International Journal of Botany

ISSN: 1811-9700

science alert

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International Journal of Botany 5 (1): 93-99, 2009
ISSN 1811-9700

Hybridization and Polyploidy: Cytogenetic Indications for *Hoffmannseggella* (Orchidaceae) Species Evolution

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Abstract: In the present research through chromosome counts utilizing root meristems and immature ovaries and/or observation of meiotic behavior from floral buds, we analysed seven *Hoffmannseggella* species and confirmed the basic number of x = 20. From the seven species analyzed, one presented polyploidy (*H. briegeri* (Blumensch. ex Pabst) V.P.Castro and Chiron, 2n = 80), one presented both diploid (2n = 40) and polyploid (2n = 80) cytotypes (*H. rupestris* (Lindley) V.P. Castro and Chiron) and the five remaining species presented n = 20/2n = 40 chromosomes. Polyploid species/cytotypes presented aneusomatic root tissues. Meiotic abnormalities, like monovalents, early disjunction of bivalents and putative tetravalents were observed in several species. We suggest that hybridization and polyploidy are, if not the major, at least very important mechanisms for the evolution of species and that these events are probably occurring in the present, possibly being responsible for many taxonomic divergences within the group.

Key words: Campos rupestres, chromosome evolution, cytotypes, *Laelia*, meiotic behavior

INTRODUCTION

Orchidaceae is one of the major Angiosperm families, with ca. 20,000 species (Cameron *et al*., 1999). The *Hoffmannseggella* genus was first described by Schlechter (1917) as *Laelia*, with most Brazilian species composing the *Parviflorae* section. Recently, Van den Berg *et al*. (2000), through molecular studies involving the ITS1 and ITS2 nuclear DNA sequences, suggested that most of the Brazilian species of *Laelia sensu lato*, especially from *Parviflorae* section, should be circumscribed under the *Sophronitis* genus. However, in a recent review, Chiron and Castro Neto (2002) using morphological and environmental characteristics, together with molecular data presented by Van den Berg *et al*. (2000) suggested that the genus *Hoffmannseggella*, which is earlier proposed by Jones (1968) should be reconsidered to accommodate Brazilian rupiculous species. However, the taxonomic delimitation of *Hoffmannseggella*, with approximately 32 species described, is under discussion and the delimitation of species inside the genus is complicated by high intra-specific variability and existence of individuals presenting intermediary characteristics between species (Barros, 1990).

Hoffmannseggella species are rupiculous species, distributed mainly throughout the Espinhaço Range, on the state of Minas Gerais, Southeast Brazil. Earlier studies suggested that the genus had its origin through rapid evolutionary events, with transition from epiphytic to rupiculous habit and colonization of a new habitat, the campos rupestres, a vegetation type that could hardly be translated into rocky savannas. As presented by Giulietti and Pirani (1988), the campos rupestres are characterized by a mosaic of related communities under the control of local topography, angle of declivity, microclimatic influences and nature of the substrate. Campos rupestres are located at least 900 m above sea level and have reduced soil depth, due to a high degree of outcropping.

Together with transition from epiphytic to terrestrial habitat and the colonization of campos rupestres, species of the genus would have suffered a shift on pollinators, indicated by reduction of elements and color pattern change of the flowers (Blumenschein, 1960; Brieger, 1960, 1961, 1966).

The only chromosome number documentation found for *Hoffmannseggella* species were made by Blumenschein (unpublished data) who suggested that events of polyploidy and interspecific hybridization were also important for the origin of the genus. From 12 species of *Hoffmannseggella* studied by the researcher, five presented polyploidy, with 2n = 80 chromosomes

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(*H. briegeri*, *H. caulescens*, *H. longipes*, *H. rupestris* and *H. tereticaulis*). He also suggested the basic number of x = 20 for the genus and observed meiotic abnormalities in three species (*H. crispata*, *H. mixta* and *H. cinnabarina*). According to Pabst and Dungs (1975), there are at least eight natural interspecific hybrids documented for *Hoffmannseggella* and also intergeneric hybrids between *Laelia sensu lato* and *Cattleya* species.

With artificial crossing experiments among *Laelia sensu lato*, *Cattleya*, *Brassavola*, *Epidendrum*, *Schomburgkia* and *Sophronitis*, Stort (1984) obtained several hybrids with high tetrad viability and successful artificial crosses between F_1 hybrids.

Through chromosome number and meiotic behavior analysis of seven *Hoffmannseggella* species (*H. angereri*, *H. briegeri*, *H. cinnabarina*, *H. crispata*, *H. fournieri*, *H. liliputana* and *H. rupestris*), mainly distributed on Southern and Central Espinhaço Range, this study aimed not only the characterization and differentiation of species but also evaluate the importance of polyploidy for evolution and speciation and observe putative evidences of interspecific hybridization events, specially between sympatric or neighbor species.

MATERIALS AND METHODS

Root meristems, floral buds and immature ovaries from seven species of *Hoffmannseggella* (*H. angereri*, *H. briegeri*, *H. cinnabarina*, *H. crispata*, *H. fournieri*, *H. liliputana* and *H. rupestris*), presented in Table 1, were collected on the field (mainly in Southern and Central Espinhaço Range, in the State of Minas Gerais, Brazil) and from cultivated material (obtained through transplanted plants), between years 2002 and 2006. Vouchers are deposited at UEC Herbarium, from Universidade Estadual de Campinas and numbers are presented in Table 1. Species were identified by specialist M.Sc. C.F. Verola.

Roots and immature ovaries were pretreated in 8-Hq (8-hidroxiquinolein) 0,002 M for 24 h at 4°C. Roots, immature ovaries and floral buds were fixed in Carnoy's solution (ethanol: acetic acid: chloroform; 6:3:1) for at least 24 h at room temperature and stored in freezer.

For mitotic analysis, roots and immature ovaries were hydrolyzed in HCl 5 N for 20 min., rinsed in distilled water and squashed in a drop of 45% acetic acid solution. Slides were stained with Giemsa 2% solution (Guerra, 1983). At least 20 cells from each species, from distinct clones, were observed for determination of chromosome number, except for *Hoffmannseggella liliputana*, *H. crispata* and populations 1, 2 and 3 of *H. rupestris*, due to material scarcity.

For meiotic analysis, pollinia were collected from floral buds and squashed in aceto-carmine 1.2%. For tetrad normality evaluation at least five clones were sampled, totalizing at least 1000 tetrads observed for each population and SD calculated among clones and populations. Slides were observed and best chromosome spreadings photographed under photomicroscope Olympus BX51 using BW ISO 25 Imagelink (Kodak) or BW ISO 50 PanF (Ilford) films.

RESULTS

Mitotic analysis: We obtained chromosome counts for one population of *Hoffmannseggella briegeri*, *H. cinnabarina*, *H. crispata*, *H. fournieri* and *H. liliputana*; two populations of *H. angereri* and six populations of *H. rupestris* (Table 1). Five species (*H. angereri*, *H. cinnabarina*, *H. crispata*, *H. fournieri* and *H. liliputana*) presented n = 20 and/or 2n = 40 chromosomes (Fig. 1a-e). *Hoffmannseggella briegeri* presented aneusomatic root tissues with variation from 64-84 chromosomes (Fig. 1f). Of 20 cells analysed, the most frequent number was 2n = 78 (25%).

Table 1: *Hoffmannseggella* species studied - with voucher number, populations, number of bivalents at metaphase I (n), mitotic chromosome number (2n) and previous data found in literature (Reference)

Species	Voucher	Population	n	2n	Reference
H. angereri (Pabst) V.P.Castro and Chiron*	CL27	Diamantina/MG Cachoeira dos Cristais	20	40	Present study
	CL28	Milho Verde/MG órrego do Inferno		40	Present study
H. briegeri (Blumensch. ex Pabst) V.P.Castro and Chiron*	C.F.V. 0056	Serro/MG istrito de Pedro Lessa		64-84	2n = 80, Blumenschein (1960)
H. cinnabarina (Batem. ex Lindley) H.G. Jones*	C.F.V. 0069	Catas Altas/MG Serra do Caraça		40	Present study
H. crispata (Thunb.) H.G. Jones	C.F.V. 0110	São Thomé das Letras/MG Serra de São Thomé	20		2n = 40, Blumenschein (1960)
H. fournieri (Cogniaux) V.P.Castro and Chiron	J.Y.C. 03/052	Ouro Preto/MG Serra do Itatiaia	20	40	Present study
H. liliputana (Pabst) H.G. Jones	J.Y.C. 03/059	Ouro Branco/MG Serra do Ouro Branco	20	40	Present study
H. rupestris (Lindley) V.P. Castro and Chiron	J.Y.C. 03/020	Diamantina/MG Biribiri	ca.40	52-75	2n = 80, Blumenschein (1960)
	J.Y.C. 03/032	Diamantina/MG Afloramento de Soberbo	ca.40	54-76	
	J.Y.C. 03/043	Diamantina/MG Caminho dos Escravos		80	
	pop 1*	Francisco Sá/MG		60-71	
	pop 2*	Botumirim/MG		40	
	pop 3*	Grão Mogol/MG		40	

*Species or populations studied from greenhouse individuals

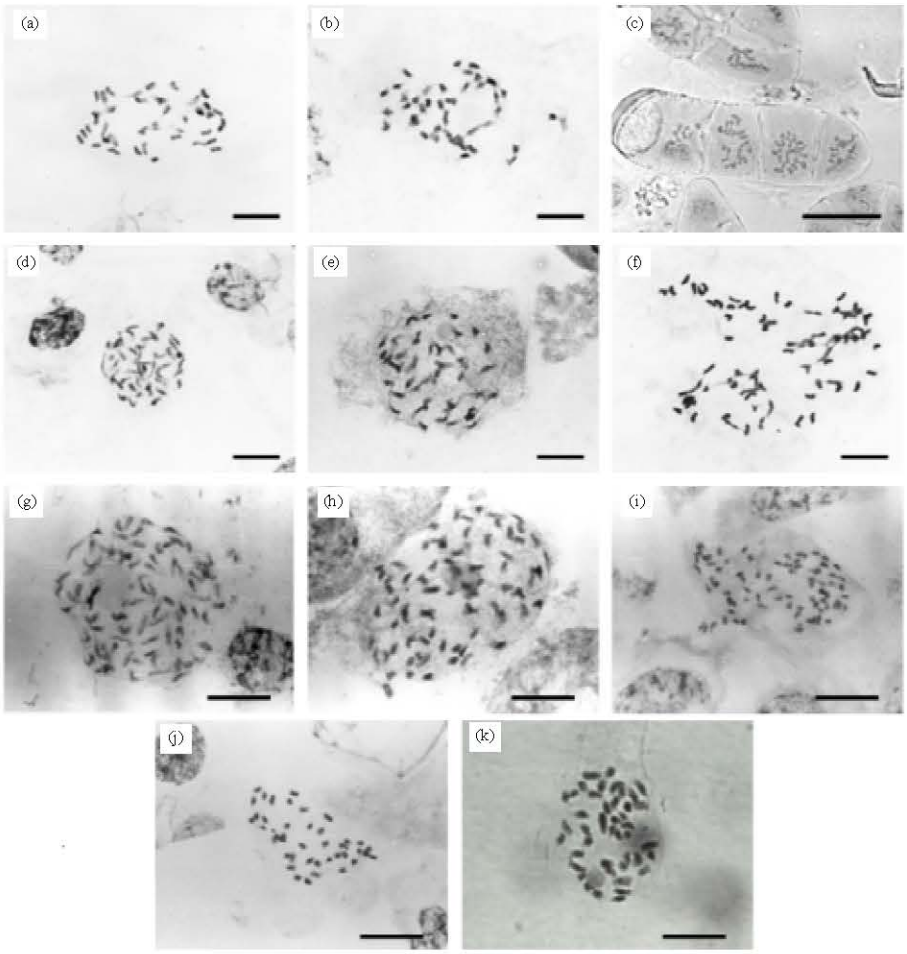

Fig. 1: Mitotic metaphase chromosomes of *Hoffmannseggella* species/populations. (a) *H. angereri* (2n = 40), (b) *H. cinnabarina* (2n = 40), (c) *H. crispata*, pollinic mitosis (n = 20), (d) *H. fournieri* (2n = 40), (e) *H. liliputana* (2n = 40), (f) *H. briegeri*, cell with 2n = 73, (g) *H. rupestris* population from Biribiri (Diamantina/MG) with 2n = 75, (h) *H. rupestris* population from Soberbo (Diamantina/MG) with 2n = 72, (i) *H. rupestris* population from Francisco Sá (MG) with 2n = 70, (j) *H. rupestris* population from Botumirim (MG) with 2n = 40 and (k) *H. rupestris* population from Grão Mogol (MG) with 2n = 40. Bars = 10 μm

Hoffmannseggella rupestris presented both polyploid (2n = 80) and diploid (2n = 40) cytotypes (chromosome races). *H. rupestris* also presented aneusomatic root tissues, varying between 52-76 chromosomes in two populations from Diamantina/MG (Fig. 1g, h) and between 60-71 chromosomes in one population from Francisco Sá/MG (Fig. 1i). Of 40 cells analysed, the most frequent numbers were 2n = 66 (10%), 2n = 69 (12.5%) and 2n = 74 (10%). *H. rupestris* also presented two diploid (2n = 40) populations from Botumirim/MG (Fig. 1j) and Grão Mogol/MG (Fig. 1k).

Meiotic analysis: Meiotic abnormalities were observed in *Hoffmannseggella angereri*, *H. fournieri*, *H. liliputana* and *H. rupestris* (for both populations analyzed). Unfortunately, due to material scarcity, meiotic process was not observed in *H. briegeri*, *H. cinnabarina*, *H. crispata* and for diploid populations of *H. rupestris*. *H. angereri* presented mainly n = 20II, but variations occurred, with cells presenting n = 19II, n = 19II + 2I, n = 19II+3I, n = 20II+1I, n = 20II+2I and n = 21II (Fig. 2a-g). *H. fournieri* presented always n = 19II + 2I (Fig. 2i) and early migration of bivalents during metaphase I (Fig. 2j). *H. liliputana* presented n = 20II (Fig. 2k) but chromosome laggards in anaphase I (Fig. 2l). *Hoffmannseggella rupestris* presented variation on the number of bivalents, with putative tetravalents and monovalents observed at metaphase I (Fig. 2m, o) and

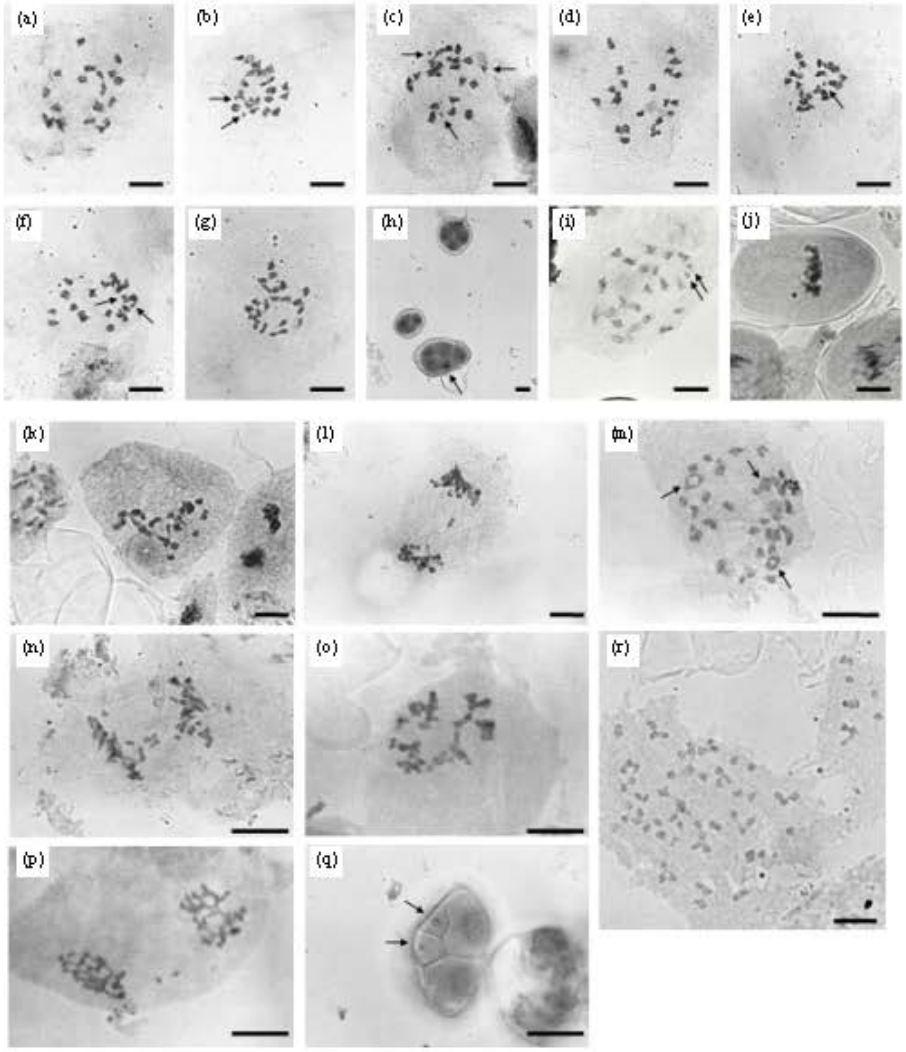

Fig. 2: Meiotic chromosomes of *Hoffmannseggella* species/populations. (a-h) *H. angereri*, with n = 19II (a), 19II + 2I (b), 19II + 3I (c), 20II (d), 20II+1I (e), 20II + 2I (f), 21II (g), (arrows point to monovalents) and tetrad with one microcyte (h), (i-j) *H. fournieri* with n = 19II + 2I (arrows) (i) and early migration of one bivalent at metaphase I (j), (k-l) *H. liliputana* with n = 20II (k) and chromosome laggard at anaphase I (l), (m-n) *H. rupestris* population from Biribiri (Diamantina/MG) with n = ca. 27II and 3IV (arrows) (m) and chromosome laggards at anaphase I (n), (o-q) *H. rupestris* population from Soberbo (Diamantina/MG) with n = ca. 40 (o), chromosome laggards at telophase I (arrows) (p) and tetrad with two microcytes (arrows) (q), (r) *H. rupestris* population from Caminho dos Escravos (Diamantina/MG) at anaphase I with 2n = 80. Bars = 10 µm

anaphase I (Fig. 2n, p). However, late anaphase I presented 2n = 80 chromosomes in another population from Diamantina (Fig. 2r). Even with meiotic abnormalities, species presented high tetrad normality, varying from 86.4% in one population of *H. rupestris* (Biribiri) to 99.3% in *H. fournieri*. Abnormal tetrads presented one or two microcytes (Fig. 2h, q). Differences observed between populations of *H. angereri* and *H. rupestris* were not significant (SD = 1, 5).

DISCUSSION

Chromosome numbers obtained in the present study support the previous hypothesis (Blumenschein, 1960) of x = 20 as the basic chromosome number for *Hoffmannseggella*. New counts are presented for *H. angereri*, *H. fournieri* and *H. liliputana* (2n = 40) and chromosome number confirmed for *H. crispata* (n = 20, 2n = 40), polyploid populations of *H. rupestris* (2n = 80)

and *H. briegeri*, which presented aneusomatic root tissues, but has 2n = 80, as previously reported by Blumenschein (1960) and observed by Yamagishi-Costa and Forni-Martins (unpublished data).

The aneusomaty of root tissues was observed only in polyploid species and apparently has no influence on species fertility, since reproductive tissues presented stability of 2n = 80 chromosomes. However, the aneusomaty could reflect ancient hybridization events that generated allopolyploid species. Lim and Loh (2003) observed different chromosome numbers among different tissues and development stage of the hybrid *Vanda* Miss Joaquim (Orchidaceae). This hybrid presented multiploid cells on leaves, roots and column, but not on floral scape apex, stem, perianth and pedicel. Variation of chromosome numbers between individuals or tissues of the same individual was also observed in *Ornithogalum divergens* Boreau (Liliaceae) by Couderc *et al*. (1985). Researchers observed, inside the same root tissue a variation between 2n = 11 and 2n = 108. Sugarcane species also represent a very well documented case of aneuploid variation in root tissues. Recently, D'Hont (2005) presented a good review of polyploidy research already made for *Saccharum* species. Two species of the genus are aneuploids, *S. barberi* (2n = 81 - 124) and *S. sinense* (2n = 116 - 120). Classical cytogenetic and morphological studies already indicated hybrid origin for both species, but genomic *in situ* hybridization (GISH) together with molecular marker studies confirmed that both represent ancient hybridization events between *S. officinarum* (female) and *S. spontaneum* (male).

Of all the mechanisms of aneuploid cells formation, the most probable is the non-disjunction of sister chromatids at centromeres during cell division (Appels *et al*., 1998). Non-disjunction of centromeres would cause the migration of both chromatids to the same cell pole, resulting in one daughter cell with 2n+1 and another with 2n-1. Appels *et al*. (1998) listed a number of factors that could be involved in the formation of aneuploid cells, like ageing of cells and tissues, environmental conditions, viral infections and physiological stress. All of them could be involved in the origin of aneusomatic root tissues of some *Hoffmannseggella* species, because these rupiculous species are fixed directly on the rocks and exposes its roots to intense solar UV radiation. Furthermore, because of the rocky substrate, plants are submitted to intense variation of temperatures. Campos rupestres are also constantly submitted to drought periods and fire. Unfortunately, there are no studies of the influence of these environmental conditions on species genetics or physiology.

The present study, also documented the existence of two cytotypes for *H. rupestris*, representing the first documentation of diploid (2n = 40) cytotypes for *H. rupestris*, but not the first documentation of different cytotypes for the genus. Blumenschein (1960) observed three different chromosome numbers in *H. longipes* (Rchb. f.) V.P. Castro and Chiron, with 2n = 40, 60 and 80. Cytotypes present a great evidence of recent evolution of species through polyploidy events. Cytotypes of *H. rupestris* are probably isolated from each other, if not for differences on chromosome numbers, at least geographically.

In a recent study, focused on autopolyploid documentation, Soltis *et al*. (2007) suggested that polyploid cytotypes should be more deeply investigated. The authors defend that many autopolyploid cytotypes may deserve the category of a new species and many of them are kept under cytotypes to facilitate taxonomists phenetic job. Even though autopolyploid cytotypes present morphological similarities with diploid cytotypes, they usually present distinct geographical distribution when compared to diploid cytotypes.

The observation of putative tetravalents on polyploid cytotypes of *Hoffmannseggella rupestris* could indicate that these cytotypes are of autopolyploid origin, because autopolyploids usually present abnormalities during meiotic chromosome pairing, resulting on multivalent formation and subsequent reduction of pollen and seed fertility (Briggs and Walters, 1997). However, multivalent formation could also derive from segmental allopolyploidy, with hybridization between parental species that present shared chromosome segments (Stebbins, 1947).

Tetravalents observed in *H. rupestris* could indicate that there are some homeologous chromosomes, representing genetic similarities between parental plants hybridized. Furthermore, other meiotic abnormalities observed, like chromosome laggards during anaphases and tetrads with microcytes, are also frequently correlated to hybrids or species of hybrid origin (Boff and Schifino-Wittmann, 2003; Hoshino, 1988; Gatt *et al*., 1998; Stort, 1984). Ramsey and Schemske (1998) observed that spontaneous polyploid frequency is higher in hybrid than non-hybrid systems and new polyploids in non-hybrid systems are rare and typically triploids.

According to Stace (2000), the degree of homology of a segmental allopolyploid result in a degree of hybrid fertility and that could be inferred for *H. rupestris* by the high percentage of tetrad normality (86.4-89.2%). It is important to reinforce that normal tetrads are not necessarily fertile, because aceto-carmine staining could only detect microcytes or micronucleus, but not

unbalanced cell division. Unbalanced cell division could be caused by tetravalents and chromosome laggards during anaphase and was observed by Blumenschein (unpublished data) in *H. rupestris*, which presented different chromosome numbers during mitotic division among the four pollen grains of some tetrads.

Meiotic abnormalities were also observed on diploid (2n = 40) species, like *H. angereri* and *H. fournieri*. In fact, meiotic abnormalities seem to be a rule, rather than the exception for *Hoffmannseggella*. Blumenschein (unpublished data) observed variation between 20-26 of what he decided to call units during metaphase I in *H. crispata*, between 20-23 units in *H. mixta* and between 20-24 units in *H. cinnabarina*. Again, despite meiotic abnormalities, species presented high tetrad normality.

It is suggested that these meiotic abnormalities could represent evidences of events of hybridization and these non-polyploid species would represent homoploid hybrid speciation. Stort (1984), studying hybrids produced between species of *Laelia sensu lato*, *Cattleya*, *Brassavola*, *Epidendrum*, *Schomburgkia* and *Sophronitis*, all with 2n = 40 chromosomes, observed that hybrids presented haploid numbers varying from 7 to 40 chromosomes, but most hybrids presented at a higher frequency the numbers 19, 20 and 21. Stort (1984) also observed high tetrad normality, reaching 99.85%. Homoploid hybrid speciation is well known for *Helianthus* (Asteraceae), in which three species, *H. anomalus*, *H. deserticola* and *H. paradoxus*, were shown to represent hybrids derived from the same parental species *H. annuus* and *H. petiolaris* (Rieseberg, 1991).

With this study, together with earlier studies (Blumenschein, 1960), 16 of 32 species described for the genus are characterized for its chromosome numbers. Of 16, five presented polyploidy (2n = 60, 80), representing 31.25% of studied species. Amazingly, when regarding all chromosome numbers listed by Blumenschein (unpublished) for all species (92) studied inside Epidendroideae, only seven presented polyploidy and three of them belonging to *Hoffmannseggella*. Other polyploids were described for species of *Cattleya* and *Epidendrum*.

In conclusion, the data presented in this study suggest that even though *Hoffmannseggella* species presented mainly 2n = 40 chromosomes (x = 20), polyploidy is an important evolutionary mechanism, being responsible either for origin of some species or of cytotypes, which can represent an initial divergence into new species. Furthermore, meiotic behavior and aneusomaty of root tissues indicates that hybridization is probably another important evolutionary force, with species presenting allopolyploidy or representing homoploid species evolution, explaining much of the morphological variation and taxonomic divergences within the group.

Obviously, chromosome data alone are not enough to prove that at least some *Hoffmannseggella* species are of hybrid origin, but they provided good indications that hybridization should be more deeply investigated. It is suggested as further steps molecular marker studies, such as microsatellites, reproductive biology and even artificial interspecific crosses, to better understand the origin of the genus and also help delimitation of species.

ACKNOWLEDGMENTS

The researchers thank M.Sc. Christiano Franco Verola for species identification and biological material disponibilization, all staff of Botanic Department and Programa de Pós Graduação em Biologia Vegetal of UNICAMP and the Instituto Estadual de Florestas (IEF), MG, for authorization to access protected areas. J.Y.-C. received a fellowship from the Fundação da Amparo à Pesquisa do Estado de São Paulo (FAPESP No. 02/07750-0).

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