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Research Article

Cytogenetics and embryology of *Eupatorium laevigatum* (Compositae)

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Abstract

Embryological studies indicate *Eupatorium laevigatum* to have *Antennaria* type diplospory with precocious embryony. The embryo sac is of the *Polygonum* type and the polar nuclei fuse before anthesis (maturation of the stamens). Endosperm development is autonomous and the central cell divides only after the initial stages of embryo formation. It is estimated that about 10% of the florets in anthesis contain an undivided egg which can be used for sexual reproduction. The study of microsporogenesis revealed abnormalities in chromosome pairing which result in the formation of univalents, bivalents, trivalents and higher polyvalents, with the consequent production of lagging chromosomes, unbalanced nuclei, micronuclei and sterile pollen. We found that, as represented by the material studied, *E. laevigatum* is an autohexaploid (2n = 6x = 60) in which each chromosome of a basic set of ten chromosomes is repeated six times and that *E. laevigatum* is an essentialy obligate apomictic.

Key words: Eupatorium, apomixis, plant cytogenetics, plant embryology, Asteraceae.

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Introduction

Apomixis is defined as the process by which certain plants produce seeds without fertilization, by parthenogenetic development of an unreduced egg or a somatic cell. It is a genetically controlled reproductive process which eliminates (or modifies) female meiosis and syngamy to produce embryos genetically identical to the mother plant (Dujardin and Hanna, 1994). There are two principal types of apomixis, adventitious embryony and gametophytic apomixis (Nogler, 1984). Adventitious embryony occurs when an embryo is formed directly from a cell of the nucellus or integument of the ovule without the occurrence of the megagametophyte generation (Nogler, 1984; Koltunow, 1993, Khush *et al*, 1994). In gametophytic apomixis there are two distinct mechanisms for producing an unreduced embryo sac, diplospory, in which the embryo sac originates from the megaspore mother cell by mitosis or modified meiosis, and apospory, in which the embryo sac is formed from a somatic cell of the ovule, generally of the nucellus. In both cases, the resultant embryo sac is unreduced and embryo development is parthenogenetic (Asker and Jerling, 1992; Koltunow *et al.*, 1995). Apomixis can be obligate or facultative, depending on whether the seeds produced were formed by entirely asexual processes or with the participation of some sexual mechanisms. Richards (2003) affirmed that only in some diplosporous species does sex completely disappear, although even in these species some variability may persist because of somatic recombination.

Endosperm initiation in apomictic species can be completely independent of pollination (autonomous apomixis) or pollination can be necessary to fertilize the polar nuclei (pseudogamy) with only the egg dividing parthenogenetically (pseudogamic apomixis) (Richards, 1986). The family Compositae heads the list of eight families which have at least 10 apomictic genera, apomixis having been registered in 2.9% of the genera which make up the Compositae. However, embryological studies have been published for only 11.1% of Compositae genera and it is possibly that the number of apomicts in this family is much greater (Czapick, 1996). One Compositae genera in which apomixis has been studied is *Eupatorium*, the first study having been carried out by Holmgren (1919) on *Eupatorium glandulosum* (*Ageratina adenophora*, according to King and Robinson, 1987) with the later studies of Fryxell (1957), Sparvoli (1960) and Sullivan (1976) having demonstrated apomixis in other northern hemisphere species. More recently, apomixis has been demonstrated in

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five Brazilian *Eupatorium* species (Coleman and Coleman, 1984, 1988; Coleman, 1989; Bertasso-Borges and Coleman, 1998) and one species from Argentina (Rozemblum *et al.*, 1988). Taken together, these studies indicate that apomixis is an important reproductive mechanism in several species and sections of *Eupatorium* and it is clear that clarification of the role of apomixis is essential for a better understanding of the systematics and evolution of *Eupatorium*. The objective of this study was to elucidate the embryology and cytogenetics of *E. laevigatum*, a perennial weed species common to much of Brazil (Lorenzi, 1991).

Material and Methods

The seven *Eupatorium laevigatum* plants used in this study were collected in the municipality of Mirassol (São Paulo state, Brazil), voucher specimens are deposited in the herbarium of the Instituto de Botânica de São Paulo (SP) and in the herbarium of the Instituto de Biociências, Letras e Ciências Exatas, São José do Rio Preto (SJRP). Buds for embryological studies were fixed in formaldehyde/acetic acid/ethyl alcohol (FAA). Ovules were dissected from the ovaries, cleared in 4½ clearing solution (Herr, 1971, 1972) and mounted directly in Hoyer's medium (Alexopoulos and Benke, 1952). Buds for the study of microsporogenesis were fixed in 1:3 acetic acid/ethanol. Preparations were stained with acetocarmine and the permanent slides prepared using Hoyer's medium. Analysis of pollen viability was done by staining pollen grains with cotton blue-lactophenol, 175 pistils were examined. Karyotypic studies were carried out using the root-tips from seedlings germinated on humid filter paper in petri dishes, the root-tips being pretreated in 0.002 M 8-hydroxyquinoline for 6 h at 15 ± 3 °C, fixed in 1:3 acetic acid/ethanol, hydrolyzed in 1 N HCl for 5 min at 60 °C, stained with acetocarmine and the apices squashed in Hoyer's medium. The slides were examined using a Zeiss Standard WL photomicroscope equipped with phase contrast and good preparations photographed and prints made at 3750x from which the metaphase chromosomes of three cells were measured. Analysis of seed production was made by direct examination of each achene (the small, one-seeded, indehiscent fruit of this plant) for the presence of a seed (embryo). Germination tests were carried out using 50 one-seeded achenes per plant (a total of 350 achenes for the seven plants tested) by placing the achenes in petri dishes containing humid filter paper and incubating them at 20-25 °C.

Results and Discussion

Of the 3632 achenes obtained from the seven *E. laevigatum* plants examined 28.2% (1025) contained seed (embryo), with values for individual plants ranging from 17.8 to 54.3% (Table 1). Germination tests on the achenes revealed that, overall, 33.42% (117) germinated (Table 1), with values for individual plants being from 20 to 52%. An analysis of 1252 ovules in the initial stages of floral development revealed total absence of megaspore (embryo sac) dyads and tetrads and, therefore, of reductive meiosis (Table 2), the megasporocyte (Figure 1a) functioning directly as a megaspore. The nucleus of the megasporocyte divides to form a binucleate embryo sac (Figure 1b), the nuclei of which migrate to opposite poles (Figure 1c). A second division produces the tetranucleate embryo sac (Figure 1d) and a third division the mature unreduced embryo sac consisting of the egg, two polar nuclei (Figure 2a), two synergids and three antipodals (not shown in Figure 2a), this arrangement being of the *Polygonum* type. The polar nuclei fuse to form the central cell (Figure 2b) and the egg and central cell divide parthenogenetically, the egg producing the embryo and central cell the endosperm. It is interesting to note that the egg initiates embryo formation before the central cell divides to form the endosperm, which results in production

Table 1 - Seed production and germination capacity of *Eupatorium laevigatum*.

Plant	Total achenes examined	Seeds			
		Produced*		Germinated**	
		N	%	N	%
1	641	348	54.3	13	26
2	517	142	27.5	26	52
3	506	118	23.3	20	40
4	506	149	29.4	10	20
5	477	85	17.8	11	22
6	497	96	19.3	19	38
7	488	87	17.8	18	36
Total	3632	1025	28.2	117	33.42

*This data refers to the number of achenes shown in the 'Total achenes examined' column.
**Based on germination tests using 50 achenes (each containing one seed) per plant.

of embryo sacs with both an embryo and a central cell (Figure 2c). The presence of both an embryo and endosperm (Figure 2d) was already observable in the ovules of florets in the initial stages of floral development (*i.e.* unopened buds) and, hence, there would have been no possibility of pollination occurring, which is strong evidence of apomixis. The fact that the embryo sac develops directly from the megasporocyte indicates that the type of apomixis occurring in *E. laevigatum* is diplospory, this diplospory being of the *Antennaria* subtype because megasporocytes with a dumbbell shaped nucleus (characteristic of meiosis with the formation of a restitutional nucleus) were not encountered and no cell wall (characteristic of the Taraxacum subtype) separated the two initial nuclei. Diplospory of the *Antennaria* subtype has been reported for other *Eupatorium* species, *e.g. E. glandulosum* (Holmgren, 1919), *E. riparium* (Sparvoli, 1960), *E. callilepis* (Coleman and Coleman, 1984), *E. squalidum* (Coleman and Coleman, 1988), *E. tanacetifolium* (Rozemblum *et al.*, 1988), *E. odoratum* (Coleman, 1989) and *E. pauciflorum* (Bertasso-Borges and Coleman, 1998).

We analyzed ovule content in three floral stages, the initial stage when the florets were judged to be more than 24 h from anthesis, the pre-anthesis stage in which the flo-

Table 2 - Contents of *Eupatorium laevigatum* ovules at different stages of floral development.

	Floral Stage		
	Initial stage	Pre-anthesis	Anthesis
Plants examined	8	8	8
Ovules analyzed	1252	317	505
Ovule contents	% of total contents		
Aborted embryo sacs	18.29	17.98	19.61
Megasporocytes	13.26	0	0
2-Nucleate embryo sacs	13.58	0	0
4- Nucleate embryo sacs	8.95	0	0
Egg and polar nuclei	15.65	3.16	0.99
Egg and central cell	18.05	21.45	9.70
Embryo and central cell	10.46	33.12	25.54
Embryos and endosperms	1.76	24.29	44.16

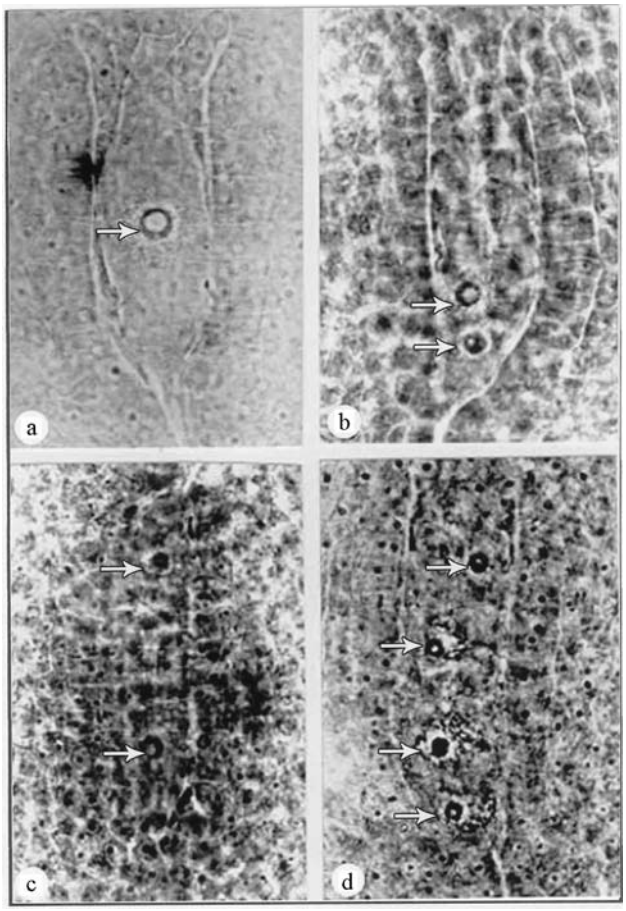

Figure 1 - Embryo sac formation in *Eupatorium laevigatum*. Key: a = Megasporocyte (500x); b = early 2-nucleate embryo sac (620x); c = late 2-nucleate embryo sac (940x); d = 4-nucleate embryo sac (620x).

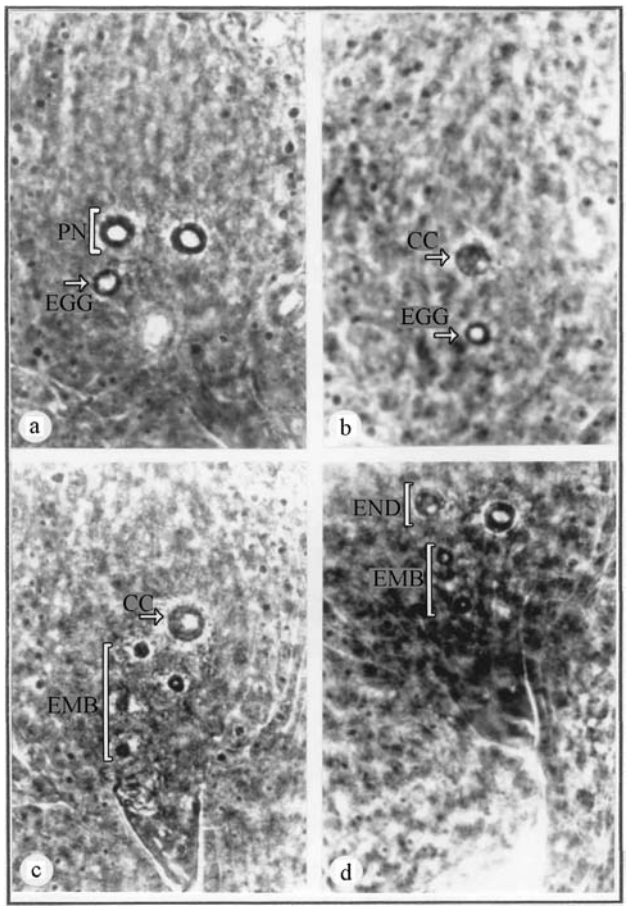

Figure 2 - Embryo sac and embryo formation in *Eupatorium laevigatum*. Key: a = Embryo sac showing egg and two polar nuclei (PN); b = Embryo sac with egg and central cell (CC); c = Embryo sac with embryo (EMB) and central cell (CC); d = Embryo sac with initial divisions of embryo (EMB) and endosperm (END). All 540x.

rets were judged to be within 24 h of anthesis, and during anthesis. Embryos were already detected in 12.22% of initial stage ovules and 57.41% of pre-anthesis ovules (Table 2) but at anthesis only about 10% of the ovules presented an egg. These results indicate precocious embryony to be a strong barrier to sexual reproduction. Of the total of 2074 ovules examined, none showed the presence of a pollen tube, although 687 (33%) ovules contained an embryo. Precocious embryony and the absence of reductive meiosis and pollen tubes indicates that reproduction was occurring apomictically (asexually).

The presence of endosperm in ovules from florets in the initial and pre-anthesis stages (Table 2) indicates that endosperm formation is autonomous. In *E. laevigatum* the two polar nuclei fuse and endosperm initiates from the resulting central cell (Figure 2b). Autonomous endosperm development is prevalent in most apomictic Compositae species although it only occurs sporadically in other families (Nogler, 1984). Autonomous initiation of endosperm from the central cell has also been reported in *E. squalidum* (Coleman and Coleman, 1988), and *E. pauciflorum* (Bertasso-Borges and Coleman, 1998), while in *E. callilepis* (Coleman and Coleman, 1984) and *E. odoratum* (Coleman, 1989) initiation occurs from the polar nuclei. In *E. laevigatum*, embryo development precedes endosperm formation, with Vijayaraghavan and Prabhakar (1984) affirming that this phenomenon is of physiological interest because it is evidence that early embryo development is not sustained by endosperm nutrients but by nutrients transferred to the embryo sac from the surrounding tissues.

Microsporogenesis in *E. laevigatum* showed aberrations in chromosome pairing during diakinesis and metaphase, with the formation of univalents, bivalents, trivalents and higher polyvalents (Figure 3a, b, c). Lagging chromosomes were frequent during anaphase I (Figure 3d) and the loss of univalents resulted in the formation of micronuclei at the first and second divisions (Figure 3d, e). Meiosis resulted in a high frequency not only of tetrads but also dyads, both with or without supernumerary microspores (Figure 4a, b). Restitutional nuclei were not observed but the presence of microspore dyads suggests that they were in fact present. A consequence of these chromosome aberrations pollen viability was very low (4%) (Figure 4c) and infrequent pollination would be expected.

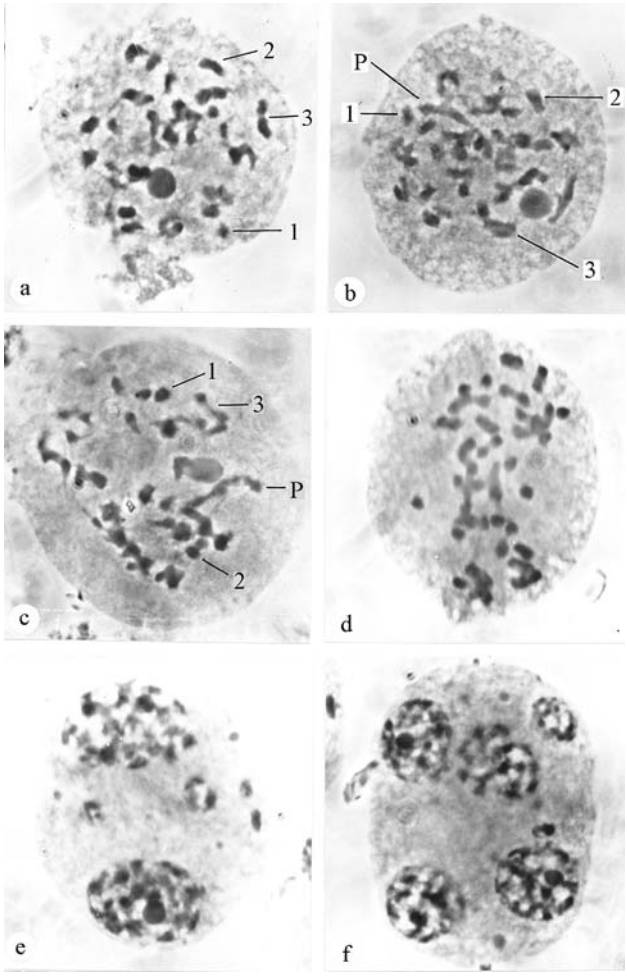

Figure 3 - Microsporogenesis in *Eupatorium laevigatum*. Key: a, b, c = Diakinesis showing univalents (1) bivalents (2), trivalents (3) and higher polyvalents (P); d = Anaphase I with lagging chromosomes; e, f = First and second divisions with micronuclei. Magnification: a = 1450x; b, c = 1350x; d, e = 1500x; f = 1600x.

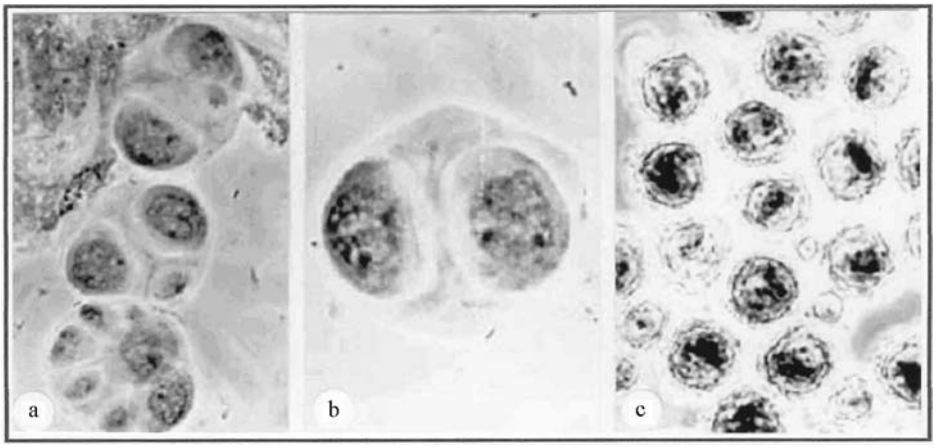

Figure 4 - Microsporogenesis and pollen in *Eupatorium laevigatum*. Key: a = two microspore dyads each with a supernumerary microspore and a polyad of microspores (580x); b = microspore dyad (950x); c = nonviable pollen grains (360x).

Although 105 (60.08%) of the 175 pistils examined showed the presence of pollen grains (Table 3) there was an average of only 1.04 pollen grains per pistil and none of the pollen grains showed a pollen tube. These factors indicate sexual reproduction to be very rare or absent in the material studied, even if some eggs are available at anthesis. Thus, in *E. laevigatum* diplospory assures embryo production and autonomous endosperm development means that male sterility is tolerable. Male sterility has also been demonstrated for *E. callilepis* and *Eupatorium bupleurifolium* (Coleman and Coleman, 1984), *E. squalidum* (Coleman and Coleman, 1988), *E. odoratum* (Coleman, 1989) and *E. pauciflorum* (Bertasso-Borges and Coleman, 1998).

Karyotype analysis of three metaphases revealed six morphologically indistinguishable genomes in the complement (Figure 5), which characterizes the species as being an autohexaploid (2n = 6x = 60). Chromosome length varied from 1.33 µm to 2.09 µm (Table 4). A consequence of the gradual variation in chromosome length was that arm length proportion was essential to recognizing the homologs. Two chromosome 5 satellite chromosomes were visible in one of the three metaphases. Our results show that, based on he classification of Levan *et al.* (1964), there were eight metacentric (1,2,4,6,7,8,9,10) and two submetacentric chromosomes (3 and 5) in the *E.*

Table 3 - Occurrence of pollen grains on *Eupatorium laevigatum* stigmas.

		Pistils with grains		Grains per pistil	
Plant	Number of pistils examined	N.	%	range	mean
1	34	23	67.6	0-4	1.2
2	31	18	58.0	0-4	1.0
3	37	29	78.3	0-6	1.8
4	38	15	39.4	0-3	0.5
5	35	20	57.1	0-3	0.7
Total	175	105	60.08	0-6	1.04

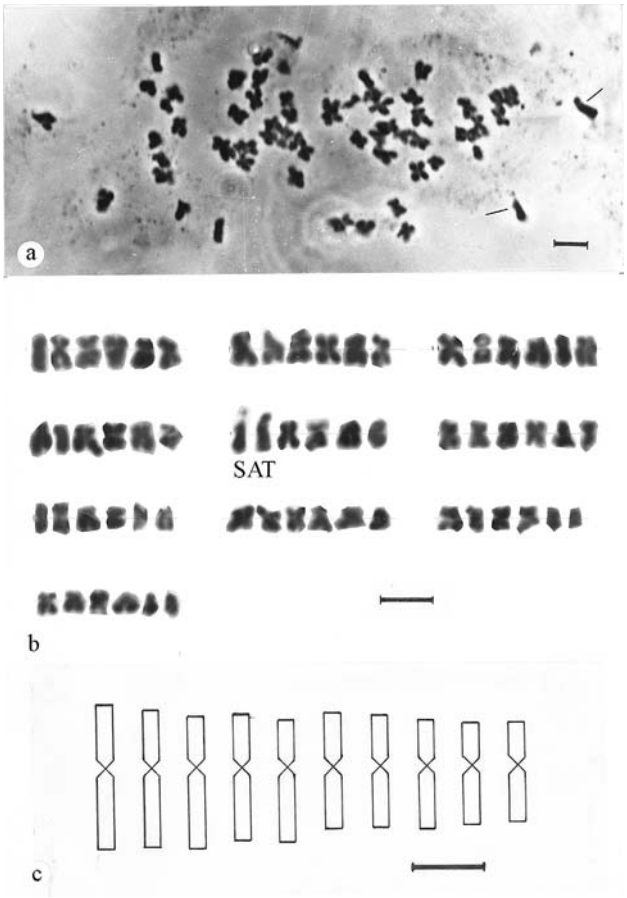

Figure 5 - Mitotic chromosomes in *Eupatorium laevigatum*. Key: a = Mitotic metaphase showing 60 chromosomes; b = karyotype; c = ideogram. Bar represents 5µm (a, b) and 1 µm (c).

Table 4 - Basic chromosome set of *Eupatorium laevigatum*.

	Arm length (Mean (µm) ± standard error)					
Chromosome	Long arm	Short arm	Total arm length	Arm ratio	% Total length of set*	Type**
1	1.19 ± 0.03	0.89 ± 0.02	2.09 ± 0.04	1.36	12.46	M
2	1.14 ± 0.05	0.80 ± 0.03	1.94 ± 0.04	1.47	11.57	M
3	1.16 ± 0.02	0.68 ± 0.02	1.84 ± 0.04	1.72	10.97	SM
4	1.06 ± 0.05	0.73 ± 0.02	1.79 ± 0.03	1.52	10.67	M
5	1.09 ± 0.03	0.61 ± 0.01	1.71 ± 0.03	1.80	10.20	SM
6	0.88 ± 0.01	0.75 ± 0.01	1.64 ± 0.03	1.17	9.78	M
7	0.88 ± 0.03	0.69 ± 0.02	1.52 ± 0.06	1.33	9.06	M
8	0.91 ± 0.01	0.60 ± 0.02	1.51 ± 0.02	1.55	9.01	M
9	0.83 ± 0.01	0.56 ± 0.01	1.40 ± 0.02	1.49	8.35	M
10	0.77 ± 0.02	0.56 ± 0.01	1.33 ± 0.01	1.40	7.93	M

*16.77 µm.
**M = metacentric , SM = submetacentric.

laevigatum karyotype and that the karyotype can be classified as symmetrical because the chromosomes vary gradually in length and the centromeres are median or submedian. Specimens of *E. laevigatum* (*Chromolaena laevigata,* King and Rob) from Ecuador have been reported as having a karyotype of n = 20 (King *et al*., 1976) while *E. laevigatum* from Argentina as having 2n = 50 (Watanabe *et al*., 1995).

Our investigation indicates that the *E. laevigatum* specimens were essentially obligatorily apomictic, as supported by the absence of reductional meiosis during megasporogenesis, precocious embryo and endosperm formation, male sterility, absence of pollen tubes in pollen grains on stigmas and the autohexaploid nature of karyogram.

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