAGG. However, AACCT (TTAGG) exhibited lower frequency compared to AAACC

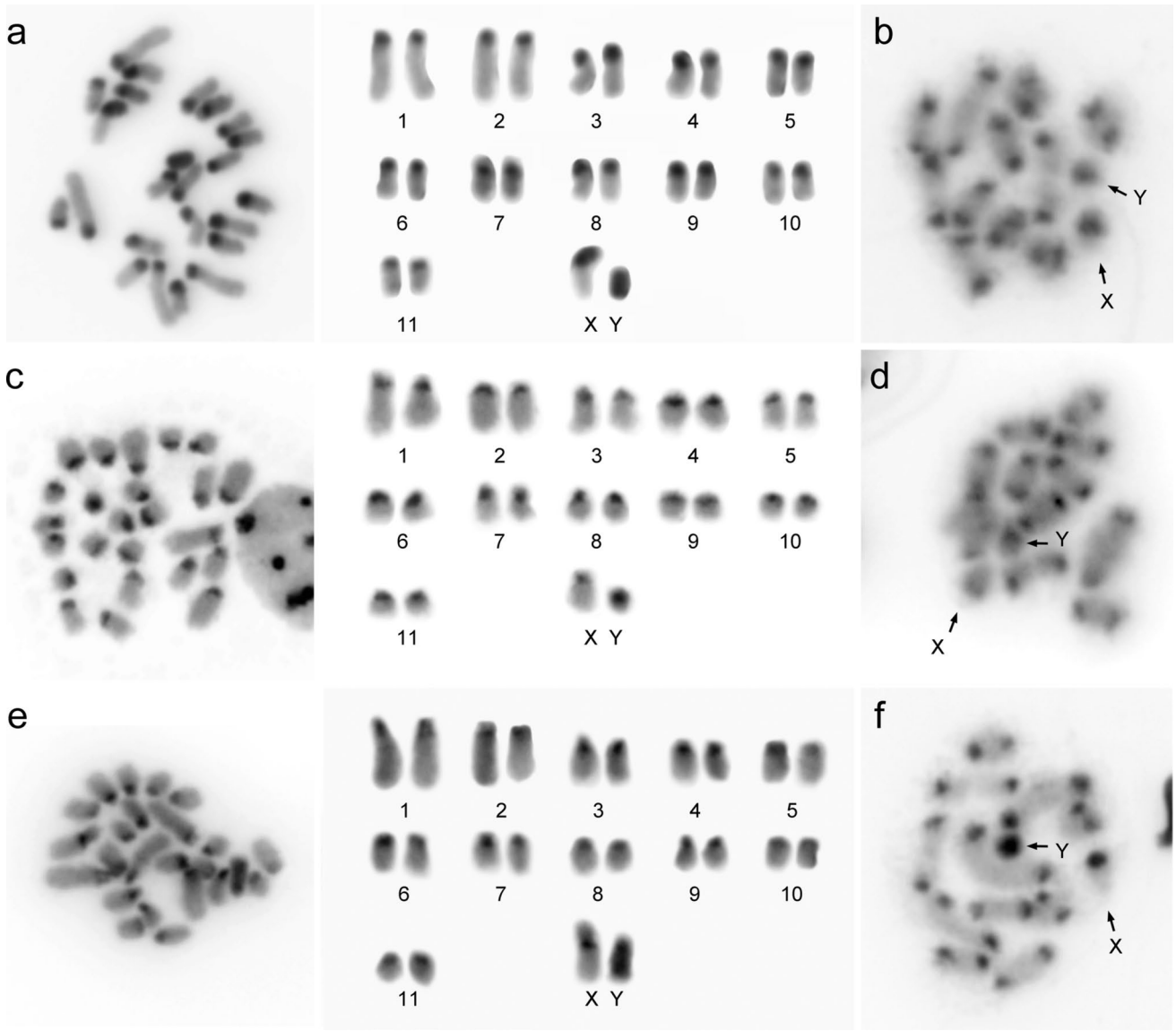

Fig. 3 *Lachnaia hirta*, **a** male mitotic metaphase after C-banding and karyotype of an adult male and **b** C-banding in meiotic metaphase I. *Lachnaia tristigma*, **c** male mitotic metaphase after C-banding and karyotype of an adult male and **d** C-banding in meiotic metaphase I. *Lachnaia vicina*, **e** male mitotic metaphase after C-banding and karyotype of an adult male and **f** C-banding in meiotic metaphase I. In all species is visible the presence of heterochromatic blocks on the centromeric region of the autosomes and on both sex chromosomes

(TTTGG) and was not identified as a candidate sequence in the other two species. These findings are consistent with the FISH results, which showed sparse hybridization signals in *L. hirta*. Watson et al. (2021) proposed that telomeric repeats should ideally span at least 300–400 bp in length, considering the minimal functional length required for the formation of t-loops, a requirement supported by several studies on telomeric sequences in various organisms (Barcenilla et al., 2023; Lukhtanov & Pazhenkova, 2023; Pazhenkova & Lukhtanov, 2023). To confirm whether the TTTGG TRM candidate exhibited tandem organization over this length, we performed a Bowtie2 analysis (Table 1). The results indicated that, in all three species, most 150-bp reads containing the TTTGG sequence in tandem were paired-end reads, suggesting that the 350-bp library fragments used in Illumina sequencing consisted entirely of the TTTGG repeat. The Bowtie2 analysis was also performed to search for reads containing the TTAGG repeat (Table 1). As expected from the results obtained by FISH, the species with the highest number of reads containing the TTAGG repeat was *L. hirta*, although the number of reads was significantly lower (190 for TTAGG compared to 8965 for TTTGG), as well as the percentage of paired reads (18.95% for TTAGG compared to 79.31% for TTTGG). These results indicate that in the

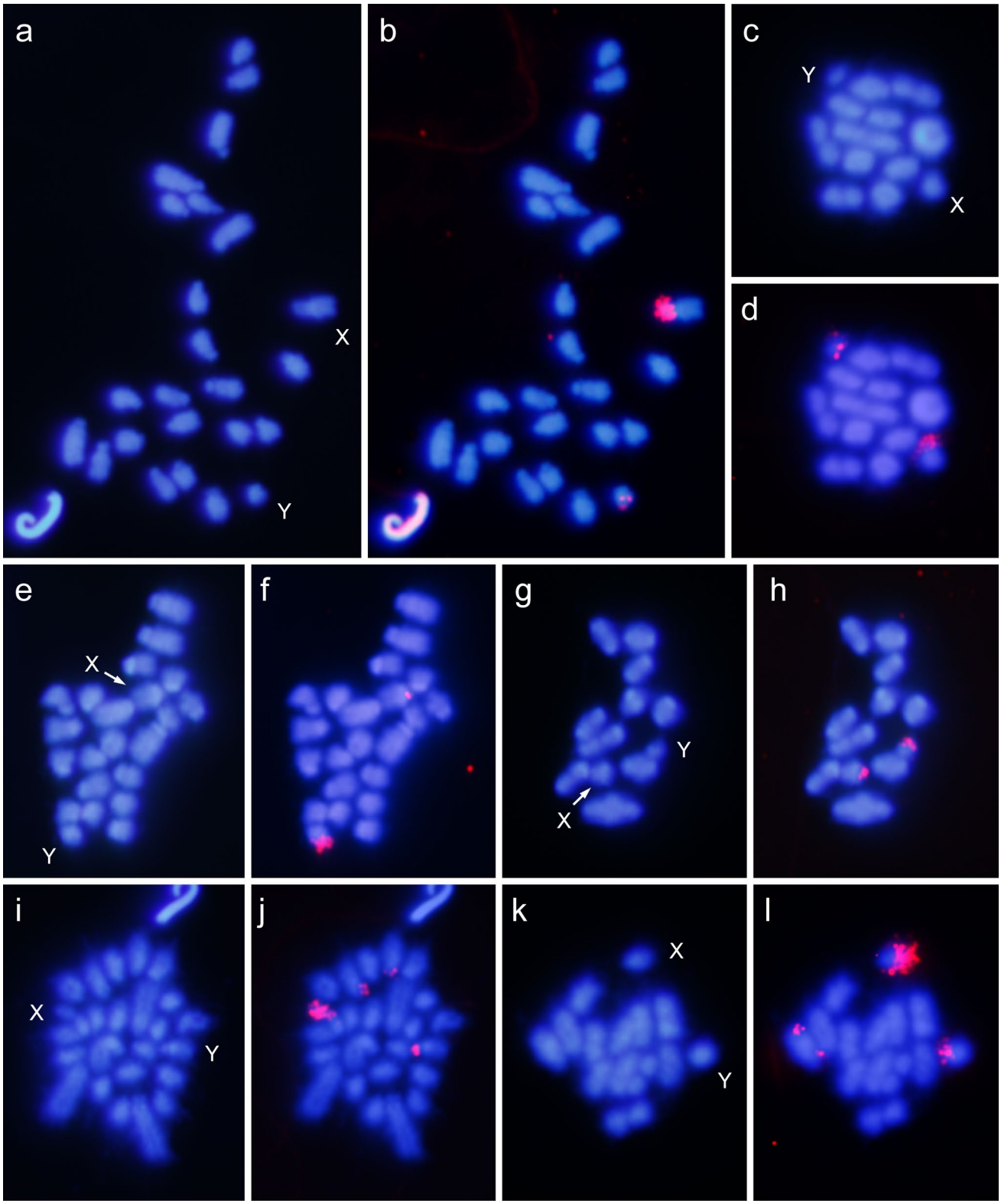

Fig. 4 FISH in *Lachnaia* species using a probe of rDNA. *Lachnaia hirta*, **a** DAPI staining and **b** FISH of male mitotic chromosomes, **c** DAPI staining and **d** FISH of male meiotic chromosomes. *Lachnaia tristigma*, **e** DAPI staining and **f** FISH of male mitotic chromosomes, **g** DAPI staining and **h** FISH of male meiotic chromosomes. *Lachnaia vicina*, **i** DAPI staining and **j** FISH of male mitotic chromosomes, **k** DAPI staining and **l** FISH of male meiotic chromosomes. Hybridization signals are visible on the sex chromosomes of the three species. In *Lachnaia vicina*, additional hybridization signals are visible on a pair of autosomes

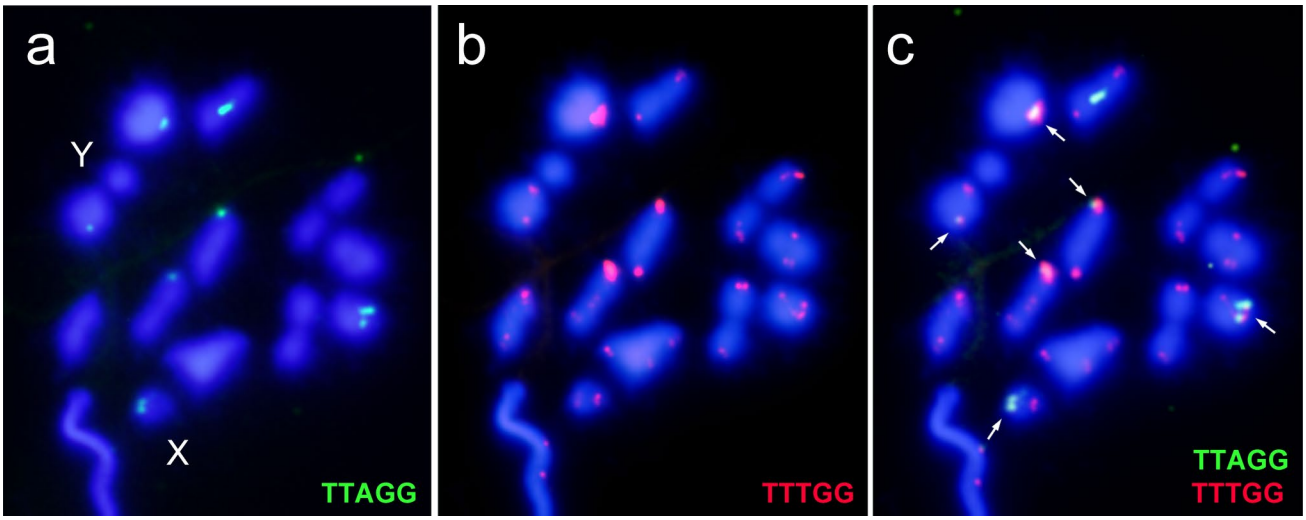

Fig. 5 Lachnaia hirta. **a** FISH with a telomeric TTAGG probe in meiotic chromosomes. **b** The same metaphase I after FISH using the TTTGG repeat as a probe. **c** Merged image. The arrows indicate the sites where hybridization signals with both probes were obtained

genome of *L. hirta*, the abundance of the TTAGG repeat is much lower than that of TTTGG and that the TTAGG arrays are likely much shorter than TTTGG arrays.

As experimental proof, we conducted FISH using the TTTGG sequence as a probe. In all three species, numerous hybridization signals were observed at the telomeric regions of the chromosomes (Fig. 7). However, not all chromosome ends showed hybridization signals. Several hypotheses can be proposed to explain these results. One possibility is that the size of the telomeric sequence in these regions without hybridization signals was smaller than in the telomeres where positive hybridization was observed. However, it is also likely that the differences in the number of hybridization signals are due to technical problems rather than differences in the amount of telomeric sequences, as has been observed in other insects (Lorite et al., 2002b). This is supported by the variability observed between homologous telomeres, where a clear hybridization signal is obtained on one chromatid's telomere but not on its sister chromatid. These differences among sister chromatids have been observed in all three *Lachnaia* species. A detailed analysis is not possible due to variability in the number of hybridization signals among cells and the similar size of the chromosomes. However, larger chromosomes and the sex chromosomes were easier to identify. The comparison of the obtained results supports the existence of technical problems. For instance, in Fig. 5b, hybridization signals with TTTGG are not visible on the Y chromosome of *L. hirta*, even though they are observed in other metaphases (Fig. 7b). Similarly, in Fig. 5b, the X chromosome shows hybridization signals at both ends, whereas in Fig. 7b, hybridization is observed at only one of the telomeres. A third cause for the absence of signal in some telomeres could be the presence of another telomeric sequence in those telomeres lacking hybridization signals with a TTTGG probe. As previously mentioned, FISH performed using the ancestral insect telomeric repeat TTAGG as a probe showed hybridization signals only in *L. hirta*. To further investigate, we performed double FISH using probes for both the TTAGG and TTTGG repeats (Fig. 7). The results showed that most regions displaying a signal with TTAGG also exhibit a signal for TTTGG, as has been reported in other insects (Zhou et al., 2022). These findings indicate that both repeats coexist in the telomeres of several chromosomes, although it cannot be ruled out that some telomeres contain only the TTAGG repeat.

The data suggest that in the analyzed *Lachnaia* species, the ancestral insect telomeric sequence TTAGG has been replaced by the TTTGG repeat, although in *L. hirta*, it remains present in some chromosomes. These findings are clearly consistent with the results of the bioinformatic analyses. The ancestral telomeric repeat in insects $(TTAGG)_n$ is only suggested by TRIP analysis as a plausible candidate in *L. hirta*, and not in the other two species. It is likely that, while this sequence is present in *L. tristigma* and *L. vicina* at lower levels, it lacks the necessary conditions to produce a detectable signal in the FISH analysis, at least under the experimental conditions used, which have nevertheless been efficient in detecting the TTTGG telomeric motif in all three species. Overall, the analyses indicated that the telomeric sequence considered ancestral in insects does not form the functional telomere in these species.

Several computational methods have been developed to identify and quantify telomere repeats from NGS data. The mean telomere length (MTL) in *Lachnaia* species was calculated using the Illumina reads and the program CompuTEL (v1.2). Using the "TTTGG" pattern, the MTL was

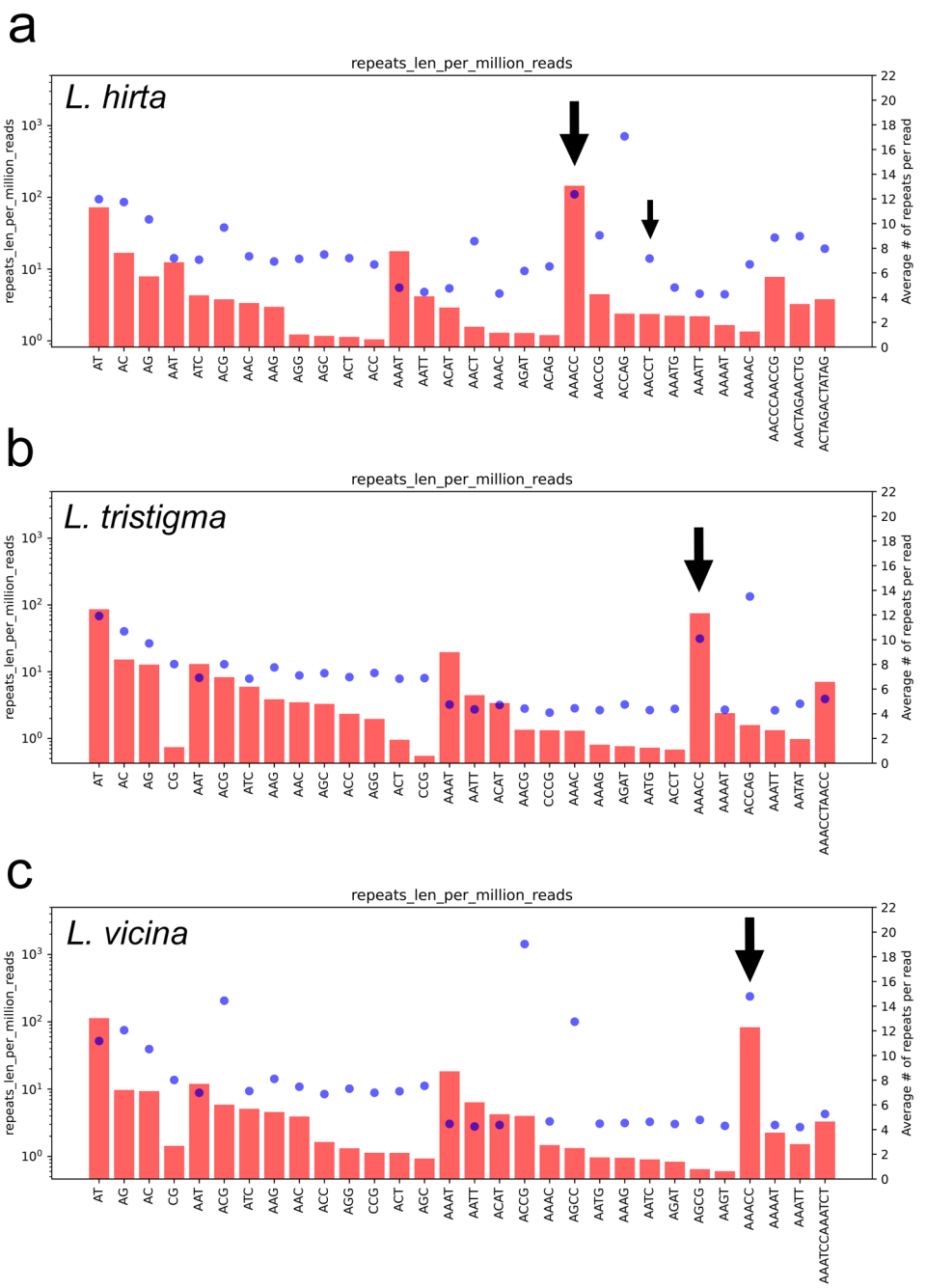

Fig. 6 Graphic output of the TRIP analysis in **a** *Lachnaia hirta*, **b** *Lachnaia tristigma*, and **c** *Lachnaia vicina* showing the best telomeric repeat motifs (TRM) candidates. On the X-axis, the top 30 candidate TRMs are represented. The red bars indicate the total repeat length per million reads for each TRM ($\log_{10}$ scale), while the blue points show the average number of repeats per read for each TRM. The TRM AAACC (TTTGG) is the most abundant and has the highest number of repeats per read simultaneously in all three species (large arrows). The small arrow indicates the TRM AACCT (TTAGG) in *L. hirta*. The AACCT is not among the top TRM candidates in *L. tristigma* or in *L. vicina*

Table 1 Results of the Bowtie2 analyses in the genome of the three *Lachnaia* species, using the TTTGG and TTAGG repeats

	TTTGG		TTAGG	
	Total reads	Paired-end reads (%)	Total reads	Paired-end reads (%)
L. hirta	8965	7010 (79.31)	190	36 (18.95)
L. tristigma	10,951	8954 (81.77)	33	26 (78.79)
L. vicina	15,563	14,028 (90.14)	0	0

estimated to range between 3.3 and 6.2 kb (Table 2). These data align with telomere size estimates in beetles, which typically range from 2 kb to more than 21 kb (Prušáková et al., 2021). In contrast, using the "TTAGG" pattern, much lower values were obtained. Only in *L. hirta*, with an MTL of 354 bp, could this repeat potentially form part of the

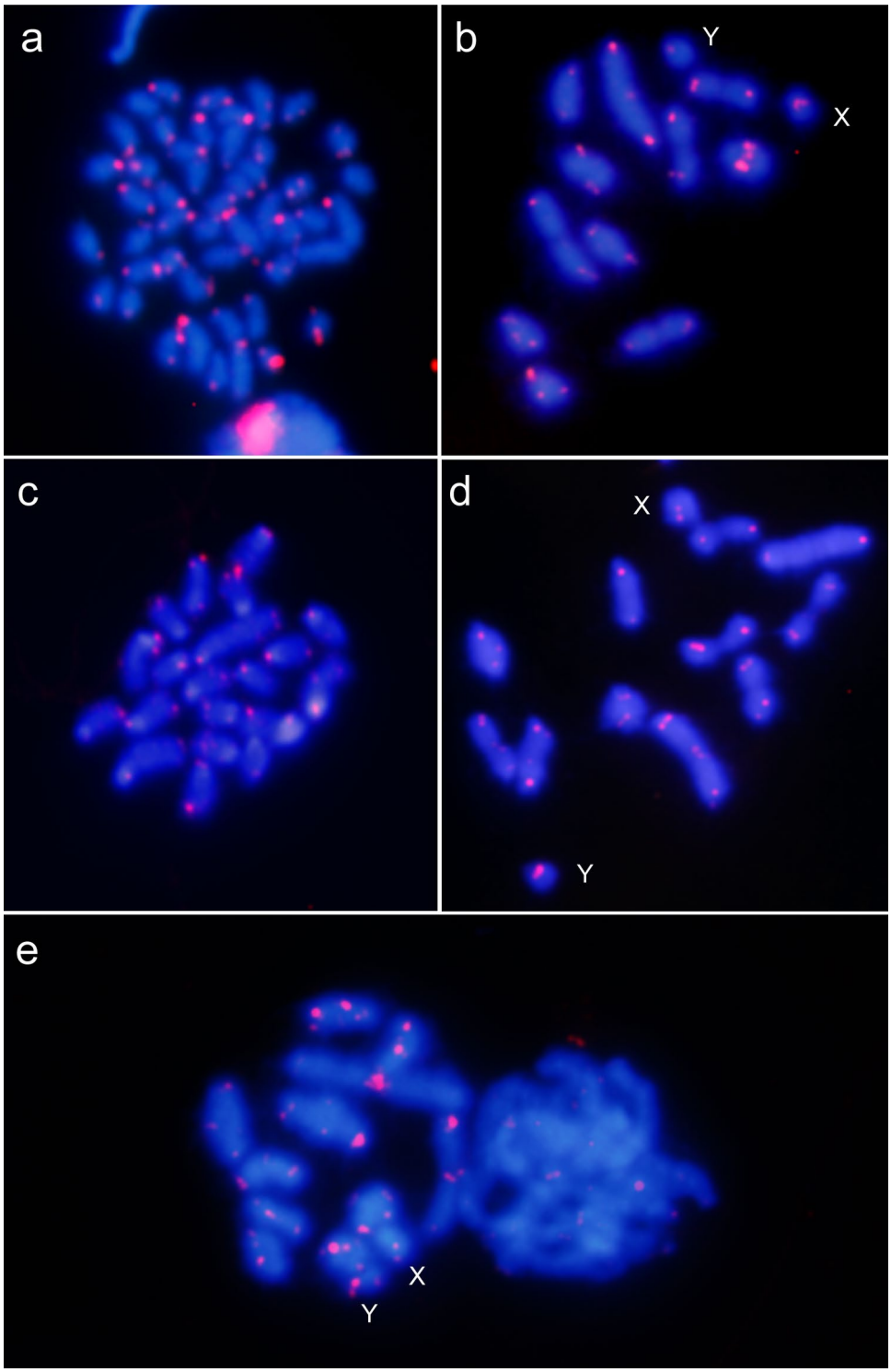

Fig. 7 FISH with a telomeric TTTGG probe in **a** mitotic and **b** meiotic chromosomes of *Lachnaia hirta*, **c** in mitotic and **d** meiotic chromosome of *Lachnaia tristigma*, and **e** in meiotic chromosomes of *Lachnaia vicina*

Table 2 Mean telomere length (in bp) in *Lachnaia* species, estimated using the CompuTEL program, with the "TTTGG" or the "TTAGG" patterns

	TTTGG	TTAGG
L. hirta	6201	345
L. tristigma	3360	16
L. vicina	4487	1

telomere, according to the previously mentioned proposal of Watson et al. (2021), which suggests a minimum size of 300–400 bp for a functional telomere. Altogether, the data obtained in this study support the hypothesis that TTTGG could represent a novel telomeric repeat motif in Coleoptera. This motif has also not been previously described in insects,

according to the data included in the TeloBase database (http://cfb.ceitec.muni.cz/telobase/) (Lyčka et al., 2024).

In recent years, a high diversity in the motifs and organization of telomeric DNA in insects has been described (Lukhtanov & Pazhenkova, 2023; Prušáková et al., 2021; Zhou et al., 2022). For instance, it has been reported that in numerous Hemiptera and Hymenoptera insects, the telomeric DNA sequence consists of long arrays of $(TTAGG)_n$ or other motifs, such as $(TTATTGGGG)_n$ or similar sequences with slight variations, regularly and specifically interrupted by the insertion of the non-long terminal repeat (non-LTR) retrotransposons (Lukhtanov & Pazhenkova, 2023; Zhou et al., 2022). This variability is further supported by studies of the telomerase RNA template region (Fajkus et al., 2023). In certain hymenopterans, different telomeric sequences have even been described at opposite chromosomal ends. For example, in *Lasioglossum lativentre* Schenck, 1853, chromosomes possess the short telomeric repeat $(TTAGGTCTGGG)_n$ at one end, while the opposite ends terminate with medium and long repeats (Lukhtanov & Pazhenkova, 2023). The coexistence of more than one telomeric motif has also been observed in other insects, such as the ant *Solenopsis geminata* (Zhou et al., 2022). In Coleoptera, the ancestral telomeric motif (TTAGG)n has been replaced by two new alternative motifs (TCAGG)n and (TTAGGG)n, across different species belonging to three superfamilies, including Chrysomeloidea, which is considered the most diverse within Metazoa (Prušáková et al., 2021), as highlighted in the introduction of this study. According to the authors, variation in telomeric sequences among different beetle taxa is positively correlated with the species richness.

Diversity and evolutionary changes in the composition and structure of telomeres have also been documented beyond insects, in other invertebrates. Olson et al. (2020) studied the telomeres in the tapeworm *Hymenolepis microstoma* Dujardin, 1845 (Platyhelminthes) using long-read sequencing, optical mapping, FISH techniques, and assembled chromosomes. The authors reported that the six chromosomes of this species possess TTAGGG, or similar, telomeric repeats at one end, while the opposite ends terminate with a novel repeat array with an average length of 179 bp, exhibiting centromeric characteristics. These findings suggest a potential functional and evolutionary relationship between centromeres and telomeres, indicating that, under specific conditions, there may be a replacement between these two types of DNA repeats (Olson et al., 2020).

A BLASTN search was conducted in DNA databases to determine whether other insect species present the TTTGG repeat as a telomeric sequence. Interestingly, we have found the TTTGG sequence in other Coleoptera species, such as the oak pinhole borer *Platypus cylindrus* Fabricius,1792 (Curculionidae), as well as in a Hymenoptera species, the wasp *Oxytorus armatus* Thomson, 1883 (Ichneumonoidea). The genomes of both species have been recently assembled at the chromosomal level (Barclay et al., 2024; Broad et al., 2024), although their telomeric sequences were not explicitly reported in these studies. The genome of *P. cylindrus* has been assembled into eight chromosomal pseudomolecules, including the X and Y sex chromosomes (Barclay et al., 2024). Analysis shows that most chromosomal pseudomolecules terminate with a TTTGG array. Similarly, the genome of *O. armatus* was assigned to 13 chromosomal-level scaffolds (Broad et al., 2024), and analysis indicates that 24 of the 26 chromosomal ends terminate with a TTTGG array, suggesting that this motif constitutes the putative telomere of this species.

In this study, the use of TRIP on the genome of three *Lachnaia* species, alongside other bioinformatic approaches and FISH results, suggests that the new motif TTTGG constitutes the telomeres in *L. hirta*, *L. tristigma*, and *L. vicina*. The detection of this motif in species of two Coleoptera families and in a Hymenoptera species supports this hypothesis and may indicate that this telomeric repeat emerged independently in distant insect groups. Future sequencing using long reads and high-quality genome assemblies in a broader range of insect species could definitively confirm the findings presented in this study.

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Declarations

Conflict of interest The authors declare no competing interests.

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