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Chromosome evaluation of southern South American species of *Camptosema* and allied genera (Diocleinae – Phaseoleae – Papilionoideae – Leguminosae)

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The genera *Camptosema*, *Galactia* and *Collaea* are grouped in a complex with exomorphological similarities and different criteria have been adopted to circumscribe them. The neotropical genus *Camptosema* in its southern distribution is represented by four species of which only the type, *C. rubicundum,* shows the diagnostic features of the genus. The other three taxa, *C. paraguariense*, *C. praeandinum* and *C. scarlatinum* are related morphologically to *Camptosema s.s.*, *Galactia* and *Collaea*. In the subtribe Diocleinae, *Camptosema* is characterized by $n = 11$ chromosomes and *Galactia* and *Collaea* by $n = 10$. The aim of this study was to analyse cytological characters with special emphasis on the species of uncertain taxonomy. The most relevant character is chromosome number, which in the conflicting species of *Camptosema* is the same as in *Galactia* and *Collaea*. In this paper the chromosome numbers of *C. praeandinum* ($2n = 20$), *C. paraguariense* ($n = 10$) and *C. scarlatinum* ($n = 10$) are reported for the first time. These results, together with the morphological affinity and the phylogenetic hypotheses of other authors, would be of use for revising the current circumscription of these species. *Botanical Journal of the Linnean Society*, 2006, **152**, 235–243.

ADDITIONAL KEYWORDS: Ag-NOR sites – chromosome numbers – FISH – karyotype – rDNA sites.

INTRODUCTION

The genera *Camptosema* Hook. & Arn., *Galactia* P. Browne and *Collaea* DC., as currently recognized, are grouped in a complex with exomorphological similarities. Several criteria have been adopted to delimit them (de Candolle, 1825; Bentham, 1859; Grisebach, 1874; Taubert, 1894; Burkart, 1970, 1971; Lackey, 1981). In recent phylogenetic studies of the subtribe Diocleinae based on morphology, the hypothesis of an artificial generic delimitation was corroborated (de Queiroz, Fortunato & Giulietti, 2003; Maxwell & Taylor, 2003). Most of the genera analysed by these authors, including *Galactia* and *Camptosema,* were found not to be monophyletic. Nevertheless, the taxonomic criteria proposed by Burkart (1970, 1971) are accepted currently. About 20 neotropical species are recognized for the genus *Camptosema.* These are distributed from eastern and central Brazil to north-eastern/eastern Paraguay, north-eastern Argentina and western/south-western Uruguay (Chodat & Hassler, 1904; Burkart, 1952, 1970; Cowan, 1961; Irwin & Arroyo, 1974; Lackey, 1981; Izaguirre & Beyhaut, 1998; de Queiroz *et al*., 2003).

In its southern geographical distribution, *Camptosema* is represented by four species (Burkart, 1970; Fortunato, 1999). *Camptosema rubicundum* Hook. & Arn., the type species, is the only one which has all the diagnostic features characterizing the

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genus. The other three taxa: *C. paraguariense* (Chodat & Hassl.) Hassl., *C. praeandinum* Burkart and *C. scarlatinum* (Mart. ex Benth.) Burkart have a floral morphology related in part to *Camptosema s.s.* and also to *Galactia* and *Collaea* (Lackey, 1981; de Queiroz *et al*., 2003). These morphological similarities, together with the different criteria adopted to circumscribe the genera, have caused taxonomic confusion.

Chromosome numbers have proved to be of great value in revising and improving the classification of the Leguminosae and in understanding evolution in the family (Goldblatt, 1981). In the tribe Phaseoleae x = 11 is the prevailing basic chromosome number. Similarly to most genera in the subtribe Diocleinae, *Camptosema* is characterized by $n = 11$ and the allied genera *Galactia* and *Collaea* by $n = 10$ (Goldblatt, 1981), with the latter originating from x = 11 by descending dysploid changes via structural re-patterning.

There are a few counts reported for *Camptosema* species: $n = 11$ in *C. coriaceum* (Nees & C. Mart.) Benth. (Coleman & Smith, 1969) and *C. tomentosum* Benth. (Turner & Irwin, 1961), also $n = 10$ in *C. tomentosum*, which was said to represent an aneuploid condition in the material studied (Coleman, 1982).

Recently, Sede *et al*. (2003), analysed for the first time the chromosome numbers of *Camptosema rubicundum* ($2n = 22$), *Galactia fiebrigiana* Burkart ($2n = 20$) and *G. latisiliqua* Desv. ($2n = 20$), and confirmed previous reports of $2n = 20$ for *Collaea stenophylla* (Hook. & Arn.) Benth., *Galactia striata* (Jacq.) Urban and *G. texana* (Scheele) A. Gray. The karyotypes of the six species were also reported for the first time. Those of *Galactia* and *Collaea* species had no particular characteristics, while *C. rubicundum* presented a distinctive karyotype morphology, as well as a different chromosome number.

At the chromosome level, it was thus possible to differentiate *Camptosema rubicundum* from the rest of the species of the group, so we have continued to analyse other representatives of the complex, with special attention to those from the austral region of South America with conflicting taxonomy.

MATERIAL AND METHODS

Plant material

Localities and voucher numbers of the species studied are recorded in Table 1.

Mitotic studies

Root tips were pretreated for 3 h in 0.002 M 8 hydroxyquinoline at 20 ± 2 °C, fixed in absolute ethanol: acetic acid (3:1) and stained in Feulgen after 40 min of hydrolysis in 5 N HCl at 20 °C. Slides were prepared using the squash technique. Five metaphase cells of each species were photographed. Negatives were digitized as monochrome images using a Umax scanner (4800 dpi). Images were analysed using the Zeiss KS400 program to determine the following parameters: total chromosome area (TCA), total chromosome length (TCL), the smallest and the largest chromosome, and centromeric index. The chromosome types were designated according to the position of the centromere (Levan, Fredga & Sandberg, 1964) as: median

Table 1. Species, chromosome numbers, voucher numbers and localities

Taxa	$2n$	n	Voucher number	Locality
Galactia sect. *Odonia* (Bertol.) Burkart				
G. latisiliqua Desv.	20		BAB 98089	Argentina, Entre Ríos: Concordia
G. striata (Jacq.) Urb.	20		BAB 92096	Argentina, Entre Ríos: Concordia
G. dubia DC.	20		RF 5931 (BAB)	Argentina, Salta: Campo Quijano
G. fiebrigiana Burkart	20		RF 7190 (BAB)	Argentina, Salta: 18 km south from Guachipas
G. longifolia (Jacq.) Benth.	20		RF 6237 (BAB)	Argentina, Formosa: El Colorado
Galactia sect. *Collaearia* (Benth.) Burkart				
G. boavista (Vell.) Burkart	20		SS 4 (BAB)	Argentina, Misiones: Parque Teyucuaré
Camptosema rubicundum Hook. et Arn.	22		JG 25 (BAB)	Argentina, Buenos Aires: Isla Martín García
C. paraguariense (Chod. et Hassl.) Hassl.		10	LP 7605 (BAB)	Paraguay, Alto Paraguay: Fortín Patria
C. praeandinum Burkart	20		RF 6936 (BAB)	Argentina, Salta: 7 km north from Quebrada del Toro
C. scarlatinum (Mart. ex Benth.) Burkart		10	RF 8003 (BAB)	Argentina, Misiones: ruta 4, 3 km from Bonpland
Collaea stenophylla (Hook. et Arn.) Benth.	20		SS 1 (BAB)	Argentina, Misiones: San Ignacio, Cerro Sta. Ana

(m), submedian (sm), subterminal (st) and terminal (t). In order to estimate karyotype asymmetry, two numerical parameters, A_1 (intrachromosomal asymmetry index) and A_2 (interchromosomal asymmetry index), were used according to Romero Zarco (1986):

$$A_1 = 1 - \frac{\sum_{i=1}^{n} \frac{bi}{Bi}}{n}$$

and

$$A_2 = \frac{\text{standard deviation}}{\text{mean length}}$$

Both indexes are independent of chromosome number and size. A_1 ranges from 0 to 1 and lower values are obtained when chromosomes tend to be metacentric; n is the number of homologous chromosome pairs or groups, b_i is the average length for short arms in every homologous chromosome pair or group, and B_i is the average length for long arms in every homologous chromosome pair or group. A_2 estimates karyotype asymmetry by comparing the size of different chromosomes: it is Pearson's dispersion coefficient that expresses the ratio between the standard deviation and the mean of chromosome length for each sample.

Statistical analysis for TCA, TCL, smallest and largest chromosome and asymmetry indexes (A_1 and A_2) was performed according to one-way ANOVA followed by comparisons using Scheffeé's test (Sokal & Rohlf, 1981).

Meiotic studies

For meiotic counts, flower buds were fixed in absolute ethanol : chloroform : acetic acid (6:3:1) and the anthers squashed in 2% acetic haematoxylin. Squashes were observed using a Leitz Westlar microscope equipped with a Leica camera.

In situ hybridization

Slides were prepared by digesting fixed root tips in 2% cellulase, 10% pectinase in citrate buffer at 37 °C for 2 h, followed by squashing in 45% acetic acid. After coverslip removal, slides were stored and desiccated at –20 °C before processing.

The probe used was p*Ta*71, which contains a 9-kb *Eco*R1 fragment isolated from wheat, *Triticum aestivum* (Gerlach & Bedbrook, 1979). This fragment contains the 18S, 5.8S and 26S rRNA subunits and nontranscribed spacer sequences. It was labelled with biotin-14-dUTP (Life Technologies) by nick translation according to the manufacturer's instructions.

The procedure was carried out according to Cuadrado & Jouve (1995) with minor modifications. In order to detect labelled probes, fluoresceinated avidin (Vector Laboratories) was used, and one amplification with biotinylated antiavidin D was performed as described by Schwarzacher *et al*. (1989). The slides were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (1 μg/mL in Mc Ilvaine's, citrate buffer, pH = 7), mounted in Vectashield mountant (Vector Laboratories) and examined using a Leica Westlar epifluorescence microscope. Fluorescein-labelled biotinylated DNA and DAPI fluorescence was made visible by exciting at 450–490 nm and 365 nm, respectively, with adequate filter blocks.

Silver staining of NORs

Silver staining followed the technique described by Neves, Heslop-Harrison & Viegas (1995). Root tips were pretreated as for mitotic studies and fixed in FAA 1:18:1 (v/v) formaldehyde 37% : ethanol 50% : glacial acetic acid for 3 days at 4 °C. The whole root tips were washed in distilled water and immersed in 15% $AgNO_3$ solution at 60 °C overnight, washed in distilled water and developed in 1% hydroquinone : 10% formaldehyde (1:1) for 5–10 min at room temperature and fixed in photographic fixative. Slides were prepared by squashing in 45% acetic acid. The numbers of silver-stained metaphase NOR (Ag-NOR) chromosomes and of nucleoli per interphase cell were scored in four species.

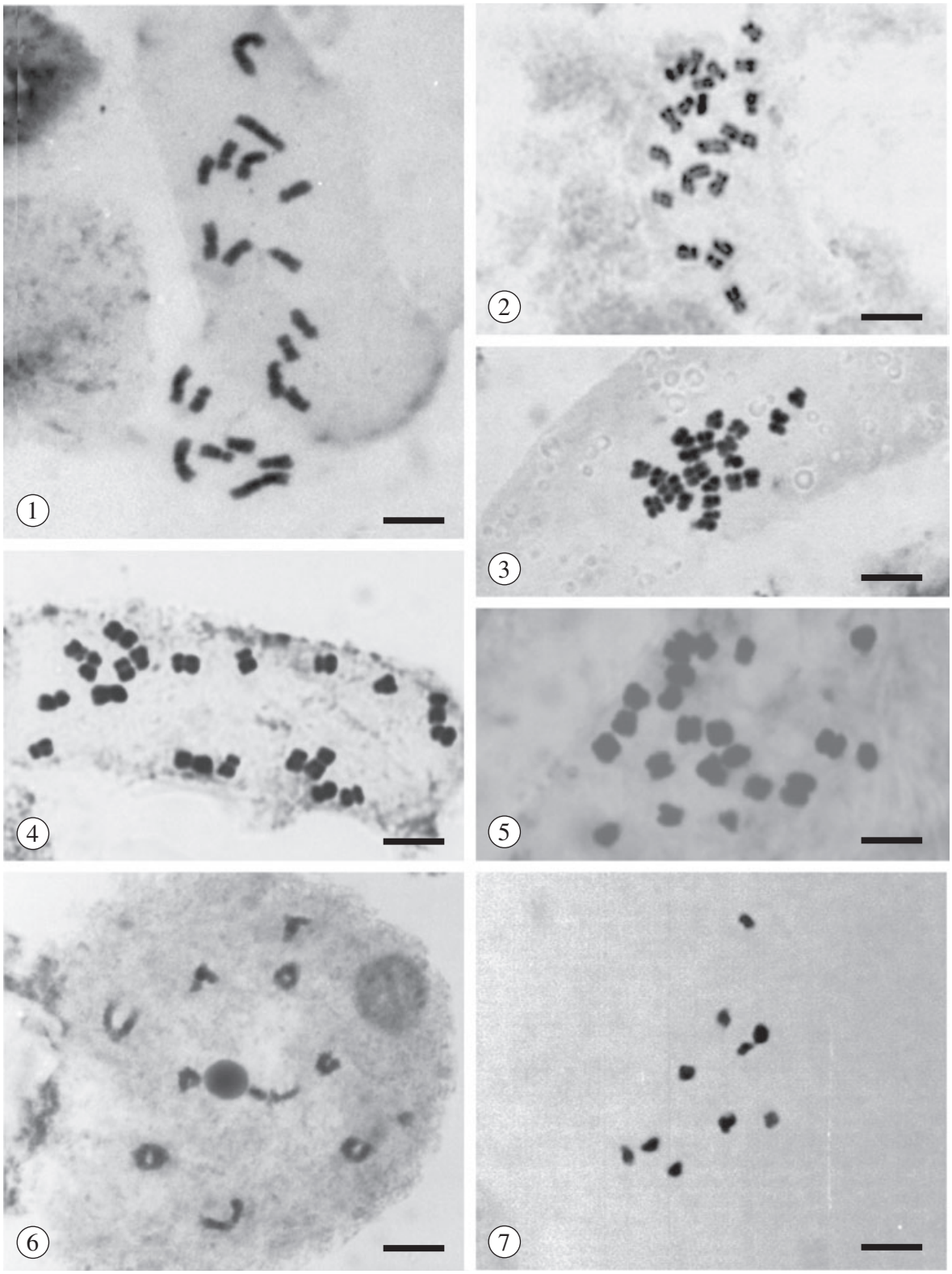
RESULTS AND DISCUSSION

Chromosome numbers of *Camptosema praeandinum* ($2n = 20$), *C. paraguariense* ($n = 10$) and *C. scarlatinum* ($n = 10$) are reported for the first time, while those of *Galactia longifolia* (Jacq.) Benth., *G. dubia* DC. and *G. boavista* (Vell.) Burkart $2n = 20$ confirm previous reports (Kumar & Hymowitz, 1989; Seijo & Vanni, 1999; Bossi & Daviña, 2000) (Table 1; Figs 1–4, 6, 7).

The karyotype formulae, TCL, TCA and asymmetry indexes of *Camptosema praeandinum, Galactia longifolia, G. dubia* and *G. boavista* were analysed and compared with the data reported by Sede *et al*. (2003) (Table 2).

Camptosema praeandinum has the same chromosome number as the analysed species of *Galactia* and *Collaea*. Its karyotype shows a greater proportion of submetacentric chromosomes in relation to the *Galactia* species (Table 2). On the other hand, when *C. praeandinum* is compared with *C. rubicundum*, the latter not only differs in chromosome number ($2n = 22$), but also in karyotype morphology (12m + 2m–sm + 4sm + 4st–t) (Table 2; Figs 1, 5).

Camptosema praeandinum shows the largest total chromosome area (TCA) and total chromosome length (TCL) of all the species studied herein. The TCA of



Figures 1–7. Mitotic (Figs 1–5) and meiotic metaphases (Figs 6, 7). Fig. 1. *Camptosema praeandinum*, $2n = 20$. Fig. 2. *Galactia dubia*, $2n = 20$. Fig. 3. *G. boavista*, $2n = 20$. Fig. 4. *G. longifolia*, $2n = 20$. Fig. 5. *Camptosema rubicundum*, $2n = 22$. Fig. 6. *C. scarlatinum*, $n = 10$. Fig. 7. *C. paraguariense*, $n = 10$. Figs 1–4, 6, 7: scale bar = 5 μm. Fig. 5: scale bar = 3 μm.

Table 2. Karyotype formulae, chromosome size: TCA (total chromosome area, μm^2), TCL (total chromosome length, μm), length of smallest/largest chromosome (μm) and asymmetry indices (A_1 and A_2) for *Galactia*, *Collaea* and *Camptosema* species. Values with different letters mean that they are significantly different (comparisons follow Scheffeé's test, $P < 0.05$)

Species	Karyotype formula	Chromosome size				Asymmetry indices	
		TCA	TCL	L (smallest)	L (largest)	A_1	A_2
*Galactia striata**	16m ± 4m–sm	39.03 ± 4.98ab	38.07 ± 4.42a	1.54 ± 0.13ab	2.79 ± 0.25a	0.27 ± 0.03abc	0.20 ± 0.01
G. dubia	16m ± 2m–sm	43.97 ± 16.13ab	42.21 ± 7.71a	1.58 ± 0.27ab	3.37 ± 0.65ab	0.26 ± 0.04ac	0.24 ± 0.04
*G. texana**	16m ± 4m–sm	38.73 ± 5.84ab	34.07 ± 7.36a	1.34 ± 0.27a	2.45 ± 0.65a	0.26 ± 0.04abc	0.19 ± 0.04
*G. latisiliqua**	16m ± 4m–sm	29.36 ± 6.82b	30.25 ± 8.32a	1.13 ± 0.19a	2.22 ± 0.77a	0.23 ± 0.09a	0.17 ± 0.06
G. longifolia	16m ± 4sm	36.75 ± 5.57ab	39.39 ± 1.86ab	1.47 ± 0.03ab	2.89 ± 0.47ab	0.25 ± 0.01abc	0.22 ± 0.03
G. boavista	16m ± 4sm	45.85 ± 10.24ab	36.77 ± 5.55ab	1.36 ± 0.20ab	2.62 ± 0.60ab	0.26 ± 0.01abc	0.18 ± 0.03
*G. fiebrigiana**	16m ± 4sm	35.63 ± 8.22b	38.99 ± 4.92a	1.43 ± 0.13a	3.00 ± 0.44a	0.27 ± 0.04abc	0.23 ± 0.04
*Collaea stenophylla**	14m ± 2m–sm ± 4sm	42.44 ± 4.03ab	40.91 ± 6.29ab	1.50 ± 0.06ab	3.04 ± 0.79ab	0.29 ± 0.02abc	0.20 ± 0.05
*Camptosema rubicundum**	12m ± 2m–sm + 4sm ± 4st–t	29.94 ± 3.71b	33.43 ± 4.18a	1.07 ± 0.11a	2.33 ± 0.41a	0.37 ± 0.06b	0.20 ± 0.03
C. praeandinum	12m ± 2m–sm ± 6sm	60.40 ± 16.63a	56.92 ± 11.87b	2.12 ± 0.56b	4.50 ± 0.98b	0.35 ± 0.02bc	0.27 ± 0.03

*Data taken from Sede *et al.* (2003).

C. praeandinum differs from the smaller values shown by some species of *Galactia* ($P < 0.001$). When compared with *C. rubicundum*, the TCA of *C. praeandinum* also showed differences ($P < 0.001$) (Table 2). *Camptosema praeandinum* differs in TCL from the other species ($P < 0.001$) (Table 2) by showing the longest chromosomes (2.12–4.50 μm) and thus differs from most species of *Galactia* ($P < 0.001$). It also differs from *C. rubicundum* in TCL and chromosome length range ($P < 0.001$) (Table 2).

Camptosema praeandinum and *C. rubicundum* have the highest values of the asymmetry index A_1, showing more asymmetrical karyotypes. They differ from *G. latisiliqua* ($P < 0.001$), which shows the most symmetrical karyotype (Table 2). Although A_2 differences were found when all the species where compared, it was not possible to detect the origin of these differences by means of partial comparisons.

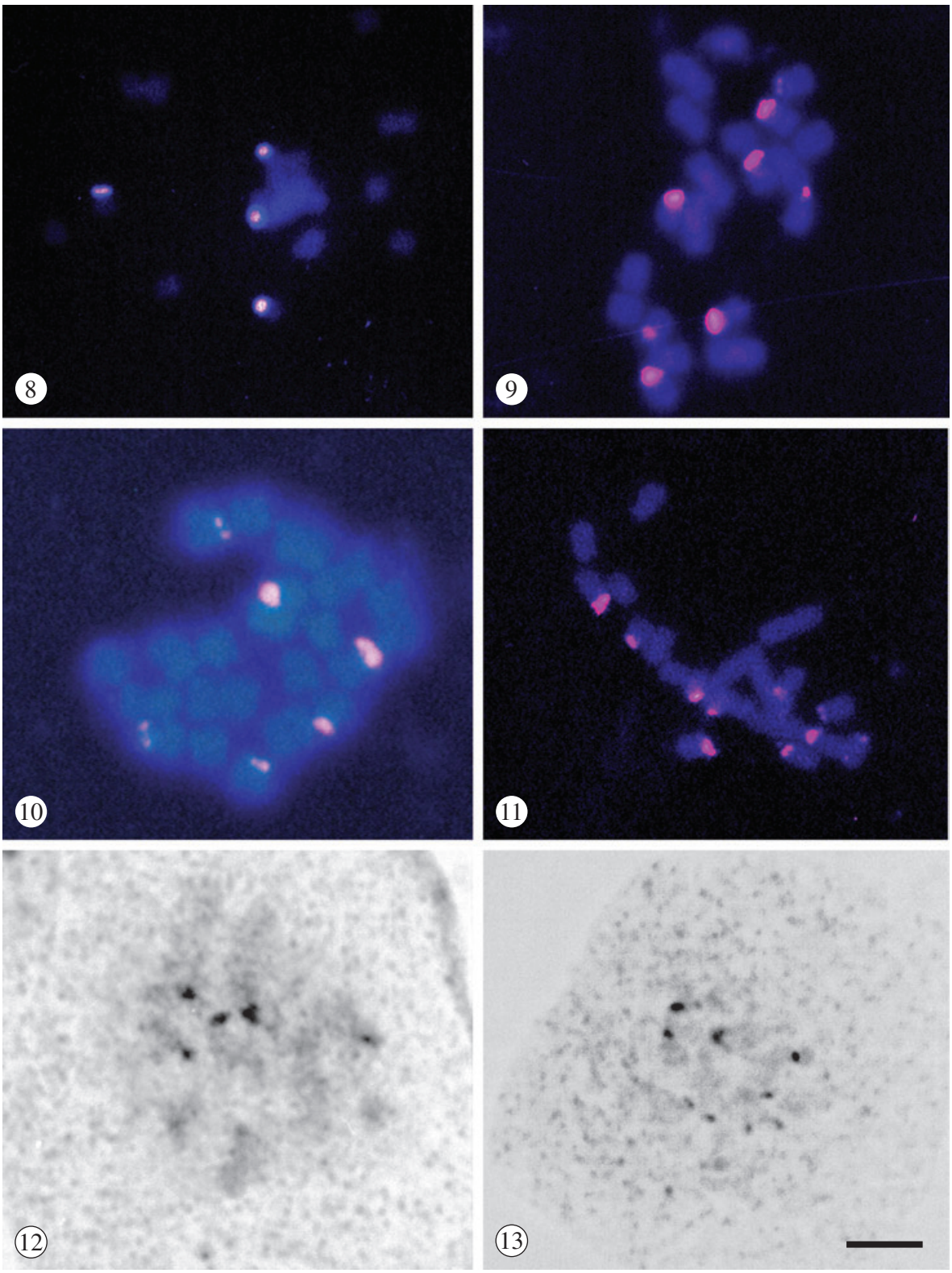
Chromosome numbers of *Camptosema praeandinum* ($2n = 20$), *C. paraguariense* ($n = 10$) and *C. scarlatinum* ($n = 10$) (Table 1; Figs 1, 6, 7) do not agree with either the proposed number for the genus ($n = 11$ Goldblatt, 1981) or that of the type species *C. rubicundum* ($2n = 22$) (Sede *et al.*, 2003).

The present work confirms differences between *C. praeandinum* and *C. rubicundum* not only in karyotype formula, but also in values of TCA and TCL (Table 2).

The number of rDNA zones detected by fluorescent *in situ* hybridization (FISH) using the probe p*Ta*71 showed interspecific variation, from four signals in *G. dubia* to ten in *Camptosema praeandinum* (Table 3; Figs 8–11). Owing to small size and similar morphology of the chromosomes it was not possible to map these sites physically. However, it was observed that

Table 3. Number of rDNA sites revealed by *in situ* hybridization using p*Ta*71 as a probe, number of NORs detected by silver staining and maximum number of nucleoli at interphase in *Galactia*, *Camptosema* and *Collaea* species analysed. (–, not determined)

Species	No. of rDNA sites	No. of Ag-NOR sites	Maximum no. of nucleoli at interphase
C. rubicundum	6	4/6	4
C. praeandinum	10	6/8	5
C. stenophylla	8	4/6	4
G. fiebrigiana	8	6/8	5
G. latisiliqua	6	–	–
G. striata	6	–	–
G. dubia	4	–	–



Figures 8–13. Mitotic metaphases. Figs 8–11. Metaphases following *in situ* hybridization with p*Ta*71 probe (rDNA sites are shown in red). Figure 8. *Galactia dubia.* Figure 9. *G. fiebrigiana*. Fig. 10. *G. latisiliqua.* Fig. 11. *Camptosema praeandinum*. Figs 12, 13. Silver-stained metaphases showing NORs. Fig. 12. *C. rubicundum*. Fig. 13. *G. fiegrigiana*. Scale bar = 5 μm.

the signals were always present in the terminal zones of the chromosomes and that in no case did they appear in the two major pairs of chromosomes of the *Galactia* species studied (Figs 8–10). *Camptosema praeandinum* differs from *C. rubicundum* in the number of rDNA zones. Nevertheless, the intraspecific variation in *Galactia* suggests that these data must be considered carefully if they are to be used for taxonomic purposes.

There are some reports on a marked variation in number and localization of rDNA sites between species of the same genus (Schubert & Wobus, 1985; Guerra, Kenton & Bennett, 1996; Adams *et al*., 2000; Široký *et al*., 2001). According to Schubert & Wobus (1985) and Adams *et al*. (2000), one of the possible mechanisms to explain the interspecific variation in the number of rDNA zones could be the amplification and differential fixation of the sequences in different chromosome sites.

With the aim of determining whether the rDNA zones detected by FISH were active, the number of Ag-NORs was counted in metaphases of *Camptosema rubicundum*, *C. praeandinum, Collaea stenophylla* and *G. fiebrigiana*. *Camptosema rubicundum* and *Collaea stenophylla* showed 4–6 regions, but *C. praeandinum* and *G. fiebrigiana* showed 6–8 (Table 3; Figs 12, 13). The Ag-NOR technique showed that most of these zones were active. When comparing the number of Ag-NORs at metaphase with the number of nucleoli at interphase, the latter was always smaller than the former, probably as a result of fusion between them in interphase nuclei (Table 3). The frequency of interphase cells containing the maximum number of nucleoli was always smaller than 1.5% and the percentage of interphase cells with only one nucleolus was above 82% in all cases. The existence of numerous active rDNA zones is not frequent in diploid plants (Carnide, Orellana & Do Valle Ribeiro, 1986; Garrido-Ramos *et al*., 1992; Moscone *et al*., 1995).

The comparison of the exomorphology between the conflictive species of *Camptosema* and other representatives of the complex is summarized in Table 4. It is notable that:

1. *C. praeandinum* ($2n = 20$) has several features related to *Galactia* spp. (form and pubescence of calyx), but others nearer to *Galactia* sect. *Collaearia* (corolla and androecium type), or to *Camptosema s.s.* (corolla colour and presence of gynophore).
2. *C. paraguariense* ($n = 10$) is related in part to the species of *Camptosema s.s*. (glabrate petals, reflexed standard, pseudomonadelphous androecium and long gynophore), but also to some species of *Galactia* sect. *Odonia* (form and pubescence of the calyx and standard). In this section its closest

Table 4. Exomorphology of *Camptosema*, *Galactia* sects. *Odonia* and *Collaearia*, and *Collaea* species

Taxon	Calyx		Androecium	Gynophore	Standard		
	Form	Pubescence			Colour	Pubescence	At anthesis
Camptosema rubicundum	Tubular	Finely and sericeous strigulose	Pseudomonadelphous	Present, 4 mm	Red	Glabrate	Reflexed
C. paraguariense	Campanulate	Pubescent to glabrous	Pseudomonadelphous	Present, 2–3 mm	Red	Glabrate	Reflexed
C. scarlatinum	Campanulate	Strigose-pubescent to villous	Pseudomonadelphous	Present, 2–2.5 mm	Red	Glabrate	Reflexed
C. praeandinum	Campanulate	Sericeous – pubescent-hispid	Pseudomonadelphous–diadelphous	Present, 1.2–2 mm	Red	Pubescent	Reflexed
Galactia sect. *Odonia*	Campanulate	Pubescent-strigose	Diadelphous	Absent–present	Rose–lilaceous	Glabrate	Not reflexed
Galactia sect. *Collaearia*	Campanulate	Hispid-sericeous pubescent	Diadelphous	Absent–present	Rose–lilaceous	Pubescent	Not reflexed
Collaea spp.	Campanulate	Sericeous	Pseudomonadelphous	Absent–present	Red	Pubescent	Reflexed

relative is *G. longifolia*, which shares a geographical distribution in southern South American regions (Chaco region of Paraguay and Argentina).

3. *C. scarlatinum* ($n = 10$) has calyx and corolla related to species of *Galactia* sect. *Odonia* (Bertol.) Burkart and it has the same floral characters as in *C. paraguariense*, showing affinity with *Camptosema s.s.* species.

Of the four species from southern South America recognized by Burkart, *C. rubicundum* is the only one with a tubular calyx, the most relevant diagnostic character of the genus *Camptosema*.

In the parsimony analysis of the subtribe Diocleinae based on morphological characters (de Queiroz *et al.*, 2003), the analysed species of *Camptosema* do not form a monophyletic group.

CONCLUSIONS

The present study indicates that chromosome number is a resolving character in the conflicting taxonomy of *Camptosema* species. The numbers of rDNA zones and of Ag-NOR sites are very variable characters to be considered of use in the generic delimitation of the studied species.

By means of this analysis, it was observed that the similarity of the chromosome numbers of *Camptosema praeandinum*, *C. paraguariense* and *C. scarlatinum* to that of the species of *Galactia s.l.* is correlated with an affinity in certain morphological characters. These observations, together with the phylogenetic hypothesis based on morphology (de Queiroz *et al.*, 2003) make it necessary to revise the current circumscription of these species. A molecular phylogenetic analysis is being undertaken in our laboratory and data from morphology, cytology and DNA will be combined to propose a new taxonomic delimitation of the species.

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