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Cytogenetic and Molecular Characterization of Three Mimetic Species of the Genus *Alagoasa* Bechyné 1955 (Coleoptera: Alticinae) from the Neotropical Region

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Abstract
Coleoptera is a mega-diverse order, but only about 1% of its species have been analyzed cytogenetically. In this order, the subfamily Alticinae presents many identification problems, mainly due to the occurrence of mimicry. The objective of this work was to cytogenetically characterize 3 very similar species of the genus *Alagoasa* (*A. pantina*, *A. areata*, and *A. scissa*). We used classical and molecular cytogenetic as well as molecular genetic techniques. All 3 species showed a diploid chromosome number of 2n = 22 (20+X+y), but differences in the morphology of the chromosomes. All had a meiotic formula of 2n = 10II+X+y and an X+y sex determination system with giant, fully asynaptic sex chromosomes, concordant characteristics observed in the subtribe Oedionychina. FISH demonstrated the presence of 18S and 5S rDNA clusters in 1 pair of autosomes, syntenic and colocalizing in the 3 analyzed species. However, in *A. areata*, heteromorphism between the cistrons was observed. The telomeric $(TTAGG)_n$ probe showed signals in all 3 species, with proximal signals in the X and dispersed signals in the y chromosome of *A. areata*, and 2 proximal signals in the X chromosome of *A. scissa*. Molecular analysis of the *COI* gene indicated that they are 3 distinct species, corroborating the observed cytogenetic characteristics.

The Alticinae subfamily comprises about 8,000 described species [Nadein, 2013] and presents several species identification problems. In addition to this matter, the occurrence of mimicry has been reported in this group [Begosi and Benson, 1988].

Only 240 species of the Alticinae subfamily have been analyzed cytogenetically [Petitpierre et al., 1988; Virkki and Santiago-Blay, 1996; Almeida et al., 2006, 2009; Petitpierre, 2006; Goll et al., 2018]. The diploid chromosome number in these species ranges from 2n = 8 in *Homoschema nigriventre* to 2n = 64 in *Disonycha bicarinata*, and varied systems of sex determination are found [Virkki and Purcell, 1965; Smith and Virkki, 1978; Vidal, 1984; Petitpierre et al., 1988; Virkki, 1988; Petitpierre, 1989; Segarra and Petitpierre, 1989; Virkki et al., 1991; Virkki and Santiago-Blay, 1998]. However, the ancestral chromosome number considered for Alticinae is 2n = 24 and a Xy_p sex determination system [Virkki, 1970].

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The Oedionychina subtribe of Oedionychini in the Alticinae subfamily showes increased sex chromosome sizes in relation to autosomes, with an asynaptic behavior. Besides the fully separated sex chromosomes, a chromosome number of 2n = 22 (10II+X+y) has been maintained in the majority of species [Virkki, 1970, 1988; Petitpierre, 1988; Virkki et al., 1991]. The genus *Alagoasa* Bechyné 1955 belongs to Oedionychina. Up to now, in which 34 species of this genus have been cytogenetically analyzed, most of them presenting 2n = 22. These all have a sex determination system of the X+y type, with giant sex chromosomes [Virkki, 1961, 1970, 1985, 1988; Smith and Virkki, 1978; Petitpierre et al., 1988; Virkki et al., 1991; Virkki and Santiago-Blay, 1993]. Variations in the diploid number in this genus were observed due to the presence of supernumerary chromosomes or due to centromeric fusion. *Alagoasa oblecta* shows 2n = 22 + 15B, *A. transparente* 2n = 22 + 2B, *A. arcifera* 2n = 16, *A. equestris* 2n = 12 + 1B, and *A. parana* 2n = 16 [Smith and Virkki, 1978; Petitpierre et al., 1988; Virkki, 1988; Virkki and Santiago-Blay, 1993].

Regarding the 45S multigene family, Schneider et al. [2007] proposed that 1 pair of autosomes carrying this marker could represent the ancestral and/or a more stable condition in Coleoptera. In Alticinae, using FISH, Almeida et al. [2010] and Goll et al. [2018] found the cluster associated with 1 pair of autosomes in *Omophoita octoguttata*, *O. personata*, *O. abbreviata*, and *O. aequinoctialis* (cytotype 1), with 2 pairs in *O. magniguttis*, and with 3 pairs in *O. aequinoctialis* (cytotypes 2 and 3). Thus, Almeida et al. [2010] proposed that 18S rDNA may be an important marker in this group in order to demonstrate karyotypic differences.

The location of 5S rDNA in chromosomes is variable in Coleoptera; it may be present in one or several pairs of autosomes. In Alticinae, Goll et al. [2018] demonstrated that in *O. abbreviata* and *O. aequinoctialis* (cytotype 1), the 5S cistrons are located in 1 pair of autosomes, while in *O. aequinoctialis* (cytotypes 2 and 3), they are associated with 3 pairs. Moreover, these cistrons usually are colocated with some genes belonging to another multigenic family [Cabral-de-Mello et al., 2010, 2011a; Goll et al., 2015, 2018].

Telomeres are structural components at the ends of eukaryotic chromosomes that prevent fusions and the gradual diminution of their termini during successive cycles of semiconservative DNA replication [Blackburn, 1991]. These sequences may contribute to the identification of chromosomal rearrangements, indicating regions of chromosomal fusion [Slijepcevic, 1998]. Most insects

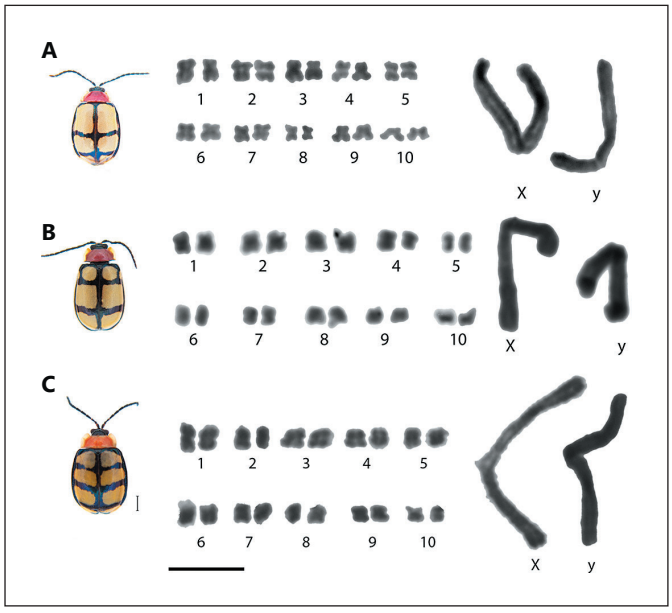

Fig. 1. Species of the genus *Alagoasa* analyzed in this work and their respective karyotypes. **A** *Alagoasa pantina*. **B** *Alagoasa areata*. **C** *Alagoasa scissa*. Horizontal bar, 10 μm; vertical bar, 1 mm.

present $(TTAGG)_n$ repeats as a telomeric sequence [Frydrychová et al., 2004], and in only 2 species of Alticinae these regions have been mapped [Goll et al., 2018].

In this sense, the aim of this study was to characterize and differentiate 3 very similar, possibly mimetic, species of the genus *Alagoasa* through a comparative analysis using conventional and molecular cytogenetic techniques and partial sequencing of the cytochrome C oxidase subunit I gene (*COI*).

Materials and Methods

Male *Alagoasa* adults were collected from natural populations in Ponta Grossa, PR, Brazil (S 25°07′10″, W 49°56′24″). We collected 12 individuals of *A. pantina* Bechyné 1958 (Fig. 1A), 10 of *A. areata* Germar 1824 (Fig. 1B), and 10 of *A. scissa* Germar 1824 (Fig. 1C). Specimens were sent to a specialist, Carlos Campaner (USP Zoology Museum), for identification. All individuals were kept in the Genetic and Evolution Laboratory, Universidade Estadual de Ponta Grossa, Brazil.

Mitotic and meiotic chromosomes were obtained by dissection of the insects according to Almeida et al. [2000], with modifications of Rosolen et al. [2018]. The slides were stained with 3% Giemsa in phosphate buffer pH 6.8 for 12 min, washed with distilled water, and air-dried.

The 18S rDNA probes were labeled by PCR with biotin-16-dUTP using the 18S partial gene cloned in pTZ from *O. octogut-*

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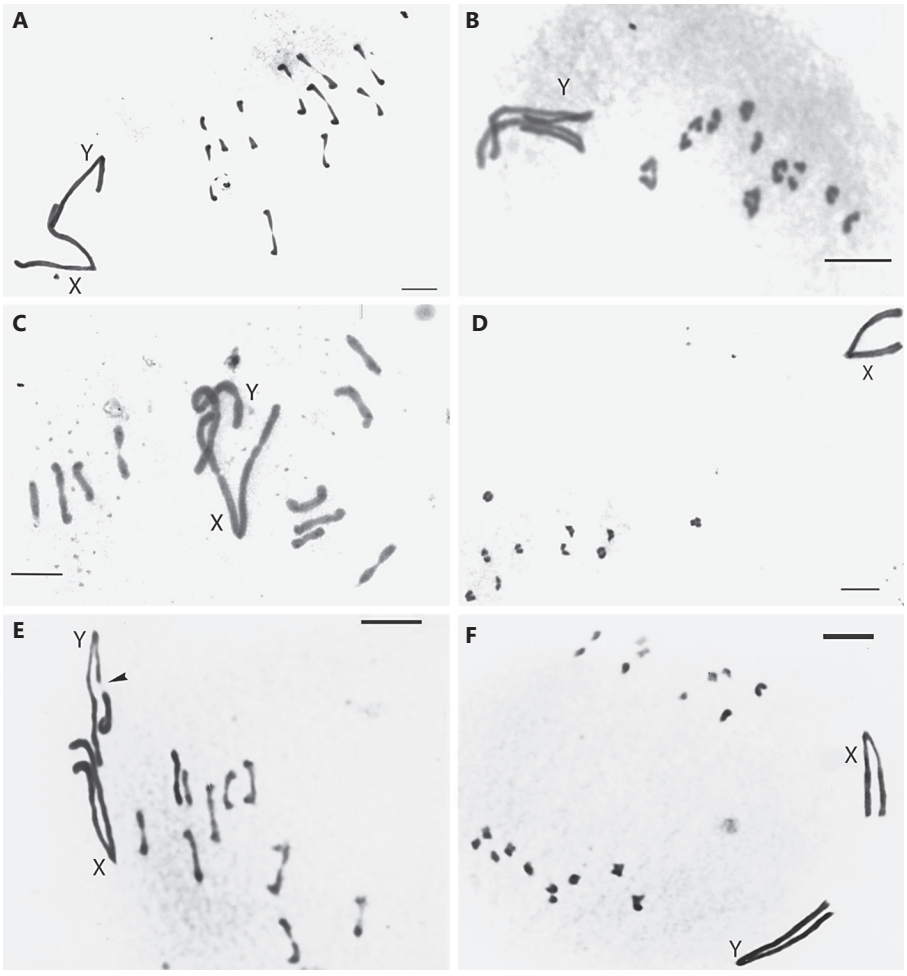

Fig. 2. Giemsa-stained meiotic chromosomes from the *Alagoasa* species analyzed. **A**, **B** *A. pantina*. **A** Anaphase I with 10+X+y. **B** Anaphase II with 10+y, showing the segregating chromatids. **C**, **D** *A. areata*. **C** Metaphase I with 10+X+y. **D** Anaphase I with 10+X. **E**, **F** *A. scissa*. **E** Metaphase I with 10+X+y; **F** Anaphase I with 10+X and 10+y. The arrow head in **E** indicates a secondary constriction in the y chromosome. Scale bars, 10 μm.

tata (HM036738.1) according to Almeida et al. [2010]. The 5S rDNA probes were labeled by PCR with digoxigenin-11-dUTP, using the 5S gene of *O. octoguttata* cloned in pTZ (KX858924.1). The telomeric probe was obtained from a PCR reaction containing 1× *Taq* Reaction Buffer (200 mM Tris pH 8.4, 500 mM KCl), 1.5 mM $MgCl_2$, 0.16 mM dNTP mix, 0.5 μM of each primer (forward 5′(TTAGG)$_6$3′ and reverse 5′(CCTAA)$_6$3′) [Sahara et al., 1999], and 1U of *Taq* DNA polymerase, in a final volume of 50 μL, following the program proposed by Ijdo et al. [1991]. The PCR product was labeled with digoxigenin-11-dUTP using the DIG-Nick Translation Mix (Roche Applied Science) according to the manufacturer's recommendations. FISH was performed according to Pinkel et al. [1986], with minor modifications described by Almeida et al. [2010]. Chromosome preparations were analyzed using the bright field and epifluorescence microscope Olympus Bx41, equipped with the DP71 digital image capture system (Olympus). The images were analyzed with Adobe Photoshop CS6 software. The chromosomes were organized into karyotypes according to Levan et al. [1964].

Genomic DNA from 3 *A. pantina* individuals (3860, 3960, and 3972), 3 *A. areata* individuals (3959, 4010, and 4011), and 2 *A. scissa* individuals (3790 and 4003) was extracted by fragmentation of the head, pronotum, and posterior pair of legs in liquid nitrogen following the protocol of Murray and Thompson [1980]. For amplification of the *COI* gene fragment, the primers described by Kim et al. [2003] were used, and the reactions contained 1× *Taq* Reaction Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1.0 μM of each primer, 2.5 U *Taq* DNA polymerase, and 40 ng of DNA in a final volume of 35 μL. PCR was performed in a thermocycler with an initial denaturation cycle of 4 min at 95°C, followed by 35 cycles of 94°C for 60 s, 53°C for 30 s, and 72°C for 30 s, and a final step of 72°C for 10 min. PCR products were purified using the Illustra GFX PCR DNA and Gel Band Purification (GE Healthcare) and sequenced by an ABI-PRISM 3100 Genetic Analyzer automated sequencer.

The sequences were verified and corrected using Geneious v.7.1.3 [Kearse et al., 2012] and aligned with the outgroups *O. octoguttata* (AF479430.1), *Chrysomela tremula* (KM452126.1), and *Timarcha tenebricosa* (KC185782.1), using the algorithm Clustal W, implemented in Geneious. A Bayesian inference tree was constructed in MrBayes 3.2.6 [Huelsenbeck and Ronquist, 2001] with 1,000,000 Markov Chain Monte Carlo (MCMC) and burning in 100,000. The genetic distances were calculated using the Kimura-2-parameter model and 1,000 bootstrap replicates in MEGA v.7.0 [Kumar et al., 2016] for the *Alagoasa* sequences.

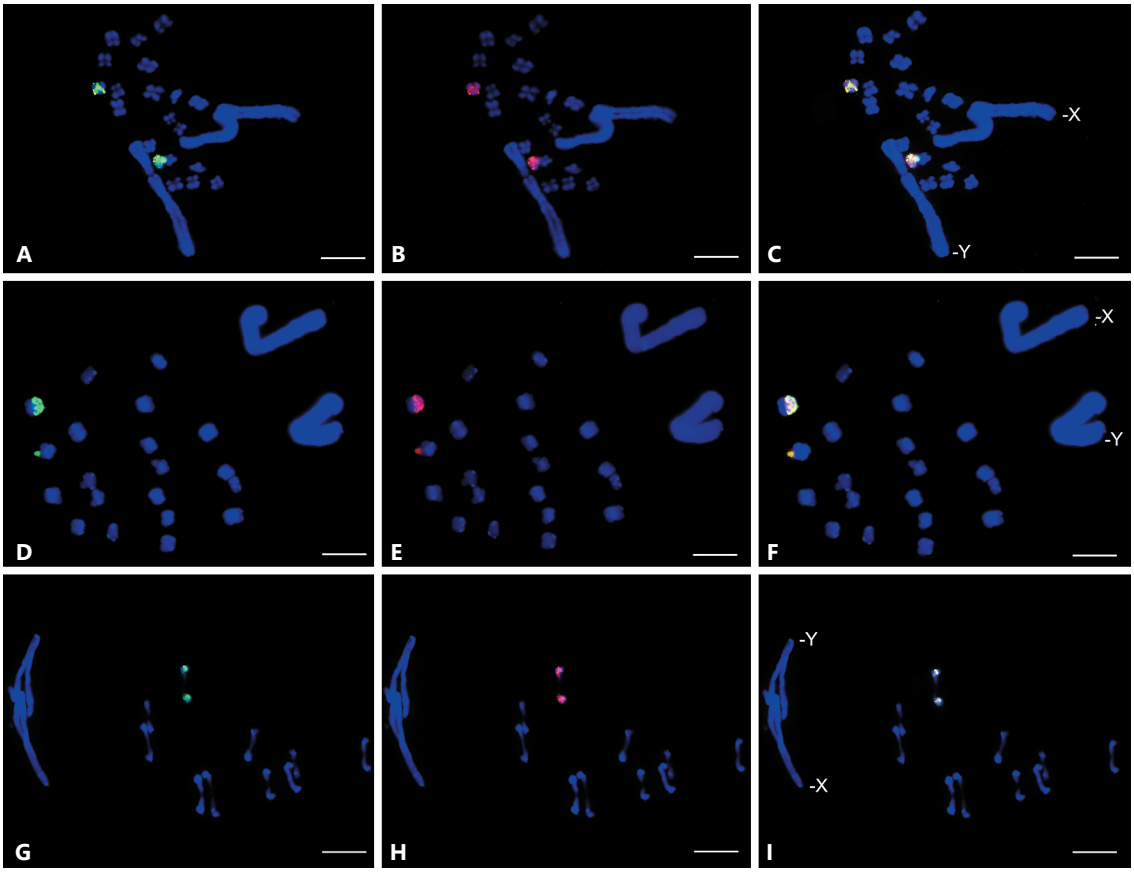

Fig. 3. Testicular cells submitted to FISH with 18S (green) and 5S (red) rDNA probes. **A**–**C** Spermatogonial metaphase of *Alagoasa pantina*. **D**–**F** Spermatogonial metaphase of *Alagoasa areata*. **G**–**I** Metaphase I of *Alagoasa scissa*. The merged images of both probes (**C**, **F**, **I**) demonstrate the synteny and colocalization of 18S and 5S rDNA in all species. Scale bars, 10 μm.

Results

Alagoasa pantina

The spermatogonial metaphases of *A. pantina* showed a karyotype of 2n = 22, giant sex chromosomes, and an X+y sex determination system (Fig. 1A). The autosomes are metacentric to submetacentric, with 1 acrocentric pair. The X is metacentric, and the y is submetacentric. The cells in anaphase I demonstrated 2n = 10II+X+y and asynapsis of the sex chromosomes (Fig. 2A). The anaphase II evidenced the correct segregation of the sex chromosomes, with the formation of haploid n = 10+X and n = 10+y cells (Fig. 2B). In Figure 2B, the segregation of autosomal and y chromosomal chromatids is shown.

FISH with 18S rDNA (Fig. 3A) and 5S rDNA (Fig. 3B) probes in *A. pantina* showed that both rDNA clusters are associated with the same autosomal pair in spermatogonial metaphases. The merged images demonstrate that they are syntenic and colocalize (Fig. 3C). The analysis of meiotic cells submitted to FISH with the telomeric probe revealed telomeric signals in 5 autosomal pairs (Fig. 4A) and in the y chromosome (Fig. 4B) in metaphases I, while the X chromosome and the other 5 autosomal pairs displayed no signals (Fig. 4A, C).

Alagoasa areata

The spermatogonial metaphases of *A. areata* showed a karyotype of 2n = 22, with giant sex chromosomes and X+y sex determination system. The autosomes have a meta- and submetacentric morphology, whereas the sex chromosomes are submetacentric (Fig. 1B). The observed meiotic formula was 2n = 10II+X+y, with asynaptic sex chromosomes (Fig. 2C), and metaphases II confirmed the formation of haploid cells with n = 10+X (Fig. 2D) and n = 10+y).

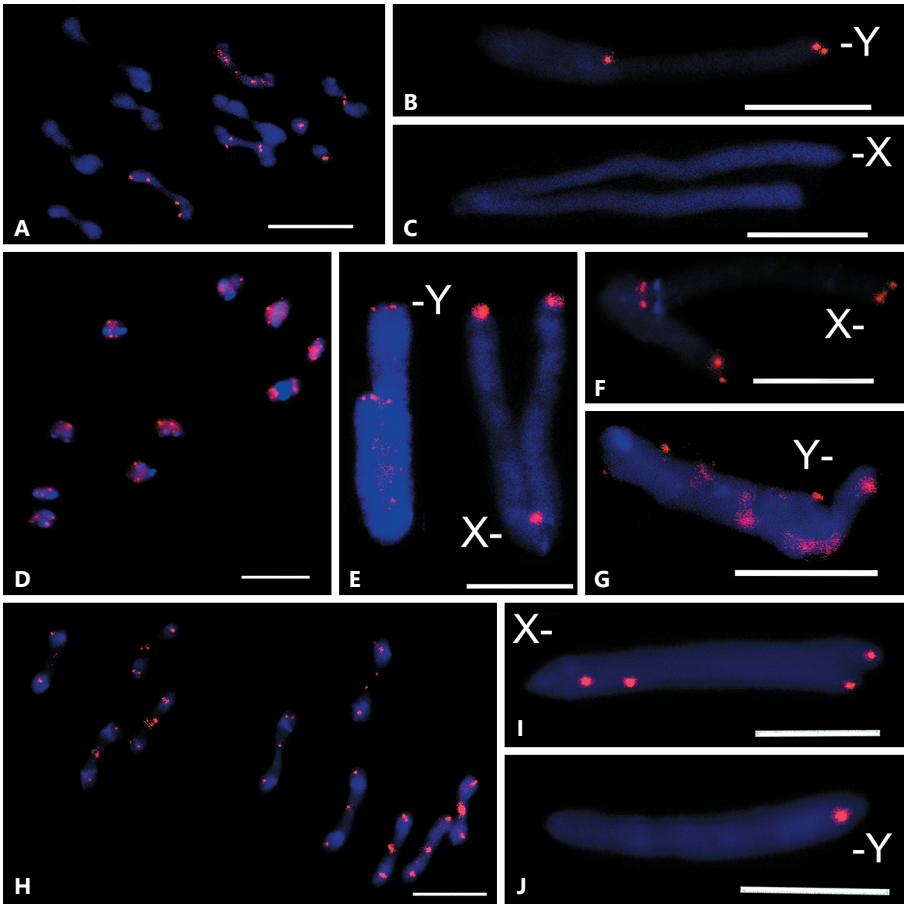

Fig. 4. FISH with a telomeric probe on meiotic chromosomes of the *Alagoasa* species. **A**–**C** *A. pantina*. **A** Autosomal bivalents in metaphase I. **B**, **C** Sex chromosomes y (**B**) and X (**C**). **D**–**G** *Alagoasa areata*. **D** Autosomal chromosomes in metaphase II. **E**–**G** Sex chromosomes X and y (**E**), X (**F**), and y (**G**). **H**–**J** *A. scissa*. **H** Autosomal bivalents in metaphase I. **I**, **J** Sex chromosomes X (**I**) and y (**J**). Scale bar, 10 μm.

FISH demonstrated the presence of 1 pair of autosomes carrying the 18S rDNA cluster (Fig. 3D) and the 5S rDNA (Fig. 3E) in spermatogonial metaphase. In addition, we observed a size heteromorphism of the 18S and 5S cistrons between the chromosomes of the pair. The merged image indicates the synteny and colocalization of these clusters (Fig. 3F). After FISH with the telomeric probe, metaphases II showed signals in all chromosomes (Fig. 4D, E). The X chromosome displayed signals in the terminal regions and a proximal interstitial signal (Fig. 4E, F); the y chromosome also showed terminal hybridizations as well as scattered signals along the short arm (Fig. 4E, G).

Alagoasa scissa

The spermatogonial metaphases of *A. scissa* showed that this species has a diploid number of 2n = 22 and a sex determination system of the X+y type, with giant sex chromosomes. The autosomes are meta- and submetacentric, the sex chromosomes are metacentric (Fig. 1C). The observed meiotic formula was 2n = 10II+X+y, with asynaptic sex chromosomes (Fig. 2E). Anaphase I demonstrated correct segregation of the sex chromosomes and formation of haploid n = 10+X and n = 10+y cells (Fig. 2F).

Regarding 18S and 5S rDNA probes, in metaphase I, this species presents 1 pair of autosomes carrying a cluster of each multigene family (Fig. 3G, H), and the merged image shows that they are syntenic and colocalize in this pair (Fig. 3I). FISH with telomeric probes in metaphases I of *A. scissa* demonstrated hybridizations in all chromosomes (Fig. 4H–J). The X chromosome presents 2 proximal interstitial signals (Fig. 4I).

Molecular Analyses

The partial *COI* sequences of the 3 species had approximately 500 bp and were deposited in GenBank (MN506227 to MN506234). In the phylogenetic tree, *A. pantina*, *A. areata*, and *A. scissa* are a sister group of *O. octoguttata*, the nearest external group (probability of

Table 1. Estimates of evolutionary divergence over sequences pairs and among groups in the *Alagoasa* species

		A. pantina			*A. areata*			*A. scissa*	
		3860	3960	3972	3959	4010	4011	3790	4003
A. pantina	3860	–			**0.1040 (0.0147)**			**0.0872 (0.0136)**	
	3960	0.0000 (0.0000)	–						
	3972	0.0020 (0.0019)	0.0020 (0.0019)	–					
A. areata	3959	0.1040 (0.0151)	0.1040 (0.0151)	0.1063 (0.0153)	–			**0.0904 (0.0134)**	
	4010	0.1016 (0.0148)	0.1016 (0.0148)	0.1040 (0.0151)	0.0020 (0.0020)	–			
	4011	0.1040 (0.0151)	0.1040 (0.0151)	0.1063 (0.0153)	0.0000 (0.0000)	0.0020 (0.0020)	–		
A. scissa	3790	0.0856 (0.0132)	0.0856 (0.0132)	0.0833 (0.0130)	0.0900 (0.0139)	0.0877 (0.0137)	0.0900 (0.0139)	–	
	4003	0.0903 (0.0134)	0.0903 (0.0134)	0.0880 (0.0132)	0.0923 (0.0140)	0.0900 (0.0138)	0.0923 (0.0140)	0.0040 (0.0027)	–
Intraspecific distance		0.0013 (0.0013)			0.0013 (0.0013)			0.0040 (0.0027)	

The numbers of base substitutions per site from averaging over all sequence pairs among groups are shown. Analyses were conducted using the Kimura 2-parameter model. The analysis involved 8 nucleotide sequences. Codon positions included were 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated. There were a total of 497 positions in the final dataset. Bold values represent the distance among groups. Standard errors are given in parentheses.

0.99). The 3 species are supported as clades in the analysis (probability of branches of 0.69). The first clade includes the individuals belonging to *A. pantina* and represents a sister group of the specimens belonging to *A. areata*. Samples of *A. scissa* were grouped in a third clade and are a sister group of *A. pantina* and *A. areata* (Fig. 5). The pairwise distance among the obtained sequences ranged from 0 to 0.1063. The intraspecific distance varied from 0.13 to 0.40% in the analyzed species, and the interspecific distance from 8.72 to 10.40% (Table 1).

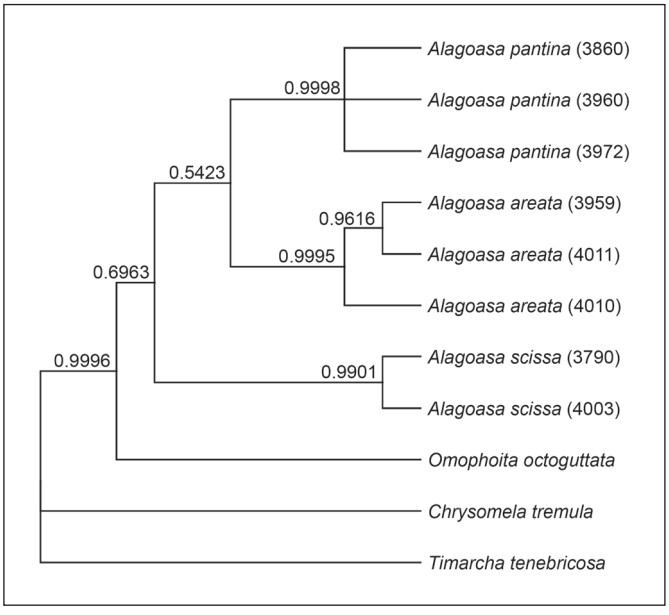

Fig. 5. Tree of phylogenetic relationships constructed with the partial sequence of the *COI* gene using the Bayesian method (numbers on the branches are posterior probability).

Discussion

The subfamily Alticinae is an interesting group for cytogenetic and evolution studies, since species in this group share and maintain many cytogenetic characteristics, while others show derivations in chromosome number and behavior and size of the sex chromosomes in relation to most species of Coleoptera [Almeida et al., 2010]. Another aspect concerns the taxonomy of the species and the group. Many species including the genera *Alagoasa* and *Omophoita* have been addressed in previous studies, without analyses or interpretation of the male or female

genitalia, a main character for the identification of the species. This lack of information hampers the interpretation and construction of a phylogenetic relation of the group. The use of molecular markers and cytogenetics could clarify these relationships.

According to Begosi and Benson [1988], the very similar coloration of several Oedionychina, mainly from the genera *Omophoita* and *Alagoasa* suggests that these may be mimetic. The species *O. octoguttata*, *Alagoasa* cf. *pantina*, *A. scissa*, and *A. areata* have 3 or 4 pairs of large cream-colored elytral spots bordered by black, a pale orange pronotum, and are sympatric [Begosi and Benson, 1988]. The specimens collected for this work occur in sympatry with other species of *Alagoasa* and *Omophoita,* including *O. octoguttata*, reinforcing the case of mimicry between these species.

The species analyzed here present a diploid chromosome number of 22 with giant and asynaptic X+y sex chromosomes, conserved characteristics in the genera of the subtribe Oedionychina [Virkki, 1968, 1970, 1988; Smith and Virkki, 1978; Virkki et al., 1991]. Changes in the chromosome number in the genus *Alagoasa* were observed in only 5 species and correspond to the presence of supernumerary chromosomes or centric fusions [Smith and Virkki, 1978; Petitpierre et al., 1988; Virkki, 1988; Virkki and Santiago-Blay, 1993].

In many genera of Alticinae, karyotype characteristics are very similar [Smith and Virkki, 1978; Segarra and Petitpierre, 1985, 1988; Petitpierre et al., 1988; Petitpierre, 2006], which makes it difficult to differentiate between these by conventional cytogenetic techniques. With the exception of *A. pantina*, which presents a pair of acrocentric chromosomes, the morphology of the autosomes is metacentric or submetacentric in the analyzed species. However, the morphology of the sex chromosomes differs between these: in *A. pantina*, the X is metacentric and the y submetacentric; in *A.* areata, X and y are submetacentric; and in *A. scissa*, X and y are metacentric. The occurrence of submetacentric chromosomes in the species is associated with pericentric inversions, common in the chromosomal evolution of Coleoptera [Virkki, 1984], and thus explains the alteration of the condition of metacentric chromosomes proposed as ancestral for Chrysomelidae by Petitpierre et al. [1988] and also for the order Coleoptera in general [Smith and Virkki, 1978]. *O. octoguttata,* the fourth species in the mimetic complex proposed by Begosi and Benson [1988], differs from the species analyzed here by the presence of 10 pairs of acrocentric chromosomes and metacentric X+y sex chromosomes [Almeida et al., 2006].

The use of 18S and 5S rDNA probes did not show differences among the species; all of them presented a cluster associated with 1 autosomal pair for each multigene family. In other species, these markers showed differences [Almeida et al., 2010; Goll et al., 2018]. However, *A. areata* presented heteromorphism in the cistron size between the homologous chromosomes of the pair. Mechanisms such as unequal crossing-over, transpositions or other rearrangements, including deletions and/or duplications, involve segments of homologous chromosomes and often attribute to structural modifications of nucleolus organizer regions (NOR) [Galetti et al., 1995].

The presence of a single pair of autosomes carrying 18S and 5S rDNA, observed in the analyzed species, is considered an ancestral condition for Coleoptera [Schneider et al., 2007; Cabral-de-Mello et al., 2010, 2011a]. But the dispersion of these genes is not uncommon in Alticinae, as observed in the genus *Omophoita*, and indicates derivations of the karyotypes [Almeida et al., 2010; Goll et al., 2018]. This work is the first report localizing 18S and 5S rDNA and telomeric probes in the genus *Alagoasa*. However, in contrast to *Omophoita*, which is considered to be the sister group of *Alagoasa*, the analyzed species did not present variations in the number of rDNA sites. Cytogenetic studies in *Alagoasa* species are restricted to the description of the diploid number, morphology of chromosomes, and meiotic formulae.

The species analyzed presented synteny and colocalization of the 18S and 5S genes. Such synteny and colocalization have already been described in Scarabaeidae [Cabral-de-Mello et al., 2011b; Oliveira et al., 2012], Tenebrionidae [Goll et al., 2015], and Chrysomelidae [Goll et al., 2018]. The reason for this association is not clear yet [Goll et al., 2018] but may play a role in regulating the expression of multigene families and, especially, in the rapid organization of the nucleolus.

Telomeric hybridization signals were observed in all 3 species, indicating the occurrence of $(TTAGG)_n$ sequences in the genus *Alagoasa*, as observed in *Omophoita* [Goll et al., 2018]. *A. pantina* showed telomeric signals in only 5 autosomal pairs and in the y chromosome. FISH with telomeric probes was repeated 3 times in *A. pantina*, including different specimens and probes, and in all experiments, the same results were obtained. The absence of telomeric signals on the other chromosomes must rather be related to technical problems of the procedure than to the lack of the telomeric sequences, or its replacement by others. The most likely possibility is that the telomeric sites are small and thus were not detected by this technique.

A. areata and *A. scissa* demonstrated telomeric signals in all chromosomes, but differed by the occurrence of intrachromosomal telomeric sequences (ITSs) in the sex chromosomes. In the first species, signals were scattered along the short arm of the y chromosome and the X displayed a proximal signal. In the second species, 2 proximal interstitial signals were present in the X chromosome. The ITSs observed may represent remnants of past chromosomal fusion events. Smith and Virkki [1978] considered the diploid number of 2n = 24 as ancestral for Chrysomelidae. Thus, the reduction in the diploid number to 22 is probably due to a fusion event between an autosome and the X chromosome. *A. pantina* must have lost this ITS, as well as the species of the genus *Omophoita* analyzed by Goll et al. [2018]. However, the analysis of more species of the subfamily Alticinae is necessary to corroborate this hypothesis.

On the other hand, the telomeric sequences scattered along the short arm of the y chromosome of *A. areata*, the second signal on the X chromosome of *A. scissa*, as well as the marks mentioned above, may have originated by the insertion of telomeric sequences during the repair mechanism of DNA double-strand breaks associated with fragile DNA sites, as proposed by Ruiz-Herrera et al. [2005, 2008]. The repetitive fractions of DNA can often act as hotspots for the occurrence of these breaks, promoting chromosomal rearrangements [Kidwell and Lisch, 2000, 2001; Kidwell, 2002]. According to Mello et al. [2014] and Rosolen et al. [2018], in *Omophoita*, a large proportion of the sex chromosomes is composed of repetitive DNAs and/or transposable elements. Additionally, the loss of homology between the sex chromosomes could be due to differentiation processes caused by transposable elements. Rosolen et al. [2018] showed the occurrence of transposons on sex chromosomes and inferred that they contributed to their length increase and differentiation. Alternatively, the invasion of these transposable elements could be responsible for transferring sequences of the telomeres to the interior of chromosomes.

Sequence analysis of the *COI* gene can help in the initial identification of species [Hebert et al., 2003]. Despite the low number of sequences analyzed in this work, the phylogenetic relationship demonstrates separation of these in the 3 species, since the individuals belonging to each species were grouped in different clades. The same topology of the Bayesian inference tree was observed using the Maximum Likelihood (bootstrap = 85%), Maximum Parsimony (bootstrap = 79%), and Neighbor Joining (bootstrap = 79%) methods (data not shown). In addition, the observed values of interspecific divergence are 10× greater than those found in intraspecific divergence, which, according to Hebert et al. [2004] would separate the species under study. A threshold of 3% divergence between groups was initially proposed for separation of insect species by Hebert et al. [2003]. This value was successfully used to discriminate species of well-known beetle fauna [Raupach et al., 2010; Astrin et al., 2012] and was also confirmed by Papadopoulou et al. [2013]. Thormann et al. [2016] tested different molecular methods to explore the leaf beetle fauna from Ecuador, including Alticinae, and they showed that the threshold value of 3% identified a greater number of species, or molecular operational taxonomic units (MOTUs), than the previously identified morphospecies. For Alticinae, Thormann et al. [2016] identified 37 morphospecies, but molecular analysis revealed 47 MOTUs. Our results demonstrate values >3% for the distance between the analyzed species: *A. pantina* and *A. areata* presented 10.4% of distance; *A. pantina* and *A. scissa* presented 8.7%; and *A. areata* and *A. scissa* showed a distance of 9%.

Numerous taxonomy problems in the subfamily Alticinae and a limited number of taxonomists make an identification and classification of species in this group difficult. Therefore, cytogenetics and molecular biology can help in these processes. Although there are no differences in the diploid number in the species analyzed in this work, as well as in the number of rDNA sites, there are differences in the morphology of autosomes and sex chromosomes, in the presence of ITSs in the sex chromosomes, and in the analysis of the fragment of the *COI* gene. These data indicate the occurrence of 3 different species and, adding to the data of Almeida et al. [2006], the 4 species considered mimetic by Begosi and Benson [1988] can be differentiated.

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Statement of Ethics

The collection of the individuals was authorized by the Chico Mendes Institute for Biodiversity Conservation (ICMBio) through license number 15402.

Disclosure Statement

The authors have no conflicts of interest to declare.

Author Contributions

All authors made substantial contributions to this work and approved the final manuscript.

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