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Cytogenetics of *Hypericum caprifoliatum* Cham. & Schltdl. (Clusiaceae) populations and other species of the genus

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ABSTRACT - *Chromosome numbers and meiotic behavior are presented for the first time for* Hypericum caprifoliatum *(2n=48),* H. cf carinatum *(2n=48) and* H. polyanthemum *(2n= 32) and confirmed the* H. perforatum *(2n=32) number. Chromosomes are very small (ca. 1 to 2 mm). Meiotic irregularities, such as uni and multivalents, un-oriented chromosomes, bridges and laggards were very frequent in* H. caprifoliatum *and* H. cf carinatum. *Meiotic indexes ranged from 44.0 to 73.0% in* H. caprifoliatum, *66.0 to 77.0% in* H. cf carinatum *and was 80.0% for the* H. polyanthemum *population. Pollen fertility ranged from 42.0 to 88.0% in* H. caprifoliatum, *65.0 to 88.0% in* H. cf carinatum *and was 89.0% for* H. polyanthemum. *Together with literature and meiotic behavior data, the great variability in pollen size and fertility within and among populations of* H. caprifoliatum *and* H. cf carinatum *could suggest that, as many other* Hypericum *species, these taxa are apomictic.*

Key words: *Hypericum*, chromosome number, meiotic behavior, pollen fertility, apomixis.

INTRODUCTION

The genus *Hypericum* L. (Clusiaceae) has around 460 species, divided in 30 sections (Robson 1981), characterized by several types of secretory structures, where synthesis and/or accumulation of biologically active substances occur. Originated in Europe and Asia, the genus is widely distributed across tropical and subtropical regions, absent only in extremely dry or humid areas (Robson 2006). Many species are widely used in folk medicine, due to their phytotherapic properties. The most studied and used species is the Eurasian *H. perforatum* L. (St. John's wort) (section *Hypericum*) due to its anti-inflammatory, analgesic and anti-depressive properties (Couceiro et al. 2006). In Brazil, mainly in the southern and southeastern regions, there are around 20 native *Hypericum* species, belonging to the sections *Brathys* and *Trigynobrathys*. Based on chemical and pharmacological evaluations, it was observed that extracts of *H. caprifoliatum* Cham & Schlecht ("escadinha") have potential anti-depressive activity (Daudt et al. 2000, Viana et al. 2005). Other phytotherapeutic effects of other Brazilian *Hypericum* species have also been reported, such as anti-microbial

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(Dall'Agnol et al. 2003), anti-septical (Avancini and Wiest 2002), anti-proliferative (Ferraz et al. 2004) and anti-oxidant (Bernardi 2007) action.

The chromosome numbers in the genus are known of around 115 (25.00%) species and the pollen viability has been estimated for less than 10% of the species, but meiotic studies are even rarer. Despite some suggestions of a variety of basic chromosome numbers of x= 7, 8, 9, 10, 12 or even higher, the most common and accepted basic chromosome number is x=8. (Nielsen 1924, Robson and Adams 1968, Kogi 1984, Matzk et al. 2003, Moraes 2007). The diploid species are normally obligate sexual whereas polyploid species may be sexual or apomictic (generally facultative). *H. perforatum* is facultative apomictic, with diploid (2n=2x=16), tetraploid (2n=4x=32) and hexaploid (2n=6x=48) populations (Matzk et al. 2003, Mayo and Lamgridge 2003). Specifically for the Brazilian species information is available on the chromosome numbers, meiotic behavior and pollen fertility of *H. brasiliense* Choisy (2n=16), *H. cordatum* Vell. (2n=56) and *H. ternum* A. St. Hill (2n=36) (Moraes 2007) and pollen fertility of *H. caprifoliatum* and *H. connatum* Lam (Clarke 1975).

This study aimed to determine the chromosome number, analyze meiosis and estimate pollen fertility in three native Brazilian species: *H. caprifoliatum, H.* cf *carinatum* Griseb., *H. polyanthemum* Klotzsch ex Reichardt and the exotic *H. perforatum*.

MATERIAL AND METHODS

A total of 17 populations of three species native to southern Brazil: *H. caprifoliatum* (14 populations), *H.* cf *carinatum* (two populations), *H. polyanthemum* (one population) and one of *H. perforatum* (commercial seeds) were analyzed. Young flowers and, when possible, whole living plants, were collected from natural or cultivated populations in Rio Grande do Sul, southern Brazil (Table 1).

The plants were transplanted to pots with garden soil in a greenhouse. For *H. perforatum,* the seeds were germinated in Petri dishes and then transferred to pots, but the plants did not flower.

Table 1. *Hypericum* populations analyzed, collection site (county) and population type

Population number	Collection site	Population type
H. caprifoliatum		
1	Porto Alegre, RS (-30º 01', -51º 13')	anthropogenic
2	Eldorado do Sul, RS (-30º 05', -51º 36')	wild
3	Porto Alegre, RS (-30º 01', -51º 13')	wild
4	Sananduva, RS (-27º 56', -51º 48')	wild
5	Viamão, RS (-30º 04', -51º 01')	wild
6	Lagoa Vermelha, RS (-28º 12', -51º 31')	wild
7	Galópolis, RS (-29º 23', -51º 17')	wild
8	Campestre da Serra, RS (-28º 47', -51º 05')	wild
9	Marcelino Ramos, RS (-27º 27', -51º 54')	wild
10	Triunfo, RS (-29º 56', -51º 43')	wild
11	Estrela, RS (-29º 30', -51º 57')	wild
12	Lajeado, RS (-29º 28', -51º 57')	wild
13	Boa Vista do Sul, RS (-29º 21', -51º 40')	wild
14	Carlos Barbosa, RS (-29º 17', -51º 30')	wild
H. cf *carinatum*		
15	Eldorado do Sul, RS (-30º 05', -51º 36')	wild
16	Tabaí, RS (-29º 38', -51º 40')	wild
H. polyanthemum	Porto Alegre, RS (-30º 01', -51º 13')	antropogenic
17		
H. perforatum	Commercial Seed	cand ultivated
18		

[a] geographical coordinates

Somatic chromosome numbers were determined in the root-tip cells. Roots with a length of about 0.5-1.0 cm were pre-treated in saturated solution of paradichlorobenzene at 4 ºC for 24 hrs, fixed in 3:1 ethanol-acetic for 24 h, and stored in 70% ethanol below 0º C until required. The roots were treated with 1N HCl at 60 ºC for 6 min, stained by the Feulgen method and squashed in 2% propionic carmine. At least 10 well-spread cells were analyzed per population

To determine the gametic number and study meiosis, young flowers collected in the field or from the plants in the greenhouse were fixed in a mixture of 3:1 ethanol-acetic for 24 h and stored in 70% ethanol below 0 ºC. Slides were prepared by squashing the anthers in 2% propionic carmine. The gametic chromosome number was determined in at least 10 cells. For *H. caprifoliatum* and *H.* cf *carinatum* the gametic chromosome number was best determined at anaphase. In the meiotic behavior analyses, where all available meiotic phases were examined, the number of cells analyzed varied greatly among populations. Due to the difficulty of a good resolution of all meiotic configurations in *H. caprifoliatum* and *H.* cf *carinatum*, all cells with chromosomes not aligned at the equatorial plate, with bridges, laggards or micronuclei were recorded as abnormal. Meiotic indexes were estimated following Love (1949). Tetrads with 4 equal-sized cells were considered normal and any deviation as abnormal. Pollen fertility was estimated, as traditional in cytogenetics, by stainability. However, since all grains were stained (2% acetic orcein) and a great variation in pollen size had been observed in preliminary tests for *H. caprifoliatum* and *H. cf. carinatum*, pollen fertility was estimated by pollen size (mean diameter of the longitudinal and transversal axis) of 150 mature grains per population. Grains in the most frequent size classes were considered as potentially fertile, while very small or very big grains were excluded. To validate this assumption of estimating pollen fertility by pollen grain size, an *in vitro* germination test was performed, following the methodology described by Moraes (2007), with one population of *H. caprifoliatum* (population 1), and one of *H. polyanthemum* (population 17) and the final pollen germination percentage was measured after 24 h.

Results were recorded by interpretative drawings, photomicrographs and digital images.

RESULTS AND DISCUSSION

These are new pieces of information on chromosome number and meiotic behavior for *H. caprifoliatum, H.* cf *carinatum* and *H. polyanthemum*, as well as estimates of pollen fertility for *H.* cf *carinatum* and *H. polyanthemum.* Pollen fertility (stainability) of *H. caprifoliatum* has been studied by Clarke (1975).

Data of chromosome number determinations (2n and n) are presented in Table 2. The chromosomes are small (ca 1.0-2.0 mm), in agreement with Moraes (2007). The somatic chromosome number was determined in three populations: one population of *H. caprifoliatum* (Figure 1A-B), one of *H.* cf *carinatum*, both with 2n=8x=48 and one of *H. perforatum* (2n=6x=32). These were the only plants of *H. caprifoliatum* and *H.* cf *carinatum* that survived transplantation from the field to the greenhouse. The chromosome number of the other populations was estimated in pollen mother cells in meiosis.

If the basic chromosome number of *Hypericum* is accepted as x=8, *H. caprifoliatium* and *H.* cf. *carinatum* are hexaploid and *H. polyanthemum* (2n=4x=32) is tetraploid These results agree with those of Matzk et al. (2003) and Moraes (2007) who evaluated other species of the *Trigynobrathys* section and found x=8 for *H. brasiliense, H. cordatum* and *H. japonicum* Thunb. The *H. perforatum* population studied was tetraploid (2n=4x=32).

H. polyanthemum had a predominantly regular meiotic behavior and 16 bivalents were observed at diakinesis and metaphase I in most cells analyzed (Table 2, Figure 1D, 1G). For *H. caprifoliatum* and *H.* cf *carinatum*, during the meiotic analysis (Table 2), the exact chromosome associations in most cells at diakinesis (Figure 1C), and metaphase I (Figure 1 F) as well as the exact pattern of chromosome segregation in many anaphases and telophases could not be clearly interpreted. Irregularities such as un-oriented chromosomes (Figure 1E), univalents and multiple associations at metaphase I (Figure 1F-G), laggards and bridges at anaphase and telophases I and II (Figure 1H), and micronuclei at telophase II (Figure 1I) were observed.

There are few studies on meiosis in *Hypericum.* Moraes (2007), found scarce meiotic irregularities for *H. brasiliense* (2n=2x=16), but a predominantly abnormal meiosis for *H. cordatum* (2n=7x=56) and *H.*

Table 2. Chromosome number, meiotic behavior, meiotic indexes, mean diameter of pollen grains, grain size limits of possible fertile pollen and estimated pollen fertility in *Hypericum* species

Population	2n	n	Number of cells at Metaphase I[a] Telophase I[b]	Number of cells at Anaphase I	Number of cells at Metaphase II[b] Telophase II[b]	Number of cells at Anaphase II	Meiotic index (%)	MD (µm)[c] Mi	Ma	Size limits of potentially fertile grains(µm)	Estimated Pollen fertility (%)
H. caprifoliatum											
1	48	24	83 (55)	45 (13)	1	127 (57)	54.0	7.5	38.0	20.0 - 30.0	59.0
2		24	19 (10)	14 (8)	-	42 (21)	56.0	5.0	38.5	20.0 - 30.0	44.0
3		24	74(66)	17 (8)	13 (7)	78 (24)	68.0	5.0	56.0	17.0 - 29.0	69.0
4		24	271 (256)	101 (48)	28 (16)	54 (17)	73.0	5.0	32.5	18.5 - 29.8	88.0
5		24	325 (202)	22 (9)	12 (7)	32 (11)	71.0	5.0	31.5	20.4 - 31.4	68.0
6		24	51 (32)	1 (1)	-	18 (7)	68.0	10.0	31.0	20.2 - 30.4	71.0
7		24	266 (245)	19 (18)	5 (2)	30 (16)	51.0	5.0	31.0	19.0 - 29.0	64.0
8		24	69 (64)	10 (10)	8 (6)	33 (17)	50.0	8.0	37.5	19.5 - 28.7	51.0
9		24	108 (66)	38 (24)	3 (3)	36 (11)	70.0	5.0	33.5	18.5 - 29.8	57.0
10		24	491 (221)	20 (16)	2 (1)	90 (29)	71.0	9.0	32.0	19.8 - 30.8	83.0
11		24	109 (95)	11 (11)	-	33 (21)	44.0	11.5	66.0	20.0 - 32.7	42.0
12		24	71 (61)	38 (29)	2 (1)	34 (18)	56.0	10.0	35.0	20.0 - 30.0	48.0
13		24	56 (19)	2 (1)	-	47 (23)	54.0	8.0	29.0	19.9 - 30.1	57.0
14		24	46 (36)	6 (3)	-	54 (28)	58.0	10.0	54.0	17.0 - 31.0	45.0
H. cf *carinatum*											
15	48	24	203 (194)	84 (69)	31 (21)	69 (26)	77.0	7.5	40.0	18.1 - 31.4	88.0
16		24	19 (17)	8 (5)	-	38 (24)	66.0	12.0	48.5	18.0 - 33.0	65.0
H. polyanthemum											
17		16	-	7 (3)	3 (2)	1(1)	80.0	18.5	25.0	20.0- 23.5	89.0
H. perforatum											
18	32		-	-	-	-	-	-		-	-

[a] No. of cells with unoriented chromosomes shown in brackets
[b] No. of cells with bridges and laggards shown in brackets
[c] MD: mean diameter of pollen grains; Mi- minimum; Ma-maximum

ternum (2n=4x=36), and reported some of the irregularities described here. The same author studied the mode of reproduction of these species and concluded that *H. brasiliense* is preferentially allogamous and that reproduction by apomixis could not be ruled out for *H. cordatum*. Hoar and Haertl (1932) reported meiotic irregularities for the apomictic polyploid *H. perforatum* and an apparent regular meiosis for the sexual diploid *H. arnoldianum*, *H. lobocarpum*, *H. prolificum* and *H. gentianoides*.

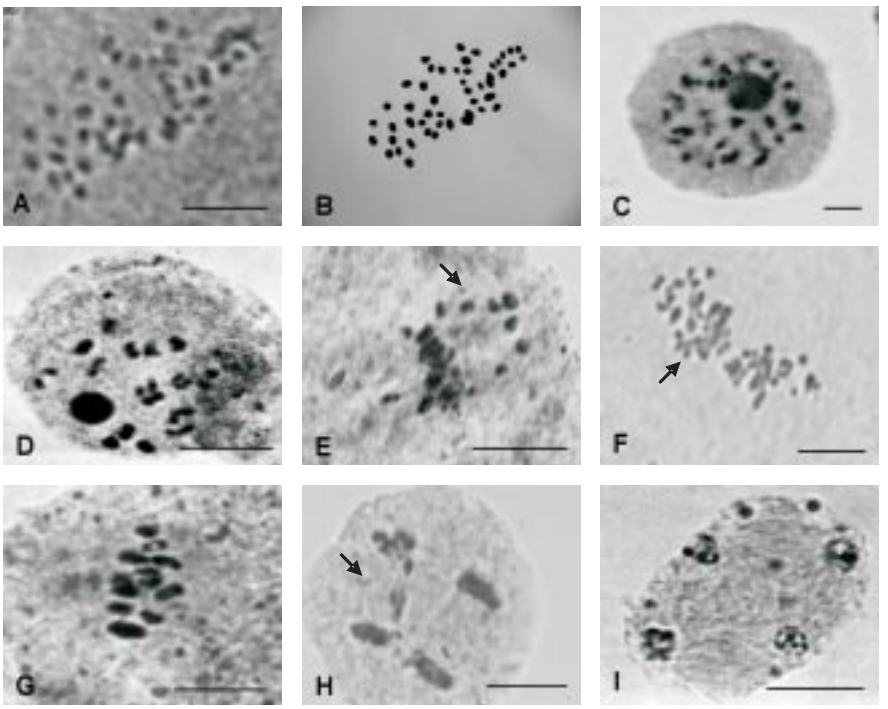

Figure 1. A) population 1 of *H. caprifoliatum*, 2n=48; B) schematic drawing of figure 1A; C) diakinesis in population 1 of *H. caprifoliatum*; D) diakinesis in population 17 of *H. polyanthemum*, with 16 bivalents; E) metaphase I with unoriented chromosomes in population 4 of *H. caprifoliatum* (arrow); F) population 1 of *H. caprifoliatum* metaphase I with non identified chromosome associations (arrow); G) population 17 of *H. polyanthemum*, metaphase I; H) anaphase II with bridges and laggards in population 1 of *H. caprifoliatum* (arrow); I) telophase II with micronuclei in population 11 of *H. caprifoliatum*. Scale bar equal to 10 µm

Meiotic indexes ranged from 44.0% (*H. caprifoliatum*, population 11) to 80.0% (*H. polyanthemum* population 17) (Table 2). Several abnormalities at the tetrad stage, such as tetrads with microcytes, polyads, dyads and tryads, were frequent (Figure 2A-D). The variation in pollen size within and among populations was wide (Table 2, Figure 2E-G), with the lowest value of 5.0 µm (several populations of *H. caprifoliatum*) and the highest of 66.0 µm (population 11 of *H. caprifoliatum*). The intrapopulation variation in pollen grain size was highest (11.5 to 66.0 µm) in population 11 of *H. caprifoliatum*. The *H. polyanthemum* population was relatively uniform for pollen grain size (18.5 to 25.0 µm) (Figure 2H). Pollen fertility (estimated based on the most frequent pollen-size classes, as explained in Material and Methods) ranged from 42.0% (population 11) to 89.0% (population 17). There was generally a good correspondence between meiotic indexes and the estimated pollen fertility (Table 2).

Clarke (1975) observed morphologically irregular pollen grains (ranging from 50.0 to 100.0%) in several *Hypericum* species, including *H. caprifoliatum.* Matzk et al. (2003) reported pollen stainability ranging from 58 to 97% among species and sections. The highest values was found for sexual and the lowest for apomictic species, with no observation of intraspecific pollen-size variation. Moraes (2007) determined pollen viability (stainability) for three species of sections of *Trigynobrathys , H. brasiliense* (83.0%), *H. cordatum* (66.0%) and *H. ternum* (61.0%) and reported the occurrence of some very small grains in the latter two species.

However, to our knowledge, no other study reports such an enormous variation in pollen grain size in *Hypericum* species as found here for *H. caprifoliatum* and *H.* cf. *carinatum*. This wide variation is probably due to the irregular meiotic behavior of these species.

The *in vitro* germination of pollen grain (detailed data not shown) for the *H. polyanthemum* population 17 reached a maximum of 44% germination, corresponding to nearly 50% of the fertility estimated by stainability, which was 89.0%. For the *H. caprifoliatum* population 1, pollen tube emission (only

in grains considered as potentially fertile based on their size) began at the end of the 24 h observation period, although after this time the germination medium began to dehydrate and disintegrate. It was therefore not possible to determine the exact percentage of germinated grains. The pollen grain germination protocols for this species must be optimized as a follow-up to this study.

Moraes (2007) found 43.0% pollen grain germination for *H. brasiliense* (2n=2x=16, sexual) and over 80.0% pollen fertility estimated by stainability, similar to our results for *H. polyanthemum.* For the apomictic *H. perforatum* Arda et al. (2006) reported 83.0% pollen fertility estimated by stainablity and 12.8% *in vitro* pollen germination, and 72.0% and 64.42%, respectively, for *H. rumeliaceum* (diploid sexual). In other genera, such as *Citrus* (Cavalcante et al. 2000) *in vitro* pollen germination ranged from 1.5% to 100% in different plants, and there were high positive correlations between the different predictors of male fertility: 0.86 between meiotic index and pollen stainability, 0.77 between meiotic index and *in vitro* pollen germination and 0.80 between pollen stainability and *in vitro* pollen germination.

Apomictic species support rather low pollen fertilities, since pollen is not necessary for embryo formation. However, for pseudogamous apomictic species, a certain pollen fertility is necessary for endosperm formation, as in the facultative apomictic *Hypericum* (Matzk et al. 2003) and other apomictic species such as *Paspalum notatum* Flügge (Dahmer et al. 2008) and *P. nicorae* Parodi (Reis et al. 2008).

The mode of reproduction of *H. caprifoliatum*, *H.* cf *carinatum* and *H. polyanthemum* has not yet been characterized. The diploid species of the genus are normally obligate sexual whereas polyploidy species may be sexual or apomictic (generally facultative) (Matzk et al. 2003).

The meiotic behavior of young natural and induced polyploids tends to be irregular due to polyploidy *per se*, but it is well known that in nature, the well-established sexual auto (e.g. alfalfa) as well as allopolyploid species (e.g. wheat and white clover) normally have regular meiotic behavior, due to diplodization, a process both at the chromosome and at the genome level, by which the polyploid is reorganized to behave as a diploid species (Leitch and Bennet 1997, Soltis and Soltis 1999). At the chromosome behavior

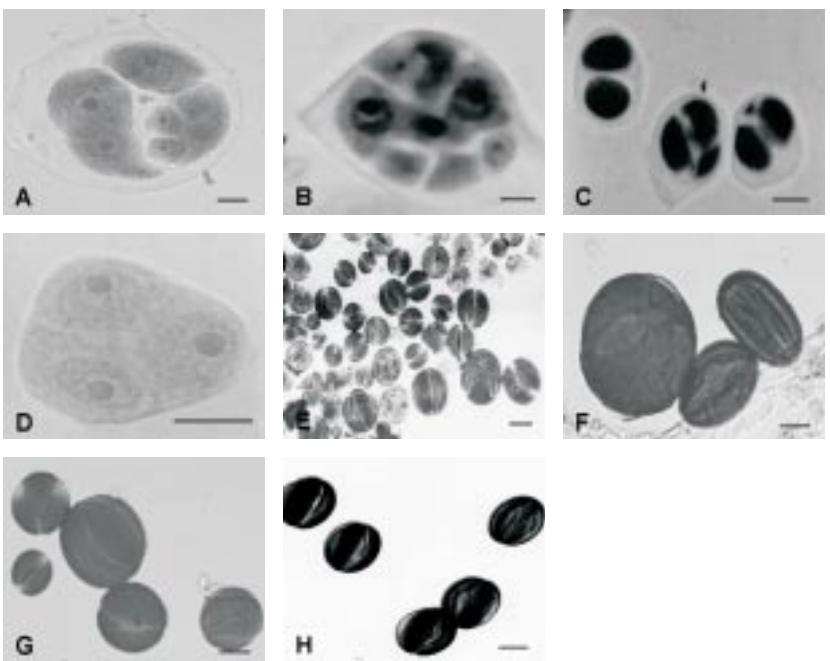

Figure 2. A) Tetrads with microcytes in population 4 of *H. caprifoliatum*; B) polyad in population 7 of *H. caprifoliatum*; C) dyad and dyad with microcyte in population 4 of *H. caprifoliatum*; D) tryad in population 1 of *H. caprifoliatum*; E) population 13 of *H. caprifoliatum* displaying wide pollen grain size variation; F and G) different-sized pollen grains in populations 14 and 6 of *H. caprifoliatum*; H) equal-sized pollen grains in population 17 of *H. polyanthemum*. Scale bar equal to 10 µm

level, diploidization implies in a regularization of chromosome pairing after polyplodization (Stebbins 1970, Ramsey and Schemske 2002), and is a rather fast process, as shown by Ramsey and Schemske (2002) in their review on neopolyploid. The extensive and frequently rapid genome restructuring at the DNA level includes sequence loss, gene silencing, action of transposable elements and epigenetic regulation (Adams and Wendel 2004, Levy and Feldman 2004, Adams 2007).

On the other hand, the meiotic irregularities in natural apomictic polyploid species are numerous and well-documented for a number of genera, as for example *Paspalum* (Moraes-Fernandes et al. 1968, 1973, 1974, Pagliarini et al. 2001, Adamovski et al. 2005, Dahmer et al. 2008). In these species, a regularization of the meiotic behavior would not be essential for species propagation and success, as the embryo is formed by apomixis, provided that, in the pseudogamous species, there is some fertile pollen to ensure endosperm formation.

Therefore, the meiotic behavior *per se* does not clearly indicate the mode of reproduction. Nevertheless, based on the studies of Hoar and Haertl (1932) and Moraes (2007), who found regular meiosis in the sexual *Hypericum* and meiotic irregularities in the apomictic species, together with the rather regular meiosis, high pollen viability and homogeneity in pollen grain size observed for *H. polyanthemum* it could be suggested that this species reproduces sexually. On the other hand, the irregular meiosis, lower pollen fertility and high variation in pollen grain size *of H. caprifoliatum* and *H.* cf *carinatum* could indicate that these two species are apomictic. These results are not conclusive but indicative.

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Citogenética de populações de *Hypericum caprifoliatum* Cham. & Schltdl. (Clusiaceae) e outras espécies do gênero

RESUMO - *Números cromossômicos e comportamento meiótico são apresentados para* Hypericum caprifoliatum *(2n=48),* H. cf carinatum *(2n=48) e* H. polyanthemum *(2n= 32) e confirmado 2n=32 para* H. perforatum. *Os cromossomos são muito pequenos (ca. 1 a 2 mm). Irregularidades meióticas como uni, multivalentes, cromossomos não orientados, pontes e retardatários foram muito freqüentes em* H. caprifoliatum *e* H. cf carinatum. *Índices meióticos variaram de 44,0 a 73,0% em* H. caprifoliatum, *de 66,0 a 77,0% em* H. cf carinatum *e foi 80,0% em* H. polyanthemum. *Fertilidade do pólen variou de 42,0 a 88,0% em* H. caprifoliatum, *65,0 a 88,0% em* H. cf carinatum *e foi 89,0% em* H. polyanthemum. *Juntamente com dados da literatura e comportamento meiótico, a grande variabilidade no tamanho e fertilidade do pólen dentro e entre as populações de* H. caprifoliatum *e* H. cf carinatum, *poderia sugerir que, assim como muitas outras espécies de* Hypericum, *estes táxons sejam apomíticos.*

Palavras-chave: *Hypericum*, número cromossômico, comportamento meiótico, fertilidade do pólen, apomixia.

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