Anais da Academia Brasileira de Ciências (2012) 84(4): 1029-1037
(Annals of the Brazilian Academy of Sciences)
Printed version ISSN 0001-3765 / Online version ISSN 1678-2690
www.scielo.br/aabc

Karyological studies in Brazilian species of *Lippia* L. (Verbenaceae)

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Manuscript received on September 15, 2011; accepted for publication on November 14, 2011

ABSTRACT

The genus *Lippia* (Verbenaceae) comprise around 160 species spread out mainly in South and Central Americas with few African species, some of them with potential medicinal use. Brazil is one the most important centers of diversity with approximately 75% of the species described so far. Innumerous species are endemic and poorly studied especially at a cytological level. Here, chromosomal length, karyomorphology and chromosome asymmetry of twelve Brazilian species of *Lippia* were evaluated [*L. alba* (Miller) N.E.Brown, *L. diamantinensis* Glaz., *L. florida* Cham., *L. hermanioides* Cham., *L. lacunosa* Mart. & Schauer, *L. lupulina* Cham., *L. pohliana* Schauer, *L. pseudothea* (St. Hil) Schauer, *L. rosella* Moldenke, *L. rotundifolia* Cham., *L. rubella* Moldenke and *L. sidoides* Cham.]. The analysis suggested that the genus has a variable chromosome number (from 2n = 20 to 2n = 56) originated by dysploidy and polyploidy. This is the first description of chromosome morphology for 11 of the 12 *Lippia* species studied.

Key words: chromosomes, cytology, dysploidy, karyology, *Lippia*, polyploidy.

INTRODUCTION

Cytogenetic studies have long been contributing to increase evolutionary, phylogenetic and taxonomic inferences about various plant groups (Stebbins 1971, Pellicer et al. 2007, Guerra 2008, Kissling et al. 2008). The karyotype can be considered as the phenotypic aspect of the chromosome complement at mitotic metaphase. For several decades, it was the prime cytological trait used to infer species relationships (Levin 2002). In a general way, the description of the karyotype includes: the chromosome number, the total length of chromosome complement (genome size), the absolute and relative size of chromosomes, the symmetry of each chromosome (as dictated by the position of the centromere on each chromosome), the number and positions of satellites associated with the nucleolar-organizing regions and the distribution of heterochromatin segments. In addition, modern molecular cytogenetic techniques, as FISH (Fluorescent in situ Hybridization) and GISH (Genome *in situ* hybridization) permit the mapping of specific sequences in chromosomes and the study of genome constitution, respectively (Levin 2002, Guerra 2008).

Very often chromosome number variation has been used to understand karyoptype evolution and three basic mechanisms could be suggested to explain those variations at genus level. Polyploidization is

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one the most important mechanisms of angiosperms evolution, probably due to the success of the hybrids comparing with their diploids parents (Stebbins 1971, Ramsey and Schemske 1998, Guerra 2008, Soltis et al. 2009). Another possible mechanism that is not a consensus as an important evolutionary event is aneuploidy. According to Guerra (2008), mainly due to the induction of lethality or subvitality, this kind of chromosome variation has apparently no evolutionary meaning and has been largely documented in cultivated plants and in human chromosome disorders. Besides, aneuploidy in natural plant populations occurs in isolated individuals, asexual clones, cryptic species, interespecific hybrids, B chromosomes or any particular mechanism, but not as a general event. The third mechanism, the dysploidy, in contrast to polyploidy, induces an alteration on the average length of the chromosomes, altering (when structural changes occur) or not the total chromosome length (Jones 1998, Guerra 2008).

Lippia L., an important genus of the Verbenaceae family, possesses many species mainly distributed throughout South and Central America and Tropical Africa. Brazil, one of the most important centers of diversity possesses nearly 75% of the 160 described species that can be easily found in Brazilian rocks environments with high altitudes (Salimena 2000, Viccini et al. 2006). Although many species have been considered as an important source of chemical constituents for pharmaceutical, food and cosmetic industry, few studies have been done in order to amplify their biological, anatomical, morphological, physiological, phenological, genetic and evolutionary knowledge (Salimena 2000, Pascual et al. 2001, Viccini et al. 2006, Sousa et al. 2009, Campos et al. 2011).

Few studies have been done about the karyology of the genus *Lippia*. Most of them only reported the chromosome numbers (Bose and Choudhury 1960, Smith 1966, Navaneethan 1982, Coleman 1982, Filippa 1984, Kumar and Dutt 1989, Brandão et al. 2005, Viccini et al. 2006, Campos et al. 2011). Moreover, the authors used mainly meiotic and non-mitotic chromosomes, due to the difficult of obtaining meristematic roots (Viccini et al. 2006, Campos et al. 2011).The chromosome number described for the genus vary from n = 10 for *L. rubella* (Viccini et al. 2006) to n = 30 for *L. turbinata* and *L. fissicalyx* (Pastoriza and Andrada 2006). Studies with somatic chromosomes were done only for *L. turbinata*, *L. fissicalyx* (Pastoriza and Andrada 2006) and *Lippia alba* (Brandão et al. 2007, Sousa et al. 2009, Pierre et al. 2011). For *L. alba*, the most studied species, the karyological studies reveal the centromer position, number of secondary constrictions, heterochromatin distribution and the map of rDNA sites (45S and 5S) by FISH (Brandão et al. 2007, Sousa et al. 2009). More yet, Pierre et al. (2011) related intraspecific chromosome number showing polyploidy and mixoploidy for different accessions of *L. alba*.

Considering the small number of species karyomorphologically studied in *Lippia*, the present paper reports karyomorphological aspects of twelve Brazilian species of *Lippia*, with eleven of them studied for the first time.

MATERIALS AND METHODS

Plant materials. All species were collected at Espinhaço Range of Minas Gerais state (MG), Brazil. Only *L. alba* was collected directly from the germplasm collection of Universidade Federal de Juiz de Fora, Minas Gerais, Brazil. The collection data and Voucher number of each species are described in Table I. The Vouchers are deposited at the Herbarium CESJ of Universidade Federal de Juiz de Fora. Approximately three individuals were analyzed per species.

Chromosome preparation. Meristematic cells from root tips and apical buds meristems were pre-treated with 8-hydroxyquinoline (2 and 3mM) during 6-14h at 4°C and fixed in 3:1 methanol: acetic acid solution for at least 24h before the slide preparation.

Slide preparation (root meristems). Pre-treated root tips were macerated in an enzymatic solution

TABLE I
Collection data of *Lippia* species from Espinhaço Range, Minas Gerais (MG) State, Southeast, Brazil.

Species	Origin Place	Latitude	Longitude	Herbarium number
Lippia alba (Miller) N.E. Brown	Juiz de Fora-MG	21°23'21"S	42°41'48"W	47,724
Lippia diamantinensis Glaz.	Diamantina-MG	18°14'48"S	43°36'6"W	34,734
Lippia florida Cham.	Diamantina-MG	18°14'48"S	43°36'6"W	34,738
Lippia hermanioides Cham.	Serra do Cipó-MG	19°27'47"S	43°33'10"W	34,739
Lippia lacunosa Mart. & Schauer	Diamantina-MG	18°14'48"S	43°36'6"W	42,705
Lippia lupulina Cham.	Diamantina-MG	18°14'48"S	43°36'6"W	34,742
Lippia pohliana Schauer	Diamantina-MG	18°14'48"S	43°36'6"W	39,360
Lippia pseudothea (St. Hil) Schauer	Diamantina-MG	18°14'48"S	43°36'6"W	34,340
Lippia rosella Moldenke	Diamantina-MG	18°14'48"S	43°36'6"W	34,746
Lippia rotundifolia Cham.	Diamantina-MG	18°14'48"S	43°36'6"W	34,338
Lippia rubella Moldenke	Diamantina-MG	18°14'48"S	43°36'6"W	42,706
Lippia sidoides Cham.	Serra do Cipó-MG	19°27'47"S	43°33'10"W	34,737

[4% cellulase (Sigma) plus 40% pectinase (Sigma) diluted in 0.001M citric acid-sodium citrate pH 4.8 buffer] at 37°C (4h). The slides were prepared according to Carvalho and Saraiva (1993, 1997).

Slide preparation (buds meristems). Due great difficulty in the obtaining of root meristem in some species of *Lippia* (*L. lacunosa*, *L. pseudothea*, and *L. diamantinensis*), bud meristems were also used for chromosome achievement. The slides were prepared according to Viccini et al. (2006) with some modifications. Nearly ten pre-treated meristematic apical buds were placed in a 0.5 mL microtube adapted with a 60 μm nylon mesh. The material was washed three times in distilled water to remove fixative solution. The adapted tube containing the apical buds meristems was immersed in another normal microtube (1.5 mL) with concentrated enzymatic solution (Pectinex NOVO FERMENT™). The material was placed in an incubator at 34°C for 20 min. After enzymatic maceration, the material was washed again in order to remove the excess of enzymatic solution. The adapted microtube was then placed into another microtube (1.5 mL) containing distilled water. The meristems were mechanically fragmented with an adapted pin in order to permit the dissociation of the tissue. The cellular suspension obtained was then centrifuged three times at 2,000 rpm for 12 min. In each step, the supernatant was removed and the volume of the microtube was completed with distilled water. After the last centrifugation, the volume of the microtube was replenished with fresh fix. The cellular suspension was carefully resuspended. The final suspension was stored at -20°C. For slide preparation, about six drops of the suspension were dropped onto a clean slide. The slides were air-dried and stained with Giemsa solution (5%) for 3 min.

Karyotype analysis. At least five metaphases by individual were used to determine the length of the short (*si*) and long arm (*li*) of each chromosome (*i*). The chromosome length ($t = li + si$) and arm ratio ($r = li / si$) were calculated. The chromosomes were classified on the basis of arm ratio using the standard nomenclature (Levan et al. 1964): m: median ($r = 1.01$-1.69); sm: submedian (r = 1.70-3.00); st: subterminal ($r = 3.01$-7.00); and t: terminal ($r = 7.01$-8.00).

Karyotype asymmetry, the length of heterogeneity or degree of asymmetry of each chromosome (*Ai*) and the degree of karyotype asymmetry (*A*) were estimated as proposed by Watanabe et al. (1999):

$Ai = (li - si) / (li + si)$ and $A = (1 / n) \Sigma Ai$

where *n* is the haploid chromosome number with *A* ranges from zero (completely symmetrical) to one (completely asymmetrical).

Chromosomes were measured using Image ProPlus software (Media Cybernetics™) and the ideograms arranged in order of decreasing short arm length.

RESULTS

Karyotype Constitution

The karyotypic constitution of *Lippia* species was highly variable between the 12 species studied (Fig. 1). In general, *Lippia* karyotypes have mainly median and submedian chromosomes. Only *L. diamantinensis* (2n = 52) and *L. lacunosa* (2n = 56) showed chromosomes with sub-terminal

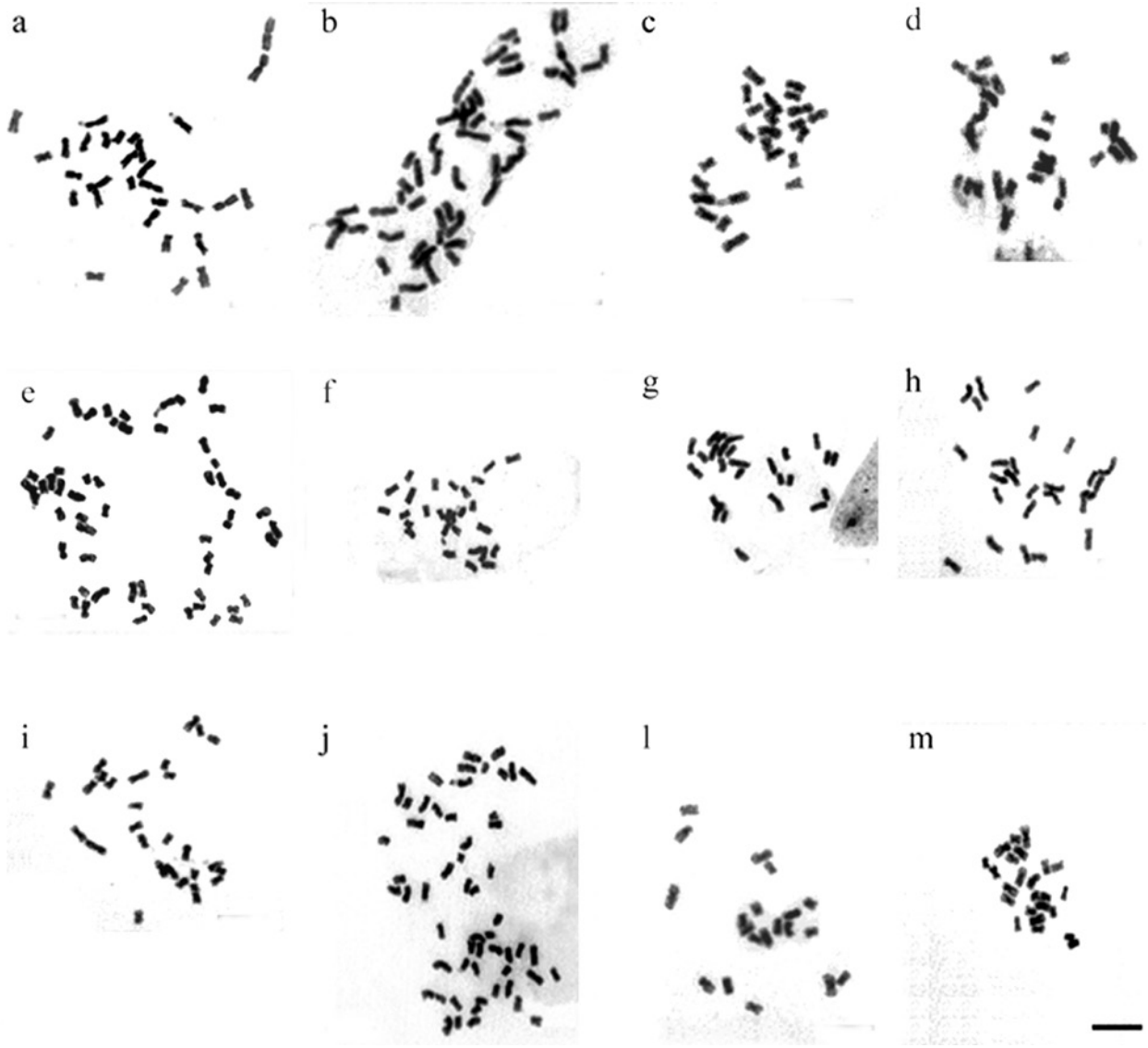

Fig. 1 - Metaphase chromosomes of **(a)** *L. alba*, 2n = 30; **(b)** *L. diamantinensis*, 2n = 52; **(c)** *L. florida*, 2n = 24; **(d)** *L. hermanioides*, 2n = 26; **(e)** *L. lacunosa*, 2n = 56; **(f)** *L. lupulina*, 2n = 28; **(g)** *L. pohliana*, 2n = 24; **(h)** *L. pseudothea*, 2n = 26; **(i)** *L. rosella*, 2n = 28; **(j)** *L. rotundifolia*, 2n = 56; **(l)** *L. rubella*, 2n = 20; and **(m)** *L. sidoides*, 2n = 24;. Bars = 5μm .

centromers. The percentage of median chromosomes varied from 42% in *L. diamantinensis* (2n = 52) to 100% in *L. rubella* (2n = 20) (Table II).

Chromosome Length

The chromosome length of those twelve *Lippia* species was relatively small, with *L. diamantinensis* showing the smallest and the bigger chromosomes, ranging from 0.42 μm to 2.47 μm (Table II). A direct relationship between the average of chromosome length and the chromosome number was not observed. Nevertheless, the total chromosome length (TCL) increase as the chromosome number increased (Fig. 2a). The TCL varied from 16.96 in *L. rubella* to 67.96 μm in *L. diamantinensis* and the average of TCL of all species was 37.40 μm (Table II).

Karyotype Asymmetry

As the degree of asymmetry (*A*) increased, the chromosome number increased accordingly (Fig. 2b). Species with chromosome number smaller than 2n = 30 showed an asymmetry (*A*) ranging from 0.09 in *L. rubella* (2n = 10) to 0.15 in *L. alba* (2n = 30), while species with higher chromosome number showed values varying from 0.21 to 0.26. *L. diamantinensis* karyotype was the most asymmetric one (Table II).

Secondary Constrictions

Six species exhibit secondary constrictions (SC): *L. alba*, *L. florida*, *L. hermanioides*, *L. diamantinensis*, *L. lacunosa* and *L. rotundifolia*. The number of SC varied among the species, ranging from one pair in *L. hermanioides*, *L. florida* and *L. lupulina* to four in *L. diamantinensis*. Moreover, the positions of SC varied among the species. Some of them showed this structure only in the short chromosome arms while others showed the same structure in both arms (Fig. 3).

DISCUSSION

Previous cytogenetic studies in the genus Lippia indicated that the genus has a wide range of chromosome numbers: 2n = 20, 24, 26, 28, 30, 32, 52 and 56 (Viccini et al. 2006, Campos et al. 2011). The present study confirmed the previous chromosome numbers (Viccini et al. 2006, Bose and Choudhury 1960) for all species that were now investigated by mitotic chromosomes.

TABLE II

Karyotype characters of 12 *Lippia* species. Abbreviations: 2n = chromosome number; PMC = percentage of median chromosomes; TCL = total chromosome length; ACL = average chromosome length; LCL = longest chromosome length; SCL = shortest chromosome length; *A* = Karyotype asymmetry; KF = karyotype formulae.

Taxon	2n	PMC (%)	TCL (μm)	ACL (μm)	LCL (μm)	SCL (μm)	*A*	KF
L. rubella	20	100	16.96	1.54	1.36	0.43	0.09	10m
L. pohliana	24	75	19.78	0.82	1.54	0.44	0.12	9m+3sm
L. florida	24	75	24.26	1.01	1.54	0.51	0.12	9m+3sm
L. sidoides	24	92	26.40	1.10	1.41	0.58	0.11	11m+1sm
L. hermanioides	26	77	29.70	1.14	1.48	0.72	0.12	10m+3sm
L. pseudothea	26	62	38.70	1.49	2.00	1.03	0.14	8m+5sm
L. lupulina	28	71	28.00	1.00	1.52	0.66	0.14	10m+4sm
L. rosella	28	72	33.22	1.19	1.95	0.51	0.15	11m+3sm
L. alba	30	67	34.00	1.13	2.01	0.48	0.15	10m+ 5sm
L. diamantinensis	52	42	67.96	1.31	2.47	0.42	0.26	11m+13sm+2st
L. lacunosa	56	64	62.28	1.11	1.78	0.43	0.21	18m+7sm+3st
L. rotundifolia	56	50	67.56	1.20	1.83	0.46	0.22	14m+14sm

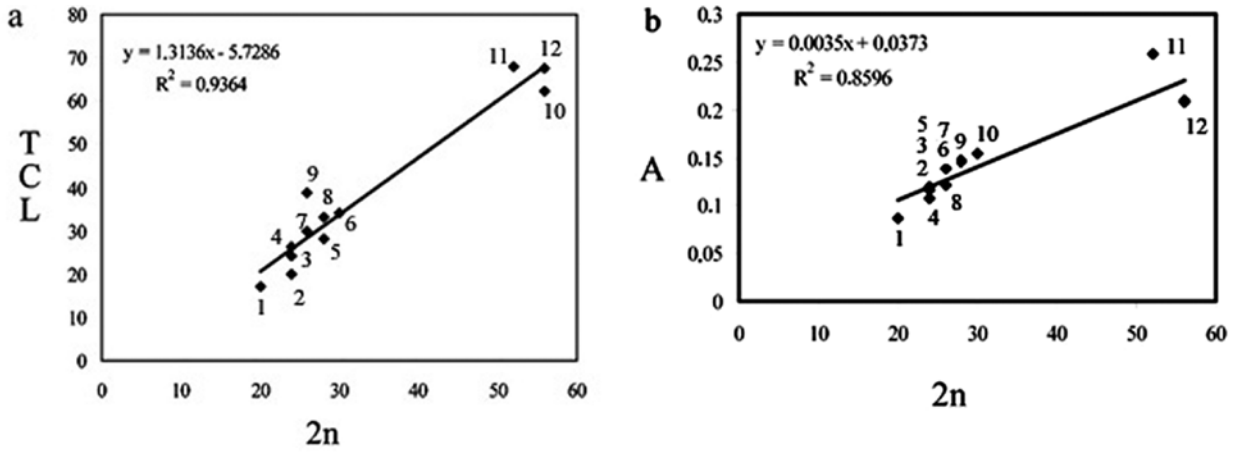

Fig. 2 - (a) Relationship between total chromosome length (TCL) and chromosome number. **(b)** Relationship between asymmetry **(A)** and the chromosome number. **(1)** *L. rubella*, **(2)** *L. pohliana*, **(3)** *L. florida*, **(4)** *L. sidoides*, **(5)** *L. hermanioides*, **(6)** *L.pseudothea*, **(7)** *L. lupulina*, **(8)** *L. rosella*, **(9)** *L. alba*, **(10)** *L.diamantinensis*, **(11)** *L. lacunosa*, **(12)** *L. rotundifolia*.

Regarding karyomorphological aspects, symmetrical karyotypes have been considered as less derived and asymmetrical karyotypes as the more derived ones (Stebbins 1971). The present data revealed that asymmetry of *Lippia* karyotypes increase as the chromosome number increase, suggesting that an increment of the chromosome number constitutes an important factor in the evolution of *Lippia* chromosomes.

According to Sanders (2001), the hypothesis of x = 5 as an ancestral chromosome number for the family Verbenaceae seems to be the most reasonable. According to the same author, the numbers x = 6 and 7 could have arisen by ascending aneuploidy and x = 10 by polyploidy, which, by descending aneuploidy, could have originated the numbers x = 9 and 8. Viccini et al. (2006) suggested two mechanisms for karyotypic evolution within the genus *Lippia*: polyploidy and aneuploidy. Although *Lippia* species with n = 5 have not been described, Campos et al. (2011) suggested that an ancestral species with 2n = 10 could rise species with 2n = 20 (by chromosome duplication, autopolyploidy), which could have originated numbers 2n = 16 and 18 through descending dysploidy. Additionally, ascending dysploidy could be responsible for the origin of 2n = 12 and 2n = 14, from which chromosome duplication could lead to 2n = 24 and 2n = 28, respectivelly. The species with 2n = 26 and 2n = 30 could have arisen by hybridization between species with 2n = 12 and 14 or 2n = 18 and 2n = 16, respectively, followed by genome duplication. Species with 2n = 52 and 2n = 56 could be formed from another event of autopolyploidy or allopolyploidy. Here we observed differences among average length of *Lippia* chromosomes in addition to variation in the centromer position, in the number and position of secondary constriction and also an increase of total chromosome length (mainly due to polyploidy). Dysploidy plus polyploidy can be considered the most important events in chromosome evolution of several plant groups (James 1981, Otto and Whitton 2000, Guerra 2008). Although the descending dysploidy series is more common (Stebbins 1971, Levin 2002, Guerra 2008), some groups show an ascending series as described in *Hemizonia* (Asteraceae) (Kyhos et al. 1990), *Gentiana* (Gentianaceae) (Yuan et al. 1998), *Sideritis* (Lamiaceae) (Barber et al. 2000), and in *Exacum* (Gentianaceae), *Ornichia* (Gentianaceae), *Sebaea* (Gentianaceae) and *Tachiadenus* (Gentianaceae) (Kissling et al. 2008). The same fact was here

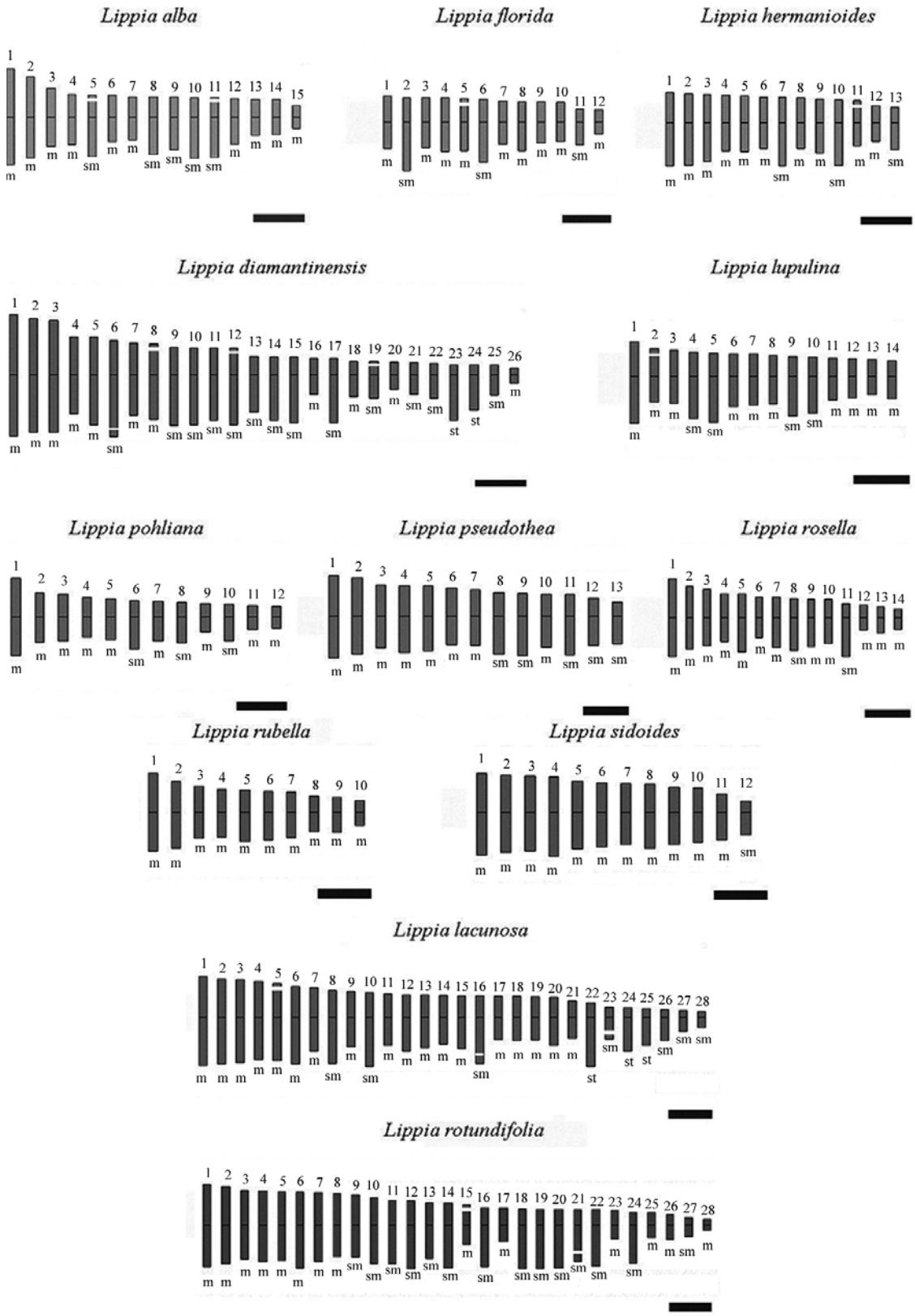

Fig. 3 - Haploid idiograms of 12 *Lippia species*. Bars = 1μm.

observed and seems to be the same case in the genus *Lippia*. Our data reinforce the hypothesis proposed by Campos et al. (2011), that dysploidy followed by polyploidy could be the most important mechanisms of *Lippia* chromosome evolution.

Taxonomically, according to Troncoso (1974), the Brazilian *Lippia* species are divided in five sections: *Dipterocalyx*, *Goniostachyum*, *Zapania*, *Rhodolippia*, *Pseudoaloysia* and *Dioicollipia*. In our study *L. rubella*, *L. hermanioides*, *L. alba*, *L. diamantinensis*, *L. lacunosa* and *L. rotundifolia* belong to *Zapania* section; *L. florida*, *L. pseudothea*, *L. lupulina* and *L. rosella* belong to *Rhodolippia* section; while only *L. pohliana* and *L. sidoides* belong to *Goniostachyum* section. According to our analysis, the section *Zapania* is the most variable regarding chromosome number and size. Moreover, this is the unique section where we observed secondary constrictions in both chromosome arms, while in the other sections this structure were observed only in the short arm of the chromosomes. According to Campos et al. (2011), Viccini et al. (2006) and Salimena (2000), the *Zapania* section should be reappraised considering the higher variation in chromosome number, DNA content and plant morphology. The variation in chromosome size and position of the secondary constriction among species here observed reinforce that suggestion.

Regarding the absence of secondary constrictions in some species, these structures are formed in active sites with 45S rDNA (NORs) and sites with restricted NOR activation can, but do not have to form secondary constrictions, thus hindering the real determination of their number and position through conventional techniques (Marcon et al. 2005). For this, the application of FISH and AgNOR banding (two more refined techniques for NOR visualization) in the species where secondary constriction were not observed. It will be very important in the future for a better knowledge of the distribution of these structures among the species studied here, as well as their activity in the precedent interphases.

The present data constitutes the first karyomorphological study for eleven of twelve species here investigated, and were important to corroborate previous hypothesis and to reveal new aspects of *Lippia* chromosomes, especially mitotic ones. Nevertheless, additional studies including more species and integrating karyology, phylogeny, distribution, specific divergence time, geographical and climatics changes would be desirable in order to extend our conclusions and consequently better understand the evolution of the genus.

RESUMO

O gênero *Lippia* (Verbenaceae) possui cerca de 160 espécies distribuídas principalmente nas Américas Central e do Sul com poucas espécies Africanas, algumas delas com potencial uso medicinal. O Brasil é um dos mais importantes centros de diversidade com aproximadamente 75% das espécies descritas. Inúmeras espécies são endêmicas e pouco estudadas, principalmente a nível citológico. Aqui, o comprimento cromossômico, a cariomorfologia e a assimetria cromossômica de doze espécies brasileiras de *Lippia* foram avaliados [*L. alba* (Miller) N.E.Brown, *L. diamantinensis* Glaz., *L. florida* Cham., *L. hermanioides* Cham., *L. lacunosa* Mart. & Schauer, *L. lupulina* Cham., *L. pohliana* Schauer, *L. pseudothea* (St. Hil) Schauer, *L. rosella* Moldenke, *L. rotundifolia* Cham., *L. rubella* Moldenke e *L. sidoides* Cham.]. As análises sugeriram que o gênero tem um número cromossômico variável (de 2n = 20 a 2n = 56) originado por disploidia e poliploidia. Esta é a primeira descrição da morfologia cromossômica para 11 das 12 espécies de *Lippia* estudadas.

Palavras-chave: cromossomos, citologia, disploidia, cariologia, *Lippia*, poliploidia.

ACKNOWLEDGMENTS

The authors thank the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for the financial support.

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