Genetics and Molecular Biology, 28, 1, 111-116 (2005)

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Research Article

A study of the constitutive heterochromatin and nucleolus organizer regions of *Isocopris inhiata* and *Diabroctis mimas* (Coleoptera: Scarabaeidae, Scarabaeinae) using C-banding, $AgNO_3$ staining and FISH techniques

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Abstract

Meiotic and mitotic chromosomes of *Isocopris inhiata* and *Diabroctis mimas* were studied by standard staining procedures, C-banding, silver nitrate staining and FISH using *Apis mellifera* 28S rDNA as probe. *Isocopris inhiata* presented a 2n = 18 (8II+ Xy_p) karyotype, composed of meta-submetacentric chromosomes with gradual reduction in size. The karyotype of *D. mimas* was 2n = 20 (9II+ Xy_p), composed of meta-submetacentric (pairs 1, 2, 3, 4 and 7) and acrocentric (pairs 5, 6, 8 and 9) chromosomes, with gradual reduction in size. Analysis of constitutive heterochromatin revealed similar C-banding patterns for both species, showing pericentromeric and telomeric bands and diphasic chromosomes. In addition, the X chromosomes of these species were found to be almost completely heterochromatic. The presence of chromocenters was checked in one or more phases of prophase I of these species. All heterochromatin reacted positively for the silver stain. By FISH analysis we were able to locate the rDNA in medium-size autosome pairs in both species and in the X chromosome of *D. mimas*.

Key words: Coleoptera, heterochromatin, NORs, FISH.

Received: December 19, 2003; Accepted: June 25, 2004.

Introduction

Scarabaeidae constitutes the largest family within the superfamily Scarabaeoidea, forming a cosmopolitan group and one of the richest in species number within the order Coleoptera. It comprises approximately 2,300 genera and 27,000 species of worldwide distribution. About 204 genera and 1,800 species have been described for Brazil (Crowson, 1967; Costa *et al*., 1988). The Scarabaeinae constitute a highly diverse subfamily that comprises about 5,000 described species belonging to 234 genera spread widely in the world (Hanski and Cambefort, 1991).

Representatives of about 320 species comprising 21 Scarabaeidae subfamilies are cytogenetically characterized to date. The chromosome numbers and morphology in this family are highly conserved, predominantly presenting a diploid number of 2n = 20, a sex chromosome mechanism of the "parachute" Xy type (Xy_p), and metacentric, submetacentric and acrocentric chromosomes (Smith and Virkki, 1978; Yadav and Pillai, 1979; Colomba *et al*., 1996; Bione, 1999; Moura *et al*., 2003). This chromosome number is considered as being the most primitive within the order Coleoptera. Most of the 85 Scarabaeinae species which are known cytogenetically have a chromosome number varying from 2n = 12 to 2n = 20, with the Xy_p type being the most prevalent sex chromosome determination system (Smith and Virkki, 1978; Vidal, 1984; Colomba *et al*., 2000a).

The order Coleoptera has the highest species diversity within the animal kingdom, yet cytogenetic data using specific banding techniques are still scarce. C-banding data have revealed a preferential localization of autosomal constitutive heterochromatin (CH) in the centromeric area and less so observed in interstitial and telomeric areas. Sex chromosomes also show a variable CH distribution, as it has been observed in the pericentromeric region or along the entire chromosome (Ennis, 1974; Vidal *et al*., 1977; Angus, 1983; Drets *et al*., 1983; Virkki, 1983; Juan and Petitpierre, 1989).

$AgNO_3$ staining of both mitotic and meiotic chromosomes of eukaryotic species has been a very useful approach for the analysis of the structure and variability of nucleoli, nucleolar organizer regions (NORs) and kinetochores (Goodpasture and Bloom, 1975; Virkki and Denton, 1987; Virkki *et al*., 1991). Although in Coleoptera this technique has been considered unsuitable to identify active rDNA clusters (Colomba *et al*., 2000a), it is gener-

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ally accepted that silver impregnation is indicative of rRNA synthesis and that it stains functionally active NORs (Miller *et al*., 1976; Hubbell, 1985). However, in most genomes transcriptionally active and inactive rRNA genes usually coexist (López-León *et al*., 1999).

Recently, the FISH method has been employed in a great number of species to locate specific DNA sequences directly on the chromosomes. This approach has been used mainly for mapping different types of repetitive DNA sequences, including ribosomal DNA (Vitturi *et al*., 1999; Colomba *et al*., 2000a, 2000b; Moura, 2002). Due to its high resolution, the use of *in situ* hybridization with a ribosomal DNA as probe makes the precise detection of active and inactive rDNA clusters possible, even in cases with minute amounts of ribosomal genes (López-León *et al*., 1999).

In the present study, meiotic and mitotic chromosomes of male *Isocopris inhiata* and *Diabroctis mimas* specimens were analyzed, in order to better characterize the chromosomes of the Scarabaeinae subfamily, especially in terms of distribution and variability of CH and NORs.

Material and Methods

Meiotic chromosomes of 15 *Isocopris inhiata* (Germar, 1824) and 2 *Diabroctis mimas* (Linnaeus, 1758) male specimens were analyzed. The material was collected in Ribeirão Preto (*I. inhiata*), State of São Paulo (21°10'24"S and 47°48'24"W), and Igarassú (*D. mimas*), State of Pernambuco (7°50'20''S and 35°00'10''W), a south-eastern and a north-eastern region of Brazil, respectively. The insects were anaesthetised with ether [$(C_2H_5)_2O$], and their testes were removed and fixed in ethanol-acetic acid (3:1), using the classic technique of testicular follicle squashing and staining with 2% lacto-acetic orcein for standard karyotype analysis.

C-banding was performed by the method described by Sumner (1972). The material was incubated with 0.2 N HCl, followed by treatment with 5% barium hydroxide and 2 x SSC at 60°C. Silver nitrate staining was performed as described by Rufas *et al*. (1987), with the slides being pre-treated with 2 x SSC at 60 °C for 10 min and stained with silver nitrate (1 g/L mL) at 70-80 °C.

FISH was performed according to the methodology described by Natarajan *et al*. (1998) and Sakamoto-Hojo *et al*. (1999). The rDNA fragment used as probe for NOR detection was obtained from an *Apis mellifera* 28S clone (GenBank accession number AJ302936). First this fragment was amplified by polymerase chain reaction (PCR) and then used as template to amplify biotinylated products by another PCR round.

Results

Standard staining and C banding

This was the first time *Isocopris inhiata* and *Diabroctis mimas* were analyzed cytogenetically. The *I. inhiata* males presented a 2n = 18 karyotype, with a meioformula of 8II + Xy_p. The chromosomes of this species were meta-submetacentric and showed a gradual reduction in size. Pair 1 was considerably larger than the other chromosomes (Figure 1). The chromosome number of the *D. mimas* males was 2n = 20, with a meioformula of 9II + Xy_p (Figure 2). This species presented both meta-submetacentric (pairs 1, 2, 3, 4, and 7) and acrocentric (pairs 5, 6, 8, and 9) chromosomes, with gradual size reduction. The sex determination mechanism turned out to be of the "parachute" type, and the y chromosome had a metacentric dot configuration in both species (Figure 1).

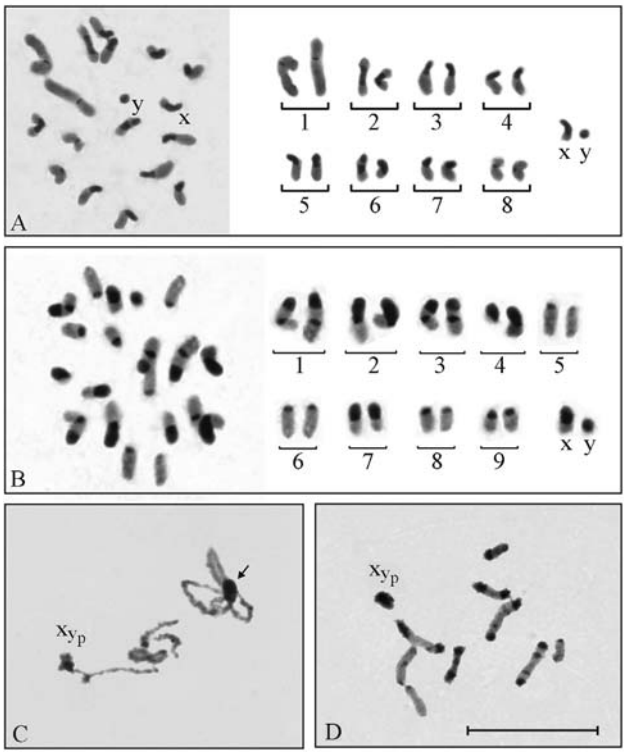

Figure 1 - CH distribution pattern in spermatogonial metaphases of *I. inhiata* (**A**) (2n = 18) and *D. mimas* (**B**) (2n = 20). **A** and **B** present the C-banded karyotype showing the presence of diphasic chromosomes (pairs 3, 4, 5 and 7 in **A**, and 2, 4 and 7 in **B**) and terminal bands (pair 6 in **A**, and pairs 1 and 3 in **B**). **C** and **D** show, respectively, pachytene and metaphase I of *D. mimas*. Note the chromocenter in **C** (arrow) and the heterochromatic sex bivalent (Xy_p) in **C** and **D**. Bar = 10 µm.

Constitutive heterochromatin (CH) analysis revealed similar C-banding patterns for both species, despite the diphasic chromosomes, i.e., large paracentromeric blocks observed on each short arm of pairs 3, 4, 5 and 7 of *I. inhiata* and pairs 2, 4 and 7 of *D. mimas* (Figure 1A and 1B). In addition, *I. inhiata* showed telomeric blocks on pair 6 (Figure 1A), and *D. mimas* on pairs 1 and 3 (Figure 1B). The X chromosomes of these species were found to be almost completely heterochromatic. Non-homologous heterochromatic associations forming chromocenters between the bivalent autosomes were visible during the meiotic prophase and persisted until the end of pachytene (Figure 1C).

$AgNO_3$ staining

Amorphous masses corresponding to argyrophilous proteins were observed upon $AgNO_3$ staining in the Xy_p bivalent of the two species studied. These masses were observed from the beginning of prophase until the end of pachytene or the beginning of diplotene. $AgNO_3$ staining also detected blocks similar to those observed by C-banding during the different phases of meiosis (Figures 2A and 3B).

FISH analysis revealed rDNA clusters in one autosome pair of *Isocopris inhiata* (Figure 2D) and in two autosome pairs plus in the X chromosome of *Diabroctis mimas* (Figure 3C and D). In both species, these clusters were located in chromosomes of medium size.

Discussion

The chromosome number found in *D. mimas* was in accordance to that observed in most species of the suborder Polyphaga. Therefore, the presence of acrocentric chromosomes in this karyotype (Figure 1B) suggests the occurrence of pericentric inversions. The reduction in chromosome number and the presence of a larger pair observed in *I. inhiata* (Figure 1A) may be due to inversion followed by fusion mechanisms between autosomes, a fact that may explain the alteration in karyotype without modifying the type of sex-determining system. Such changes have been widely reported in the literature, including the karyotype evolution types proposed for Coleoptera by Yadav and Pillai (1979).

D. mimas and *I. inhiata* presented an interesting distribution pattern of CH blocks, as well as a type of heterochromatic association forming chromocenters between some bivalent autosomes (Figure 1C). Non-homologous heterochromatic associations and chromocenter fusions involving various or all chromosomes seem to be a very common phenomenon in insect meiosis. The heterochromatic segments of several coleopteran species present different

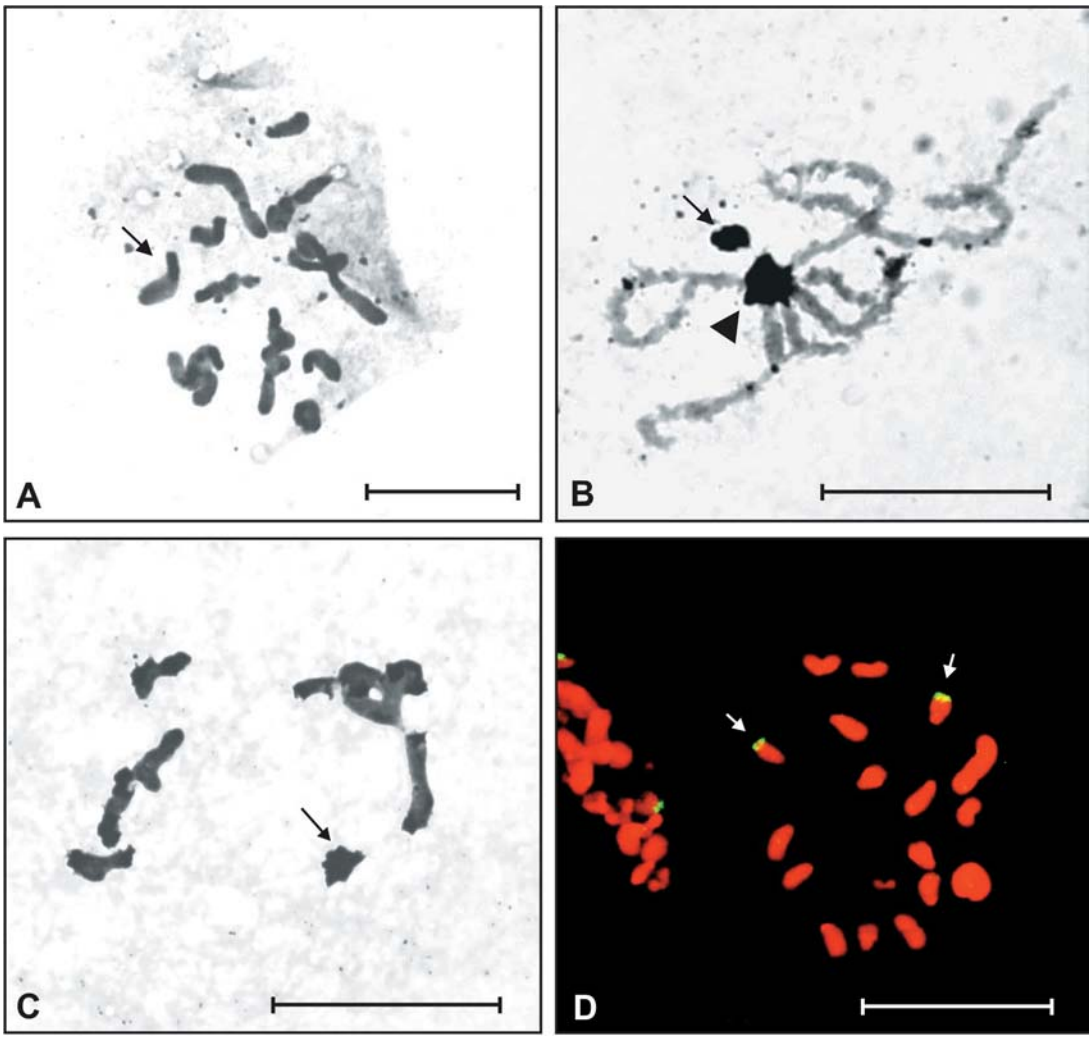

Figure 2 - Silver staining and FISH in *I. inhiata*. Spermatogonial metaphase (**A** and **D**), pachytene (**B**) and metaphase I (**C**). Note the sex bivalent (black arrows) and CH affinity for silver in **A** (arrow), **B** and **C**. rDNA sites (white arrows) were detected by FISH in one autosome pair, as shown in **D**. The arrowhead in **B** indicates the chromocenter. Bar = 10 µm.

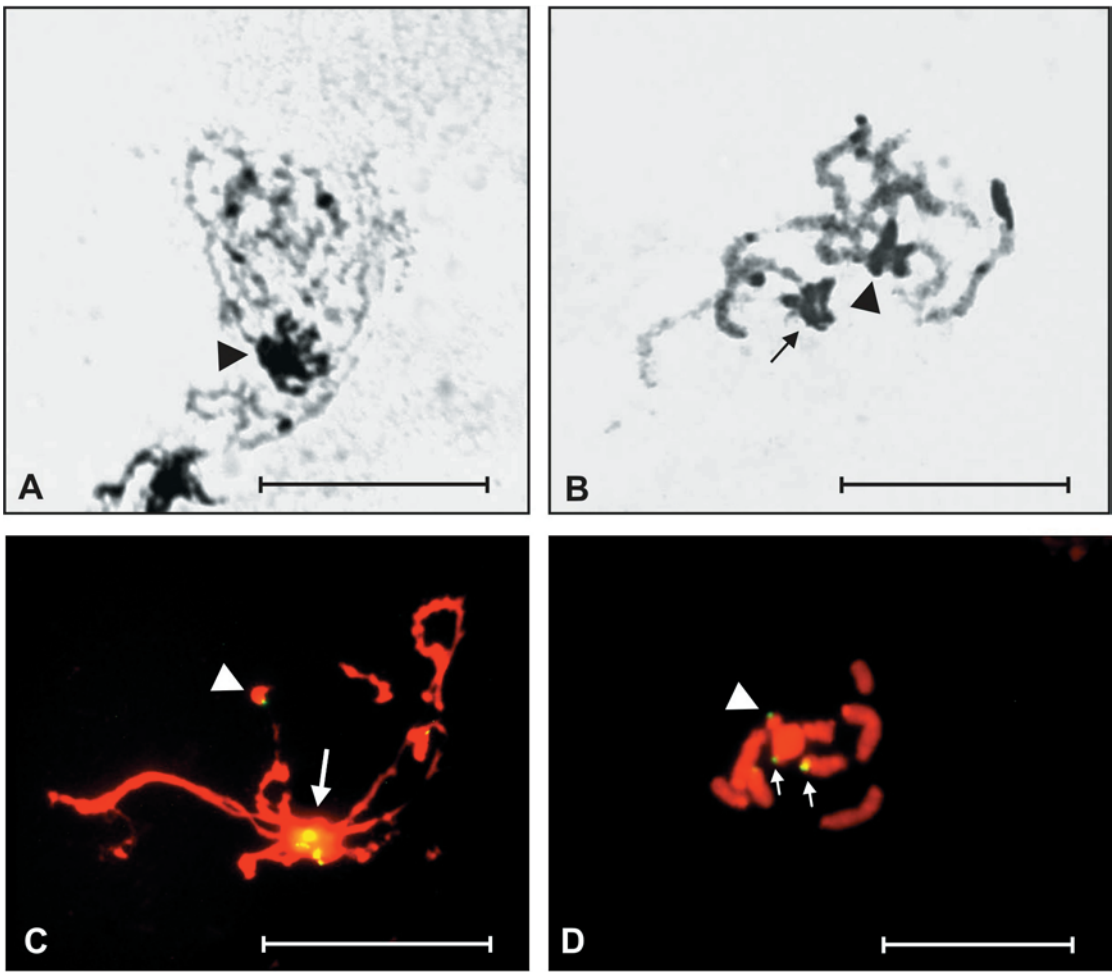

Figure 3 - Silver staining and FISH in *D. mimas*. Zygotene (**A**), pachytene (**B** and **C**) and metaphase I (**D**). Note the sex bivalent (black arrows) and chromocenter affinity for silver in **A** and **B** (arrowheads). rDNA sites were detected by FISH in two autosome pairs (white arrows) and in the X chromosome (white arrowheads), shown in **C** and **D**. Bar = 10 μm.

degrees of ectopic pairing, leading to the formation of chromocenters. This type of association seems to play an important role in nuclear organization and segregation of meiotic chromosomes in beetles (Smith and Virkki, 1978; Drets *et al*., 1983).

Although the CH in these species was seen mostly in pericentromeric regions, as in other beetles analyzed by the C-banding technique (Rees *et al*., 1976; Vidal and Giacomozzi, 1978; Virkki, 1983; Vidal and Nocera, 1984; Postiglioni and Brum-Zorrilla, 1988; Colomba, *et al*., 1996; Bione, 1999; Moura, 2002), *Diabroctis mimas* and *Isocopris inhiata* showed some telomeric blocks and diphasic chromosomes with one heterochromatic and one euchromatic arms (Figures 1A and 1B). According to previous reports, in addition to pericentromeric C-bands, distal C-bands were also observed in the tenebrionids *Misolampus goudoti* (Juan and Petitpierre, 1989) and *Palembus dermestoides* (Almeida *et al*., 2000), while exclusively telomeric blocks were observed in the carabid *Bembidion minimum* (Rozek and Rudek, 1992). The additional C-bands in these beetles and in *D. mimas* and *I. inhiata* probably arose by small tandem duplications, as proposed by King and John (1980).

Data on localization of NORs in Coleoptera suggest that an autosome pair functioning as nucleolus organiser appears to be widely distributed in this order (Virkki, 1983; Virkki *et al*., 1984; Postiglioni and Brum-Zorrilla, 1988; Colomba *et al*., 2000a; Moura *et al*., 2003). This stands in contrast to most Scarabaeidae, where the NOR is found in the sex bivalent (Bione, 1999; Moura, 2002)

NOR activity at the beginning of the meiotic prophase is widely observed in a large number of organisms, including Coleoptera species. However, this activity was observed during a restricted period of time only, declining rapidly and disappearing in the middle of the diplotene phase. Nevertheless, the nucleolar masses produced can persist for a longer period of time, especially in species with a prolonged diplotene (Virkki and Denton, 1987; Virkki *et al*., 1991). This phenomenon was clearly observed in the two species studied here.

Studies on some curculionid beetles investigating the development and segregation of the Xy_p showed that, even when the NORs are autosomal, the lumen of the sex bivalent is filled with a proteinaceous substance with affinity for silver, and this affinity persists from diakinesis to anaphase I. This substance is probably similar to that present in the synaptonemal complex and in the chromosome skeleton. It has been suggested that this substance may play an adhesive role, thus controlling the correct separation of the sex chromosomes (Virkki *et al*., 1990, 1991). The fact that the sex bivalents of the species analyzed here continued to be labelled with silver after the disappearance of the nucleolus, as well as the high affinity of their heterochromatin for silver, suggests that the Xy_p association is not necessarily due to the NOR, but possibly to the presence of argyrophilic proteins associated with the heterochromatin of these species. These results are supported by data obtained for representatives of Geotrupinae (Vitturi *et al*., 1999), Scarabaeinae (Colomba *et al*., 2000b), Rutelinae and Dynatinae (Bione, 1999) and Melolonthinae (Moura *et al*., 2003) showing that the entire heterochromatin stained by C-banding possessed high affinity for silver, regardless of its base pair composition.

Juan *et al*. (1993) demonstrated that in *Tenebrio molitor* only the Xy_p bivalent was labelled upon $AgNO_3$ staining. However, FISH analysis using an rDNA as probe revealed 6 NORs in the mitotic metaphase chromosomes of this species, located on two different autosome pairs and on the X_p and y_p chromosomes. Similarly, *D. mimas* presented two pairs of autosomes and an X chromosome carrying nucleolus organizer regions. On the other hand, due to the great affinity of the constitutive heterochromatin of *I. Inhiata* and *D. mimas* for the $AgNO_3$ substrate (Figures 2 and 3), we were unable to determine which NORs were active during meiosis in these species.

I. inhiata and *D. mimas* showed differences in number and localization of rDNA clusters, as evidenced by the FISH method. At least for *I. Inhiata*, our data are in concordance with the hypothesis that an autosome pair functions as nucleolus organizer in Coleoptera. The fact that the nucleolus organizer of *D. mimas* is localized in the chromocenter could have favoured the "dislocation" of the rDNA clusters and its subsequent amplification on the other chromosomes that form the chromocenter. A plausible explanation for the presence of an rDNA cluster in the X chromosome could be the occurrence of an autosome fission followed by the translocation of the cluster to this chromosome.

Although *I. inhiata* and *D. mimas* belong to distinct tribes (Coprini and Phanaeini, respectively) and present differences in their chromosome number and morphology, the data collected here show similarities between the karyotypes of these two species in respect to the amount, localization and behavior of the CH during meiosis, as well as to the presence of diphasic chromosomes. Our results suggest that a similar evolutionary pathway was followed by these two species. The use of banding and FISH methods is necessary to establish a real karyotype overview in beetles. Further on, such studies will shed more light on the chromosome evolution in this subfamily.

Acknowledgments

This work was supported by grants from the Brazilian agency Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). We are grateful to Dr. Sérgio Ide from Instituto Biológico, São Paulo, and Fernando Vaz de Melo from Universidade Federal de Lavras (UFLA), for the taxonomic identification of the species used in this study. We also thank Angel Roberto Barchuk for help in preparing the rDNA probe and Dr. Adriana Mendes Nascimento and Dr. Rita de Cássia de Moura for critically reading and commenting on a previous version of this manuscript.

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Associate Editor: Yatiyo Yonenaga-Yassuda