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Meiotic Studies in Ten Species of *Hybanthus* Jacq. (Violaceae) from South America

Micaela Noemi Seo*

Lab. de Citogenética y Evolución, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Int. Güiraldes, Pab. II, Ciudad Universitaria, Cdad. de Buenos Aires, Argentina.

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Summary Chromosome numbers and meiotic behaviour of 10 native species of *Hybanthus* in South America were analyzed. 3 different chromosome numbers were found: $2n=16$ for *Hybanthus atropurpureus* (A. St.-Hil.) Taub., *H. bigibbosus* (A. St.-Hil.) Hassl. and *H. paraguariensis* (Chodat) Schulze-Menz, $2n=32$ for *H. bicolor* (A. St.-Hil.) Baill., *H. calceolaria* (L.) Oken, *H. communis* (A. St.-Hil.) Taub., *H. hasslerianus* (Chodat) Hassl., *H. leucopogon* Sparre and *H. nanus* (A. St.-Hil.) Paula-Souza, and $2n=48$ for *H. longistylus* Schulze-Menz. This is one of the first reports for 8 analyzed species. Nowadays, the basic chromosome numbers in the South American *Hybanthus* species are under discussion. The presence of 1 tetravalent in *H. atropurpureus*, *H. bigibbosus* and *H. paraguariensis* ($2n=16$) would suggest the duplication of chromosome sets, and therefore would indicate that the basic chromosome number is $x=4$, at least, for this group of South American *Hybanthus* species. The studied species would be tetraploids ($2n=4x=16$), octoploids ($2n=8x=32$) and dodecaploids in *H. longistylus* ($2n=12x=48$). In light of these findings, such polyploidy would likely have played an important role during the speciation of this heterogenerous generic assembly.

Key words Argentina, Basic chromosome number, *Hybanthus* subgenus *Ionidium*, Meiotic behaviour, Polyploidy, South America.

The genus *Hybanthus* Jacq. (Violaceae) comprises over 100 species which are distributed throughout the tropical and subtropical areas of the New and Old World. Most of them are concentrated in Latin America with nearly 70 species, 20 species in Africa, 5 in Asia and 11 in Oceania (Bennett 1972). In South America this genus comprises more than 50 species, most of them occuring in Argentina, Brazil, Paraguay and Bolivia (Sanso *et al*. *in press*).

According to the traditional infrageneric classification of the Violaceae, *Hybanthus* belongs to subfamily Violoideae, tribe Violeae (Hekking 1988), and it was divided in 2 subgenera by Schulze-Menz (1936): *Hybanthus* Subg. *Hybanthus* for the species of North and Central America and *Hybanthus* Subg. *Ionidium* (Vent.) Schulze-Menz mainly for South America.

In a recent molecular analysis, several authors (Ballard *et al.* 2005, Tokuoka 2008) showed that *Hybanthus sensu lato* is a non-monophyletic group based on chloroplast DNA data set. According to this analysis, Ballard *et al.* (2005) proposed to split the genus into 7 genera. The status and position of many species of Latin America are still doubtful in relation of the lack of information in cytology and taxonomy, and further studies will be necessary to confirm the classification among the various hybanthoid lineages (H. E. Ballard, *pers. comm.*).

From a cytological viewpoint, the chromosome numbers reported in *Hybanthus* have not been investigated deeply. Most of the comprehensive studies in cytogenetics were apported by Bennett (1972), based on Australian species, who suggested base numbers like $x=4$ and $x=6$ for this genus. Other chromosome numbers from North American species were provided by Turner and

* Corresponding author, e-mail: micaseo@ege.fcen.uba.ar

Escobar (1991). However, chromosomal information in relation to South American species of *Hybanthus* has been poorly known.

The main objectives of the present study were to report new chromosome numbers and meiotic behaviour in some South American native species of *Hybanthus* and to analyze the relation with the other species of the genus. In addition, systematic and evolutionary aspects of this genus are discussed in the light of these findings, with emphasis on the basic chromosome numbers, ploidy levels and the relationships between some of the taxonomical sections of the genus.

Materials and methods

The species were collected from wild populations of northeastern Argentina, in the provinces of Misiones, Corrientes and Entre Rios, between the summers of 2005 and 2007. The provenance and vouchers of each species are summarized in Table 1. Voucher specimens are deposited in the Herbaria BAFC (Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina) and SI (Instituto de Botánica Darwinion, Argentina).

Meiosis eas studied in young floral buds were fixed in ethanol–chloroform–glacial acetic acid (6 : 3 : 1) for at least 24 h, transferred into 70% ethanol and stored at 4–5°C until required. Immature anthers were then squashed into a drop of propionic acid haematoxylin (2%) using ferric citrate as a mordant. The analyses were carried out using, at least, 5–20 pollen mother cells per individual in different meiotic stages and 5 individuals per population when it was possible. Photographs of the different stages of each specimen were taken using a digital camera.

Results

The analysis revealed 3 different chromosome numbers (Table 2), $2n=16$ for *Hybanthus atropurpureus*, *H. bigibbosus* and *H. paraguariensis*, $2n=32$ for the remaining analyzed species, except in *H. longistylus* which presented $2n=48$. The meiotic configurations with their frequencies and the chromosome numbers of 10 species of *Hybanthus* are detailed in Table 1. The meiosis was fairly normal and the formation of bivalents (II) was frequent in the species analyzed although from 1 to 4 quadrivalents (IV) and 2 univalents (I) were observed in some species.

In *Hybanthus atropurpureus*, the most frequent meiotic configuration in diplotene and diakinesis was 8 II ($2n=16$) with other meiotic configurations such as 6 II+1 IV (Fig. 1A) or 7 II+2 I (Table 1).

The presence of 8 II ($2n=16$) was observed in *Hybanthus bigibbosus* (Fig. 1B, Table 1) at diplotene and diakinesis. However, other meiotic configurations such as 7 II+2 I or 6 II+1 IV (Fig. 1C, Table 1) were detected in some cells in diplotene. Similar meiotic configurations were observed in *H. paraguariensis*, 8 II (Fig. 1D, Table 1) were observed in a 98% of the cells analyzed.

The presence of 16 II ($2n=32$) was the most frequent meiotic configuration in several species, such as *Hybanthus bicolor*, *H. calceolaria*, *H. communis* (Fig. 1F), *H. hasslerianus* (Fig. 1G), *H. leucopogon* and *H. nanus* (Table 1). However in a low frequency of cells other meiotic configurations such as 14 II+1 IV in *H. bicolor* (Fig. 1E) and *H. nanus* (Fig. 1H), or 12 II+2 IV in *H. leucopogon* (Fig. 1J). At metaphase II, 2 groups of 16 chromosomes could be observed in 1 cell of *H. calceolaria* (Fig. 1I).

In *Hybanthus longistylus* Schulze-Menz several chromosome configurations were observed, such as 24 II, 23 II+2 I or 22 II+1 IV (Fig. 1G; Table 1) at diplotene and diakinesis.

The presence of 1 to 4 B-chromosomes (Table 1) was observed in 3 species: *H. atropurpureus* (Fig. 1A), *H. communis* (Fig. 1F) and *H. hasslerianus* (Fig. 1G).

Table 1. Meiotic configurations, provenance and voucher specimens of the *Hybanthus* studied species. In the third column, numbers in parentheses indicate the number of cells and studied individuals in each taxon

Species	Meiotic configurations	Voucher details
Hybanthus atropurpureus (A. St.-Hil.) Taub.	8 II+0–4 Bs (87.6%), 7 II+2 I+0–4 Bs (7.3%), 6 II+1 IV+0–4 Bs (5.1%)	Argentina, Tucumán, San Miguel de Tucumán, Fund. M. Lillo, cultivated at the Botanical Garden. M. N. Seo 55 (BAFC). (90, 10)
Hybanthus bicolor (A. St.-Hil.) Baill.	16 II (53.5%), 15 II+2 I (3.5%), 14–8 II+1–4 IV (43%)	Argentina, Corrientes, Department Santo Tomé, Virasoro, M. N. Seo 2, 36, 62 (BAFC). (28, 10)
Hybanthus bigibbosus (A. St.-Hil.) Hassl.	8 II (61%), 7 II+2 I (35.6%), 6 II+1 IV (3.4%)	Argentina, Misiones, Department Iguazú, National Park Cataratas of Iguazú, M. N. Seo 7, 12, 23, 47 (BAFC). (87, 7)
Hybanthus calceolaria (L.) Oken	16 II (95%), 15 II+2 I (5%)	Argentina, Corrientes, Department Ituzaingó, Ituzaingó, M. N. Seo 1, 17, 59 (BAFC). (21, 20)
Hybanthus communis (A. St.-Hil.) Taub.	16 II+0–1 B (55.8%), 15 II+2 I+0–1 B (4.7%), 14 II+1 IV+0–1 B (39.5%)	Argentina, Misiones, Department Iguazú, National Park Cataratas of Iguazú, M. N. Seo 13, 20, 39 (BAFC), Argentina, Misiones, Department San Ignacio, San Ignacio; M. N. Seo 43, 49 (BAFC). (43, 20)
Hybanthus hasslerianus (Chodat) Hassl.	16 II+0–1 B (57.5%), 15 II+2 I+0–1 B (7.5%), 14 II+1 IV+0–1 B (35%)	Argentina, Misiones, Department San Ignacio, San Ignacio; M. N. Seo 3, 48 (BAFC). (40, 5)
Hybanthus leucopogon Sparre	16 II (58.8%), 14 II+1 IV (31.4%), 12 II+2 IV (9.8%)	Argentina, Corrientes, Department Mercedes, Mercedes, M. N. Seo 34, 58 (BAFC). (51, 1)
Hybanthus longistylus Schulze-Menz	24 II (78.3%), 23 II+2 I (13%), 22 II+1 IV (8.7%)	Argentina, Misiones, Department San Ignacio, San Ignacio, M. N. Seo 61 (BAFC). (23, 1)
Hybanthus nanus (A. St.-Hil.) Paula-Souza	16 II (45.4%), 15 II+2 I (9.1%), 14–12 II+1–2 IV (45.5%)	Argentina, Entre Ríos, Department Colón, National Park El Palmar, M. N. Seo 29, 54 (BAFC). (11, 8)
Hybanthus paraguariensis (A. St.-Hil.) Schulze-Menz	8 II (98%), 6 II+1 IV (1%), 7 II+2 I (1%)	Argentina, Corrientes, Department Ituzaingó, Ituzaingó, M. N. Seo 35, 60 (BAFC SI). (27, 8)

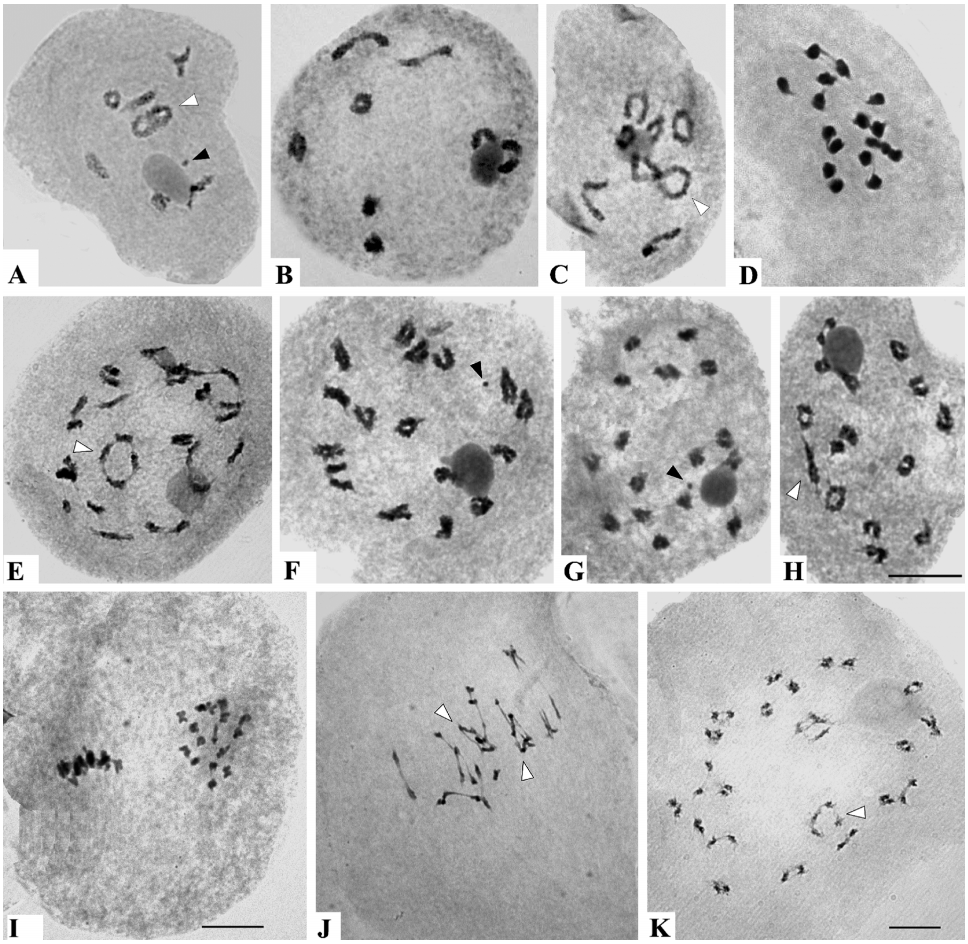

Fig. 1. Meiotic chromosomes in 10 species of *Hybanthus*. A. *H. atropurpureus*. Diakinesis, 6 II+1 IV+1 B. B–C. *H. bigibbosus*. B. Diplotene, 8II. C. Diplotene, 6 II+1 IV. D. *H. paraguariensis*. Diakinesis, 8 II. E. *H. bicolor*. Diplotene, 14 II+1 IV+1 B. F. *H. communis*. Diakinesis, 16 II+1 B. G. *H. hasslerianus*. Diakinesis, 16 II+1 B. H. *H. nanus*. Diakinesis, 14 II+1 IV. I. *H. calceolaria*. Metaphase II, 2 groups of 16 chromosomes. J. *H. leucopogon*. Metaphase I, 12 II+2 IV. K. *H. longistylus*. Diplotene, 22 II+1 IV. Bar.=10 μm. B chromosome: black arrow head. Quadrivalent (IV): white arrow head.

Discussion

The chromosome numbers of the South American *Hybanthus* species has been poorly known; this is one of the first reports with meiotic information of several species in this genus (Table 2), with emphasis on the Argentinean species (Seo 2008). Previously, only the data of *H. attenuatus*, *H. atropurpureus*, *H. communis* and *H. parviflorus* had been reported (Table 2). The chromosome numbers found (Table 2), $2n=16$, $2n=32$ and $2n=48$ are in agreement with data observed in other Latin American species, as well as reported previously (Table 2).

The basic chromosome numbers in the *Hybanthus* lineages is nowadays under discussion with different values previously postulated like $x=4$, 6 and 8.

Bennett (1972) apported the chromosome numbers in several species of Australia (Table 2), and according with this information (Table 2) this author postulated $x=4$ and $x=6$ as the basic

Table 2. Chromosome numbers, ditribution and references of species of *Hybanthus* from America

Species	2*n*	Distribution	References
H. atropurpureus (A. St.-Hil.) Taub.	16	South America	Pinto-Maglio *et al.* 1997, Seo 2008
H. attenuatus (Humb. et Bonpl.) Schulze-Menz	32, 24	Center and South America	Davidse 1971, Sundberg and Dillon 1986, Turner and Escobar 1991
H. aurantiacus (Benth.) F. Muell.	16	Oceania	Bennett 1972
H. bicolor (A. St.-Hil.) Baill.	32	South America	Seo 2008
H. bigibbosus (A. St.-Hil.) Hassl.	16	South America	Seo 2008
H. bilobus C. A. Gardner	24, 48	Oceania	Bennett 1972
H. calceolaria (L.) Oken	32	South America	Seo 2008
H. calycinus (DC.) F. Muell.	12, 24	Oceania	Bennett 1972
H. communis (A. St.-Hil.) Taub.	32	South America	Gadella *et al.* 1969, Seo 2008
H. concolor (T. F. Forst.) Spreng.	48	North America	Kovanda 1978
H. cymulosus C. A. Gardner	12	Oceania	Bennett 1972
H. enneaspermus (L.) F. Muell.	16, 32	Oceania, Asia	Bennett 1972, Peng and Chen 1985, Sarkar *et al.* 1980
H. epacroides (C. A. Gardner) Melch.	24	Oceania	Bennett 1972
H. floribundus (Lindl.) F. Muell.	12, 24, 48	Oceania	Bennett 1972
H. hasslerianus (Chodat) Hassl.	32	South America	Seo 2008
H. leucopogon Sparre	32	South America	Seo 2008
H. longistylus Schulze-Menz	48	South America	Seo 2008
H. monopetalus Domin.	8	Oceania	Bennett 1972
H. nanus (A. St.-Hil.) Paula-Souza	32	South America	Seo 2008
H. paraguariensis (Chodat) Schulze-Menz	16	South America	Seo 2008
H. parviflorus (Mutis ex L. f.) Baill.	24, 12	South America	Heilborn 1926, Di Fulvio 1977
H. suffruticosus (L.) Baill.	32	Asia	Koshy and Mathew 1990
H. verticillatus (Ortega) Baill.	16, 32	North America	Turner and Escobar 1991
H. volubilis E. M. Benn.	8	Oceania	Bennett 1972

chromosome numbers for the genus. Turner and Escobar (1991) provided additional counts (Table 2) and a chromosomal review and supported the basic chromosome number. This presumes *Hybanthus* to represent a single monophyletic genus and does not account for the suggestions from recent molecular phylogenetic studies (Ballard *et al.* 2005). The counts of the widespread Old World *Hybanthus enneaspermus* complex were $2n=16$ and 32 (Table 2), so possibly the basic chromosome number in this lineage would be $x=8$ (Ballard *pers. comm.*). Whereas the Australian species with $2n=12$, 24 and 48 would have a basic chromosome number of $x=6$ (Bennett 1972) and the North-American *H. concolor* Spreng. with $2n=48$ (Kovanda 1978) would be a polyploid species also based on $x=6$. (Ballard, *pers. comm.*).

The chromosome numbers $2n=16$, 32 and 48 were reported for several Latin species, with the exception of one record for *Hybanthus attenuatus* ($2n=24$) and *H. parviflorus* ($2n=12$, 24) (Table 2). This would suggest that most Latin American segregates of "*Hybanthus*" may be based on $x=8$ (Ballard *pers. comm.*). However, the presence of one IV in 3 species with $2n=16$, such as *H. atropurpureus*, *H. bigibbosus* and *H. paraguariensis* (Table 1), would suggest the duplication of chromosome sets and therefore the ocurrence of polyploidy during the evolution of this group. And according with these findings would support $x=4$ as a plausible basic chromosome number for, at least, this group of South American *Hybanthus* species. The studied species would be so mainly octoploids ($2n=8x=32$), tetraploids ($2n=4x=16$) and dodecaploids in *H. longistylus* ($2n=12x=48$).

The chromosome rearrangement in this genus has clearly occurred throughout complex events of polyploidy, and this process would likely have played an important role during speciation of this heterogeneous generic assemblage of Violaceae. Further analysis on more species of *Hybanthus* will heighten our understanding of how evolution has proceeded in this group.

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