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EMBRYOLOGY AND CYTOGENETICS OF SEXUAL TETRAPLOID *Eupatorium inulaefolium* H.B.K.

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ABSTRACT

Embryological studies show *Eupatorium inulaefolium* to regularly undergo reductive meiosis with tetrad formation during megasporogenesis, followed by monosporic embryo sac development of the polygonum type. The polar nuclei mostly fuse before anthesis, but endosperm formation is generaly initiated only after the embryo is already several-celled. Meiosis of microsporogenesis results in the regular formation of 20 bivalents; subsequent stages of microsporogenesis are normal, and stigmatic loads indicate the regular occurrence of pollination with viable, functional grains. Karyotypic studies revealed a complement of 40 chromosomes separable into 20 pairs, 15 with median and five with submedian centromeres, which have mean lengths ranging from 2.65 μm to 3.64 μm. The complement shows two SAT-chromosomes which are characterized by having the highest arm ratio. It is concluded that *E. inulaefolium*, as represented by the material studied, is of allotetraploid origin and is probably strictly sexual.

INTRODUCTION

Apomixis has been demonstrated in several species of *Eupatorium* (*sensu lato*), most frequently being associated with triploidy (Holmgren, 1919; Sparvoli, 1958; Sullivan, 1976; Coleman and Coleman, 1984, 1988; Rozenblum *et al.*, 1988) but also having been demonstrated in tetraploid (Sullivan, 1976) and hexaploid (Coleman, 1989) material. Although apomixis is clearly of widespread occurrence in the genus, its exact systematic distribution is far from evident. Indeed, the very systematics of the group is controversial, as a consequence of the extensive redefini-

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tion of the genera of the Eupatorieae initiated by King and Robinson some 25 years ago (see King and Robinson, 1987).

The determination of apomixis or strict sexuality in species of different ploidy levels is fundamental for a better understanding of the evolution and systematics of the group, since diploid and sexual species can be expected to be ancestral to polyploid and apomictic species. Analyses of karyotype and pairing behavior are of considerable potential significance in determining auto- or allopolyploidy and, consequently, whether hybridization was intra- or interspecific, as well as in suggesting possible ancestral species or species groups of both apomictic and sexual polyploids, and their interrelationships.

The purpose of the present paper is to report on the embryology and cytogenetics of sexual tetraploid (2n =4x = 40) *Eupatorium inulaefolium* H.B.K. (= *Austroeupatorium inulaefolium* (H.B.K.) R.M. King and H. Robinson.

MATERIAL AND METHODS

Material of *Eupatorium inulaefolium* used in this study was collected in the city of Mirassol, state of São Paulo, Brazil. Voucher specimens are deposited in the Herbarium of the Jardim Botânico de Rio de Janeiro (RJ). Buds for embryological studies were fixed in FAA. Ovules were dissected from the ovaries, cleared in 4 1/2 clearing solution (Herr, 1971) and mounted directly in Hoyer's medium (Alexopoulos and Benke, 1952). Ovule content was analyzed in three floral stages: initial stages (judged to be more than 24 hours from anthesis), pre-anthesis (judged to be within 24 hours of anthesis) and anthesis. Buds for the study of microsporogenesis were fixed in 1:3 acetic acid-ethanol. Staining was done with acetocarmine and slides were made permanent with Hoyer's medium. Karyological studies were done using root tips of seedlings obtained by germinating achenes on humid filter paper in petri dishes. Root tips were pretreated in 0.002 M 8-hydroxyquinoline during 7 hours at 12° to 18°C and fixed in 1:3 acetic acid-ethanol. Hydrolysis was done in 6% HCl for 5 minutes at 60°C. After staining with acetocarmine, the apices of the root tips were squashed directly in Hoyer's medium. Selected cells were photographed and prints for analysis made at 3750X; metaphase chromosomes of five cells were measured. A Zeiss Standard WL photomicroscope equipped with phase contrast was used for all microscopical work.

RESULTS AND DISCUSSION

Eupatorium inulaefolium is a perennial herb or subshrub widely distributed in South America and widely adventive in the eastern hemisphere (King and Robin-

son, 1975). The species is treated by Baker (1876), as *E. pallescens* DC., in section Heterolepis (Critinioides) and by King and Robinson (1975) in the genus *Austroeupatorium*. Regardless of the treatment followed, apparently no close relative of *E. inulaefolium* has previously been studied embryologically or karyotypically, nor has the species itself.

The analysis of the contents of clarified ovules showed that the megasporocyte (Figure 1A) undergoes a first meiotic division to form a dyad (Figure 1B), which in turn rapidly undergoes a second meiotic division (Table I) to produce a tetrad of presumedly reduced megaspores (Figure 1C). The high frequency of tetrads observed (Table I) indicates reduction to be a regular feature of megasporogenesis, as well as a stage of relatively long duration, and the material studied to be strictly sexual.The functional chalazal megaspore enlarges to form a 1-nucleate embryo sac which crushes the three micropylar megaspores (Figure 1D). The embryo sac passes relatively rapidly through the 2- and 4-nucleate stages (Figure 1E,F; Table I), resulting in an 8-nucleate embryo sac composed of two sinergids, the egg cell, two polar nuclei (Figure 2A) and three antipodals (Figure 2D). Embryo sac formation is thus of the polygonum type, which is apparently typical for the genus.

Table I - Contents of ovules of *Eupatorium inulaefolium* at different stages of floral development.

Contents	Floral stage					
	Initial stages		Pre-anthesis		Anthesis	
Plants examined	5		5		5	
Ovules analyzed	874		382		574	
Aborted embryo sacs	9	(1.03%)	3	(0.79%)	18	(3.14%)
Megasporocytes	39	(4.46%)	0		0	
Dyads	13	(1.49%)	0		0	
Tetrads	169	(19.34%)	0		0	
1-nucleate embryo sacs	53	(6.06%)	1	(0.26%)	0	
2-nucleate embryo sacs	44	(5.03%)	2	(0.52%)	0	
4-nucleate embruo sacs	70	(8.01%)	5	(1.31%)	0	
Egg and polar nuclei	211	(24.14%)	48	(12.57%)	2	(0.35%)
Egg and central nucleus	266	(30.34%)	323	(84.55%)	344	(59.93%)
Egg and endosperm	0		0		1	(0.17%)
Embryo and central nucleus	0		0		7	(1.22%)
Embryo and endosperm	0		0		202	(35.19%)

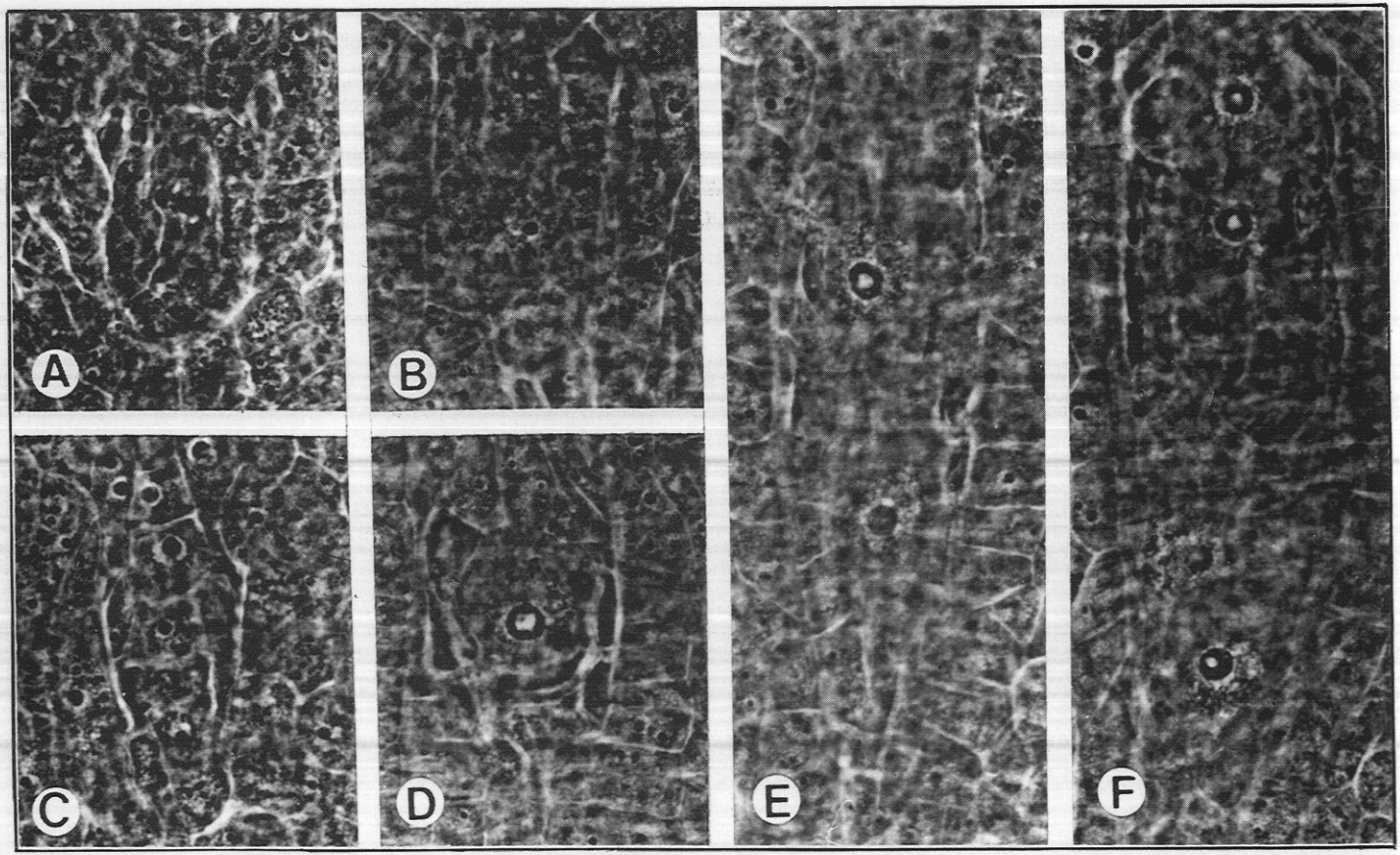

Figure 1 - Embryo sac formation in *Eupatorium inulaefolium*. A, Megasporocyte, MI; B, dyad; C, tetrad of megaspores; D, 1-nucleate embryo sac with crushed megaspores; E, 2-nucleate embryo sac; F, 4-nucleate embryo sac. A, E and F, 1250X; B, C and D, 1000X.

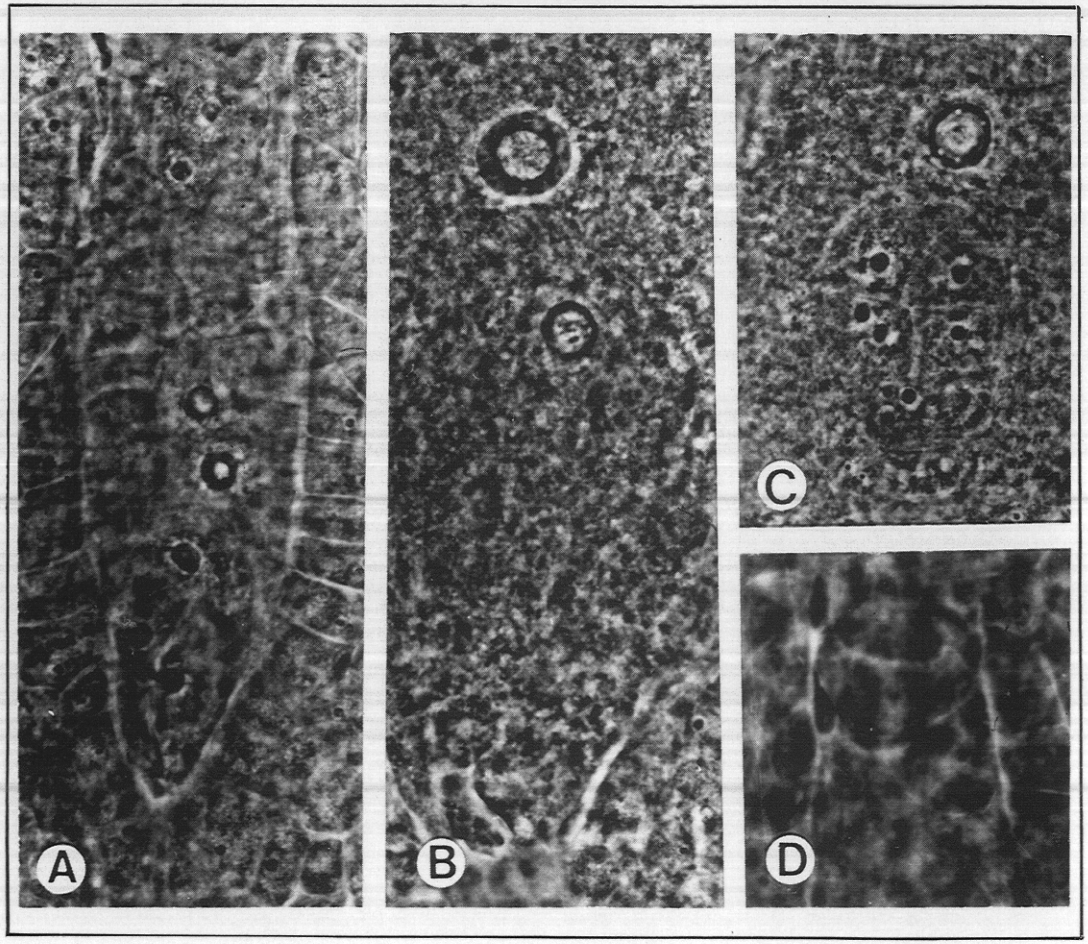

Figure 2 - Mature embryo sac structure and embryo formation in *Eupatorium inulaefolium*. A, Mature embryo sac showing sinergids, egg and polar nuclei; B, embryo sac with egg and central nucleus; C, embryo and central nucleus; D, three antipodals. A and B, 1250X; C, 750X; D, 450X.

The polar nuclei regularly fuse to form the central cell nucleus (Figure 2B), usually before anthesis is reached (Table I). The egg cell invariably attains anthesis still undivided (Table I), thereby precluding the possibility of precocious embryony, which has been observed in most of the apomictic species of the genus thus far studied embryogically. Endosperm formation is mostly delayed until after the embryo is already several-celled (Figure 2C; Table I), a feature which has also been observed in diplosporous triploid *E. squalidum* (Coleman and Coleman, 1988). Relatively few cases of embryo formation preceding endosperm initiation have been reported, and some of these have later been proved to be erroneous (Brink and Cooper, 1947). The further substantiation of this phenomenon is of physiological interest since it provides irrefutable evidence to support the position that early embryo development is not sustained by nutrients furnished by the endosperm, but rather by nutrients transferred into the embryo sac from surrounding tissues (Vijayaraghavan and Prabhakar, 1984).

The analysis of chromosome pairing at diakinesis and metaphase I in 33 microsporocytes of five plants of *E. inulaefolium* revealed the uniform occurrence of 20 bivalents (Figure 3). Although chromosome stickiness was a common feature, all aspects of microsporogenesis and pollen formation were normal. Coleman (1968) reported 10 bivalents for two collections identified as *E. pallescens* DC. from mountainous regions of the states of São Paulo and Rio de Janeiro. It is uncertain whether those collections were conspecific with the material of the present study. At any rate, diploid populations evidently occur within the *E. inulaefolium* species group and possibly within the species itself.

Figure 3 - Metaphase I of microsporogenesis in *Eupatorium inulaefolium* showing 20 bivalents. 1750X.

The regularity of microsporogenesis and pollen formation indicate high male fertility for the material studied, which is in accord with its probable sexual nature. Also, the determination of pollen loads for 25 stigmas for each of five plants showed that 71.2% of all stigmas carried one or more apparently germinated grains, the individual plants having 52% to 88% of stigmas with grains. These figures indicate the regular occurrence of pollination with viable, functional grains.

The karyotype of the material studied showed 40 chromosomes which could be separated into 20 putative pairs (Figure 4). Mean chromosome length ranged from 2.65 μm to 3.64 μm (Table II). Because of the gradual variation in chromosome length, the additional use of arm ratio was essential for the matching of homologs, which nevertheless was frequently arbitrary. Following the terminology of Levan *et al.* (1964), 15 chromosomes of the haploid set have median and five have submedian centromeres. Few cells showed the presence of SAT-chromosomes and none showed more than two. The satellites apparently occur on the chromosome pair with the highest arm ratio, which is also one of the shortest of the complement (Figure 4), corresponding to chromosome 17 (Figure 5; Table II).

Table II - Mean arm length values (μm) with standard errors and percent of total length (62.82 ± 2.63) for the basic chromosome set of *Eupatorium inulaefolium*.

Chromosome number	Long arm	Short arm	Total length	Arm ratio	% total length of set
1	2.04 ± 0.08	1.59 ± 0.08	3.64 ± 0.16	1.29 ± 0.02	5.79 ± 0.37
2	2.24 ± 0.09	1.25 ± 0.04	3.49 ± 0.13	1.79 ± 0.04	5.56 ± 0.30
3	2.08 ± 0.11	1.40 ± 0.07	3.48 ± 0.18	1.49 ± 0.03	5.54 ± 0.43
4	2.00 ± 0.04	1.48 ± 0.03	3.48 ± 0.07	1.35 ± 0.03	5.55 ± 0.15
5	1.92 ± 0.06	1.54 ± 0.04	3.46 ± 0.09	1.25 ± 0.03	5.50 ± 0.23
6	2.10 ± 0.06	1.26 ± 0.04	3.36 ± 0.08	1.68 ± 0.06	5.35 ± 0.17
7	1.89 ± 0.05	1.39 ± 0.03	3.28 ± 0.08	1.36 ± 0.02	5.21 ± 0.19
8	1.76 ± 0.08	1.52 ± 0.08	3.28 ± 0.16	1.16 ± 0.02	5.23 ± 0.37
9	2.16 ± 0.08	1.11 ± 0.05	3.27 ± 0.13	1.96 ± 0.06	5.20 ± 0.30
10	1.63 ± 0.05	1.50 ± 0.05	3.13 ± 0.11	1.08 ± 0.01	4.99 ± 0.25
11	1.72 ± 0.05	1.40 ± 0.04	3.12 ± 0.09	1.24 ± 0.02	4.97 ± 0.21
12	1.82 ± 0.11	1.21 ± 0.06	3.03 ± 0.17	1.49 ± 0.02	4.82 ± 0.40
13	1.70 ± 0.04	1.29 ± 0.40	2.99 ± 0.08	1.31 ± 0.02	4.77 ± 0.18
14	1.59 ± 0.04	1.37 ± 0.05	2.96 ± 0.08	1.17 ± 0.02	4.70 ± 0.17
15	1.68 ± 0.06	1.24 ± 0.05	2.92 ± 0.10	1.37 ± 0.03	4.64 ± 0.23
16	2.07 ± 0.10	0.83 ± 0.04	2.90 ± 0.13	2.49 ± 0.06	4.61 ± 0.29
17	2.08 ± 0.06	0.77 ± 0.03	2.86 ± 0.08	2.71 ± 0.10	4.55 ± 0.17
18	1.76 ± 0.08	1.05 ± 0.03	2.81 ± 0.11	1.67 ± 0.05	4.47 ± 0.25
19	1.82 ± 0.07	0.91 ± 0.03	2.73 ± 0.09	2.02 ± 0.06	4.34 ± 0.22
20	1.56 ± 0.06	1.09 ± 0.04	2.65 ± 0.10	1.42 ± 0.03	4.22 ± 0.24

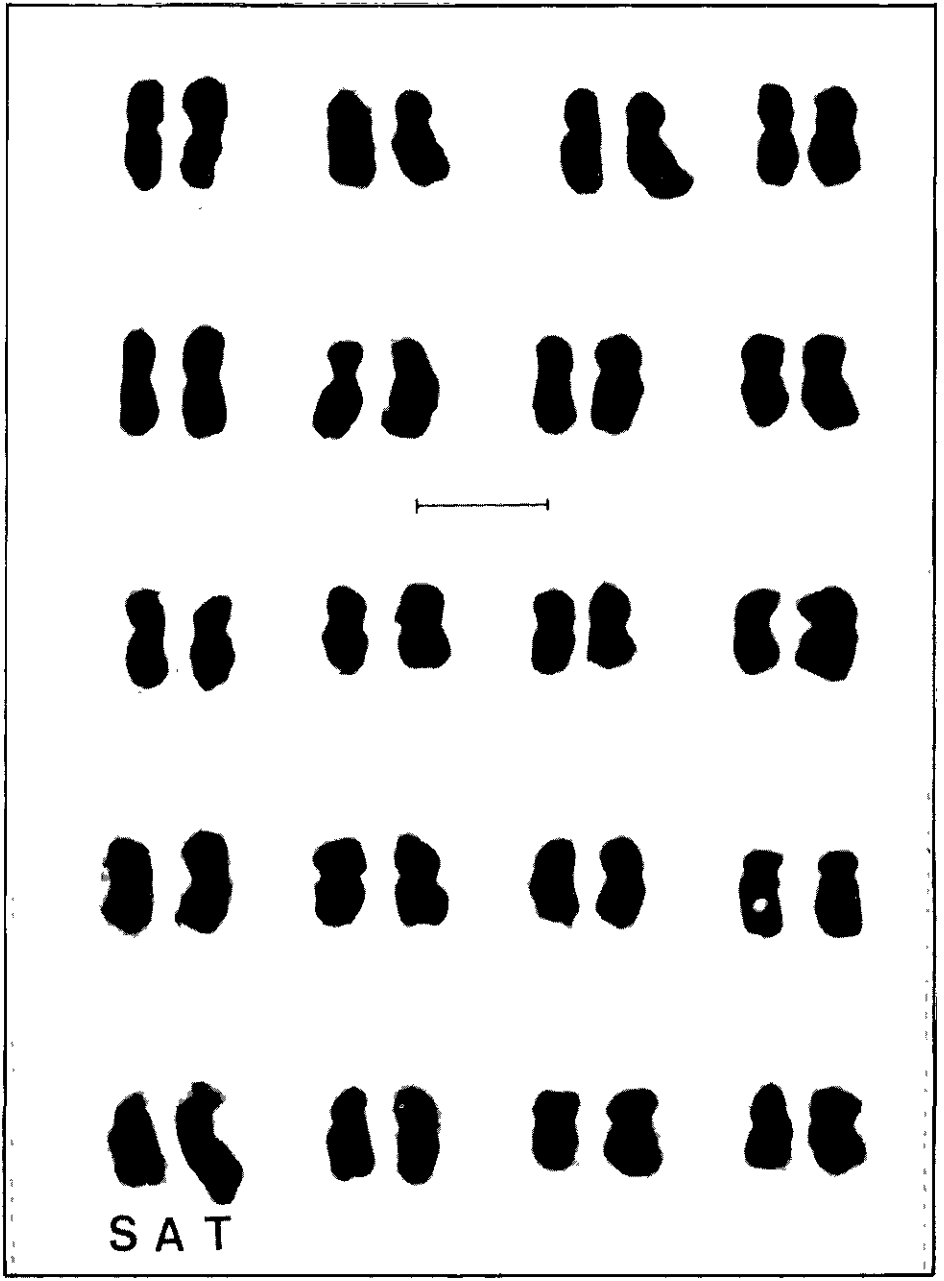

Figure 4 - Karyotype of *Eupatorium inulaefolium* indicating presence of 40 chromosomes, two of which bear satellites. Bar represents 5 μm.

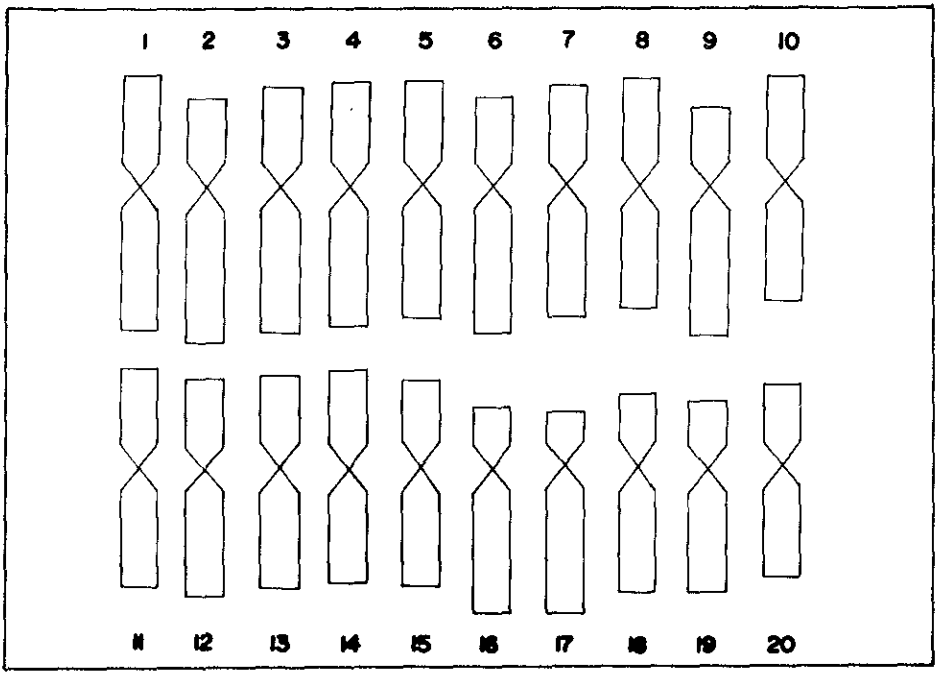

Figure 5 - Ideogram of *Eupatorium inulaefolium*.

The regular formation of bivalents during microsporogenesis indicates the material to be of allotetraploid origin. The karyotype supports this conclusion, although also indicating that the two chromosome sets involved are relatively undifferentiated morphologically. This contrasts with the situation of diplosporous *E. squalidum* and *E. odoratum* which were determined to be, respectively, autotriploid and autohexaploid (Coleman and Coleman, 1984; Coleman, 1989) and of aposporous *E. bupleurifolium*, also determined to be autotriploid (Coleman and Coleman, 1984).

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RESUMO

Estudos embriológicos de *Eupatorium inulaefolium* demonstraram a ocorrência regular de meiose redutiva com a formação de tétrades durante a megasporogênese, seguida pela formação monospórica do saco embrionário do tipo polygonum. Os núcleos polares geralmente se fundem antes da ântese, porém a formação do endosperma geralmente se inicia apenas quando o embrião já possui várias células. Meiose de microsporogênese revelou a formação regular de 20 bivalentes; as fases subseqüentes da microsporogênese, bem como a formação de pólen, são normais, e os estigmas indicaram a presença de grãos de pólen viáveis e funcionais. Estudos de cariótipo revelaram que o complemento é constituído de 40 cromossomos, separáveis em 20 pares, 15 com centrômeros medianos e cinco com centrômeros submedianos, e com comprimentos que variam entre 2,65 μm e 3,64 μm. O complemento possui dois cromossomos SAT que são caracterizados por terem a razão dos braços mais elevada do complemento. Conclui-se que *E. inulaefolium*, como representada pelo material estudado, é de origem alotetraplóide e é provavelmente estritamente sexuada.

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