



Comparative cytogenetic analysis of two grasshopper species of the tribe Abracrini (Ommatolampinae, Acrididae)

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Abstract

The grasshopper species *Orthoscaphus rufipes* and *Eujivarus fusiformis* were analyzed using several cytogenetic techniques. The karyotype of *O. rufipes*, described here for the first time, had a diploid number of $2n = 23$, whereas *E. fusiformis* had a karyotype with $2n = 21$. The two species showed the same mechanism of sex determination (XO type) but differed in chromosome morphology. Pericentromeric blocks of constitutive heterochromatin (CH) were detected in the chromosome complement of both species. CMA₃/DA/DAPI staining revealed CMA₃-positive blocks in CH regions in four autosomal bivalents of *O. rufipes* and in two of *E. fusiformis*. The location of active NORs differed between the two species, occurring in bivalents M₆ and S₉ of *O. rufipes* and M₆ and M₇ of *E. fusiformis*. The rDNA sites revealed by FISH coincided with the number and position of the active NORs detected by AgNO₃ staining. The variability in chromosomal markers accounted for the karyotype differentiation observed in the tribe Abracrini.

Key words: constitutive heterochromatin, fluorochromes, FISH, Orthoptera, ribosomal DNA.

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Introduction

The grasshopper subfamily Ommatolampinae (Acrididae) comprises nine tribes and more than 50 genera that have a wide geographic distribution, with most of them being found in North, Central and South America (Amedgnato, 1974; Carbonell, 1977). Although numerous studies have dealt with species of the family Acrididae, the analysis of most Neotropical species, especially of the subfamily Ommatolampinae, has been restricted to conventional staining (Ferreira *et al.*, 1980; Mesa *et al.*, 1982; Mesa and Fontanetti, 1983); at least 20 species of Ommatolampinae have been studied using conventional techniques (Carbonell *et al.*, 1980; Ferreira *et al.*, 1980; Mesa *et al.*, 1982; Mesa and Fontanetti, 1983; Cella and Ferreira, 1991). The use of specific techniques for chromosome identification has been applied only to the species *Abracris flavolineata* (Cella and Ferreira, 1991). Despite the small number of species studied so far, the data obtained indicate that this subfamily is characterized by a significant number of species (> 40%) with derived karyotypes originating from

centric fusions or other rearrangements. These karyotypes are found in species of the genera *Pycnosarcus* and *Ligidacris* (17, XO) – Pycnosarcini, *Bucephalacris* (21, XO) – Dellini, *Abracris*, *Eujivarus* and *Omalotettix* (21, XO), and *Jodacris* and *Siltaces* (19, XO) – Abracrini (Carbonell *et al.*, 1980; Ferreira *et al.*, 1980; Mesa *et al.*, 1982; Mesa and Fontanetti, 1983).

Constitutive heterochromatin (CH) accounts for a significant part of the genome in grasshoppers and is characterized by a low gene density and the presence of highly repetitive sequences. Some studies have shown extensive polymorphism in this type of chromatin in grasshoppers, including variation in the location and size of the CH blocks, as well as heterogeneity in these regions. The use of base-specific fluorochromes has contributed to the characterization of CH (King and John, 1980; Santos *et al.*, 1983; John *et al.*, 1985), although for Neotropical grasshoppers the data obtained with such probes are limited to a few species of the families Acrididae and Romaleidae (Souza *et al.*, 1998; Loreto and Souza, 2000; Pereira and Souza, 2000; Souza *et al.*, 2003; Rocha *et al.*, 2004; Loreto *et al.*, 2005; Souza and Melo, 2007). Other techniques, such as fluorescent *in situ* hybridization (FISH), have identified important differences in the composition of CH that involve variable quantities of repetitive DNA sequences in these regions, in-

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cluding satellite or ribosomal DNA (rDNA) (Rodríguez Iñigo *et al.*, 1993, 1996; Loreto *et al.*, 2008). Since, in contrast to silver nitrate (AgNO₃) staining, FISH does not depend on the presence of a transcription product it permits the identification of active or inactive rDNA sequences in the genome (López-León *et al.*, 1999).

In this study, we used the C-banding technique and staining with base-specific fluorochromes (CMA₃ and DAPI) to examine the distribution of CH and the proportion of GC and AT base pairs in karyotypes of the grasshoppers *Orthoscapheus rufipes* and *Eujivarus fusiformis*; silver nitrate impregnation and FISH were used to determine the position and variability of the nucleolar organizer regions (NORs). The results described here improve our understanding of chromosomal organization in these species and display new light on the chromosomal phylogeny of the Ommatolampinae.

Material and Methods

Specimens of *O. rufipes* and *E. fusiformis* were collected in two rainforest areas in Pernambuco State, north-eastern Brazil. Fifteen male and nine female specimens of *O. rufipes* and seven male and two female specimens of *E. fusiformis* were collected in Gurjaú forest, in the municipality of Cabo (8°17'12" S; 35°2'6" W). Ten male and eight female *E. fusiformis* specimens were also collected in the Dois Irmãos zoological-botanical garden, in the municipality of Recife (8°3'14" S; 34°52'52" W).

Cytological preparations were obtained from testes and ovarioles by the classic squashing technique. The ovarioles were pretreated with 0.1% colchicine for 6 h prior to preparation. The material was fixed in ethanol:acetic acid (3:1, v/v). Conventional staining was done with 2% lacto-acetic orcein.

C-banding, triple staining with CMA₃/DA/DAPI and AgNO₃ staining were done as described by Sumner (1972), Schweizer *et al.* (1983) and Rufas *et al.* (1987), respectively. FISH was done as described by Moscone *et al.* (1996) using 18S and 25S rDNA probes from *Arabidopsis thaliana* (Unfried *et al.*, 1989; Unfried and Gruendler, 1990). The probes were labeled with bio-11-dUTP by nick translation (Life Technologies) and detected using rat anti-biotin (Dakopatts M0743, Dako) and TRITC (tetramethylrhodamine isothiocyanate)-conjugated anti-antibiotin (Dakopatts R0270, Dako) antibodies. The preparations were counterstained with DAPI (2 µg/mL) and mounted with Vectashield H-1000 (Vector).

For fluorescent *in situ* hybridization (FISH), images of cells were captured with a Cytovision system coupled to an Olympus BX51 microscope. For the other techniques, the cells were photographed with a Leica microscope. The images were mounted using CorelDraw Graphics Suite 12.

Results

The karyotype of *O. rufipes*, described here for the first time, consisted of a diploid number of 2n = 23 and an XO sex determination mechanism for males, and 2n = 24 and XX for females. The chromosomes of *O. rufipes* (Figure 1a-c) were acrotelocentric and were classified according to size into two large pairs (L₁-L₂), six medium sized pairs (M₃-M₈) including the X chromosome, and three small pairs (S₉-S₁₁). In contrast, *E. fusiformis* had a diploid number of 2n = 21 and an XO sex determination mechanism. The L₁ pair was submetacentric and the other chromosomes were acrotelocentric. There were two large pairs (L₁-L₂), five medium sized pairs (M₃-M₇) including the X chromosome, and three small pairs (S₈-S₁₀) (Figure 1b-d). In both species, the X chromosome showed variable heteropyknotic behavior during meiotic prophase I (Figure 1c,d).

The CH blocks were located in pericentromeric regions of the chromosomes and varied in size between and within the species studied (Figure 2a,c). In *O. rufipes* (Figure 2a), the CH blocks were small and located on all chromosomes of the complement, whereas in *E. fusiformis* (Figure 2c) these blocks were very small and found only on some bivalents. CMA₃/DA/DAPI staining revealed CMA₃-positive blocks on four bivalents of *O. rufipes*, including one interstitial block on L₂, proximal and telomeric blocks on M₅, and pericentromeric blocks on M₆ and S₉ (Figure 2b). The CMA₃-positive blocks of the L₂ and M₅ bivalents were not detected by C-banding. *Eujivarus fusiformis* had two medium sized chromosomes (M₆ and

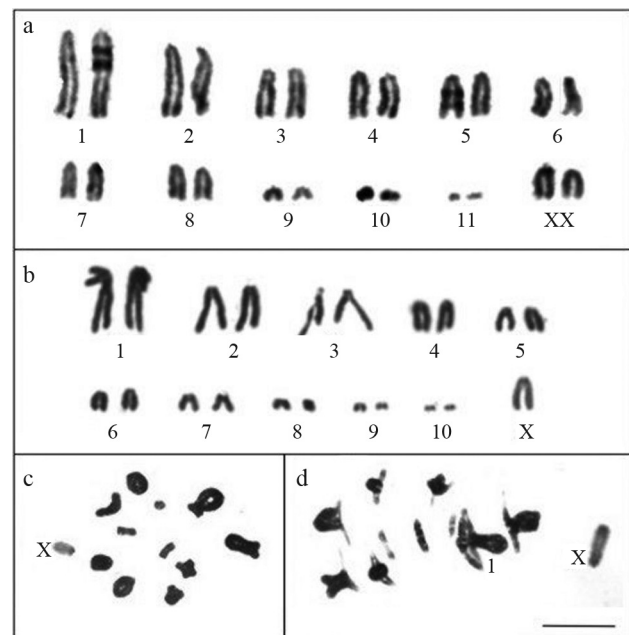


Figure 1 - Conventional staining of *O. rufipes* (a,c) and *E. fusiformis* (b,d) cells. (a) and (b), karyotypes mounted based on female mitotic metaphase chromosomes and male anaphase I chromosomes, respectively; c, diakinesis; d, metaphase I. Note the submetacentric chromosome L₁ (b,d). Bar = 5 µm.

M₇) with CMA₃-positive blocks (Figure 2d). In both species, DAPI staining was homogenous throughout all of the chromosomes (data not shown).

Active NORs were observed during prophase I (pachytene-diplotene) in both species, and were located in bivalents M₆ and S₉ of *O. rufipes* and in M₆ and M₇ of *E. fusiformis*. At both sites, the NORs coincided with CMA₃-positive blocks. Additionally, the rDNA sites detected by FISH coincided with the results of AgNO₃ staining in both species (Figure 3a-d). Table 1 summarizes the data obtained with conventional staining, C-banding, base-specific fluorochromes, AgNO₃ staining, and FISH for the two species studied.

Discussion

Variations in the karyotypes of grasshoppers have been reported, with the most frequent being chromosomal rearrangements such as inversions, reciprocal translocations and centric fusion/fission, in addition to variability in the pattern of CH distribution and the occurrence of extra chromosomal material. Among the ten Neotropical Acrididae subfamilies studied cytogenetically by Mesa *et al.* (1982), the Copiocerinae, Melanoplinae and Ommatolampinae were characterized by derived karyotypes (81.8%, 48.4% and 42.9%, respectively) that resulted mainly from centric fusions and inversions.

The karyotypes of 31 species of the subfamily Ommatolampinae are known, with 21 of them belonging to the tribe Abracrini (Carbonell *et al.*, 1980; Ferreira *et al.*, 1980; Mesa *et al.*, 1982; Mesa and Fontanetti, 1983; Cella and Ferreira, 1991; present study). Despite the small number of species studied so far, more than 40% of them show variations in diploid number, a finding that identifies this subfamily as an important group for studying chromosome evolution. Table 2 summarizes the chromosome number,

sex mechanism and chromosome morphology of representatives of the tribe Abracrini (Ommatolampinae, Acrididae).

Orthoscaphus rufipes had a basic karyotype (23, XO) consisting of acrocentric chromosomes, which was similar to that widely found in the family Acrididae (Mesa *et al.*, 1982; Santos *et al.*, 1983; Bugrov, 1996). On the other hand, the karyotype of *E. fusiformis* (2n = 21, XO) is considered to be a derived karyotype among acridoid grasshoppers. Of the 21 Abracrini species listed in Table 2, 14 have derived karyotypes. These species belong to the genera *Abracris* and *Eujivarus* (21, XO, one pair of metacentric autosomes), *Omalotettix* (21, XO, pairs 3 and 6 submetacentric, pair 5 subacrocentric, and the remaining chromosomes acrocentric), *Jodacris* (19, XO, acrocentric chromosomes) and *Sitalces* (19, XO, two pairs of metacentric autosomes). Among the three species of *Abracris* studied cytologically, *Abracris* sp. is the only one with 2n = 21 and XO, with one metacentric pair originating from the centric fusion of two medium sized chromosomes. *Abracris dilecta* and *A. flavolineata* have 2n = 23 and XO. However, whereas *A. dilecta* has acrocentric chromosomes, *A. flavolineata* has chromosomes with two arms throughout the karyotype complement, in contrast to the basic karyotype of Acridoidea (Mesa *et al.*, 1982; Cella and Ferreira, 1991).

An ancient fusion appears to have been involved in the phylogeny of *Eujivarus* since four of the five species whose karyotype has been studied show a reduction in the diploid number to 2n = 21 with XO (Ferreira *et al.*, 1980; Mesa *et al.*, 1982). In contrast, more recent fusions and polymorphisms in this type of chromosomal rearrangement have been observed in grasshoppers such as *Cornops aquaticum* (Mesa *et al.*, 1982), *Eyrepocnemis plorans*

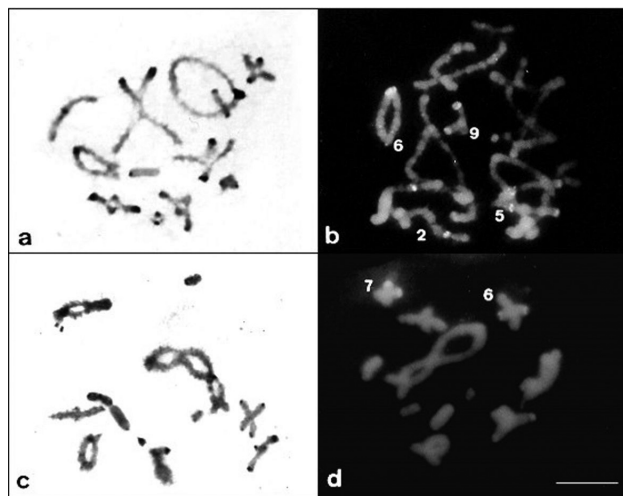


Figure 2 - Distribution pattern and characterization of CH by C-banding (a,c) and CMA₃/DA staining (b,d) in diplotenes of *O. rufipes* (a,b) and *E. fusiformis* (c,d). Note the chromosomes with CMA₃-positive blocks in (b) and (d). Bar = 5 μ m.

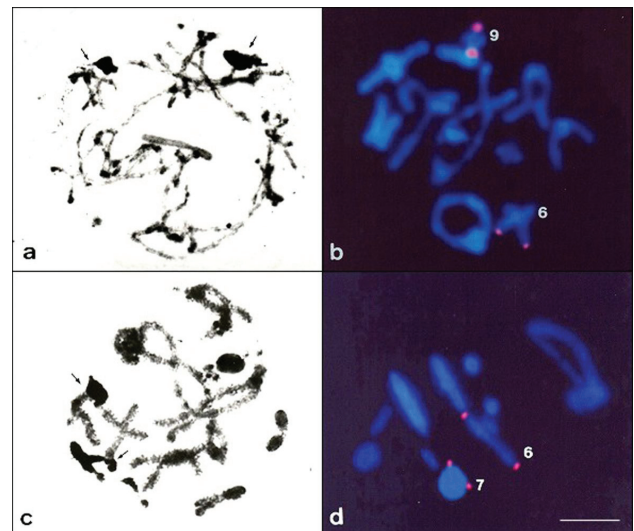


Figure 3 - Nucleolar organizer regions detected by silver nitrate staining (arrows) and fluorescent *in situ* hybridization (FISH) in meiotic cells of *O. rufipes* (a,b) and *E. fusiformis* (c,d). Bar = 5 μ m.

Table 1 - Chromosomal data for *Orthoscapheus rufipes* and *Eujivarus fusiformis*, showing the diploid number and sex determination system, the distribution and classification of CH based on C-banding and staining with base-specific fluorochromes, and the occurrence of NORs detected by AgNO₃ and FISH.

Species	Karyotype	CB +	CMA ₃ ⁺	DAPI	NORs/FISH
<i>O. rufipes</i>	23,XOM	Pc	*L ₂ I;	0	M ₆ Pc
	24,XXF		*M ₅ Px, Tel, M ₆ Pc S ₉ Pc		
<i>E. fusiformis</i>	21,XOM	Pc	M ₆ Pc	0	M ₆ Pc
	22,XXF		M ₇ Pc		

CB+ = presence of C bands; * = constitutive heterochromatin not detected by C-banding; 0 = absence of staining; I = interstitial blocks; NORs = nucleolar organizer regions; Pc = pericentromeric blocks; Px = proximal blocks; Tel = telomeric blocks. M = male. F = female.

Table 2 - Chromosome number, sex mechanism and chromosome morphology in representatives of the tribe Abracrini (Ommatolampinae-Acrididae).

Species	Diploid number	Sex mechanism	Chromosome morphology	Reference
<i>Abracris dilecta</i>	23,24	XO, XX	ac	2, 3
<i>Abracris flavolineata</i>	23,24	XO, XX	mt, sm, st	2, 3, 5
<i>Abracris sp.</i>	21,22	XO, XX	ac, mt	2, 3
<i>Eujivarus fusiformis</i>	21,22	XO, XX	ac, mt	2, 3, 6
<i>Eujivarus vittatus</i>	23,24	XO, XX	ac	2, 3
<i>Eujivarus n.sp. A</i>	21,22	XO, XX	ac, mt	2, 3
<i>Eujivarus n.sp. B</i>	21,22	XO, XX	ac, mt	2, 3
<i>Eujivarus n.sp. C</i>	21,22	XO, XX	ac, mt	2, 3
<i>Eusitalces vulneratus</i>	23,24	XO, XX	ac	2, 3
<i>Eusitalces sp. A</i>	23,24	XO, XX	ac	2, 3
<i>Jodacris f. ferrugineus</i>	19,20	XO, XX	ac	2, 3
<i>Jodacris chapadensis</i>	19,20	XO, XX	ac	2, 3
<i>Jodacris furcillata</i>	19,20	XO, XX	ac	2, 3
<i>Ommatolampina obliquum</i>	21,22	XO, XX	ac, sm, sa	1, 3
<i>Orthoscapheus rufipes</i>	23,24	XO, XX	ac	6
<i>Psilocirtus bolivianus</i>	23,24	XO, XX	ac, sa	2, 3
<i>Psilocirtus olivaceus</i>	23,24	XO, XX	ac, sa	2, 3
<i>Psilocirtus sp. A</i>	23,24	XO, XX	ac, sa	2, 3
<i>Roppacris griseipes</i>	23,24	XO, XX	ac	4
<i>Sitalces volxemi</i>	19,20	XO, XX	ac, mt	2, 3
<i>Xiphola borellii</i>	23,24	XO, XX	ac	3

References: 1: Carbonell *et al.* (1980); 2: Ferreira *et al.* (1980); 3: Mesa *et al.* (1982); 4: Mesa and Fontanetti (1983); 5: Cella and Ferreira (1991); 6: present study. ac = acrocentric; mt = metacentric; sa = subacrocentric; sm = submetacentric; st = subtelocentric.

(Arana *et al.*, 1987), *Leptyisma argentina* (Colombo, 1993) and *Dichroplus pratensis* (Bidau and Martí, 2002).

The CH of *O. rufipes* and *E. fusiformis* was preferentially located in pericentromeric regions, in agreement with descriptions for most species of the family Acrididae (King and John, 1980; Santos *et al.*, 1983; Rocha *et al.*, 2004; Souza and Melo, 2007). In *Abracris flavolineata*, in addition to the pericentromeric regions, CH blocks also occur on the short arms of all chromosomes, except for pair M₇ (Cella and Ferreira, 1991). This divergence in the pattern of distribution suggests that rearrangements in CH, such as amplifications or losses, may contribute to karyotype evolution in the Abracrini.

Differences in the base composition of CH (detected by CMA₃/DA/DAPI staining) were observed in the species studied. In most chromosomes of both species, there was no predominance of AT or GC base pairs. However, GC-rich CMA₃-positive regions were detected in some CH blocks, especially those located in NORs. This pattern has also been reported for other grasshopper species (John *et al.*, 1985; Camacho *et al.*, 1991; Souza *et al.*, 1998; Loreto and Souza, 2000; Pereira and Souza, 2000; Rocha *et al.*, 2004). In some cases, such as in *Xyleus angulatus*, *Phaeoparia megacephala* and *Cornops frenatum frenatum*, CMA₃-positive blocks have been detected in all chromosomes of the complement (Souza *et al.*, 1998; Pereira and Souza, 2000; Rocha *et al.*, 2004). On the other hand, in most species,

GC-rich CH blocks occur on only some chromosomes of the karyotype (Schweizer *et al.*, 1983; John *et al.*, 1985; Loreto and Souza, 2000; Souza *et al.*, 2003; Rocha *et al.*, 2004; Loreto *et al.*, 2005; Souza and Melo, 2007), including *O. rufipes* and *E. fusiformis*.

Three categories of CH were identified in *O. rufipes*: (1) CH detected by C-banding, (2) CH detected by C-banding and CMA₃ staining and restricted to NORs (pairs M₆ and S₉), and (3) CH detected by CMA₃ staining, but not by C-banding, and unrelated to NORs (pairs L₂ and M₅). According to Sumner (1990), CH is not always detectable by the C-banding technique since its visualization is related to the size of the segments, with blocks less than 10⁶ or 10⁷ base pairs (bp) generally not being detected by this method. The finding that the third type of CH was GC-rich but showed no functional relationship to rDNA sites suggested a different organization for these two regions.

Two rDNA sites preferentially located on medium sized autosomes were observed in *O. rufipes* and *E. fusiformis*, a pattern commonly found among representatives of Neotropical Acrididae (Rocha *et al.*, 2004; Souza and Melo, 2007; Loreto *et al.*, 2008). The presence of NORs on bivalent S₉, as observed in *O. rufipes*, has also been described for most Neotropical species studied so far, especially those of the subfamilies Leptysminae (Rocha *et al.*, 2004) and Gomphocerinae (Loreto *et al.*, 2008). According to Loreto *et al.* (2008), bivalent S₉ probably represents the ancestral location of rDNA sites in Neotropical gomphocerine species since this pattern was identified in five species analyzed (*Rhammatocerus brasiliensis*, *R. brunneri*, *R. palustris*, *R. pictus* and *Amblytropidia* sp.). Ribosomal DNA sites on S₉ were also observed in five of six leptysmine species studied (Loreto and Souza, 2000; Rocha *et al.*, 2004) and in the ommatolampine *O. rufipes*. Together, these findings suggest a pattern of ancestry for Neotropical acridid grasshoppers.

In a broad investigation of the location and expression of ribosomal genes in 49 grasshopper species, Cabrero and Camacho (2008) showed that most Old World representatives have 1-3 rDNA sites and only six species had 5-10 sites. The predominant NOR locations were bivalents 2, 3, 6 and 9 and the X chromosome. In the subfamily Gomphocerinae, signals were detected mainly in bivalents 2, 3 and the X chromosome in species with 2n = 17, whereas in species with 2n = 23 most signals were restricted to bivalent 9. In Oedipodinae, NORs were generally found on bivalents 6 and 9. These data suggest that the location of rDNA sites on chromosome 9 represents an ancestral condition.

NORs located on large chromosomes or in the X chromosome have not yet been described for Brazilian species of the family Acrididae, in contrast to observations for the romaleid species *Radacridium nordestinum* (L₂) and *Radacridium mariajoseae* (X) (Rocha *et al.*, 1997), *Xyleus angulatus* (L₃, M₄ and X) (Souza *et al.*, 1998), and some

Old World acridid species (Rufas *et al.*, 1985; Cabrero and Camacho, 1986, 2008).

Many rDNA sites are located in pericentromeric regions of medium and/or small chromosomes (Loreto and Souza, 2000; Rocha *et al.*, 2004), as observed in the present study. However, some NORs occur in proximal regions, as observed in *Stenacris xanthochlora* and *Tucayaca parvula* (M₈) (Rocha *et al.*, 2004), or in interstitial regions, as described in *Schistocerca pallens* (M₅) and *S. flavofasciata* (M₅ and M₆) (Souza and Melo, 2007). In the Old World grasshoppers studied by Cabrero and Camacho (2008), many rDNA sites detected by FISH were found in proximal regions (52.4%), although a significant number was observed in interstitial regions (34.9%) and a minority was distal (12.7%). In addition, about 13% of the 126 rDNA sites detected by FISH were silent.

In conclusion, this study provides the first detailed cytogenetic results for *E. fusiformis* and *O. rufipes*. The two species showed significant karyotype differentiation depending on the staining method used. Our findings suggest possible pathways of chromosome evolution in these species. However, additional cytogenetic and molecular analyses of other species of the tribe Abacrini are necessary to improve our understanding of the evolutionary patterns within this group.

Acknowledgments

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