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ORIGINAL ARTICLE

Comparative cytogenetic analysis in 13 tortoise beetles (Coleoptera: Chrysomelidae: Cassidinae) from Brazil

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Key words. Coleoptera, Chrysomelidae, C-band, chromosome, karyotype, meiosis, sex chromosome system, constitutive heterochromatin

Abstract. In the present work, we have characterized the chromosomes of 13 Cassidinae beetles, belonging to four tribes, the broad aim being to increase the cytogenetic data and establish the mechanisms involved in chromosome evolution of this subfamily, which appear to be conserved karyotypically, i.e. 2n = 16 + Xy_p. The analysis of mitotic and meiotic cells revealed a high diversity of diploid numbers (2n = 18, 2n = 22, 2n = 26, 2n = 32, 2n = 36, 2n = 40, 2n = 42), and the presence of sex chromosome system of the Xy_p type in most species, with the exception of two representatives that exhibited Xy_r and XY systems. C-banding showed constitutive heterochromatin predominantly localized in the pericentromeric region of the chromosomes, but differences regarding the number of chromosomes with positive C-bands, intensity of the blocks, and presence of additional bands in autosomes and/or sex chromosomes were observed among the species investigated. Our data revealed that the karyotype 2n = 16 + Xy_p does not occur in all 13 tribes of the Cassidinae characterized cytogenetically, seeming to be only a shared feature among the species of the Cassidini. Variations in the C-band pattern, mainly in closely related species, suggest that the interspecific karyotype diversification occurred as a result of changes in the quantity and distribution of constitutive heterochromatin. The occurrence of the Xy_p sex chromosome system in the tribe Mesomphaliini, which showed the highest diversity of simple and multiple systems among the coleopteran as a whole, reinforces the view that derived systems originated by chromosome rearrangements involving the Xy_p ancestral system.

INTRODUCTION

The family Chrysomelidae represents the second largest beetle family of the suborder Polyphaga, including around 37,000 species grouped into 19 subfamilies (Reid, 1995; Chaboo, 2007). Tortoise beetles are members of one these subfamilies, the Cassidinae (Borowiec & Swietojanska, 2015), which is the second most numerous clade after Galerucinae, comprising approximately 6,000 species distributed into 43 tribes around the world (Chaboo, 2007). Many researchers have changed the taxonomic status of the cassidines. They have been ranked as tribes, subfamilies, families, and even in a superfamily (Stephens, 1829; Westwood, 1920; Chen, 1964, 1973; Seeno & Wilcox, 1982; Suzuki, 1988). Lastly, taking into account morphological characters, Chaboo (2007) proposed that the subfamilies Cassidinae sensu stricto (s. str.) and Hispinae s. str. constituted a monophyletic group and incorporated these two clades in a single subfamily, the Cassidinae sensu lato. This finding was corroborated by a phylogenetic study using molecular data (Gómez-Zurita et al., 2008).

Cytogenetically, only 117 Cassidinae species belonging to 13 tribes have been investigated to date (for revision see De Julio et al., 2010). The tortoise beetles are considered as a group with conserved karyotype, in which approximately 52% of the analyzed species showed 2n = 16 + Xy_p, with metacentric and/or submetacentric chromosomes (De Julio et al., 2010). However, cytogenetic records also demonstrated that the diploid numbers can range from 2n = 16 to 2n = 51, as well the sex chromosome systems, which can be either simple – Xy_p, X0, Xy, Xy_c, Xy_r, neoXY, or multiple – Xyy_p, $X_p$neoX neoY_p, $X_p$neoX neoy_p, neo$X_p$neoyy_p, neoX_{p1}neoX_{p2}neoX neoy_p, neoX_{p1}neoX_{p2}neoX neoy, $X_{p1}X_{p2}$-neoX neoy (for review see De Julio et al., 2010). These variations in the diploid number and sex chromosome systems are more or less frequent according to the tribe considered. In Cassidini and Mesomphaliini, 44% and 100%

of the species, respectively, exhibited chromosome number higher than 2n = 18, but in the Cassidini, the sex chromosome system of the Xy_p type was found to be conserved in 96% of the species. Contrastingly, in the Mesomphaliini a differentiation of the sex chromosome system occurs, giving rise to multiple X and/or Y chromosomes. In the tribe Hispini, all nine analyzed species analyzed to date exhibit a reduction in the diploid number and the maintenance of the Xy_p sex chromosome system (Saha & Manna, 1971; Saha, 1973; Kacker, 1976; Sharma & Sood, 1978; Sood, 1978; Alegre & Petitpierre, 1984; Petitpierre, 1988).

In Cassidinae, there are no data regarding the identification of specific chromosomal sites, such as the constitutive heterochromatin. This chromosome region comprises multiple copies of repetitive DNA sequences, which are commonly transcriptionally inactive and have importance to chromosome structure and evolution. The heterochromatin can be localized in any part of the chromosome, but is often found in the centromeric region (Charlesworth et al., 1994). The simplest technique to visualize the chromosomal distribution of the constitutive heterochromatin is the use of C-banding, which provides data about the location, amount and variations that result from chromosome rearrangements. In Chrysomelidae beetles, differences in the C-band pattern are known from species with similar karyotype features, such as the genus *Omophoita* (Mello et al., 2014) as well as between individuals of the same species, as observed in one specimen of *Diabrotica speciosa* (Schneider et al., 2002). Therefore, this specific chromosome region has been useful as a cytogenetic marker in studies on chromosome evolution (Sumner, 1972, 1992; Charlesworth et al., 1994; Mello et al., 2014).

In the Chrysomelidae, the constitutive heterochromatin occurs predominantly in the pericentromeric region (Almeida et al., 2006, 2009; Mello et al., 2014). This same pattern was also observed in the autosomal chromosomes of Coleoptera (Virkki, 1983, Schneider et al., 2006; Mello et al., 2014). In the sex chromosomes, the heterochromatin can extend to all chromosome arms or is restricted exclusively in the pericentromeric region (Almeida et al., 2000; Rozek et al., 2004).

In the present study, in order to increase cytogenetic information on the the subfamily Cassidinae (Cassidini, Goniocheniini, Ischyrosonychini and Mesomphaliini) and enhance understanding of the mechanisms involved in the chromosomal evolution of these groups, 13 species, belonging to four tribes, were analyzed. Specifically, the diploid number, chromosomal morphology, sex chromosome system, behaviour of the chromosomes during meiosis, and the distribution of constitutive heterochromatin were determined for seven species of Cassidini, one of Goniocheniini, one of Ischyrosonychini, and four of Mesomphaliini. This is the first cytogenetic description of species belonging to the genera *Microctenochira* (Cassidini) and to the tribe Ischyrosonychini.

MATERIALS AND METHODS

A sample of 52 individuals, belonging to 13 species of the subfamily Cassidinae, collected in the states of Paraná, São Paulo and Rio Grande do Sul, Brazil, were analyzed in this work (Table

Table 1. Cassidinae species analyzed in this study, including the number of individuals and the collection locality in the Brazilian states. PR – Paraná; RS – Rio Grande do Sul; SP – São Paulo.

Species	Number of specimens	Collection locality
Cassidini		
Agroiconota inedita (Boheman, 1855)	1 male	Piracicaba
	1 male	Saltinho
Charidotella immaculata (Oliver, 1790)	8 males	Saltinho
	1 male	Rio Claro
	1 male	Piracicaba
Charidotella sexpunctata (Fabricius, 1781)	1 male	Saltinho
Deloyala cruciata (Linnaeus,1758)	6 males	Saltinho
	1 male	Rio Claro
Microctenochira aciculata (Boheman, 1855)	1 male / 1 female embryo	Rio Claro
Microctenochira optata (Boheman, 1855)	1 male	Saltinho
	1 male	Rio Claro
Microctenochira quadrata (Degeer, 1775)	1 male	Saltinho
	1 male	Rio Claro
Goniocheniini		
Chlamydocassis cribripennis (Boheman, 1850)	1 male	Nonoai
Ischyrosonychini		
Cistudinella obducta (Boheman, 1854)	1 male	Nonoai
Mesomphaliini		
Botanochara tesselata (Burmeister, 1870)	1 male	Saltinho
Chelymorpha cribraria (Fabricius, 1775)	1 male	Ponta Grossa
	5 males	Rio Claro
	2 males	Saltinho
Paraselenis flava (Linnaeus, 1758)	13 males	Saltinho
Stolas redtenbacheri (Boheman, 1850)	1 male	Rio Claro

Geographical coordinates: SP; Piracicaba (22°50´S, 47°38'W), SP; Rio Claro (22°24´S, 47°3´W), SP; Saltinho (22°50´S, 47°40´W), SP; Nonoai (27°21´S, 52°45´W), RS; Ponta Grossa (25°5´S, 50°9´W), PR.

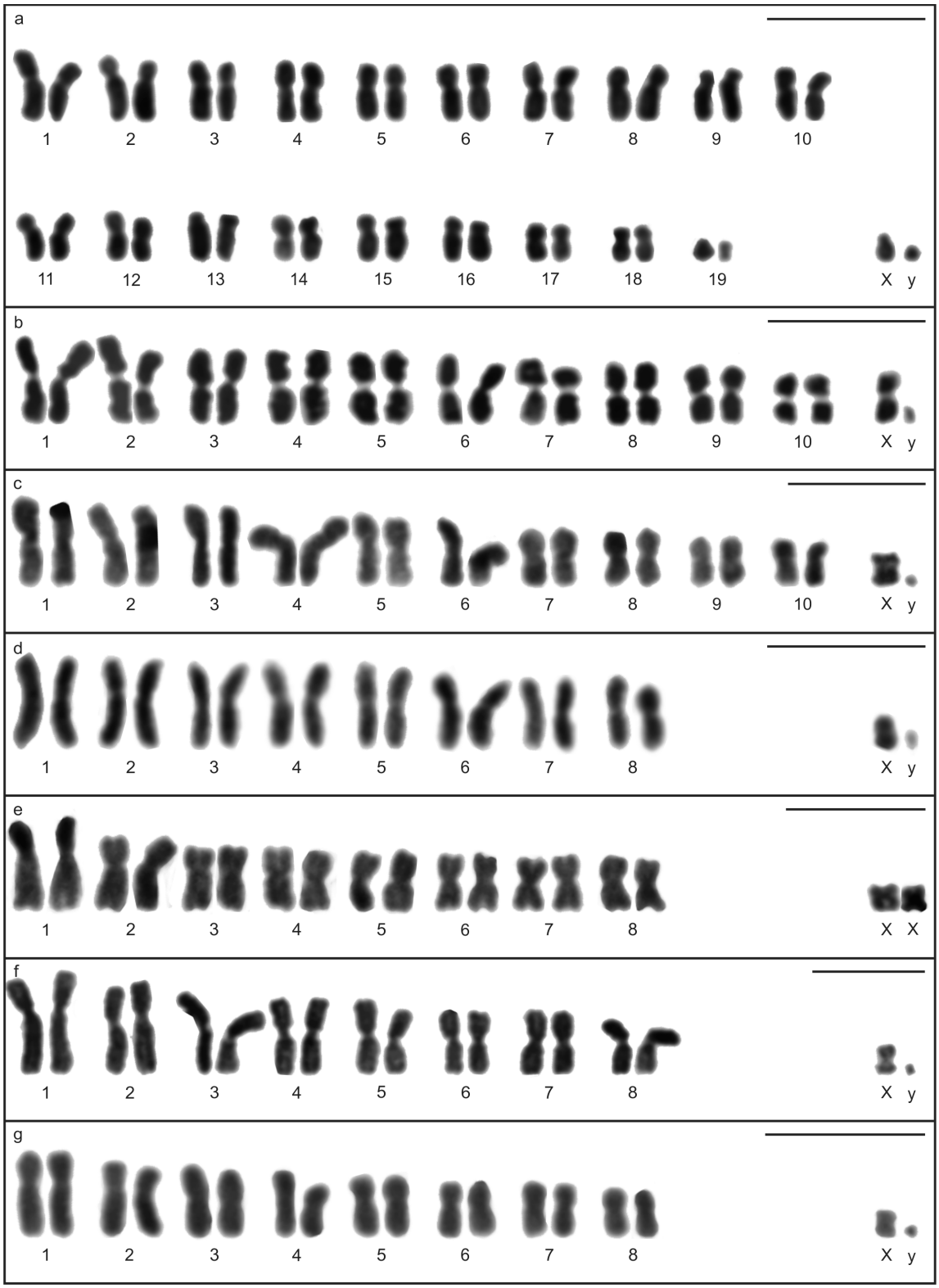


Fig. 1. Karyotypes of seven Cassidini species stained with Giemsa. a – *Agroiconota inedita* with 2n ♂ = 38 + Xy_p; b–c – *Charidotella immaculata* and *Charidotella sexpunctata*, respectively, with 2n ♂ = 20 + Xy_p; d – *Deloyala cruciata*, 2n ♂ = 16 + Xy_p; e – *Microctenochira aciculata*, 2n ♀ = 16 + XX; f–g – *Microctenochira optata* and *Microctenochira quadrata*, respectively, with 2n ♂ = 16 + Xy_p. Scale bar = 10 µm.

1). The voucher specimens used were thereafter deposited in the entomological collection of the Museu Paraense Emilio Goeldi (MPEG – curator Orlando Tobias Silveira), Belém, state of Pará, Brazil.

Chromosomal preparations were obtained from embryos and testes of adult individuals, according to the technique described by Schneider et al. (2007), with modifications in the time of hypotonization to 15 min and increase in the concentration of the acetic acid solution to 60%. The slides were stained with 3% Giemsa solution (3% commercial Giemsa and 3% phosphate buffer pH 6.8 in distilled water). Constitutive heterochromatin was detected by C-banding technique (Sumner, 1972). To obtain a better

resolution of the C-banding pattern, chromosomes were stained with the 4', 6-diamidino-2-phenylindole (DAPI) fluorochrome. Chromosomal morphology was determined according to the nomenclature proposed by Levan et al. (1964). The images of cells were captured using an Olympus BX51 light microscope coupled to an Olympus DP71 digital camera with DP Controller software.

RESULTS

The analysis of mitotic cells of seven Cassidini species revealed the diploid numbers: 2n = 40 in *Agroiconota inedita*, 2n = 22 in both species of *Charidotella*, *Cha. immaculata* and *Cha. sexpunctata*, and 2n = 18 in *Deloyala cruciata*, *Microctenochira aciculata*, *M. optata* and *M. quadrata* (Fig. 1) (Table 2). Despite the variation in the diploid number, all species exhibited a sex chromosome system of the Xy_p type. Additionally, the karyotype analysis showed the following chromosomal morphology in Cassidini species: in *A. inedita* the majority of chromosomes were submetacentric, except the pairs 7, 10, 12, 14, 17 and 18, which were metacentric, and the pair 19 and X chromosome, which were subtelocentrics (Fig. 1a). In the two *Charidotella* species studied, the chromosomes comprised metacentrics, with the exception of pair 7 in *Cha. immaculata* (Fig. 1b) and pair 10 in *Cha. sexpunctata* which comprised submetacentrics (Fig. 1c). In *D. cruciata* and in the three *Michroctenochira* species, a predominance of metacentric chromosomes was observed (Fig. 1d–g). However, in these species, some autosomes comprised submetacentrics, such as the pairs 6 and 8 of *D. cruciata*, pairs 1, 2 and 3 of *M. aciculata*, and pairs 1 and 2 of *M. optata* and *M. quadrata* (Table 2). The autosomal chromosomes of the seven Cassidini species gradually decreased in size, with the exception of *A. inedita* (Fig. 1a), in which the last autosomal pair was small, and *M. aciculata* (Fig. 1e), in which the first autosomal pair was large and the other pairs ranged in size from medium to small (Table 2). Regarding to the sex chromosomes, only *Cha. immaculata* exhibited an X chromosome of medium size, located between autosome pairs 9 and 10 (Fig. 1b). In the other species, the sex chromosomes were the smallest elements of the diploid set, whilst the y chromosome was dot-like without defined morphology.

Pachytene testicular cells of the Cassidini species (Fig. 2a) showed autosomal bivalents synapsed along their entire chromosomal length, sex chromosomes highly condensed and with positive heteropycnosis. Diplotene nuclei confirmed the chromosome number previously observed in mitotic cells and revealed the sex chromosomes to be associated in a typical parachute configuration (Fig. 2b–g). Furthermore, most autosomal bivalents exhibited one interstitial or terminal chiasma, except in *D. cruciata*, whereupon the majority of diplotene nuclei had up to five bivalents with two chiasmata (Fig. 2e). Spermatocytes in metaphase II showed chromosomes possessing a predominantly meta/submetacentric morphology and exhibiting haploid sets n = 19 + X or n = 19 + y in *A. inedita*, n = 10 + X or n = 10 + y in *Cha. immaculata* and *Cha. sexpunctata,* and n = 8 + X or n = 8 + y in *D. cruciata*, *M. aciculata*, *M. optata* and *M. quadrata* (Fig. 2h–l).

One species from each tribe of the Goniocheniini and Ischyrosonychini was analyzed in this work. In *Chl. cribripennis* (Goniocheniini), male meiotic cells allowed us to establish the karyotype formula 2n = 30 + Xy_p with meta/submetacentric chromosomes (Fig. 3a–c) (Table 2). In the pachytene nuclei, the autosomal bivalents were totally synapsed and the sex chromosomes appeared as a positive heteropycnotic block (Fig. 3a). In diplotene cells, the occurrence of only one terminal or interstitial chiasma

Table 2. Cytogenetic information of Cassidinae species analyzed in the present work: diploid number in males (2n), sex chromosome system (SCS), chromosome morphology, size of the autosomes and distribution of constitutive heterochromatin.

Species	Karyotype			Constitutive heterochromatin	
	2n and SCS	Chromosomal morphology	Autosomal size	Chromosome	Localization
Cassidini					
Agroiconota inedita	40 = 38 + Xy_p	12M + 24SM + 2ST + XST	Pairs 1–18GD; pair 19S	Pairs 1–18, X and y	P
Charidotella immaculata	22 = 20 + Xy_p	18M + 2SM + XM	GD	Pairs 2, 4 ,6, 7, 8, 9 and 10	P/T/I
Charidotella sexpunctata	22 = 20 + Xy_p	18M + 2SM + XM	GD	Pairs 1, 3, 4, 6 and y	P
Deloyala cruciata	18 = 16 + Xy_p	12M + 4SM + XM	GD	Pairs 1–8, X and y	P
Microctenochira aciculata	18 = 16 + Xy_p	10M + 6SM + XM	Pair 1L; pairs 2–8 GD	Pairs 1, 2, 3, 4, 7 and y	P/T
Microctenochira optata	18 = 16 + Xy_p	12M + 4SM + XM	GD	Pairs 1–8, X and y	P
Microctenochira quadrata	18 = 16 + Xy_p	12M + 4SM + XM	GD	Pairs 1, 2–8, X and y	P/T
Goniocheniini					
Chlamydocassis cribripennis	32 = 30 + Xy_p	26M + 2SM + 2ST	GD	15 bivalents and X	P
Ischyrosonychini					
Cistudinella obducta	18 = 16 + Xy*	12M + 4SM + XM + yM	GD	Pairs 1–8 and X	P
Mesomphaliini					
Botanochara tesselata	36 = 34 + Xy_p	20M + 14SM	Pair 1–3L / 4–7M / 8–17S	17 bivalents, X and y	P
Chelymorpha cribraria	22 = 20 + Xy_p	14M + 6SM + XM	GD	7 bivalents and y	P
Chelymorpha cribraria	22 = 20 + Xy_r	–	–	7 bivalents and y	P
Paraselenis flava	42 = 40 + Xy_p	28M + 12SM + XM	GD	Pair 20	I
Stolas redtenbacheri	26 = 24 + Xy	22M + 2SM + XM	Pairs 1L; pairs 2–12M	Pairs 1–12, X and y	P

M – metacentric; SM – submetacentric; ST – subtelocentric; GD – gradually decreased in size; S – small; L – large; M – medium; P – pericentromeric region; I – interstitial region; T – telomeric region. *Probably Xy_p sex chromosome system.

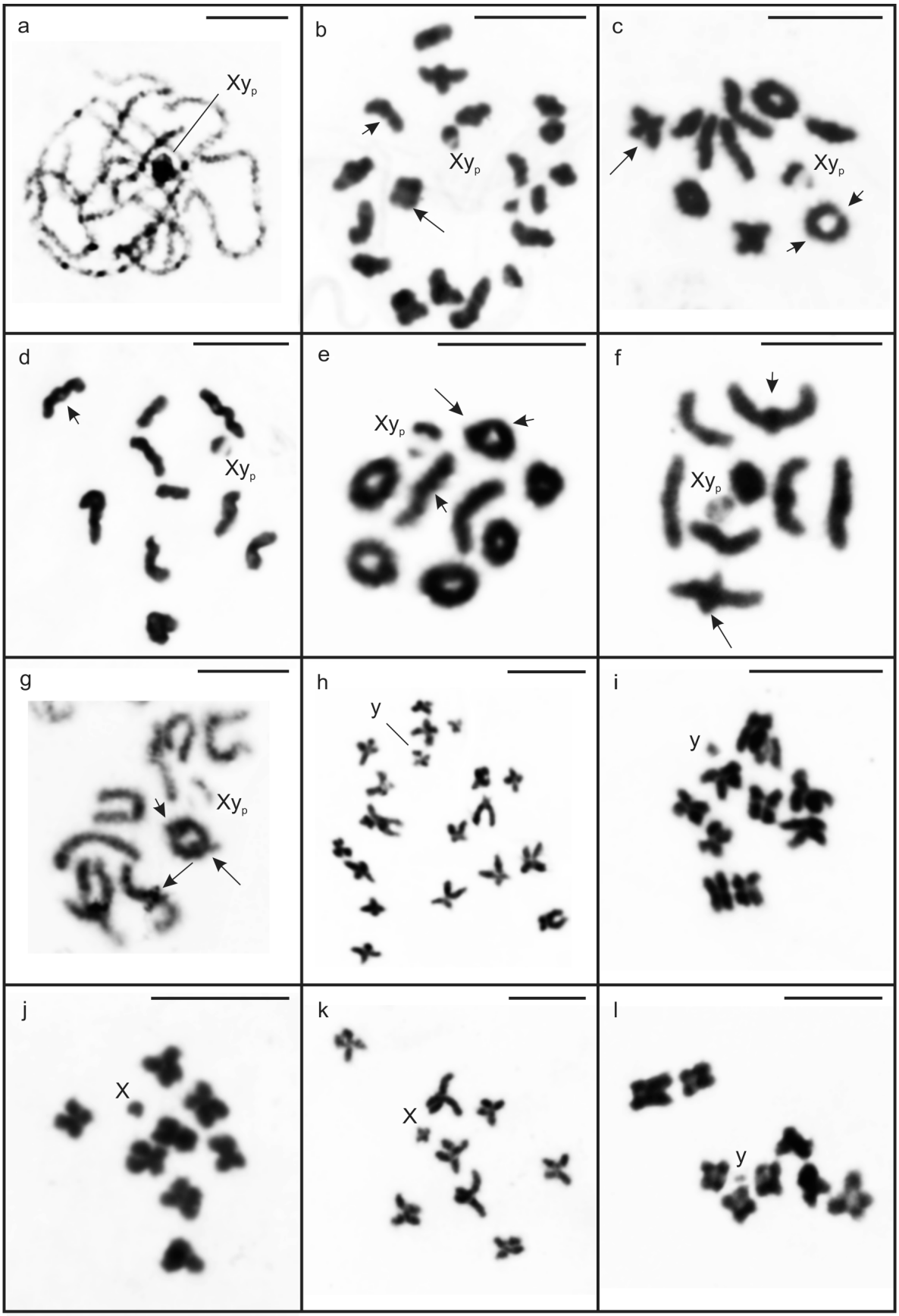


Fig. 2. Testicular cells of Cassidini species after Giemsa staining. a, b, h – *Agroiconota inedita*; c, i – *Charidotella immaculata*; d – *Charidotella sexpunctata*; e, j – *Deloyala cruciata*; f – *Microctenochira aciculata*; g, k – *Microctenochira optata*; l – *Microctenochira quadrata*. a – pachytene; b–g – diplotene nuclei with 2n = 19II + Xy_p (b), 2n = 10II + Xy_p (c, d), 2n = 8II + Xy_p (e, f, g). Large arrow – interstitial chiasma; small arrow – terminal chiasma. h–l – metaphase II cells with n = 19 + y (h), n = 10 + y (i), n = 8 + X (j, k), n = 8 + y (l). Scale bar = 10 µm.

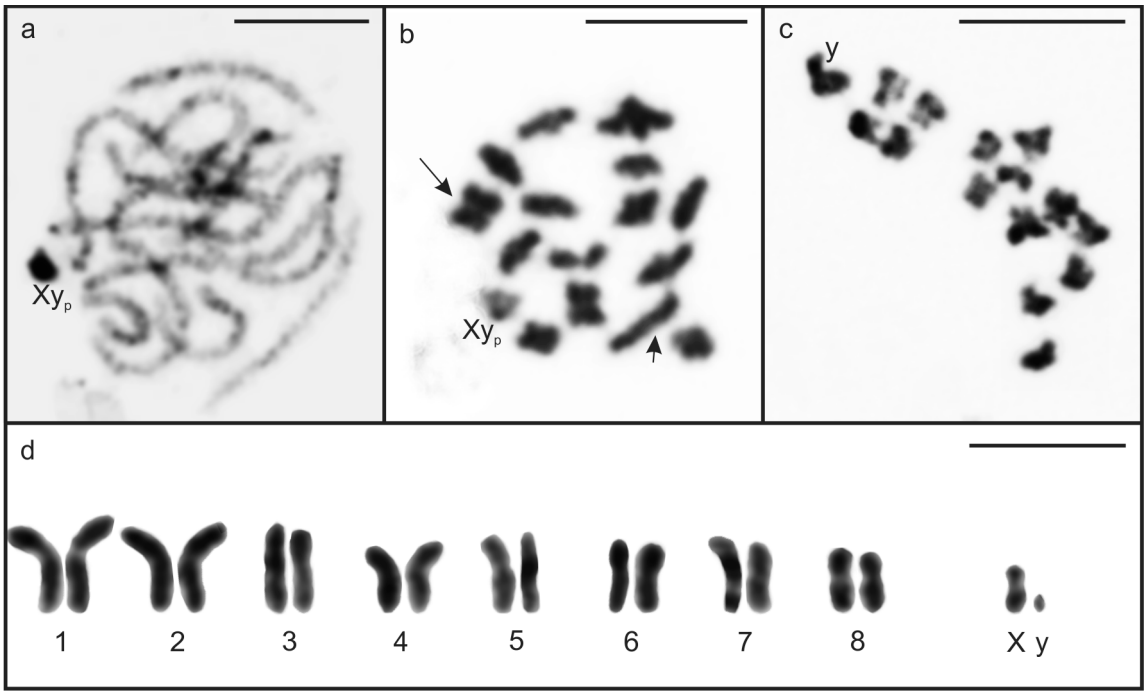


Fig. 3. Testicular cells of *Chlamydocassis cribripeninis* (a–c) and *Cistudinella obducta* (d), stained with Giemsa. a – pachytene; b – diplotene with 2n = 15II + Xy_p. Large arrow – interstitial chiasma; small arrow – terminal chiasma. c – metaphase II with n = 15 + y; d – karyotype with 2n ♂ = 16 + Xy. Scale bar 10 = μm.

was observed in the autosomal bivalents (Fig. 3b). Metaphase II cells confirmed the regular behaviour and reductional segregation of all chromosomes (Fig. 3c). In the chromosomal preparations of *Cistudinella obducta* (Ischyrosonychini), only mitotic metaphase cells were obtained, which exhibited the diploid number 2n = 18 (Fig. 3d). The karyotype analysis showed metacentric (pairs 1, 2, 3, 4, 7 and 8) and submetacentric chromosomes (pairs 5, 6 and X chromosome), which gradually decreased in size (Table 2). The sex chromosomes were the smallest elements of the set, and the y chromosome was easily identified due to its extremely small size.

Among the four Mesomphaliini species here studied, only *Stolas redtenbacheri* exhibited mitotic metaphase cells, in which the diploid number 2n = 26 was observed. All chromosomes presented metacentric morphology, except pair 10 which were submetacentric (Fig. 4a). In relation to size, chromosomes were classified as large (pair 1), medium (pairs 2–12) and small (X and y chromosomes). In the other species, cells in meiosis I showed 2n = 17II + Xy_p in *B. tesselata*, 2n = 10II + Xy_p in *Che. cribraria,* with exception of one representative from the Ponta Grossa population that exhibited 2n = 10II + Xy_r, and 2n = 20II + Xy_p in *Paraselenis flava* (Fig. 4d–g) (Table 2). Pachytene cells revealed sex chromosomes highly condensed and positively heteropycnotic (Fig. 4b–c). In the diplotene nuclei of one representative of *Che. cribraria* from Ponta Grossa, the sex chromosomes were associated in a typical rod configuration, and were classified as Xy_r. In *B. tesselata*, the other *Che. cribraria* specimens and *P. flava*, the sex chromosomes exhibited a parachute association. Additionally, the analysis of diplotene cells showed more than two bivalents with two chiasmata in *B. tesselata*, one bivalent with two chiasmata in *Che. cribraria* and *P. flava* (Fig. 4d, f–g), and bivalents with one chiasma in the specimen of *Che. cribraria* from Ponta Grossa (Fig. 4e). Metaphase II cells of *B. tesselata*, *Che. cribraria* and *P. flava* revealed haploid sets in agreement with the meioformula verified in meiosis I and exhibited meta/submetacentric chromosome morphology (Fig. 4h–j) (Table 2).

Cells of the 13 Cassidinae species investigated were also subject to the C-banding technique and subsequently stained with DAPI. In Cassidini, the chromosome of all species exhibited positive C-bands (Table 2). In *A. inedita*, *D. cruciata* and *M. optata*, heterochromatic blocks were observed in the pericentromeric region of all chromosomes, with the exception of the y chromosome of the three species that were totally heterochromatic (Fig. 5a–b, g–h, k–l). In *Cha. sexpunctata*, the pericentromeric region of pairs 1, 3, 4 and 6 showed constitutive heterochromatin, but in the 6th pair the C-band was tenuous; additionally the y chromosome was totally heterochromatic (Fig. 5e–f). In the three other Cassidini species, the heterochromatic bands were very tenuous. In *Cha. immaculata*, the positive C-bands were localized in the pericentromeric region of pairs 2, 4, 6, 7, 8, terminal region of six autosomal pairs, and interstitial region of the short arm of pair 9 and long arm of pair 10. The X chromosome showed interstitial C-band in both arms and the y chromosome was totally heterochromatic (Fig. 5c–d). In *M. aciculata*, the constitutive heterochromatin occurred in the pericentromeric regions of pairs 1, 2, 3, 4, 7 and y chromosome; an additional band was also verified in the long arm terminal region of pair 1 (Fig. 5i–j). In *M. quadrata*, the heterochromatic bands were localized in the pericentromeric region of five autosomal pairs and X chromosome, the terminal region of four

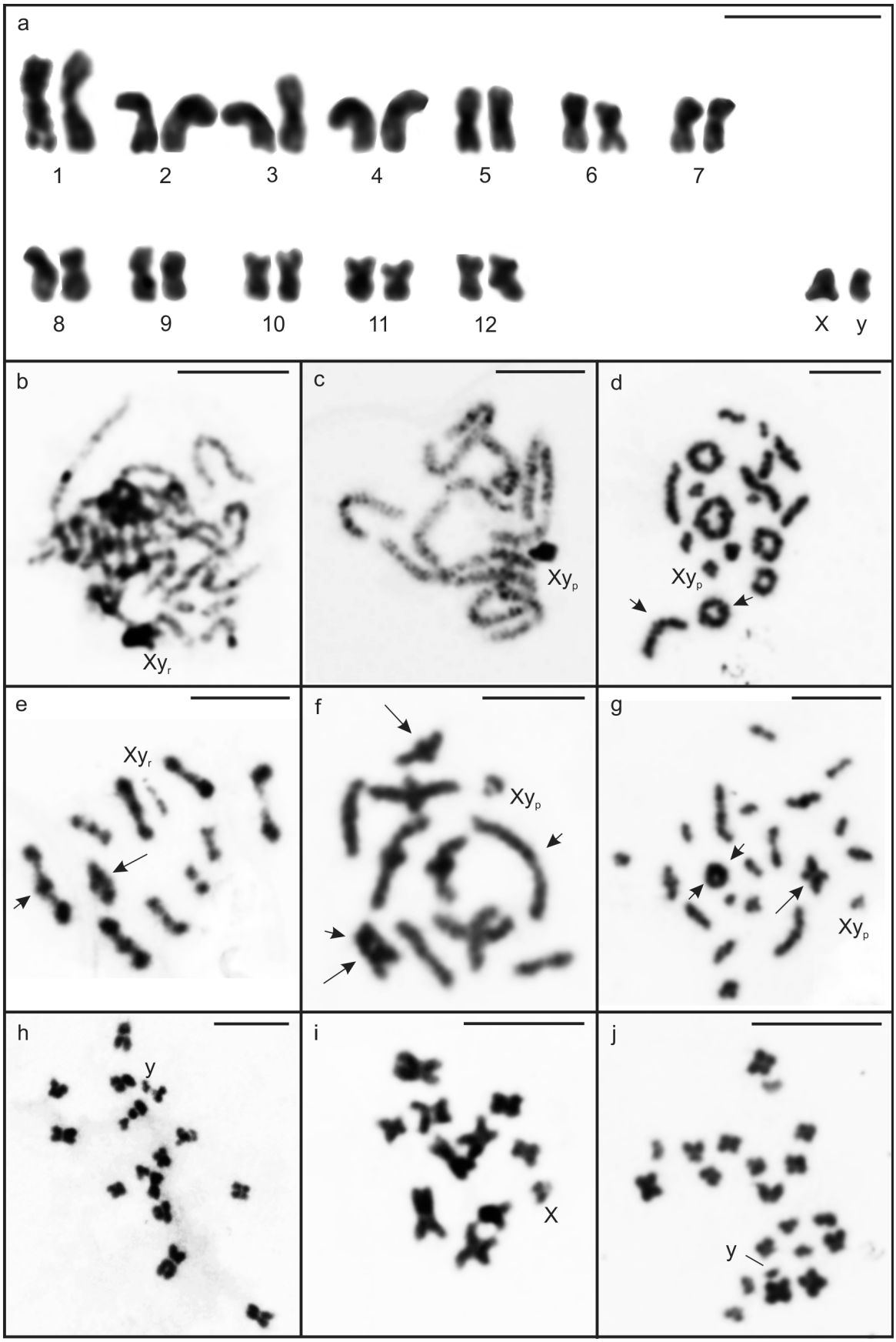
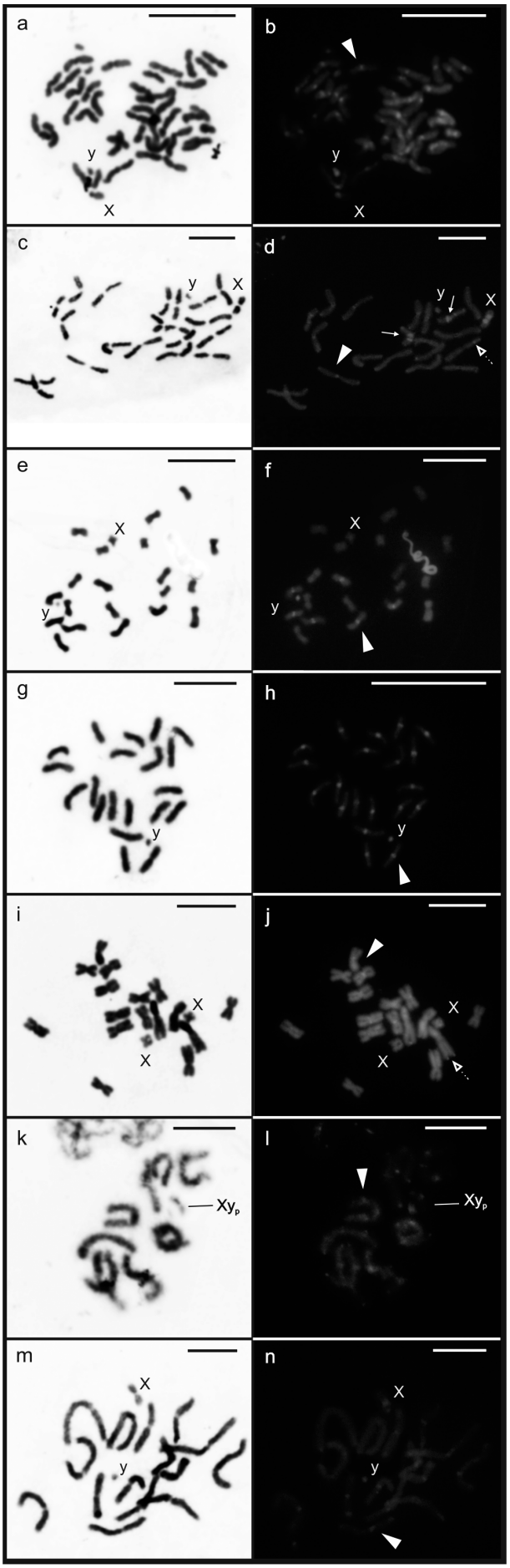


Fig. 4. Karyotype of *Stolas redtenbacheri* (a) and prophase I cells of *Chelymorpha cribraria* (2n = 10II + Xy$_r$) (b, e), *Chelymorpha cribraria* (2n = 10II + Xy$_p$) (c, f, i), *Botanochara tesselata* (d, h), and *Paraselenis flava* (g, j), stained with Giemsa. a – karyotype with 2n ♂ = 24 + Xy; b, c – pachytene; d–g – diplotene nuclei with 2n = 17II + Xy$_p$ (d), 2n = 10II + Xy$_r$ (e), 2n = 10II + Xy$_p$ (f), 2n = 20II + Xy$_p$ (g); h–j – metaphase cells II with n = 17 + y (h), n = 10 + X (i) n = 20 + y (j). Large arrow – interstitial chiasma; small arrow – terminal chiasma. Scale bar = 10 µm.



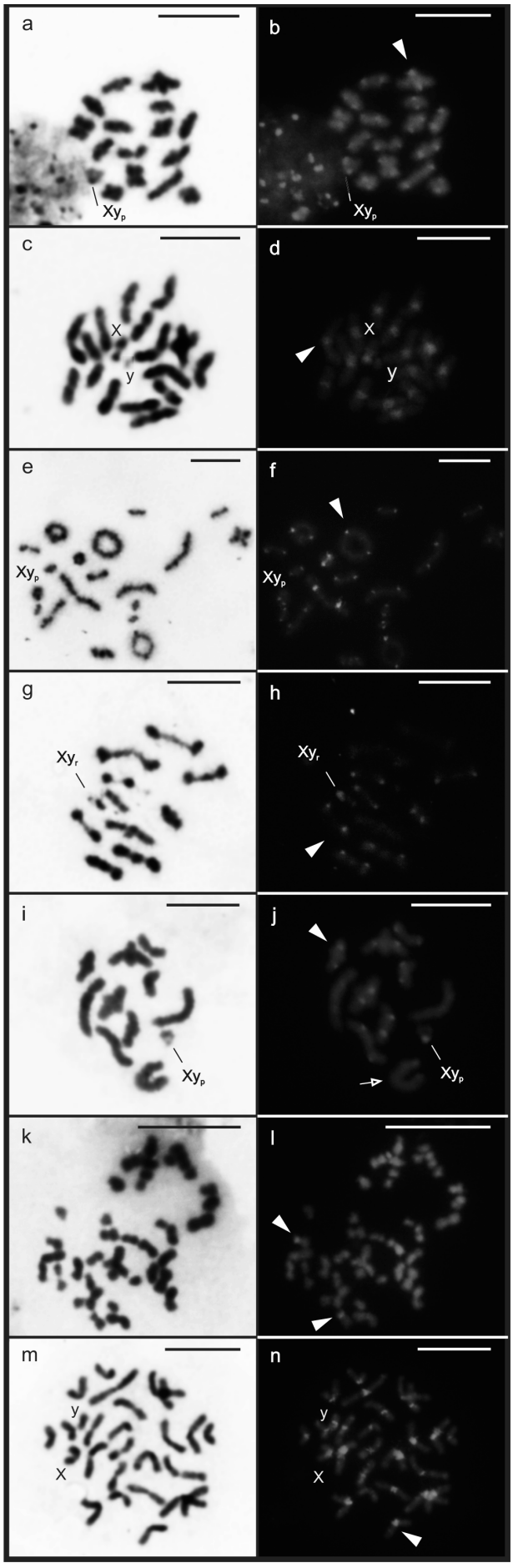
autosomal pairs, whilst the y chromosome was heterochromatic (Fig. 5m–n).

Chlamydocassis cribripennis and *Cistudinella obducta*, which belong to the tribes Goniocheniini and Ischryrosonochini, respectively, showed C-bands in the pericentromeric region of all chromosomes, except the y chromosome of both species, which were totally euchromatic (Fig. 6a–d) (Table 2). Additionally, most chromosomes of Mesomphaliini species, *B. tesselata*, *Che. cribraria* and *S. redtenbacheri*, exhibited constitutive heterochromatin in the pericentromeric region. The C-bands were absent in three autosomal bivalents and X chromosome of *Che. cribraria* (Fig. 6i–j). Furthermore, the y chromosome of all specimens of *Che. cribraria* was totally heterochromatic. In *P. flava*, the constitutive heterochromatin was localized only in the short arm of the last autosomal pair (Fig. 6k–l).

DISCUSSION

Within the family Chrysomelidae, the subfamily Cassidinae is known from earlier studies to display a conserved karyotype; however, among the nine species herein analyzed for the first time, five exhibited karyotype formula different from the expected type, namely 2n = 16 + Xy_p. The tribe Cassidini, considered a monophyletic group by Chaboo (2007), includes 16% of the diversity of species of the subfamily (around 1,000 species), but only 55 species, belonging to 16 genera, have so far been studied cytogenetically (De Julio et al., 2010). Although this number is small when compared to the total of known species, Cassidini is the tribe with the most cytogenetic information for the entire subfamily. Among these species, 36 exhibited the diploid number 2n = 18 and the sex chromosome system of the Xy_p type (present work; De Julio et al., 2010). Additionally, approximately 60% of the studied species are included in the genus *Cassida*; however, the karyotype 2n = 16 + Xy_p also occurs in 12 other genera, including *Microctenochira* (present work). This result reveals that these karyotype characteristics are shared among the species of Cassidini and may correspond to an ancestral condition for the tribe. Nevertheless, this idea will be only confirmed after a phylogenetic reconstruction of the Cassidini. The karyotype changes, when they occur, are related to the increase in diploid number, as observed in 18 species of eight distinct genera. Among these genera, three had only one species investigated (*Chirida*, *Hypocassida* and *Oocassida*), and among the other species, only *Agroiconota* and *Charidotella* presented a karyotype with a diploid number greater than 2n = 18 for all species (present work; reviewed in De Julio et al., 2010).

Fig. 5. Distribution of constitutive heterochromatin in mitotic and meiotic cells of seven Cassidini species, submitted to the Giemsa staining (a, c, e, g, i, k, m) and C-banding technique (b, d, f, h, i, l, n). a, b – *Agroiconota inedita*; c, d – *Charidotella immaculata*; e, f – *Charidotella sexpunctata*; g, h – *Deloyala cruciata*; i, j – *Microctenochira aciculata*; k, l – *Microctenochira optata*; m, n – *Microctenochira quadrata*. Arrowhead – pericentromeric C-band; small arrow – interstitial C-band; dotted arrow – terminal C-band. Scale bar = 10 μm.



The tribe Goniocheniini, with five genera and 30 taxonomically recognized species, has not yet been tested to confirm its supposed monophyly (Chaboo, 2007). The available results for two species of this tribe, *Chlamydocassis metallica* (Vidal, 1984) and *Chl. cribripennis*, revealed similarities in the diploid number 2n = 32 and Xy_p sex chromosome system. However, the investigation of representatives of other genera not yet analyzed is of the utmost importance in order to verify whether the high diploid number is a characteristic of this tribe, or rather, is restricted to representatives of the genus *Chlamydocassis*.

The tribe Ischyrosonychini, with seven genera and 68 species (Borowiec & Swietojanska, 2015), has yet to be cytogenetically studied. Data found for *Cis. obducta* in this study revealed karyotype characteristics similar to the majority of Cassidini species and to other related tribes which were also considered by Chaboo (2007) to be monophyletic.

Mesomphaliini is the third largest tribe of Cassidinae in relation to diversity, including 25 genera distributed in 526 species. Nevertheless, Chaboo (2007) did not recover the monophyly of this tribe. This result seems to be consistent with the available chromosomal information, with Mesomphaliini having the highest karyotype heterogeneity among the Cassidinae beetles (present work; De Julio et al., 2010). This heterogeneity includes different sex chromosome systems, as observed in this work in one representative of *Che. cribraria* from a population of Ponta Grossa, state of Paraná, Brazil, that exhibited the Xy_r system and was hence different from that observed in the specimens from Rio Claro and Saltinho, i.e Xy_p. This difference was also observed in the genus *Botanochara*, where the systems can vary even within a single species, e.g. *B. bonariensis* with $X_pneoXneoy_p$, $neoX_pneoyy_p$, $neoX_{p1}neoXp_2neoXneoY_p$, $X_{p1}X_{p2}neoXneoY$ (Mazzella & Panzera, 1983; Panzera et al., 1983; Postiglioni et al., 1990; Stolar & Bidau, 1997). The simple sex chromosome system of the Xy_p type in *B. tesselata* is described here for the first time for the genus, thereby adding support for the view that the multiple systems originated from the original Xy_p sex chromosome system by centric fission and/or translocation between sex chromosomes and autosomes (Smith & Virkki, 1978).

The Xy_r sex chromosome system observed in one representative of *Che. cribraria* had not previously been recorded for the tribe Mesomphaliini, occurring in only one species of Cassidinae, *Laccoptera* (*Laccopteroidea*) *nepalensis* (tribe Aspidimorphini). In *Che. cribraria* as well *L.* (*L.*) *nepalensis* the Xy_r system occurs as an interpopulational variation of the Xy_p system (Dey, 1986). In

Fig. 6. Distribution of constitutive heterochromatin in testicular cells of Goniocheniini, Ischyrosonochini and Mesomphaliini species, stained with Giemsa (a, c, e, g, i, k, m) and submitted to the C-banding technique (b, d, f, h, i, l, n). a, b – *Chlamydocassis cribripennis*; c, d – *Cistudinella obducta*; e, f – *Botanochara tesselata*; g, h – *Chelymorpha cribraria* 2n = 10II + Xy_r; i, j – *Chelymorpha cribraria* 2n = 10II + Xy_p; k, l – *Paraselenis flava*; m, n – *Stolas redtenbacheri*. Arrowhead – pericentromeric C-band; small arrow – interstitial C-band; dotted arrow – terminal C-band. Scale bar = 10 μm.

Coleoptera as a whole, the Xy_r sex chromosome system is rare, having been observed in less than 2% of the species chromosomally described in the families Carabidae, Buprestidae, Chrysomelidae, Coccinelidae, Hydrophilidae, Lucanidae, Meloidae, Scarabaeidae and Tenebrionidae (Blackmon & Demuth, 2015). The origin of the Xy_r systems seems to be related to processes of heterochromatization and inversions of the sex chromosomes of the neoXY system (Smith, 1952; Smith & Virkki, 1978). Nevertheless, in *Che. cribraria*, the y_r chromosome may have a different origin from the Xy_p system, considering that there are no records available for the neoXY system of the genus, as well as for other species of Mesomphaliini. One hypothesis is that the y_r chromosome originated from the y_p through the adding of heterochromatic material, which in turn led to the increase in size and modification in the association with the X chromosome during meiosis I.

Additionally, the results found in *P. flava* and *S. redtenbacheri* further increase the diversity of karyotypes in Mesomphaliini, once the $2n = 40 + Xy_p$ and $2n = 24 + Xy_p$ not having previously been verified for this tribe. The five species of the tribe Cassidini (*A. inedita*, *Cha. immaculata*, *Cha. sexpunctata*, *D. cruciata*) that had already been cytogenetically studied and are being analyzed in this work, exhibit karyotype characteristics similar to those previously described (Vidal, 1984; Virkki et al., 1992; De Julio et al., 2010). The exception is *A. inedita* which has $2n = 38 + Xy_p$, differing from the $2n = 40 + Xy_p$ recorded by De Julio et al. (2010) for a population from Rio Claro, state of São Paulo, Brazil. Intraspecific variations involving one autosomal pair are not rare among the cassidines, having earlier been recorded for two species of the tribe Aspidimorphini and three of the tribe Cassidini (Nowlin, 1906; Geitler, 1940; Smith, 1960; Yadav, 1973; Yadav & Pillai, 1975; Petitpierre, 1977, 1985, 1988; Gill et al., 1987; Yadav et al., 1987, 1995; Virkki et al., 1992). In *A. inedita*, this variation in diploid number may have come about through chromosomal fissions or fusions, which did not involve the last autosomal pair, which remains small and similar in the population analyzed by De Julio et al. (2010) and in the present work.

The study of meiotic cells in Coleoptera is important in order to determine the type of sex chromosome system, considering that the association of chromosomes at meiosis can vary, thereby allowing, for example, to differentiation of the Xy_p and Xy_r types. Due to the absence of cells in prophase I of *Cis. obducta* (Ischyrosonychini) and *S. redtenbacheri* (Mesomphaliini), the sex chromosome system was classified only as of the Xy type. However, it is probable that these species have different systems, because in *Cis. obducta*, the y chromosome was extremely small, such as found in the Xy_p system, whilst in *S. redtenbacheri*, the y chromosome was small in relation to the other elements of the karyotype, but similar in size to the X. This last result reinforces the great diversity of sex chromosome systems for Mesomphaliini species.

In Cassidinae, the behaviour of the bivalents during meiosis was similar to that observed in most species of Coleoptera (Smith & Virkki, 1978), i.e., autosomal bivalents totally synapsed and with one terminal or interstitial chiasma, and sex chromosomes highly condensed, and with positive heteropycnosis in early prophase I. However, four species, *D. cruciata* (Cassidini), *B. tesselata*, *Che. cribraria* and *P. flava* (Mesomphaliini) exhibited variable number of bivalents with two chiasmata. Bichiasmatic bivalents have already been observed in two species of Cassidinae, belonging to the genus *Chalepus* (Chalepini) and *Octotoma* (Uroplatini), which have distinct diploid numbers, $2n = 16 + Xy_p$ and $2n = 18 + Xy_p$, respectively (Yadav & Pillai, 1974; Virkki et al., 1992). Interestingly, in the cassidines as here examined, differences in the presence of bivalents with one or two chiasmata were observed in species with very similar karyotypes (*D. cruciata* and *M. optata*), indicating that this characteristic can be an additional criteria in discriminating species. Additionally, this result demonstrates that although the macro karyotype structure is similar, the organization of the genome and/or the controlling factors of the genetic recombination can differ among these species. Among the species of Mesomphaliini, bichiasmatic bivalents appeared in species with very distinct diploid numbers, such as *B. tesselata* with $2n = 36$, *Che. cribaria* $2n = 22$, and *P. flava* with $2n = 42$. These data show that, at least among the representatives of this tribe, the presence of bivalents with two chiasmata is no more frequent in species with lower diploid number, whose karyotypes were originated by chromosomal fusions, as suggested by Schneider et al. (2007) for other beetle families (Elateridae, Lampyridae and Melyridae).

The distribution of constitutive heterochromatin has been described for different families and subfamilies of Coleoptera, in which the C-bands are generally located in the pericentromeric region of the autosomal chromosomes (Vidal et al., 1977; Virkki, 1983; Juan & Petitpierre, 1989; Rozek & Lachowska, 2001; Zacaro et al., 2004; Bione et al., 2005; Almeida et al., 2009). The chromosomes of Chrysomelidae also exhibited this pattern (Virkki, 1983; Almeida et al., 2006, 2009; Mello et al., 2014); however, they show a lower amount of heterochromatin compared with other groups, such as Elateridae and Tenebrionidae (Juan & Petitpierre, 1989; Rozek et al., 2004; Schneider et al., 2006).

The present study is the first on the subfamily Cassidinae to reveal the distribution of constitutive heterochromatin. The 13 species analyzed showed similar pattern of pericentromeric bands, but with variations related to the number of autosomes with constitutive heterochromatin, the intensity of the bands, the presence in telomeric and interstitial regions, and the occurrence in the sex chromosomes. These C-bands variations occurred even in species of the same genus and with similar karyotypes, such as *Cha. immaculata* and *Cha. sexpunctata*, *M. optata* and *M. quadrata*. In Cassidinae, changes in the distribution of constitutive heterochromatin may ultimately have been the cause of the observed karyotype differentiation, considering that these regions can act in gene regulation and functioning (Henikoff, 2000). Furthermore, it is possible that species

that show the same C-band pattern, as in *D. cruciata* and *M. optata*, may differ with regard to the molecular constitution of the heterochromatin, such as verified in three *Omophoita* (Alticinae) species and two *Deltochilum* (Scarabaeidae) species (Cabral-de-Mello et al., 2010; Mello et al., 2014).

In conclusion, the data obtained in this work and those available in the literature have revealed that the diploid number 2n = 18 is not conserved in all Cassidinae tribes, occurring only with high frequency in Cassidini. The karyotype uniformity previously reported in some species and/or tribes is challenged by the almost species-specific pattern of constitutive heterochromatin as here found. The Xy_p sex chromosome system is present in most species and could be a shared characteristic among all the tribes, including Mesomphaliini, in which a variety of derived sex chromosomes systems has been observed.

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