Fundação Universidade Federal de Mato Grosso do Sul
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Estudo cromossômico em gafanhotos do genêro *Temnomastax* Rehn & Rehn, 1942 (Orthoptera, Caelifera, Eumastacidae, Temnomastacinae)

Eduardo Bronca Bernava

Dissertação apresentada à Fundação Universidade Federal de Mato Grosso do Sul, como requisito à obtenção do título de Mestre em Biologia Animal.
Área de concentração: Zoologia.

Orientador: Douglas de Araujo

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Campo Grande, MS

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Resumo

Eumastacidae é uma família de gafanhotos com 255 espécies descritas, das quais apenas 16 possuem algum dado citogenético, apenas com a descrição pela coloração convencional. Estes trabalhos demonstraram uma alta variabilidade no número diploide e morfologia cromossômica, apesar de possuir um sistema cromossômico sexual (SCS) conservado do tipo X0/XX. Entretanto, esta baixa quantidade de dados cromossômicos e a utilização apenas da coloração convencional impedem discussões mais elaboradas acerca da comparação entre as espécies e evolução cariotípica dentro da família. O objetivo deste trabalho foi realizar a caracterização cromossômica de quatro espécies pertencentes ao gênero *Temnomastax*: *T. hamus*, *T. otavioi*, *T. ricardoi*, e *T. tigris*. Neste trabalho, as espécies foram analisadas através da coloração convencional (Giemsa 3%), e, pela primeira vez na família Eumastacidae, outras metodologias citogenéticas de marcação molecular foram aplicadas: bandamento-C, para a visualização das regiões de heterocromatina constitutiva; fluorocromo DAPI, para a caracterização da composição do DNA; e hibridação *in situ* fluorescente para a localização das regiões contendo genes de rDNA 28S e sequências teloméricas. As quatro espécies apresentaram o mesmo número diploide (2n♂ = 25 e 2n♀ = 26), morfologia cromossômica (primeiro par subtelocêntrico e o restante dos elementos telocêntricos), e SCS (tipo X0/XX). Entretanto, algumas individualidades foram notadas em algumas espécies, *T. otavioi* apresentou um polimorfismo para inversão pericêntrica no par 4, enquanto que foi observado um cromossomo B em uma fêmea de *T. hamus*. Além disso, a quantidade, localização e composição das regiões de heterocromatina intersticial variaram entre as espécies. Sinais de hibridação com a sonda telomérica foram observados na região dos telômeros nas quatro espécies, e, com exceção de *T. hamus*, em diversos sítios intersticiais. A posição do rDNA 28S foi terminal em todas as espécies, porém a distribuição variou entre 4 a 12 cromossomos. Os resultados mostram que apesar das quatro espécies possuírem o mesmo número, morfologia cromossômica e SCS, a organização de regiões cromossômicas específicas é diferente. Diversos mecanismos podem ter ocorrido para a mudança no número e posição das regiões evidenciadas neste trabalho, sendo este o principal fator de diferenciação cariotípica entre as espécies. Além disso, cada técnica utilizada permitiu a distinção das espécies, e, com isso, sugerimos que as regiões evidenciadas por estas técnicas atuem como marcadores citogenéticos para as espécies deste gênero.

Abstract

Eumastacidae is a family of grasshoppers with 255 described species, of which only 16 have some cytogenetic data, with only description by conventional staining. These works demonstrated a high variability in diploid number and chromosome morphology, despite having the conserved sex chromosome system (SCS) of the X0/XX type. However, this low amount of chromosomal data and the use of only conventional staining restrain the discussions about the comparison between species and karyotypic evolution in this family. The aim of this study was to characterize four species belonging to the genus *Temnomastax*: *T. hamus*, *T. otavioi*, *T. ricardoi*, and *T. tigris*. In this study, the species were analyzed by conventional staining (3% Giemsa), and, for the first time in the Eumastacidae family, with other cytogenetic techniques: C-banding, for visualization of the constitutive heterochromatin regions; fluorochrome DAPI, for the characterization of the DNA composition; and fluorescence *in situ* hybridization for localization of regions containing 28S rDNA genes and telomeric sequences. The four species presented the same diploid number, 2n♂ = 25 and 2n♀ = 26, chromosome morphology, with the first pair subtelocentric and the remaining of the elements telocentric, and SCS (X0/XX). However, some individualities were noticed in some species, *T. otavioi* presented a polymorphism for pericentric inversion on pair 4, while a B chromosome was observed in one female of *T. hamus*. Furthermore, the quantity, location and composition of the interstitial heterochromatin regions varied among the species. Hybridization signals with the telomeric probe were observed in the telomere region in all four species and, with the exception of *T. hamus*, in several interstitial sites. The location of the 28S rDNA was terminal in all species, however its distribution varied between 4 and 12 bearing chromosomes. The results show that although the four species have the same diploid number, chromosome morphology and SCS, the organization of specific chromosome regions is different. Several mechanisms may have occurred for the change in the number and position of the regions evidenced in this study, which is the main factor for the karyotypic differentiation between the species. Furthermore, each used technique allowed species distinction, therefore, we suggest the regions highlighted by these techniques as cytogenetic markers for the species in this genus.

INTRODUÇÃO

Eumastacidae é uma família de gafanhotos (Orthoptera: Caelifera) conhecida como “monkey grasshoppers” e “saltamontes payaso”, que abrange 255 espécies (Cigliano et al., 2024), as quais são caracterizadas principalmente pela posição em descanso, na qual o par de pernas posteriores fica perpendicular ao eixo corporal (Fig. 1). Outras características utilizadas na identificação dos representantes desta família são a ausência de tímpano e órgão para estridulação, antena menor que o fêmur anterior e membrana cervical evidente. Porém, algumas destas características são variáveis dentro da família e podem, ainda, ser encontradas em outras famílias de Orthoptera (Rehn, 1948; Dirsh, 1961; Souza et al., 2024).

Figura 1. Exemplar da família Eumastacidae demonstrando a posição das pernas em descanso. Fonte: Orthoptera Species File.

Estudos citogenéticos nesta família são escassos, com apenas 16 espécies (aproximadamente 6%) descritas por coloração convencional (Tabela 1). O número diploide registrado nos machos é variável entre 2n= 17 e 2n= 25 e o sistema cromossômico sexual (SCS) do tipo X0♂ /XX♀ foi constante em todas as espécies. A morfologia, apesar de apresentar variação, mostrou, na maioria das espécies, cromossomos predominantemente acrocêntricos (White, 1968, 1970; Mesa & Ferreira, 1978; Rentz & Weissman, 1981; Mesa, 1984; Mesa & Mello, 1984). Devido a baixa quantidade de espécies cariotipadas e a limitação de dados obtidos pela coloração convencional, análises sobre a evolução cromossômica no grupo são prejudicadas e, muitas vezes, não conclusivas.

Tabela 1. Espécies de Eumastacidae com dados citogenéticos, incluindo suas subfamílias, número diploide (2n) em machos, SCS, fórmula cariotípica (FC), e referências. a: acrocêntrico; m: metacêntrico; sa: subacrocêntrico; sm: submetacêntrico; t: telocêntrico.

Subfamily	Species	2n	SCS	FC	References
Eumastacinae	*Eumastax pictipes* Descamps, 1971	21	X0/XX	21a	Mesa & Ferreira, 1978
	Eumastax salazari Descamps, 1971	21	X0/XX	21a	White, 1970
	Eumastax aff. *tenuis*	19	X0/XX	19a	Mesa & Ferreira, 1978
	Eumastax aff. *tenuis*	23	X0/XX	2m + 20a + Xa	Mesa & Ferreira, 1978
	Eumastax vittithorax Descamps, 1974	21	X0/XX	2m + 18a + Xa	Mesa & Ferreira, 1978
Gomphomastacinae	*Gomphomastax clavata* (Ostroumov, 1881)	19	X0/XX	2m + 2sm + 14a + Xa	White, 1968
	Phytomastax opaca (Krauss, 1898)	19	X0/XX	2m + 16a + Xa	White, 1968
Morseinae	*Daguerreacris tandiliae* Descamps & Liebermann, 1970	17	X0/XX	2m + 14sa + Xm	Mesa, 1984
	Morsea calofornica Scudder, 1898	23	X0/XX	23a	Rentz & Wiessman, 1981
Paramastacinae	*Paramastax nigra* (Scudder, 1875)	17	X0/XX	17m	Mesa & Ferreira, 1978
	Paramastax rosenbergi (Burr, 1899)	19	X0/XX	10m + 4a + 4sa + Xa	White, 1970
Parepisactinae	*Parepisactus carinatus* Giglio-Tos, 1898	19	X0/XX	10m + 6a + 2sa + Xa	White, 1970
Pseudomastacinae	*Pseudomastax carlosi* Descamps, 1973	25	X0/XX	4m + 20a + Xa	Mesa & Ferreira, 1978
	Pseudomastax imitatrix (Gerstaecker, 1889)	23	X0/XX	23a	Mesa & Ferreira, 1978
	Pseudomastax pictifrons (Bruner, 1920)	25	X0/XX	4m + 20a + Xa	Mesa & Ferreira, 1978
Temnomastacinae	*Temnomastax hamus* Rehn & Rehn, 1942	25	X0/XX	2sa + 23t	Mesa & Mello, 1984

Dentro dessa família, são alocadas atualmente oito subfamílias, majoritariamente Neotropicais, com exceção de Gomphomastacinae que ocorre na região asiática, destas apenas quatro subfamílias são encontradas no Brasil: Eumastacinae, Parepisactinae, Pseudomastacinae e Temnomastacinae (Descamps, 1973; Cigliano et al., 2024; Souza et al., 2024). Temnomastacinae é um dos grupos mais diversos e mais amplamente distribuídos da família, possuindo 46 espécies alocadas em duas tribos: Eumastacopini e Temnomastacini. Esta última está restrita à América do Sul e possui dois gêneros: *Eutemnomastax* Decamps, 1979 e *Temnomastax* Rehn & Rehn, 1942 (Descamps, 1973; Cigliano et al., 2024).

Eutemnomastax possui quatro espécies: *E. burri* Descamps, 1982, *E. caatingae* Descamps, 1982, *E. saurus* (Burr, 1899), e *E. striata* Descamps, 1982, todas com ocorrência no nordeste brasileiro (Descamps, 1982; Cigliano et al., 2024). Este gênero se diferencia de *Temnomastax* pela condição áptera, com exceção de *E. striata*, a qual é micróptera, cerco nos machos pouco especializado, tergitos 7 e 8 nos machos e 7 nas fêmeas sem coloração diferenciada, variações no formato do epífalo e esclerito endofálico curvado em vista lateral (Descamps, 1979). Nenhuma espécie de *Eutemnomastax* foi descrita citogeneticamente.

Temnomastax, por sua vez, possui nove espécies: *T. beni* Rehn & Rehn, 1942, *T. borellii* (Giglio-Tos, 1897), *T. hamus*, *T. latens* Rehn & Rehn, 1942, *T. monnei* Olivier, 2019, *T. descampsi* Olivier, 2019, *T. otavioi* Olivier, 2019, *T. ricardoi* Descamps, 1973 e *T. tigris* (Burr, 1899). Todas as espécies de *Temnomastax* possuem registro no Brasil (Descamps, 1973; Olivier et al., 2019, 2025; Cigliano et al., 2024). As espécies deste gênero são caracterizadas pelo fastígio truncado e evidente em vista lateral, tergitos 7 e 8 nos machos e 7 nas fêmeas colorido, asas presentes, e cerco nos machos comprimido ou curvado para dentro na porção apical. Porém, algumas destas características são modificadas em *Temnomastax beni*, a qual também é a única espécie macróptera do gênero (Rehn & Rehn, 1942; Rehn & Grant Jr., 1958; Descamps, 1973, 1979; Olivier et al., 2019; Cigliano et al., 2024). Entretanto, as demais espécies são muito similares morfologicamente e a identificação se baseia principalmente na placa subgenital das fêmeas e no complexo fálico e cercos nos machos, podendo ainda serem utilizados parâmetros morfométricos (Rehn & Rehn, 1942; Olivier & Aranda, 2018).

No Brasil, *Temnomastax* está amplamente distribuído, principalmente em regiões de planalto, tendo registro para sete estados: Goiás, Mato Grosso, Mato Grosso do Sul, Pará, Tocantins, Minas Gerais e São Paulo (Descamps, 1973; Olivier et al., 2019; Cigliano et al., 2024).

Temnomastax hamus é a única espécie do gênero com o cariótipo descrito, apresentando 2n= 25 nos machos, SCS X0/XX, com o cromossomo X sendo o segundo

maior elemento do cariótipo, e morfologia predominantemente telocêntrica, com exceção do primeiro par cromossômico que é subacrocêntrico (Mesa & Mello, 1984).

A citogenética é uma importante ferramenta que permite a caracterização do cariótipo das espécies, seja pela coloração convencional, que fornece dados como número diploide, morfologia cromossômica, e SCS, ou outras técnicas que marcam regiões cromossômicas específicas, sendo possível uma comparação interespecífica, o que pode auxiliar na identificação e nas discussões acerca das relações evolutivas das espécies, além de fornecer informações sobre a organização genômica (White, 1973; Araujo et al., 2015).

Dentre os marcadores cromossômicos que podem ser evidenciados por técnicas citogenéticas estão as regiões de heterocromatina, que são porções do cromossomo com sequências de DNA repetitivo que permanecem condensadas mesmo após a divisão celular (Yunis & Yasmineh, 1971; Alshire & Madhani, 2018). A heterocromatina pode ser dividida em heterocromatina facultativa, podendo variar entre um estado condensado ou descondensado, ou constitutiva, quando esta estrutura se encontra fixa em alguma região dos cromossomos, sendo constante nas células do indivíduo (Brow, 1966; Saksouk et al., 2015). A distribuição de heterocromatina constitutiva nos cromossomos pode ser identificada pela técnica de bandamento-C, podendo variar entre espécies, auxiliando como marcador na diferenciação das mesmas (John, 1988).

Além disso, a composição da heterocromatina constitutiva, abundância de bases AT ou CG, pode variar entre espécies, ou até mesmo entre diferentes cromossomos. Para a verificação da composição de bases destas regiões, o emprego de fluorocromos vem sendo utilizado em adição à técnica de bandamento-C, no qual os fluorocromos cromomicina A3 (CMA3), que detecta regiões ricas em bases CG, e o 4',6-diamidino-2-fenilindol (DAPI), que evidencia zonas ricas em bases AT, são os mais utilizados (Guerra, 2000).

Outra técnica da citogenética frequentemente utilizada, e que auxilia na caracterização cromossômica é a hibridação *in situ* fluorescente (FISH). Esta técnica se baseia na utilização de uma sonda, geralmente um fragmento de DNA com uma sequência de interesse marcada, que irá se hibridizar com o DNA em estudo, e assim, os sinais emitidos pela sonda poderão ser visualizados no microscópio, indicando a quantidade e localização destas sequências nos cromossomos (Guerra, 2004). Diferentes sondas podem ser utilizadas, a depender dos objetivos, porém, algumas sequências são frequentemente utilizadas na citotaxonomia. Dentre elas, sondas com DNA ribossômico (rDNA), como as sequências dos genes ribossomais 45S (5,8S, 18S e 28S) responsáveis pela formação e manutenção das regiões organizadoras de nucléolo, podem auxiliar como um caráter adicional que pode variar entre as espécies (Lemieux et al., 1992; Sumner, 2003). Adicionalmente, a hibridação com sondas teloméricas pode auxiliar na descrição de rearranjos cromossômicos. (Varella-Garcia et al., 2004).

Essas técnicas, amplamente difundidas dentro da citogenética, vem sendo também utilizadas em diferentes estudos com gafanhotos, especialmente na superfamília Acridoidea, visto que muitas espécies das famílias Acrididae e Romaleidae apresentam um cariótipo conservado de 2n♂ = 23, X, e cromossomos com morfologia acrocêntrica impossibilitando a diferenciação apenas pela coloração convencional (Mesa et al., 1982). Assim, com a quantidade e localização destes marcadores descritos acima, é possível diferenciar espécies filogeneticamente próximas. (King & John, 1980; Rufas et al., 1985; Cabrero & Camacho, 1986; Souza et al., 1998; Cabrero et al., 2003; Aswathanarayana & Ashwath, 2006; Cabrero & Camacho, 2008; Chadha & Mehta, 2011; Quing et al., 2012; Grzywacz et al., 2018). Entretanto, estas técnicas citogenéticas diferenciais ainda não foram aplicadas em espécies da família Eumastacidae, dificultando muitas vezes a distinção cromossômica entre espécies na família e análises da evolução cromossômica.

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Cytotaxonomy of four species of *Temnomastax* (Orthoptera, Eumastacidae) based on heterochromatin, rDNA 28S and interstitial telomeric sites

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ABSTRACT
Eumastacidae is a family of grasshoppers with 255 described species, however only 16 have some cytogenetic data, by conventional staining, showing a great variability in the diploid number and chromosome morphology, despite a preserved X0/XX sex chromosome system (SCS). Chromosome studies can be useful for species delimitation and their evolutionary relationship, using conventional and differential techniques. Here, we carried out the chromosomal characterization of four species of *Temnomastax*: *T. hamus* Rehn & Rehn, 1942, *T. otavioi* Olivier, 2019, *T. ricardoi* Descamps, 1973, and *T. tigris* (Burr, 1899), using conventional staining, and, for the first time in Eumastacidae, differential cytogenetic techniques: c-banding, DAPI fluorochrome, and fluorescent *in situ* hybridization with telomeric and 28S rDNA probes. The four species exhibited the same diploid number, chromosome morphology, and SCS (2n♂ = 25, X, telocentrics, except the subtelocentric pair 1). However, some particularities, such as polymorphism for pericentric inversion on chromosome 4 in *T. otavioi* and the presence of B chromosome in *T. hamus*, were noticed. Furthermore, differences in the number (2 to 12) of heterochromatin bands, number and location of nucleolar organizer region (NOR) (4 to 12 NOR-bearing chromosomes), and presence of interstitial telomeric sites (in three species), provided species-specific cytogenetic marker.

Keywords: cytogenetics; C-bands; FISH; monkey grasshopper; telomeres; nucleolar organizer region

INTRODUCTION

Eumastacidae is a family of grasshoppers (Orthoptera, Caelifera), known as monkey grasshoppers, with 255 species, 55 genus, and eight subfamilies (Cigliano *et al.* 2024). This family is characterized by following combination of characters: hind legs perpendicular to the body axis when resting, cervical membrane exposed, and absence of tympanum and stridulatory apparatus (Rehn 1948, Dirsh 1961, Souza *et al.* 2024). Descamps (1973), based on morphological characteristics, placed Eumastacidae along with Morabidae in Stenophalii, within the Eumastacoidea superfamily. More recent studies, using molecular data, confirmed this relationship, although they recovered Eumastacidae as paraphyletic applying the total evidence analyses (Song *et al.* 2015) or using ribosomal sequences, in which the Asian subfamily Gomphomastacinae was related to other group of Eumastacoidea (Matt *et al.* 2008). The seven other Eumastacidae subfamilies occur in the Neotropical region, and four of them, comprising 43 species, have records in Brazil: Eumastacinae, Parepisactinae, Pseudomastacinae and Temnomastacinae (Descamps 1973, Cigliano *et al.* 2024, Souza *et al.* 2024).

Cytogenetic data are available for only approximately 6% of the species of Eumastacidae i.e., 16 species, representing seven subfamilies, and they were based only on conventionally stained chromosome preparations (Table 1). In all the karyotyped species, the chromosomal morphology is predominantly acrocentric and the sex chromosome system (SCS) is X0♂/XX♀, but the diploid number in males ranges from 2n = 17 to 2n = 25 (White 1968, 1970, Mesa & Ferreira 1978, Rentz & Weissman 1981, Mesa 1984, Mesa & Mello 1984). Those works revealed a diverse chromosomal number in Eumastacidae, even in species of the same genus.

Table 1. Species of Eumastacidae that were previously karyotyped, including their subfamily, diploid number (2n) in males, SCS, karyotype formula (KF) and references. a: acrocentric; m: metacentric; sa: subacrocentric; sm: submetacentric; t: telocentric.

Subfamily	Species	2n	SCS	KF	References
Eumastacinae	*Eumastax pictipes* Descamps, 1971	21	X0/XX	21a	Mesa & Ferreira, 1978
	Eumastax salazari Descamps, 1971	21	X0/XX	21a	White, 1970
	Eumastax aff. *tenuis*	19	X0/XX	19a	Mesa & Ferreira, 1978
	Eumastax aff. *tenuis*	23	X0/XX	2m + 20a + Xa	Mesa & Ferreira, 1978
	Eumastax vittithorax Descamps, 1974	21	X0/XX	2m + 18a + Xa	Mesa & Ferreira, 1978
Gomphomastacinae	*Gomphomastax clavata* (Ostroumov, 1881)	19	X0/XX	2m + 2sm + 14a + Xa	White, 1968
	Phytomastax opaca (Krauss, 1898)	19	X0/XX	2m + 16a + Xa	White, 1968
Morseinae	*Daguerreacris tandiliae* Descamps & Liebermann, 1970	17	X0/XX	2m + 14sa + Xm	Mesa, 1984
	Morsea calofornica Scudder, 1898	23	X0/XX	23a	Rentz & Wiessman, 1981
Paramastacinae	*Paramastax nigra* (Scudder, 1875)	17	X0/XX	17m	Mesa & Ferreira, 1978
	Paramastax rosenbergi (Burr, 1899)	19	X0/XX	10m + 4a + 4sa + Xa	White, 1970
Parepisactinae	*Parepisactus carinatus* Giglio-Tos, 1898	19	X0/XX	10m + 6a + 2sa + Xa	White, 1970
Pseudomastacinae	*Pseudomastax carlosi* Descamps, 1973	25	X0/XX	4m + 20a + Xa	Mesa & Ferreira, 1978
	Pseudomastax imitatrix (Gerstaecker, 1889)	23	X0/XX	23a	Mesa & Ferreira, 1978
	Pseudomastax pictifrons (Bruner, 1920)	25	X0/XX	4m + 20a + Xa	Mesa & Ferreira, 1978
Temnomastacinae	*Temnomastax hamus* Rehn & Rehn, 1942	25	X0/XX	2sa + 23t	Mesa & Mello, 1984

1-Voucher codes, ZUFMS-ORTO: 3681-3692; 3694-3707;3724-3730; 3741; 3744-3746; 3802-3840; 3858-3877; 3895-03897

This notable variation in diploid number contrasts with the findings for other families, which include larger numbers of karyotyped species, such as Acrididae, Ommexichidae, and Romaleidae, where 23 is the common diploid number for males (e.g., 74% in Acrididae) (Mesa *et al.* 1982, Santander *et al.* 2021, Husemann *et al.* 2022). Given the conserved diploid number, differential techniques, including C-banding and fluorescent in situ hybridization (FISH), have been commonly used to characterize and differentiate closely related species in these families (Santos *et al.* 1983, Cabrero & Camacho 2008, Grzywacz *et al.* 2018).

Temnomastax is a genus of Temnomastacinae, restricted to South America. It comprises nine species, all of them are recorded in Brazil, especially at the Cerrado domain (Descamps 1973, Olivier *et al.* 2019, 2025, Cigliano *et al.* 2024). Olivier *et al.* (2019), based on morphological characteristics, separate the genus in three morphological groups: 1) *Beni* group, with only one species: *T. beni* Rehn & Rehn, 1942; 2) *Latens* group: *T. borellii* (Giglio-Tos, 1897), *T. hamus, T. latens* Rehn & Rehn, 1942, and *T. monnei* Olivier, 2019; 3) *Tigris* group: *T. descampsi* Olivier, 2019, *T. otavioi* Olivier, 2019, *T. ricardoi* Descamps, 1973 and *T. tigris* (Burr, 1899).

The Temnomastax species, especially those of the same group, are very similar morphologically with each other, and their identification is mainly based on the subgenital plate of females, phallic complex and cerci of males, and also in some morphometric parameters (Rehn & Rehn 1942, Olivier & Aranda 2018). Attempting to aid in the taxonomic identification and explore chromosomal evolution, this work characterizes the karyotypes of four Temnomastax species, using conventional staining, and, for the first time for the Eumastacidae family, the C-banding technique, DAPI fluorochrome, and FISH with telomeric and 28S rDNA probes.

MATERIALS AND METHODS

All specimens of Temnomastax were collected at Mato Grosso do Sul state in Brazil between September 2023 and April 2024, by using an entomological net at daylight. After the dissection of the individuals and removal of the gonads, the specimens were pinned and deposited at the Coleção Zoológica da Universidade Federal de Mato Grosso do Sul (ZUFMS)[1], Campo Grande, state of Mato Grosso do Sul, Brazil. A total of 99 individuals were analyzed, and 65 exhibited some results, belonging to four species: *T. hamus*, *T. otavioi*, *T. ricardoi*, and *T. tigris* (Fig. 1). Table 2 describes the analyzed material, collections sites, as well as the number of individuals and cells examined.

1-Voucher codes, ZUFMS-ORTO: 3681-3692; 3694-3707;3724-3730; 3741; 3744-3746; 3802-3840; 3858-3877; 3895-03897

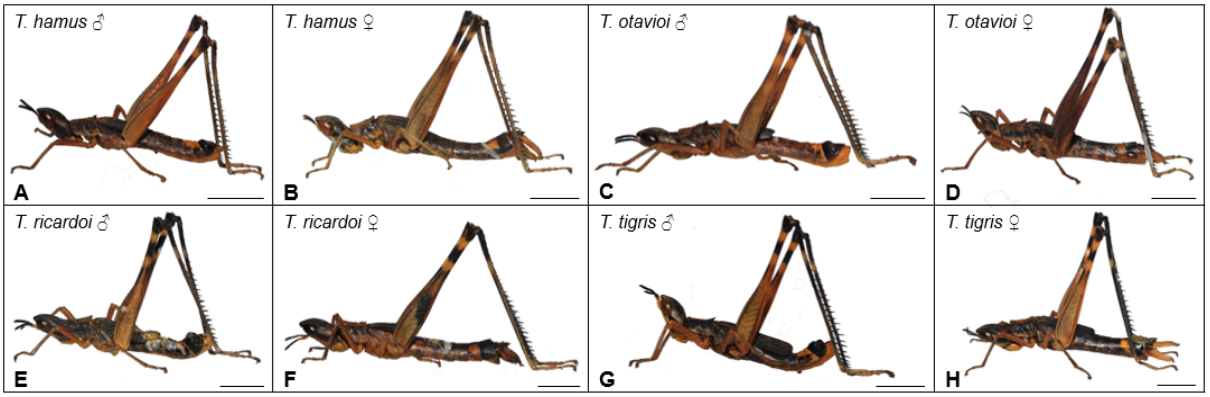

Figure 1. *Temnomastax* species analyzed. A-B, *T. hamus*. C-D, *T. otavioi*. E-F, *T. ricardoi*. G-H, *T. tigris*. Scale bars = 0,5 cm

Table 2. *Temnomastax* species analyzed in this work, their morphological group, locality within the state of Mato Grosso do Sul (Brazil), number of analyzed individuals (N), cells, and measured metaphases.

Morphological group	Species	Locality	N	Cells	Measured metaphases
Latens	*T. hamus*	Campo Grande[1]	5♂/2♀	172	22
		Aquidauana[2]	15♂/3♀		
Tigris	*T. otavioi*	Aquidauana[2,3]	13♂/3♀	225	39
	T. ricardoi	Campo Grande[1]	5♂/3♀	68	15
		Águas Claras[4]	1♀		
	T. tigris	Bonito[5]	10♂/3♀	258	21
		Corumbá[6]	4♂		

1 – Estância Sossego, Campo Grande (20º29'11.7"S, 54°30'06.9"W) –12/16/2023
2 – Morro do Paxixi, Aquidauana (20°27'00.5"S, 55°37'20.8"W) –10/20/2023; 03/21/2024
3 – Sítio Arqueológico Centro de Educação Rural de Aquidauana/Universidade Estadual de Mato Grosso do Sul (CERA/UEMS), Aquidauana (20°27'13.4"S, 55°39'22.8"W) –03/21/2024
4 – Reservatório da Usina Hidrelétrica São Domingos, Águas Claras (20°03'25.5"S, 53°10'43.8"W) –03/29/2024
5 – Recanto das Águas, Bonito (20º59'19.1"S, 56º31'45.5"W) –09/30/2023; 03/20/2024
6 – Morro Santa Cruz, Corumbá (19°12'26.7"S, 57°36'27.8"W) –04/10/2024

The chromosomal preparation using the gonads follows the procedure described by Araujo *et al.* (2008), with a 3% Giemsa staining. The C-banding technique was performed based on the methodology described by Sumner (1972), with 55 s in a 50% Ba(OH)2 solution.

FISH was carried out using telomeric and 28S rDNA probes. The DAPI-positive marking description was based on photographs with a DAPI filter (420-470 nm), after the hybridization. The telomeric-FISH were performed following the protocol of Genet *et al.* (2013), with a hybridization time of four hours at 37°C, without heat denaturing.

We applied a peptidic nucleic acid (PNA) (AATCC)3 probe (PNA Bio, Inc) that is complementary to the typical (TTAGG)n telomeric repeats of Orthoptera, labeled with Alexa fluor 488 (ThermoFisher Scientific), and mounted the slides using ProLong Diamond antifade with DAPI (ThermoFisher Scientific).

The 28S rDNA FISH assays were conducted using cloned fragments (clone 8; GenBank accession number PV172435) derived from *Cyrtophora citricola* (Forskål, 1775) and developed by Souza *et al.* (unpublished work). First, plasmid DNA was extracted following the protocol of Sambrook *et al.* (1989). The target fragment was then amplified via polymerase chain reaction (PCR) using the primers 28Sv and 28Sjj (Hillis & Dixon 1991) and the PCR DIG Probe Synthesis Kit (Roche). The labeled DNA was resuspended in a hybridization solution containing 50% formamide, 10% dextran sulfate, and 2X saline-sodium citrate (SSC), and hybridization was performed according to the methodology described by Viegas-Péquignot (1992). Probe detection was carried out using anti-digoxigenin antibodies conjugated with rhodamine (0.06 µg/mL; Roche). Finally, chromosomes were stained with DAPI (0.5 µg/mL) diluted in Vectashield (Vector).

Chromosome morphology was determined using the LEVAN plugin (Sakamoto & Zacaro 2009) from the ImageJ software (Rasband 1997–2024), which follows the nomenclature of Green & Sessions (1991).

RESULTS

Karyotype characterization

The four species of *Temnomastax* revealed karyotypes with 2n♂= 25 (Fig. 2A, C, D, F, H) and 2n♀= 26 (Fig. 2B, E, G, I). Spermatocytes exhibited 12 autosomal bivalents and one isopicnotic sex univalent (12II + X) in diplotene (Fig. 3A, D, G, H), and metaphases II with n= 12 and n= 13 = 12 + X (metaphases II not found in *T. ricardoi*) (Fig. 3B, C, E, F, I, J). Therefore, the four species possess a ♂X0/♀XX SCS. Overall, diplotene cells showed one chiasma per bivalent, with the exception of the two chiasmata in one bivalent in *T. hamus* and *T. tigris*, and three bivalents of *T. otavioi* (Fig. 3A, D, H).

The first pair of chromosomes in all the species is subtelocentric, whereas the remaining elements are telocentric. The X chromosome is the second largest element of the karyotype, and a gradual reduction in length is observed from chromosome pair 2 to 12 (Fig. 2). However, *T. otavioi* exhibits intrapopulational variation regarding the morphology of chromosome 4, with 11 individuals showing the standard karyotype morphology described above (with a homomorphic pair 4 with telocentric chromosomes) (Fig. 2C), while the remaining individuals showed subtelocentric

homologs in chromosome pair 4 (four individuals) (Fig. 2D) or a heteromorphic pair 4, consisting of a telocentric and a subtelocentric homolog (one individual) (Fig. 2E). Furthermore, within the 18 specimens from Morro do Paxixi, one female of *T. hamus* showed one small telocentric B chromosome in 15 of 18 analyzed cells (83%) (Fig. 2B, 4).

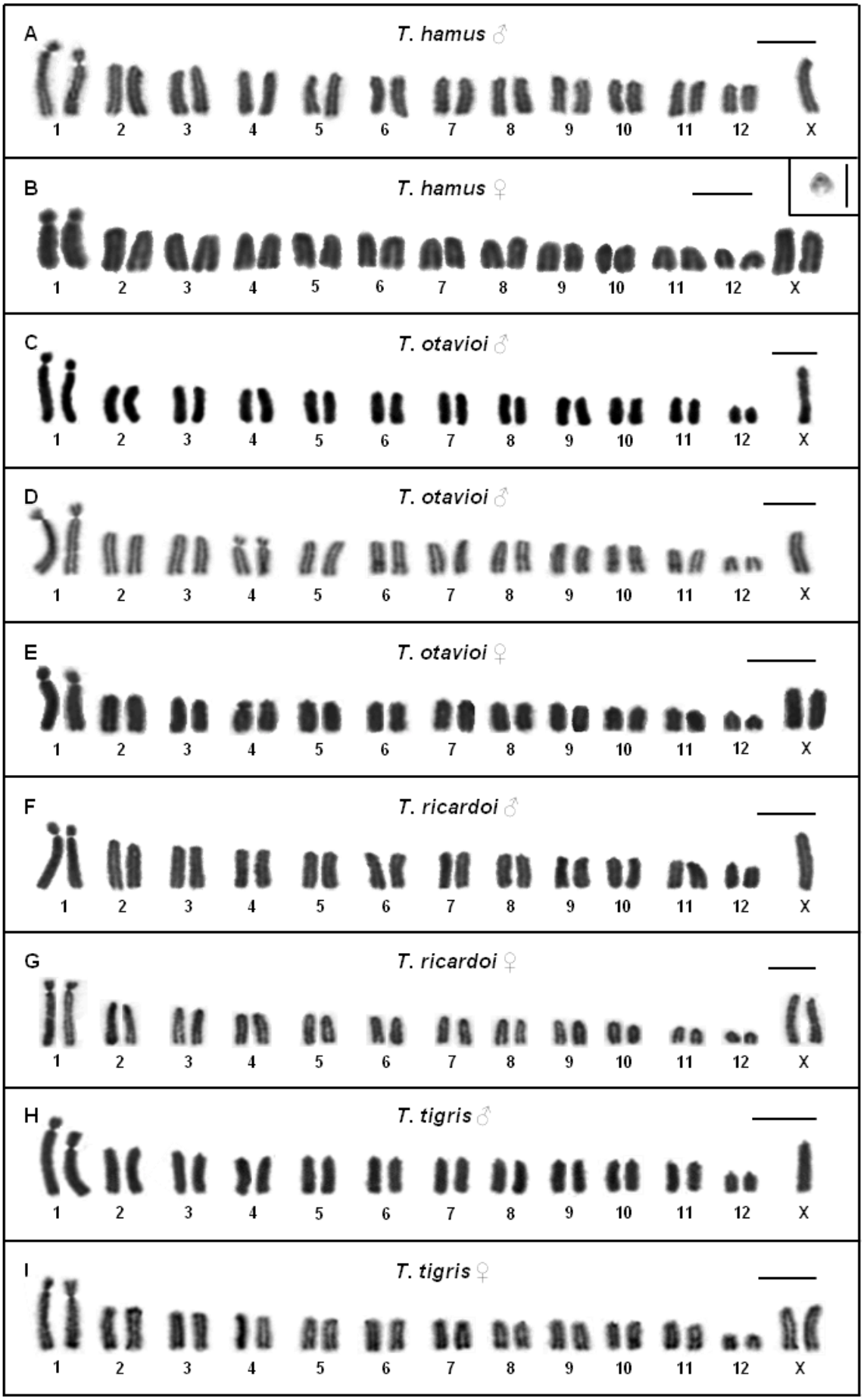
A
T. hamus ♂
1 2 3 4 5 6 7 8 9 10 11 12 X
B
T. hamus ♀
1 2 3 4 5 6 7 8 9 10 11 12 X
C
T. otavioi ♂
1 2 3 4 5 6 7 8 9 10 11 12 X
D
T. otavioi ♂
1 2 3 4 5 6 7 8 9 10 11 12 X
E
T. otavioi ♀
1 2 3 4 5 6 7 8 9 10 11 12 X
F
T. ricardoi ♂
1 2 3 4 5 6 7 8 9 10 11 12 X
G
T. ricardoi ♀
1 2 3 4 5 6 7 8 9 10 11 12 X
H
T. tigris ♂
1 2 3 4 5 6 7 8 9 10 11 12 X
I
T. tigris ♀
1 2 3 4 5 6 7 8 9 10 11 12 X

Figure 2. Karyotype of *Temnomastax* species. A-B, *Temnomastax hamus*. C-E, *T. otavioi*. F-G, *T. ricardoi*. H-I, *T. tigris*. A, C, D, F and H, spermatogonium metaphase with 2n♂= 25. B, E, G e I, oogonium metaphase with 2n♀= 26. Inset (A) shows in detail the B chromosome of *T. hamus* from another cell. Scale bars = 10μm (except for the inset, where scale bar = 5μm).

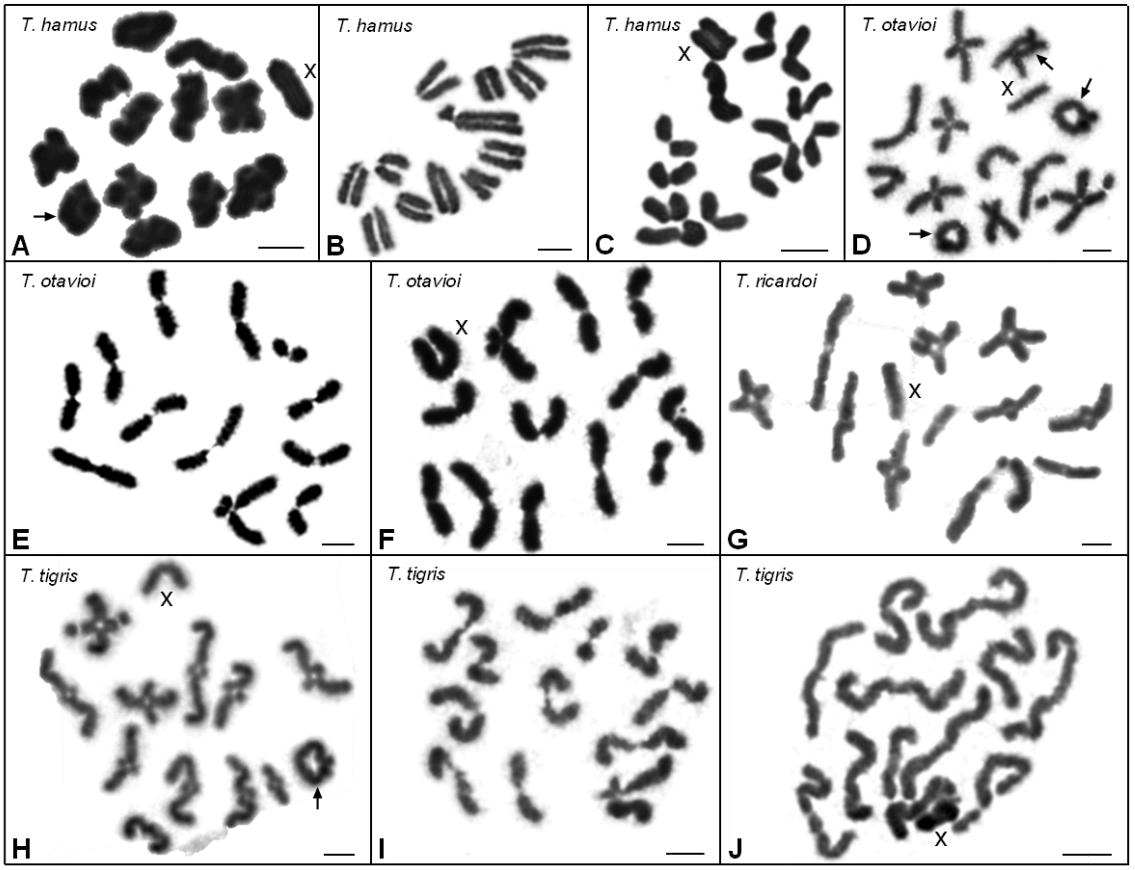

Figure 3. Meiotic cells in males of *Temnomastax*. A-C, *Temnomastax hamus*. D-F, *T. otavioi*. G, *T. ricardoi*. H-J, *T. tigris*. A, D, G, H, spermatocyte I in diplotene/metaphase with 12 autosomal bivalents and one sex univalent chromosome (12II + X). Arrows indicate bivalents with two chiasmata. B, E, I, metaphase II with n= 12. C, F, J, metaphase II with n= 13 = 12 + X. Scale bars = 5μm.

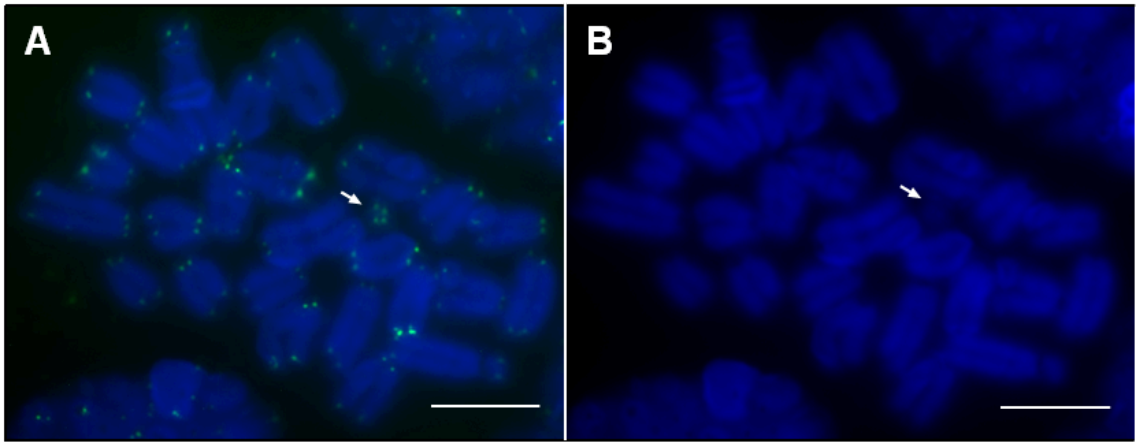

Figure 4. Oogonium metaphase of *Temnomastax hamus* (2n = 26) with *In situ* hybridization with telomeric probes (A) and DAPI (B), presenting one B chromosome (arrow). Scale bars = 10μm.

Constitutive heterochromatin

Mitotic metaphases of the four species showed pericentromeric C-bands in most chromosomes. Additionally, all species presented terminal and/or interstitial heterochromatin (Fig. 5; Supporting information, Fig. S1). *Temnomastax hamus* exhibit only a small interstitial band on chromosome pair 9 (Fig. 5A). The B chromosome did not show any interstitial heterochromatin (Fig. 5A), and it was DAPI negative (Fig. 4B).

The species of the Tigris group, on the other hand, showed more C-bands. *Temnomastax otavioi* presented interstitial heterochromatin in chromosome pairs 6, 8, 10 and 11 (Fig. 5B). All the interstitial C-bands were coincident with DAPI positive bands in metaphases subjected to FISH (Fig. 5C). The individuals with the subtelocentric chromosome 4 presented the same C-bands (Supporting information, Fig. S1).

Temnomastax ricardoi showed differences in the distribution of the constitutive heterochromatin between populations. The female from Águas Claras presented six blocks of interstitial heterochromatin, located in chromosomes 8 to 10, and one small block of terminal heterochromatin in chromosome pair 6 (Fig. 5D). The individuals from the Campo Grande population also exhibited these interstitial C-bands, with additional blocks of interstitial heterochromatin, one in each homolog of chromosome pair 6 and another in one element of pair 11 (Fig 5E).

In *T. tigris*, nine bands of interstitial heterochromatin were revealed, in chromosome pairs 8 to 11, with one chromosome of pair 9 with two bands in the same arm. However, in some cells, additional small interstitial C-bands were observed, one in each homolog of pair 7 and another one in one chromosome of pair 4. Moreover, both homologs of chromosome pair 1 exhibit one small block of terminal heterochromatin in the short arm (Fig. 5F). The DAPI fluorochrome in this species showed 21 interstitial DAPI-positive blocks, with only chromosome pairs 1, 3, 12, and the X chromosome without any DAPI band (Fig. 5G). The other elements presented one DAPI-band per chromosome arm, except chromosome pair 9 and one element of pair 2, which have 2 bands in the same arm. DAPI-positive regions of chromosome pairs 8 to 11 are coincident with interstitial C-bands (Fig. 5G).

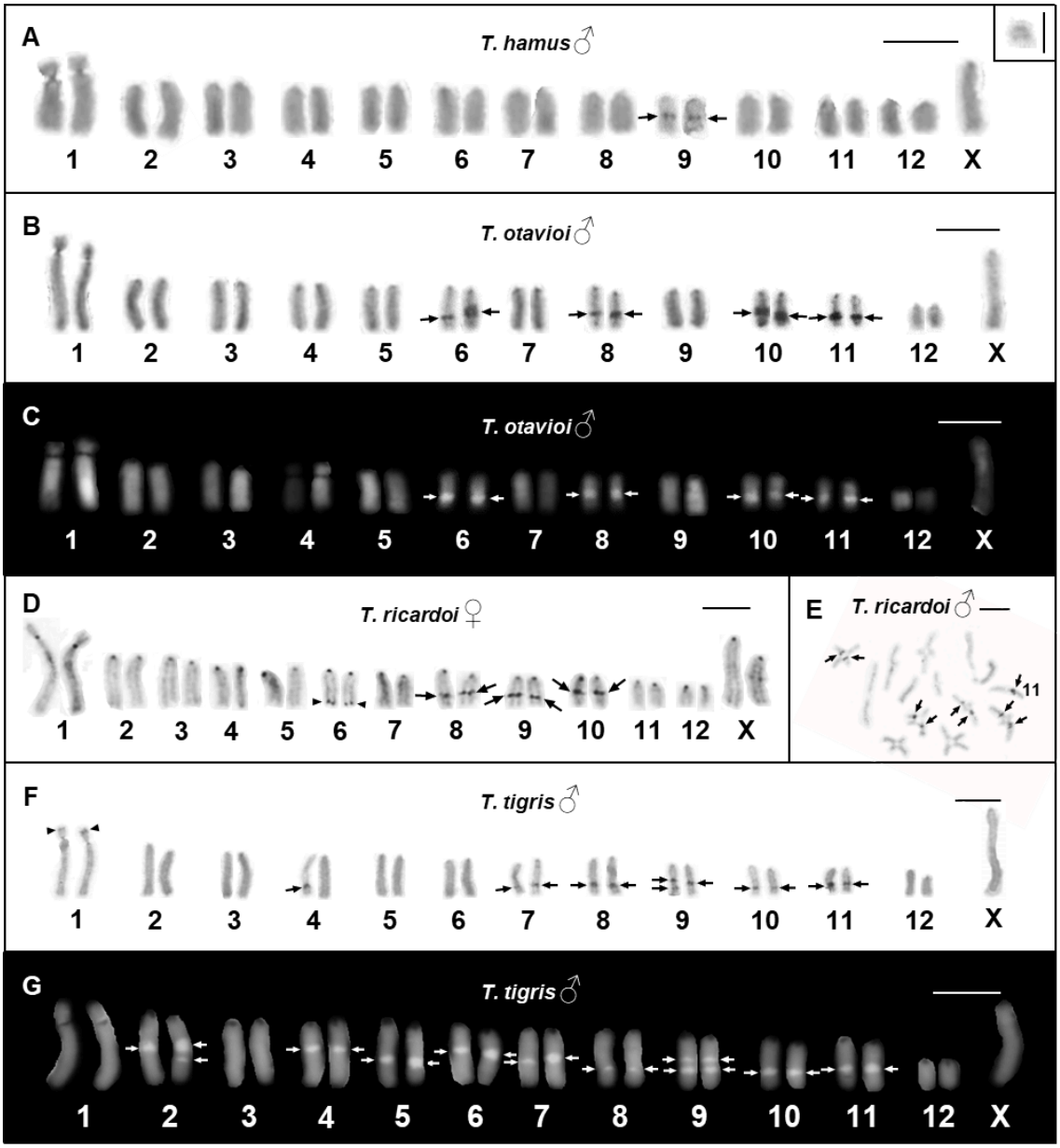

Figure 5. Heterochromatin distribution in the karyotypes (A-D, F-G) and diplotene (E) of *Temnomastax* species. A-B, D-F, C-banding technique in *T. hamus* (A), *T. otavioi* (B), *T. ricardoi* from Águas Claras (D), *T. ricardo* from Campo Grande (E), and *T. tigris* (F). Inset (A) shows in detail the B chromosome of *T. hamus* from another cell. Black arrows indicate interstitial C-bands while arrowheads indicate terminal C-bands. C, G, DAPI fluorochrome in *T. otavioi* (C) and *T. tigris* (G). White arrows indicate DAPI positive bands. scale bars = 10μm (except for the inset, where scale bar = 5μm).

Distribution of the $(TTAGG)_n$ telomeric sequence

All four species have the telomeric regions hybridized with the $(AATCC)_3$ probe (Fig. 6). Moreover, the species of the *Tigris* group presented interstitial telomeric sites (ITS). One conspicuous proximal ITS was present on chromosome pair 11 in *T. otavioi* and *T. tigris*, and pair 9 in *T. ricardoi*. Furthermore, small ITS were observed in the X chromosome, plus in 10, 8 and 4 autosomal chromosomes of *T. otavioi* (Fig. 6B), *T. ricardoi* (Fig. 6C), and *T. tigris* (Fig. 6D) respectively. The individuals of *T. otavioi* with

the subtelocentric chromosome 4 showed a pericentromeric signal in the long arm, whereas the telocentric chromosome 4 had only the telomeres detected by this probe (Fig. 6B; Supporting information, Fig. S2).

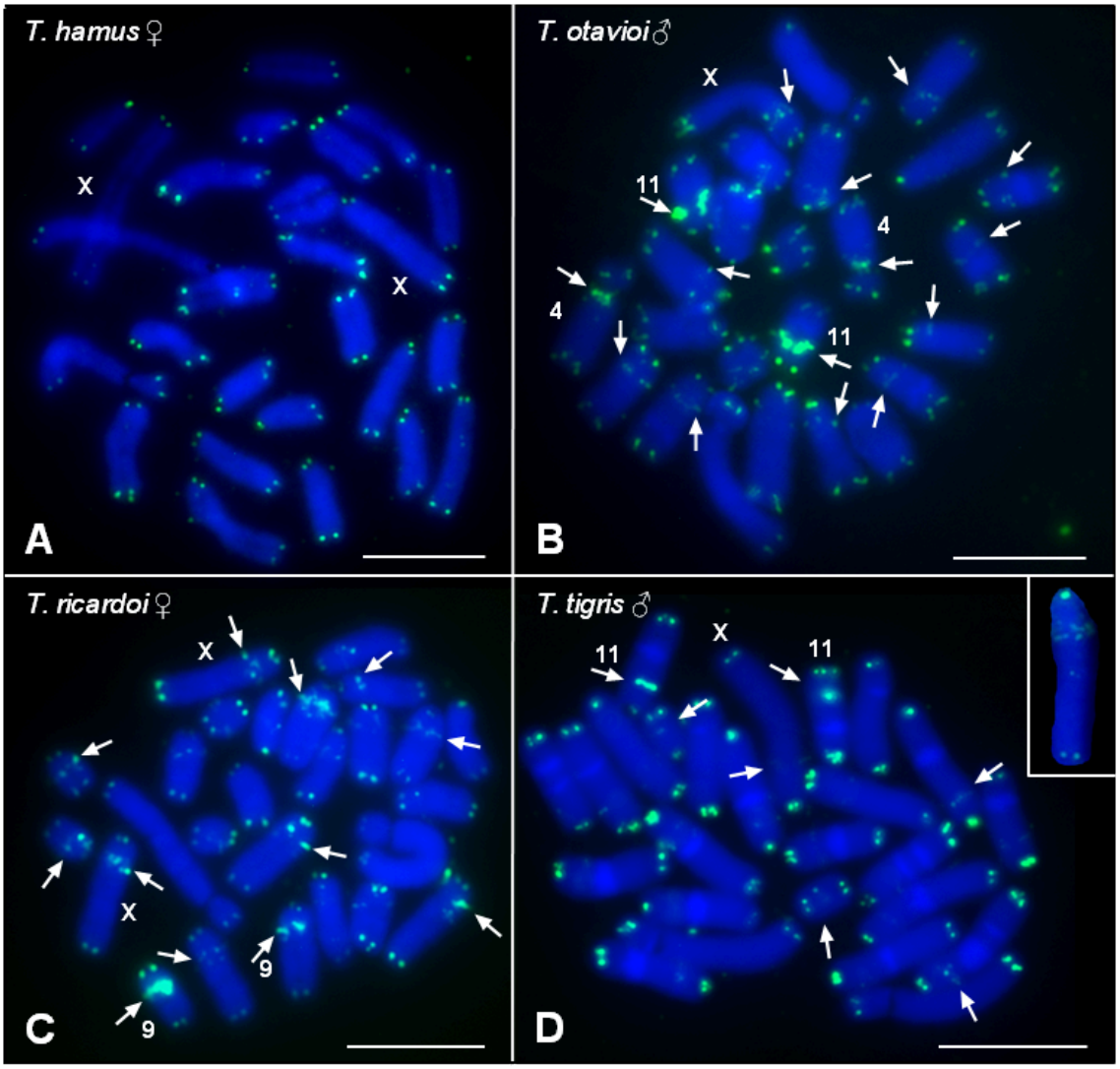

Figure 6. *In situ* hybridization of telomeric probes to mitotic metaphases of *Temnomastax*. A, C, oogonium of *T. hamus* (A) and *T. ricardoi* (C). B, D, spermatogonium of *T. otavioi* (B) and *T. tigris* (D). Inset (D) shows in detail a X chromosome with a clearer hybridization signal from another cell of *T. tigris*. White arrows indicate interstitial telomeric sites. Scale bars = 10μm.

Localization of 28S rDNA cistrons

The FISH with 28S rDNA probe showed variability in the loci number, nevertheless, for all four species, the clusters signals were terminal. In *T. hamus*, clusters of 28S rDNA were presented in chromosome pairs 7 and 10 (Fig. 7A). *Temnomastax otavioi* showed 12 hybridization signals, of which ten were strong, located on chromosome pairs 3, 5, 6, 8, and 12, and two were weaker signals, observed on chromosome pair 4 (Fig. 7B).

Four small clusters were detected in *T. ricardoi*, located in chromosomes 5 and 9 (Fig. 7C). In *T. tigris*, six signals were detected, on chromsoome pairs 2, 5, and 8 (Fig. 7D).

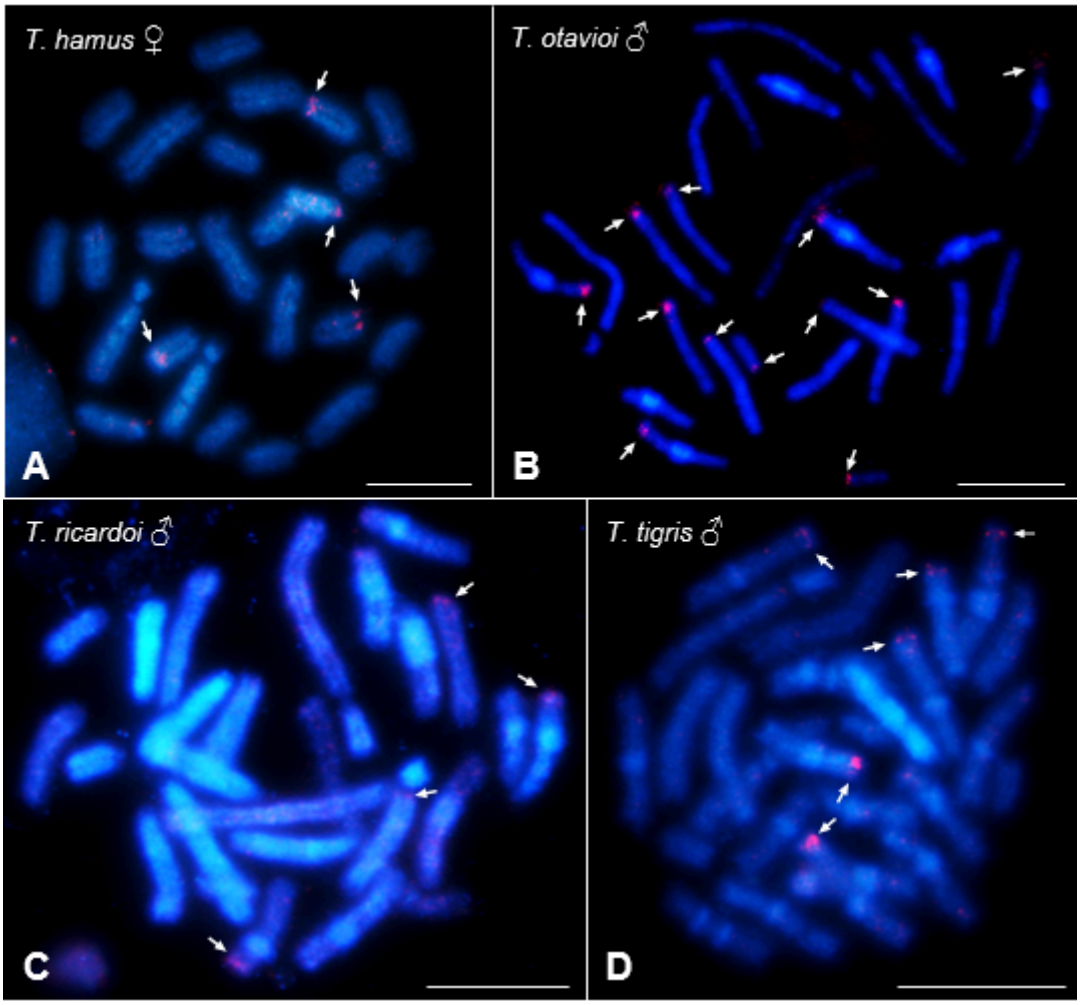

Figure 7. Fluorescence *in situ* hybridization with 28S rDNA probe in mitotic metaphases of *Temnomastax*. A, ooogonium of *T. hamus*. B-D, spermatogonium of *T. otavioi* (B), *T. ricardoi* (C), and *T. tigris* (D). White arrows indicate 28S rDNA sites. Scale bars = 10μm.

DISCUSSION

Diploid number and chromosome morphology

The diploid number, chromosome morphology, and SCS found in *T. hamus* agrees with the previously described by Mesa & Mello (1984), which analyzed the same species in a population about 800/850 km away from our collection sites. Furthermore, the other three species studied here showed the same basic characteristics with the conventional staining, demonstrating a conserved diploid number, karyotype formula, and SCS in this genus. In Eumastacidae, this diploid number was already recorded in two species of Pseudomastacinae, *Pseudomastax carlosi* Descamps, 1973 and *P. pictifrons* (Bruner, 1920), although differing in the chromosome morphology by one extra bi-armed pair (Mesa & Ferreira 1978). Rehn & Grant Jr (1958), based on

morphological characteristics, indicated “some relationship’’ between Pseudomastacinae and Temnomastacinae. The occurrence of the same diploid number (2n♂ = 25) in both Pseudomastacinae and Temnomastacinae could also suggest a closer relationship between these subfamilies. Regardless of the relation between these two families, the 2n♂ = 25 is the higher and, currently, the more common diploid number in the family. In other families of grasshopper, the diploid number is frequently lower, such as the 2n♂ = 23 in Acrididae, Ommexechidae, and Romaleidae (Mesa *et al.* 1982, Loreto *et al.* 2005, Husemann *et al.* 2022) and 2n♂ = 15, 17, and 19 in Morabidae and Proscopiidae (White 1977, 1979, Moura *et al.* 1996, Souza & Moura 2000). In addition to 2n♂ = 25, in Eumastacidae the diploid numbers 2n♂ = 17, 19, and 23, found in those others grasshoppers families, were also found (White 1968, 1970, Mesa & Ferreira 1978, Rentz & Wiessman 1981, Mesa 1984).

Temnomastax otavioi showed variation in the morphology of chromosome 4. The telocentric chromosome 4 probably represents the ancestral condition for this species, because it is found in the other three *Temnomastax* species, two of them from the same morphological group. From this chromosome, a heteromorphism could be reached by a pericentric inversion, originating the subtelocentric morphology. This rearrangement is supported by the presence of a pericentromeric ITS in the long arm of the subtelocentric chromosome 4, which is not observed in the telocentric chromosome 4. The breeding of this heteromorphic individual with individuals with the ancestral condition (i.e. the pair four telocentric), generates ½ of the individuals with the subtelocentric morphology in one chromosome of the pair 4 (heteromorphic condition). Eventually, two heteromorphic individuals could breed among them, and ¼ of the offspring would be homomorphic for the pericentric inversion. The presence of the ITS in the subtelocentric chromosome 4 and the three karyotype configurations within the same population, show that this rearrangement probably is not fixed in the population, and is recent in the evolution of *T. otavioi*. This type of rearrangement is common in Orthoptera, responsible for changes in chromosome morphology without alter the diploid number (Hewitt 1979), especially in the evolution of the karyotype of Ommexechinae (Ommexechidae) and Morabini (Morabidae) (White 1961, White *et al.* 1963, 1967, Santander *et al.* 2021).

B chromosome in Temnomastax hamus

Supernumerary chromosomes (B chromosomes) are additional elements to the standard karyotype (A complement), that follow their own evolutionary pathway, with a non-mendelian segregation. Because of this behavior, these elements can be present in only a few individuals within a population (Camacho *et al.* 2000, Vujošević *et al.*

2018). B chromosomes are common in Caelifera, with records for eight families: Acrididae, Lentulidae, Morabidae, Ommexechidae, Pamphagidae, Pyrgomorphidae, Romaleidae, and Tetrigidae (Camacho 2005, D'ambrosio *et al.* 2017). Here, we presented the first record of B chromosome in Eumastacidae, in one female of *T. hamus* from Aquidauana.

The B chromosome found in *T. hamus* was mainly euchromatic, since only pericentromeric bands were revealed by C-banding. Milani *et al.* (2021), based on the absence of heterochromatin and B-specific satellite DNA, suggested a recent origin of the B chromosome in *Abracris flavolineata* (De Geer, 1773) (Acrididae). The B chromosome in *T. hamus* could also have a recent origin, which could explain the absence of large blocks of interstitial heterochromatin. There are different possible origins for B chromosomes. In an intraspecific origin, the B element can be derived from an A chromosome by chromosomal rearrangements or unbalanced segregation. While the interspecific origin occurs by hybridization or introgression (Camacho 2005, Tosta *et al.* 2014, Ruiz-Ruano *et al.* 2017, Johnson & Reifová 2021). Based on the homology between the B chromosomes and the A complement, mostly research supposes the intraspecific origin of B chromosomes (Ruiz-Ruano 2017, Johnson & Reifová 2021).

Cytogenetic markers for Temnomastax species

All four species exhibited the same diploid number, chromosome morphology and SCS, however, the cytogenetic markers used here allowed the interspecific distinction (Fig. 8).

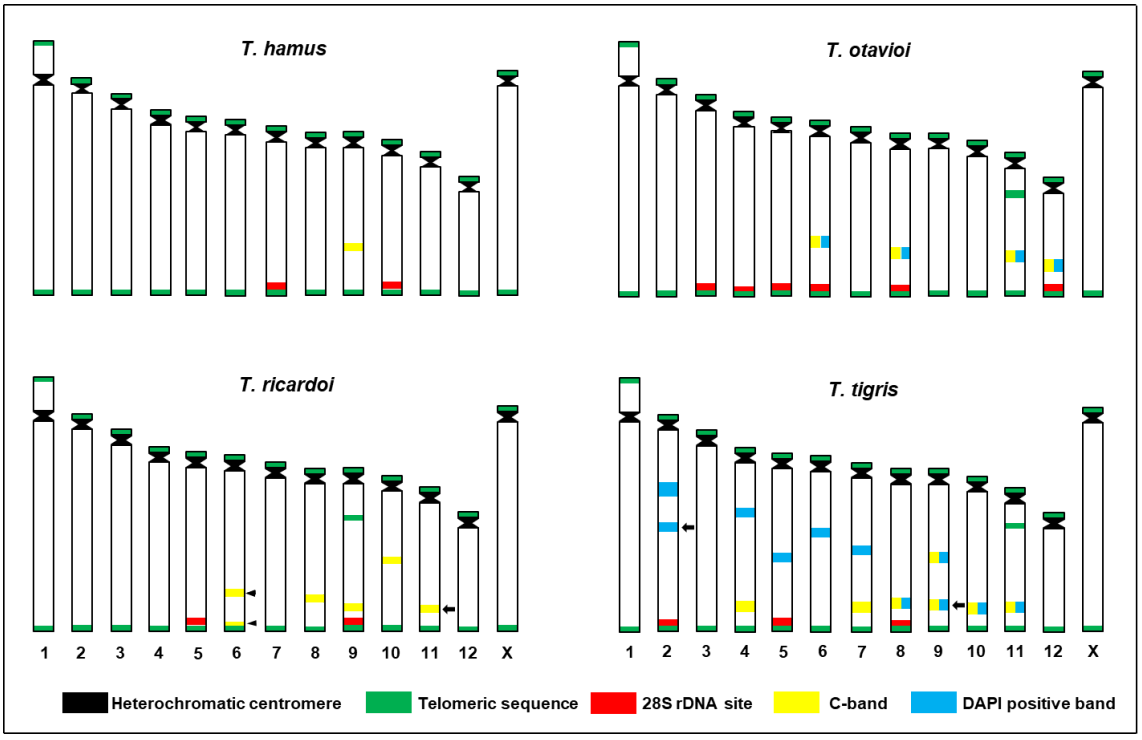

Figure 8. Schematic representation of the cytogenetic markers in the haploid karyotype of four *Temnomastax* species. Species names, components of the chromosomes, and chromosome numbers are indicated in the figure. Arrows indicate heteromorphism and arrowheads indicate interpopulational differences. Each arrowhead in chromosome 6 of *T. ricardoi* represents a different population. Short ITS are not shown. In the karyotype of *T. otavioi*, only the telocentric chromosome 4 is represented.

Heterochromatin

The presence of pericentromeric constitutive heterochromatin in *Temnomastax* is a pattern that was already found in several Acridoidea species (Santos *et al.* 1983, John *et al.* 1985, Cabrero & Camacho 1986). This pericentromeric heterochromatin is found in several other organisms and is important for chromosome segregation (Bernard 2001, Bloom 2014). The variability of the interstitial heterochromatin bands found here, although less frequent in other groups of grasshoppers, allowed the interspecific distinction, such as for some Acrididae species (King & John 1980, Santos *et al.* 1983). The *Temnomastax* species are very similar to each other. Therefore, the C-banding technique demonstrated to be a simple and useful method to differentiate the *Temnomastax* species, assisting the distinguish between the *Latens* and *Tigris* groups (by the greater amount of interstitial heterochromatin in the *Tigris* group), as well as the interspecific differentiation.

In *T. ricardoi*, interesting variations was noted with respect to the C-banding pattern of chromosomes 6 and 11. While the seven analyzed individuals from Campo Grande for this technique showed an interstitial C-band in chromosome 6, the female from Águas Claras exhibited a terminal C-band instead. This change in intrachromosomal

positioning may result from a paracentric inversion involving the heterochromatin block, and the observed difference could represent an interpopulational variation, although a larger sample is still needed to test this hypothesis. Regarding chromosome 11, the variation consisted in the presence/absence of an interstitial C-band in the long arm. All the analyzed specimens from Campo Grande were heterozygotes for this feature, whereas this C-band was absent in the individual from Águas Claras.

The chromatin composition, detected by the DAPI fluorochrome, is also different among the species. Only *T. otavioi* and *T. tigris* presented DAPI positive bands coincident with the interstitial C-band location, revealing that this constitutive heterochromatin is enriched in AT base pairs (Schweizer 1981, Kapuscinski 1995). In *T. hamus* and *T. ricardoi* no DAPI bands were revealed, suggesting that the heterochromatin in these species are not AT-rich. Furthermore, *T. tigris* showed several AT-rich regions non-coincident with C-bands. Similarly, non-correspondence of the DAPI-bands with C-bands, in the heterochromatin location, was already recorded in some Coleoptera (Insecta) (Almeida *et al.* 2006), Diptera (Insecta) (Alekseeva *et al.* 2020), Scorpiones (Arachnida) (Mattos *et al.* 2013) and Decapoda (Malacostraca) (Torrecilla *et al.* 2017). Heterogeneity and differences in the composition of heterochromatin was frequently recorded in several animals, as in the Acridoidea, whose karyotypes were analyzed by AT- and CG-specific fluorochromes (Souza & Melo 2007, De França Rocha *et al.* 2015, Santander *et al.* 2021).

Interstitial telomeric sites (ITS)

The origin of interstitial telomeric sites has been proposed as result of chromosome rearrangements or double-strand breaks (DBSs) repair mechanisms during the karyotype evolution. Fusions between two telo/acrocentric chromosomes or inversions with one of the breakpoints in the telomeres could relocate clusters of telomeric repeats to a interstitial chromosome location (Slijepcevic 1998, Rovatsos *et al.* 2011, Bolzán 2017). Alternatively, Ruiz-Herrera (2008) proposed a model based on primates and rodents, in which short ITS could be originated from the insertion of the telomeric motif during the repair of the DSB, with the influence of telomerase. Moreover, short ITS could also be formed through the homologous recombination repair mechanism, described in yeast, without the involvement of telomerase (Aksenova *et al.* 2015, Bolzán 2017). From these, amplifications of the short ITS by unequal crossing-over or replication slippage could increase the number of repetitions, making larger interstitial telomeric sites (Bolzán 2017).

Interstitial telomeric sites are frequent in Orthoptera, being recorded both in autosomal and sex chromosomes, in some cases with a formation of a neo XY (López-Fernández

et al. 2004, Jetybayev *et al.* 2012, 2017, Camacho *et al.* 2015, Grzywacz *et al.* 2019, Buleu *et al.* 2020, Warchałowska-Śliwa *et al.* 2021). In this group, based on variations in the chromosome morphology, the ITS presence was explained mainly by chromosome rearrangements, such as Robertsonian translocation, telocentric fusions or pericentric inversion (Jetybayev *et al.* 2012, 2017, Buleu *et al.* 2020, Warchałowska-Śliwa *et al.* 2021).

In the species analyzed here, the ITS from the subtelocentric chromosome 4 in *T. otavioi* seems to be a result of a pericentric inversion, with one of the breakpoints in the telomeric region of these chromosomes. The other ITS could also be related to a chromosome rearrangement, however the absence of variation in the chromosome morphology restrains the confirmation of this hypothesis. Furthermore, since *Temnomastax* presented 2n♂= 25, the higher diploid number for Eumastacidae and the sister family Morabidae (Table 1, White 1956, 1968, 1977, 1979, White *et al.* 1964, 1967, 1977, Schweizer *et al.* 1983), rearrangements such as fusions would be unlikely, because it would demand several additional rearrangements to reach the diploid number in the genus. Therefore, the remaining ITS (except the ITS from the subtelocentric chromosome 4 in *T. otavioi*) could represent remnants of breaks in the DNA strand, such as the proposed by Azzalin *et al.* (2001) and Zattera *et al.* (2019), for human cells and *Pipa carvalhoi* (Miranda-Ribeiro, 1937) (Anura), respectively. The larger ITS signals (chromosome 8 of *T. ricardoi* and chromosome 11 of *T. otavioi* and *T. tigris*) could be amplifications of the short ITS.

Ruiz-Herrera (2008) attributed the short ITS as reliable markers for evolutionary studies, since this feature can be considered as a rare genomic change that is unlikely to emerge independently during the evolution. This type of cytogenetic marker has already been used in the frog genus *Xenopus*, taking part in the karyotype evolution of *X. clivii* Peracca, 1898 (Nanda *et al.* 2008). Additionally, the results found here corroborate the grouping made by Olivier *et al.* (2019), considering that only the representatives of *Tigris* group presented those signals. Moreover, the differential location of the conspicuous ITS in chromosome 8 of *T. ricardoi* and in chromosome 11 of *T. otavioi* and *T. tigris* may also suggest a closer relationship between the latter two species.

28S rDNA

The number of chromosomal loci of 28S rDNA in *Temnomastax* ranged from 4 to 12 per diploid set. This repeated genomic element is part of the major 45S rDNA, which forms the nucleolar organizer region (NOR), and is frequently mapped on animal chromosomes, revealing a great variability, and allowing interspecific chromosomal

differentiation (Panzera *et al.* 2012, Gornung 2013, Sochorová *et al.* 2021). Cabrero & Camacho (2008) compared the distribution of the 45S rDNA in 49 grasshopper species, belonging to Acrididae and Romaleidae families, revealing a variation from 1 to 10 loci per haploid genome, with 1, 2 or 3 chromosomes bearing clusters in most species.

Therefore, the high loci variability we found here would be expected. Nevertheless, in the four species of *Temnomastax* the clusters were terminal, which differs from the standard pericentromeric or pericentromeric/interstitial location of this genes, found in different families of grasshopper (e.g. Schweizer *et al.* 1983, Loreto *et al.* 2008, Carvalho *et al.* 2011, Neto *et al.* 2013, Buleu *et al.* 2017, 2019). Changes in number and position of the rDNA loci could happen by ectopic recombination, chromosome rearrangements, such as paracentric inversions, or transposition of some rDNA sequences to different chromosomes with subsequent rDNA amplification and elimination of the original rDNA cluster (Dubcovsky & Dvorak 1995, Nei & Rooney 2005, Cabrero & Camacho 2008, Hirai 2020, Martí *et al.* 2021). Hirai (2020) proposed that terminal sites of rDNA would facilitate the dispersion of rDNA to other chromosomes by ectopic recombination, during the formation of the meiotic bouquet. Since the four species of *Temnomastax* presented only terminal clusters, the ectopic recombination could be the main factor for the repatterning of the 28S rDNA, such as proposed for moths and butterflies (Lepidoptera) (Nguyen *et al.* 2009).

The *T. hamus* chromosomes bearing 28S rDNA are not the same as the *Tigris* group species, which, along with the ITS in this group, reinforces the grouping proposed by Olivier *et al.* (2019). Furthermore, the three species of the *Tigris* group presented 28S rDNA sites in chromosome pair 5. Additionally, *T. otavioi* and *T. tigris* also shared the location of the rDNA site in chromosome pair 8. The similarity in the NOR-bearing chromosomes in *T. otavioi* and *T. tigris* could indicate a closer relationship between these two species within the *Tigris* group, such as the results from the telomeric hybridization. However, there is no data about the phylogenetic relationship of the *Temnomastax* species.

CONCLUSION

The chromosome analyses in *Temnomastax* showed that although the diploid number, karyotype formula, and SCS are the same in the four species, the chromosome organization is distinct. In each species, different evolutionary features were present, such as the presence of B chromosome in *T. hamus*, changes in chromosome morphology in *T. otavioi*, or variation in number and location of C-bands. Moreover, the location of heterochromatin, AT-rich regions, telomeric sequences and 28S rDNA

proved to be efficient cytogenetic markers for *Temnomastax* species. These markers clearly distinguish the *Latens* and *Tigris* groups, especially by the higher abundance of heterochromatin in the *Tigris* group, and the presence of ITS only in the *Tigris* group. Furthermore, the similarities in location of some 28S rDNA sites and ITS of *T. otavioi* and *T. tigris,* suggest a closer relationship between these species than with *T. ricardoi*.

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SUPPLEMENTARY DATA

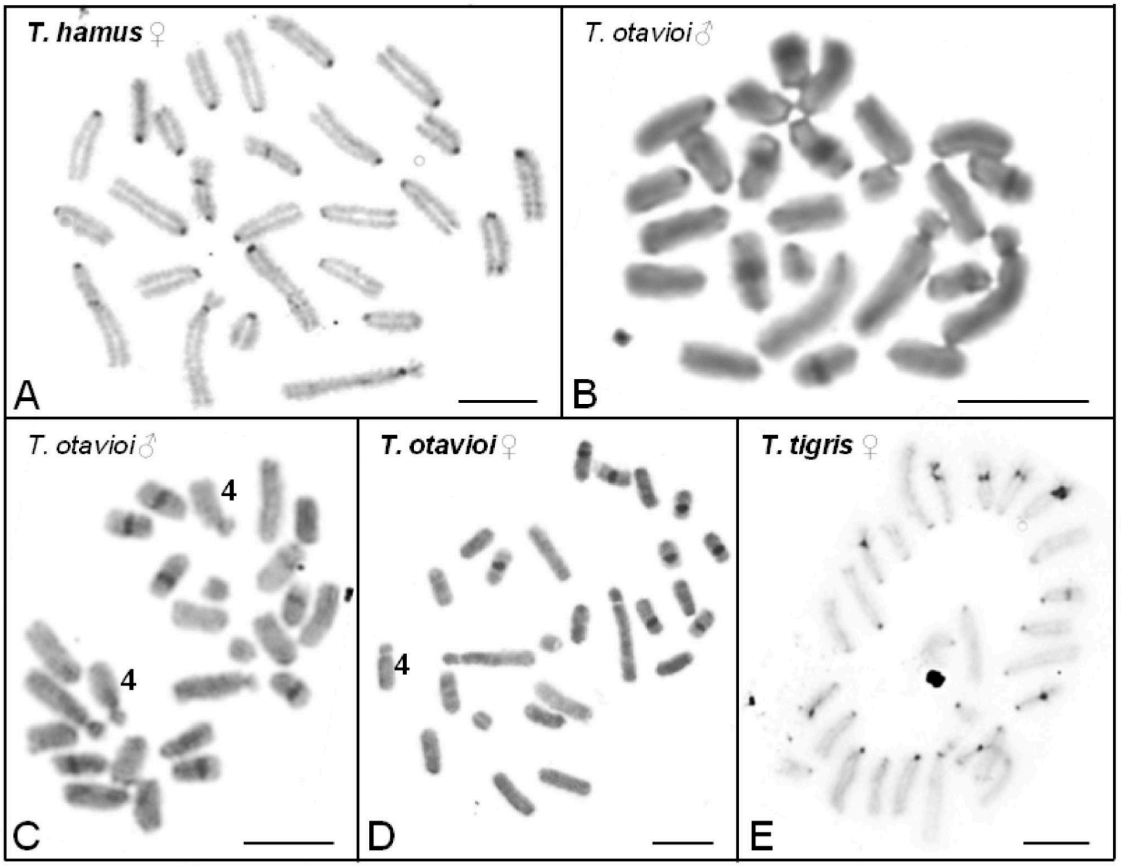

Figure S1. C-bands distribution in the metaphases of Temnomastax species, highlighting the pericentromeric heterochromatin. A, D, E, oogonium of T. hamus (A), T. otavioi (D), and T. tigris (E). B, C, spermatogonium of T. otavioi. C, D, T. otavioi with the subtelocentric chromosome 4. Scale bars = 10μm.

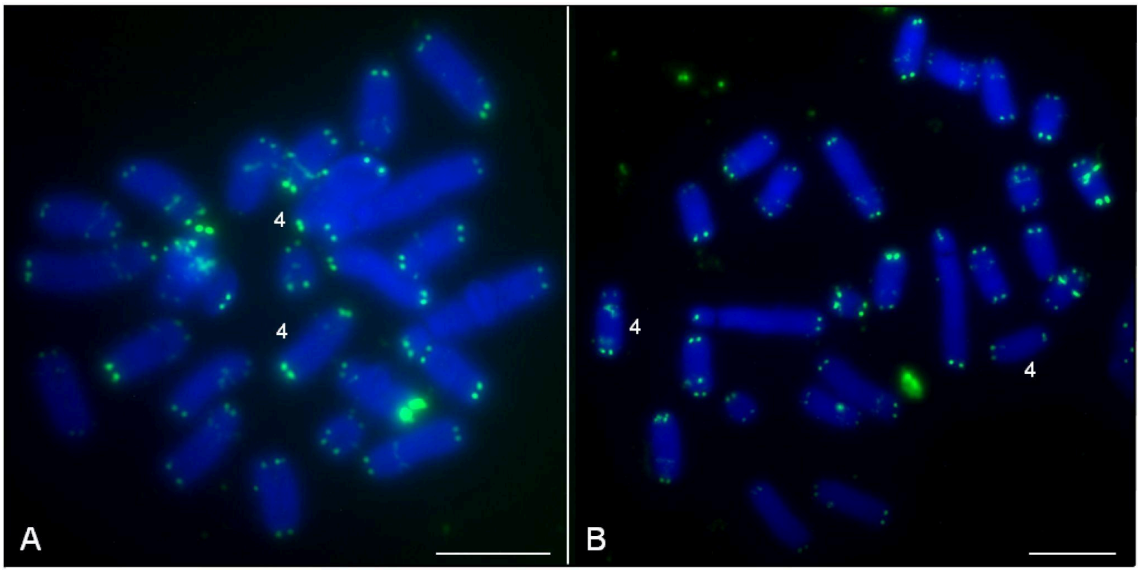

Figure S2. In situ hybridization of telomeric probes to mitotic metaphases of Temnomastax otavioi with telocentric chromosomes pair 4. A, homomorphic pair 4. B, heteromorphic pair 4, consisting of a telocentric and a subtelocentric homolog. Scale bars = 10μm.

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