

The Pollination Mechanism of *Habenaria pleiophylla* Hoehne & Schlechter (Orchidaceae: Orchidinae)

Rodrigo B. Singer^{1*} • Tiago Böer Breier² • Adriana Flach³ • Rosana Farias-Singer¹

¹ Depto Botânica, Instituto de Biociências. Universidade Federal do Rio Grande do Sul. Av. Bento Gonçalves 9500. Bairro Agronomia CEP 91570-950. Porto Alegre, RS, Brazil

² Depto de Morfologia e Patologia, Pontifícia Universidade Católica de São Paulo. Praça. Dr. José Ermírio de Moraes 290. Bairro Lageado. CEP 18030-230. Sorocaba, SP, Brazil

³ Depto Química, Universidade Federal de Roraima. Campus Paricarana. Av. Cap. Enê Garcez 2413. Bairro Aeroporto. CEP 69304-000. Boa Vista, RR, Brazil

Corresponding author: * rbsinger1@yahoo.com

ABSTRACT

Pre-existing literature reports that plants of *Habenaria pleiophylla* Hoehne & Schlechter (Orchidaceae: Orchidinae) occurring in *Eucalyptus* plantations in Rio Grande do Sul (Southern Brazil) are pollinated by diurnal Nymphalid butterflies. This is surprising, since several flower features of this orchid suggest nocturnal pollination. Field evidence obtained by the authors in a natural population occurring in Ilha Do Cardoso State Park (São Paulo State, Southeastern Brazil) clearly demonstrated pollination by crepuscular/nocturnal Lepidoptera. Thus, the aim of this contribution is to present the first detailed description of this orchid's pollination biology under natural conditions. *H. pleiophylla* flowers secrete nectar and are fragrant at dusk and during the night. Pollinators are moths of *Cosmosoma auge* (L. 1767) (Arctiidae) and *Plusia admonens* Walker, [1858] (Noctuidae, Plusiinae). Moths bore 2-9 pollinaria which adhered to the surface of their eyes. During our observations, the male efficiency factor was 1.8 (meaning that 1.8 flowers were pollinated per pollinarium removed). Individual fruiting success ranged from 61 to 100%. Possible consequences for plant reproductive success of both, flower intrinsic morphological features and pollinator behaviour are discussed. Self-pollinated flowers under epifluorescence microscopy showed normal and abundant pollen tubes, thus suggesting that flowers of *H. pleiophylla* may be self-compatible. To our knowledge, this is the first formal citation of *H. pleiophylla* for the orchid flora of Ilha do Cardoso. Unpublished, preliminary field observations made in May 1996, at Ilha do Cardoso (Paraná State) suggest that short-tongued sphingid moths (*Aellopos* sp.) may also act as pollinators.

Keywords: Arctiidae, flower morphology, moths, Noctuidae, orchids, Sphingidae

INTRODUCTION

With about 600 described species (Dressler 1993), the cosmopolitan genus *Habenaria* Willdenow (Orchidaceae: Orchidinae), is remarkable due to its diversified flower morphology (Hoehne 1940; Dressler 1993; Szlachetko and Rutkowski 2000). There is a large amount of information concerning the pollination biology of Palaeotropical and Old-World species (reviewed in van der Pijl and Dodson 1966; van der Cingel 1995, 2001). Most records indicate different kinds of Lepidoptera as pollinators, in agreement with the presence of flower features such as fragrant, long-spurred flowers (van der Pijl and Dodson 1966; van der Cingel 1995). A few species are also pollinated by mosquitoes or Tipulidae Crane-flies (Stoutamire 1968; Thien 1969; Singer 2001).

Information concerning Neotropical species is scant (van der Cingel 2001). Autogamy (automatic self-pollination) has been suggested to occur in a number of Central American species (Ackerman 1995). Pollination by sphingid moths was recorded for *H. gourlieana* in Central Argentina (Singer and Cocucci 1997). Pollination by Noctuid moths was recorded in *H. hexaptera* (as *H. hieronimyii*) in the same region (Singer and Cocucci 1997). In these two cases, the pollinators bear the pollinaria on the eyes, the only body part smooth enough for the pad-like viscidia to glue (Singer and Cocucci 1997). More recently (Singer 2001) the pollination of *H. parviflora* by both, Pyralid moths and Tipulidae crane-flies was documented. The viscidium in this species is glove-like and involute and embraces the proboscis of the pollinators (Singer 2001). Populations of *Habenaria pleiophylla* occurring in *Eucalyptus* plantations in Rio Grande do Sul (Southern Brazil) were reported as pollinated by the butterfly *Heliconius erato*

phyllis (Nymphalidae) (Moreira *et al.* 1996). Captured butterflies bore large number of pollinaria adhered onto the eyes. The pollination mechanism was elucidated offering *H. pleiophylla* inflorescences to caged insects (Moreira *et al.* 1996). In May 1996, during fieldwork in Ilha do Mel (Paraná State, Southern Brazil), a few short-tongued Sphingidae moths (*Aellopos* sp.) were sighted visiting inflorescences of *H. pleiophylla* Hoehne & Schlechter. One of these moths carried a pollinarium (this is, the pollinia plus accessory structures that adhere them onto the pollinator) adhered on an eye, but further and more detailed observations were not possible (R.B. Singer, unpublished data). During recent floristic fieldwork in Ilha do Cardoso (São Paulo State, Southeastern Brazil), a large population of *H. pleiophylla* was found. As a consequence of this fortunate finding, more detailed observations were attempted, within the limitations of our tight time schedule. These observations brought to light evidence contradicting the previously published report (Moreira *et al.* 1996). The aims of the present contribution are 1) to provide details on the pollination mechanism of *H. pleiophylla*, and 2) to discuss the probable consequences of both, flower morphology and pollinator behavior for this orchid's reproductive success.

MATERIALS AND METHODS

Field observations were carried out in Ilha do Cardoso (Cananéia Municipality, São Paulo State, Southeastern Brazil; 25° 03' 05" and 25° 18' 18" S, 47° 53' 48" and 48° 05' 42" W) from May 11 to May 13, 2003. Ilha do Cardoso is an island (area estimated in ca. 22,500 ha) with Atlantic Rain Forest vegetation. The climate is wet and annual rainfall often surpasses 3,000 mm. Annual mean temperature reaches about 21.2°C (Barros *et al.* 1991). Crepuscular-nocturnal observations took place in 11, 12 and the 13th May, 2003,

from 17:00 to 20:00 hrs. This time schedule was adopted since previous observations at Ilha do Mel (Paraná State) indicated that flowers of *H. pleiophylla* start emitting a sweet, pleasant fragrance at dusk (ca. 18 hs), suggesting crepuscular or nocturnal pollination as in previously studied species (Singer and Cocucci 1997; Singer 2001). This pattern of scent production was also confirmed for the plants of Ilha do Cardoso. Anyhow, diurnal observations were also performed from 9:00 to 13:00 hs in May 13, 2003. In total, 13 observations hours were spent.

The population of *H. pleiophylla* consisted of 82 inflorescences growing in open, grassy vegetation, less than 300 meters from the sea. Inflorescences ranged from 20 to 90 cm in height. Pollinator behavior at inflorescences was recorded with the help of a flashlight. At 10-15 min. intervals, we walked through the population and illuminated the inflorescences looking for pollinators. All available inflorescences ($n = 82$) were used for pollinator observations. Pollination behavior was recorded through field notes and photographs. In most cases, observations were made in the dark and thus, most observations refer to pollinator behavior as was perceived. It was not possible to ascertain when insects arrived or how many inflorescences they had already visited until they were noticed. Plant vouchers (T. Breier 1004) were deposited at ESA and UEC. To our knowledge, this is the first formal citation of *H. pleiophylla* for the orchid flora of Ilha do Cardoso (see Barros *et al.* 1991). Insect vouchers were deposited at ZUEC.

In May 12, 2003, the male efficiency factor (percentage of pollinated flowers divided by the percentage of flowers acting as pollen donors) (Nilsson *et al.* 1992) was calculated for 49 (59.7% of the total) inflorescences. The main criteria to select the inflorescences were accessibility and freshness. Flower stigmatic surfaces have to be unequivocally fresh in order to ascertain if they were pollinated or the flower simply withered.

Since it was uncertain whether or not we could return to the study area, three inflorescences were cut from the base and kept alive for further observations by placing them in a glass jar with water and by daily cutting a small piece at the base of the inflorescence axis. A total of 18 flowers were self-pollinated by brushing the pollinaria onto their respective stigmatic surfaces. To guarantee that these flowers were not already pollinated, only very fresh, unequivocally unpollinated flowers (stigmatic surfaces clean, without massulae) that bloomed at the laboratory were used. Nine flowers were cut and fixed 24 hours after manual pollination. The same number of flowers were cut and fixed after 48 hours after self-pollination. All these flowers were kept 48 hours in FAA (50%) and later transferred to ethanol 70%. Then, flowers were softened in 8N NaOH at 60°C overnight and washed several times with distilled water, stained with aniline blue, squashed and observed by epifluorescence microscopy. Flower features were photographed from fresh flowers obtained from the aforementioned inflorescences; with the help of a Nikon binocular stereomicroscope at the Taxonomy laboratory of the Universidade Estadual de Campinas (Unicamp). In addition, the nectar content of eight additional fresh flowers was collected with a microsyringe. Nectar concentration was ascertained with the help of a refractometer.

Fruiting success was estimated in July 3, 2003 as the number of fruits produced by 50 inflorescences (61% of the total), that produced 5,328 flowers, as a whole. The main criterion to select these inflorescences was their accessibility.

We followed the morphological and taxonomic concepts of Dressler (1993) and Chase *et al.* (2003), respectively, in this work.

RESULTS AND DISCUSSION

Flower features

Habenaria pleiophylla Hoehne & Schlechter is a herb with multiflowered, cylindrical, congested inflorescences up to 90 cm tall. Flowers are yellowish-green (Fig. 1A-B). Only flower features pertinent to pollination biology will be here discussed. Readers interested in more details on perianth parts shape and size, are referred to Pabst and Dungs (1975) or Hoehne (1940, where this species is discussed under *H. leptoceras* Lindl.). In contrast with previously (Singer and Cocucci 1997; Singer 2001) studied species, the ovary is

perpendicular to the inflorescence axis. As a whole, flower length varied from 20 to 21.8 mm (mean = 21.2 mm; $n = 10$). The concave, sturdy dorsal sepal partially holds the lateral petals. The lip is trilobed, the median lobe being strongly bent backwards. The posterior part of the lip forms an up curved, acute spur. The spur contains a column of nectar (1.7-2 mm high), the only flower reward. The nectar ($n = 8$ flowers) samples were 40% concentrated and each flower produced up to 5 μ L. Nectary structure and patterns of nectar secretion are beyond the scope of this contribution. Fine details on the nectaries and nectar properties of some related *Habenaria* species can be found in Galetto *et al.* (1997). The length of the spur varied from 21 to 23.7 mm (mean = 22.3 mm; $n = 10$). As usual in subtribe Orchidinae, the column holds two massulate pollinaria made up by the (massulate) pollinia, long hyaline caudicles and a terminal pad-like viscidium (Fig. 1C) (Dressler 1993; Szlachetko and Rutkowsky 2000). Pollinarium length varies from 4.4 to 4.8 mm (mean = 4.58 mm; $n = 10$). The presence of pollinia divided in subunits so-called massulae is a consistent feature in Orchidinae orchids (Dressler 1993). Each pollinium holds 309-375 massulae (mean = 326.2; $n = 10$). The pollinaria are held in two parallel anther sacs (Dressler 1993). When the pollinaria are removed, they leave the column through two parallel dehiscence lines. The distance between the viscidia is from 1.8 to 3.8 mm (mean = 2.68 mm; $n = 10$). Spur entrance is just below the rostellar median lobe (Fig. 1D). Rostellar median lobe is well-developed and appears as a triangular process between the two anther sacs (Dressler 1993; Singer and Cocucci 1997; Fig. 1D). There are two stigmatic surfaces in respective stalks (Fig. 1C-E). The stigmatic surface is flat to slightly convex (Fig. 1D-E). This has been interpreted as an adaptation to maximize the number of massulae that can be deposited in a pollinator visit. Most Epidendroid orchids bear entire, indivisible pollinia which are readily caught by deeply concave stigmatic cavities. Such a correlation has already been emphasized by Dressler (1993). Conversely, Orchidoid species produce massulate or granular, friable pollinia whose pollen content can be spread over several flowers and inflorescences. In correlation, these orchids show large, ample stigmatic surfaces that can receive pollen loads from different co-specific flowers. The seed content of the capsules can – in theory – have different parentals (Neiland and Wilcock 1995; Freudenstein and Rasmussen 1996; Singer and Sazima 1999, 2001a, 2001b; Singer 2001, 2002) but this matter still needs empirical proof.

The flowers start to emit a sweet, pleasant fragrance at dusk (ca. 18:00 hrs). Fragrance production apparently continues throughout the night, but pollinator activity at flowers was perceived only in a relatively brief period of time before dusk (see Pollination Mechanism and Pollinator Behaviour). The fruit is a dehiscent capsule, full of minute, dust-like seeds.

Pollination mechanism and pollinator behaviour

During observations at Ilha do Cardoso, only medium-sized moths of *Plusia admonens* Walker, [1858] (Noctuidae, Plusiinae) (Fig. 1A-B, 1F) and *Cosmosoma auge* (L. 1767) (Arctiidae) (Fig. 1G) were recorded as pollinators. A few Sphingidae moths of *Xylophanes tersa* (Linnaeus 1771) (Sphingidae, Macroglossinae) were recorded as flower visitors, but, as could be noticed in the photographs, these moths had tongues longer than the orchid spur. Consequently, they probed the flowers without touching the column and removing pollinaria. Both, Arctiidae and Noctuidae moths displayed a settling behavior, visiting 1-5 flowers and spending up to 30 seconds at each inflorescence. The moths use the lip as a landing platform, inserting the tongue into the spur. In doing so, they press the head against the column. At this moment, the viscidia contact the surface of the eyes and so the pollinaria become adhered (Fig. 1A-B, 1F-G). The pollinaria are removed when the insects leave the flowers. During the present observations, noctuid moths were rec-

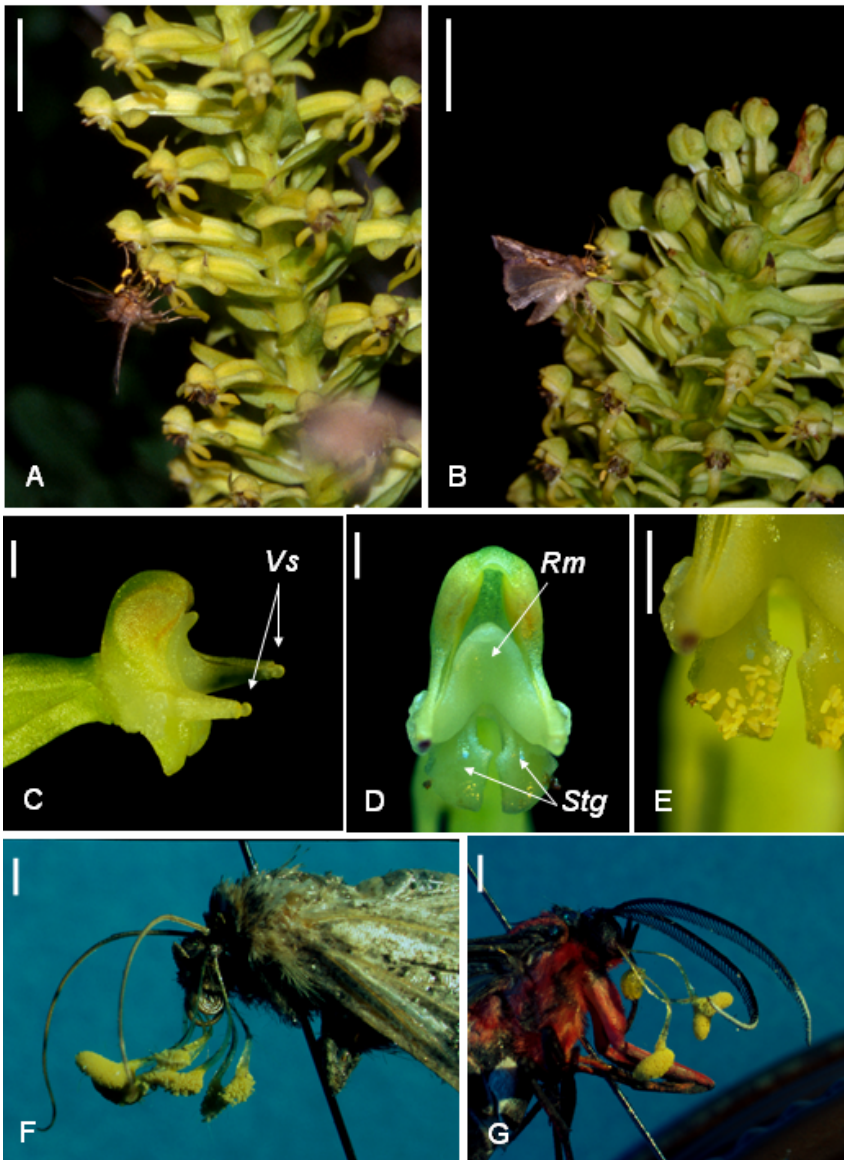


Fig. 1 (A) and (B) Inflorescences of *Habenaria pleiophylla* being visited and pollinated by moths of *Plusia admonens* (Noctuidae). Notice that in both cases moths are laden with pollinaria. (C-E) Details of the column in *H. pleiophylla* (including D). (C) Column in lateral view. (D) Column in frontal view. (E) Detail of stigmatic surfaces laden with massulae. (F) and (G) Pollinators of *H. pleiophylla* laden with pollinaria onto their eye surfaces. (F) *Plusia admonens* (Noctuidae). (G) *Cosmosoma auge* (Arctiidae). *Rm*: rostellar median lobe. *Vs*: viscidia. *Stg*: stigmatic surfaces. Scale bars = 10 mm in (A) and (B); 1 mm in (C), (D), (E), (F) and (G).

orded bearing 2-9 pollinaria (Fig. 1F). The only captured individual of *Cosmosoma auge* bore four pollinaria (Fig. 1G).

Pollination is effected when a moth laden with pollinaria visits another flower. During flower probing, the moth will brush the pollinaria surface against the stigmatic surfaces, leaving clumps of massulae. Since moths are able to carry several pollinaria (likely from different inflorescences), many stigmatic surfaces may receive pollen-loads from different individual donors (Singer 2001). In a few circumstances, the moths were observed to leave the inflorescences and visit others nearby. Many times the moths were observed to simply land or perch in the surrounding vegetation. Then, the moths either perched with their wings folded or actively tried to remove the pollinaria from their eyes with the forelegs. This clearly indicates that the pollinaria somehow disturb insects' normal behavior. All these behaviors suggest that long-distance pollen flow may not necessarily be the rule. Cut inflorescences in glass jars remain fragrant all night long. However, observed pollinator activity was restricted to a short period from dusk (ca. 18 hrs) until 19 hours (rarely, a few minutes later). A similarly restricted visitation pattern was already reported in the Malagasy *H. decaryana* (Nilsson and Jonsson 1985), which is also pollinated by Noctuid moths.

Remarkably, a previous published report on the pollination of *H. pleiophylla* indicated diurnal pollinators (Moreira *et al.* 1996). Moreira *et al.* (1996) recorded large numbers of Nymphalid butterflies, *Heliconius erato* *Phyllis*, laden with pollinaria (up to 19) of *H. pleiophylla* in *Eucalyptus*

plantations in Rio Grande do Sul (Southern Brazil). Caged insects were seen to dislodge pollinaria onto the eyes' surface while taking nectar from the spurs (Moreira *et al.* 1996). We remain somewhat skeptical, however, on the meaning of these observations. The two naturally occurring populations we know (Ilha do Mel, Paraná State and Ilha do Cardoso, São Paulo State) hold plants with flower features suggesting moth-pollination, such as greenish, crepuscular-night scented flowers. We agree with Moreira *et al.* (1996) that the observed interaction between butterflies and *H. pleiophylla* may in part be caused by the natural poverty of nectar resources in *Eucalyptus* plantations. However, there is a significant aspect to be highlighted in these apparently contradictory observations: an ecological shift from nocturnal to diurnal pollinators is possible (for whatever reasons), thus enhancing the chances of reproduction and recruitment in *H. pleiophylla*.

No diurnal pollinators or flower visitors were sought at the *Habenaria* inflorescences, even when abundant females of *Xylocopa* (Apidae) bees and *Vanessa* (Nymphalidae) butterflies were recorded in the nearby vegetation.

Male efficiency factor

Male efficiency factor (Nilsson *et al.* 1992) was estimated in May 12, 2003. Then, 385 fresh flowers were counted, distributed in 49 inflorescences. During our observations, 334 flowers (86.75%) had their stigmas pollinated and 187 (48.75%) acted as pollen donors. Yet, 107 flowers (27.79%) had only one pollinarium removed. Only 80 flowers

(20.8%) had both pollinaria removed. The male efficiency factor was 1.779. This means that ca. 1.8 flowers were pollinated per pollinarium removed. This value compares favorably with previously reported data for a community of *Habenaria* spp. in Central Argentina (Singer and Cocucci 1997) (0.30 for *H. gourlieana*, 1.15 for *H. hieroyimi*, 0.60 for *H. montevidensis* and 0.79 for *H. rupicola*). The sectile texture of the pollinarium *per se* would suggest that the pollen content of a single pollinarium should be spread along several consecutively visited flowers. On the other hand, Nilsson's coefficient may (in our opinion) underestimate the real pollination efficiency in orchids that produce two pollinaria, since in these orchids "asymmetrical" (one pollinarium only) donations are possible. On the other hand, Neiland and Wilcock (1995) have demonstrated that relatively small pollen loads may be enough to trigger fruit and seed set in some European terrestrial orchids with flower features (e.g. sectile pollinaria) similar to those of *H. pleiophylla*.

The fact that pollinators are able to carry pollinaria from different inflorescences may have deep consequences on a plant's reproductive biology and genetic diversity. As already emphasized by Freudenstein and Rasmussen (1996), each massula contains the progeny of a single pollen mother cell. This is, each massula represents a different genotype. Thus, pollinators carrying pollinaria from different plants may leave clumps of massulae from different progenitors on the stigmatic surface of the visited flowers. Consequently, fruits with contributions from different pollen progenitors may be produced (Neiland and Wilcock 1995; Freudenstein and Rasmussen 1996). This idea still needs empirical support. It is still possible (but also needs to be empirically tested) that even if plants are self-compatible (which seems to be the case for most plants of the subfamily Orchidoideae; Dressler 1993; Neiland and Wilcock 1995; van der Cingel 1995, 2001; Singer 2003), pollen from cross-pollinations may have a selective advantage, reaching the ovules faster. In theory, the broad stigmatic surface could act as a selective tract where faster/more vigorous pollen tubes may prevail in their way toward the ovules. This is a matter we expect to study in detail in the near future. Neiland and Wilcock (1995) have demonstrated that European orchids with similar flower features (including massulate pollinia) can remain receptive for up to eight days after a pollination event, thus increasing the chances of successive pollinations, cross-pollinations and multi-parental seed set.

Preliminary observations of manually self-pollinated flowers using epifluorescence microscopy suggest that *H. pleiophylla* may be self-compatible. Twenty four hours after self-pollination, great quantities of pollen tubes were recorded emerging from the massulae. At this moment, pollen tubes were recorded either at the median part of the stilar channels or almost reaching the proximal part of the ovary. Forty-eight hours after pollination, all examined flowers had pollen tubes reaching the first portion of the ovary. All observed pollen-tubes were likely normal (without deformities). Self-compatibility was recently reported for *Habenaria parviflora* (Singer 2001) and it may be widespread in the genus. Self-compatibility is likely a rule in the whole tribe Orchideae, owing to the great number of reports (reviewed in Dressler 1993; van der Cingel 1995, 2001).

Fruiting success

Fruiting success was remarkably high. From a total of 5,328 flowers produced by 50 individuals, 4,619 of them developed into fruits (86.7%). Individual fruiting success ranged from 61 to 100% (mean = 87%). These high values are in contradiction with the statements of Neiland and Wilcock (1998) which suggested that tropical orchids (either nectariferous or nectarless) usually display fruit-sets lower than 40%. The high fruiting success of *H. pleiophylla* could, in our opinion, be explained by a sum of factors. These fac-

tors include intrinsic flower features of *H. pleiophylla* such as the sectile nature of the pollinarium and (presumably) self-compatibility. On the other hand, the fact that pollinators are apparently abundant, visit several flowers and are able to carry a high number of pollinaria, may also account for a significant part of the fruit set.

Remarks on other Brazilian *Habenaria* species

A brief discussion on flower features of other Brazilian *Habenaria* spp. seems opportune. Some additional species occurring in southeastern Brazil have flowers with very similar features to those of *H. pleiophylla* and we believe they may be pollinated very much in the same way. These species are *H. leptoceras* Lindl. (Fig. 2A-B) and *H. petalodes*. These two species share significant flower features with *H. pleiophylla*, such as the yellowish-green flowers perpendicular to the inflorescence axis and the up curved spur (Fig. 2A). The column general ground plan is also very similar in these species. These plants also emit fragrances after dusk, and, in our opinion, may also be pollinated by guilds of adequately sized moths. A cultivated plant of *H. leptoceras* (L. Freitas. s.n., Unicamp) has shown to be self-compatible (20 fruits obtained from 20 self-pollinations). Still, many seedlings developed after fruit ripening though did not reach reproductive age as yet (R.B. Singer, pers. obs.). Flower morphological features suggest that the pollinaria of these *Habenaria* species may also adhere onto the eyes of Lepidopteran pollinators.

On the other hand, the flowers of other Brazilian *Habenaria* spp. show sets of floral features suggesting moth-pollination, but with additional and significant morphological

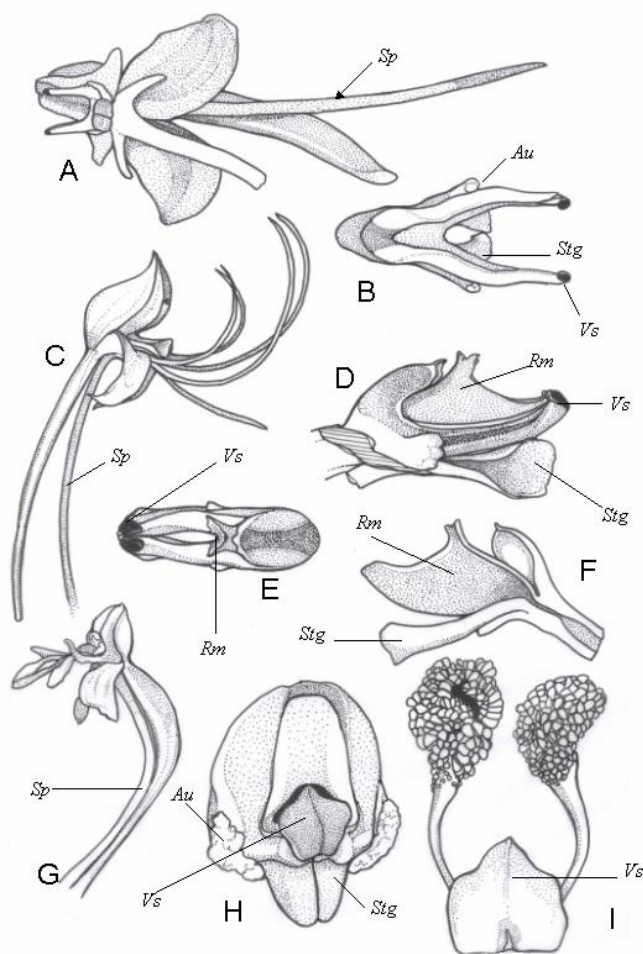


Fig. 2 Floral features in other Brazilian *Habenaria* spp. (A) and (B) *Habenaria leptoceras* Hook. (C) and (F) *H. johannensis* Barb. Rodr. (G) and (I) *H. rodeiensis* Barb. Rodr. Au: auricles. Rm: rostellar median projection. Sp: spur. Stg: stigmatic surface. Vs: viscidium.

modifications. Two of these species are *H. johannensis* Barb. Rodr. (Fig. 2C-F) and *H. rodeiensis* Barb. Rodr (Fig. 2G-I). In *H. johannensis*, the median rostellar projection is particularly well developed and boat-shaped (Fig. 2D-F). Remarkably, in this species the two viscidia are facing up (Fig. 2D-E). The flowers or *H. rodeiensis* are remarkable in that there is a single, broad viscidium; and, consequently, a single pollinarium. Studies on the floral biology of both species seem worthwhile to understand the biological meaning of such noteworthy morphological modifications.

ACKNOWLEDGEMENTS

Authors wish to thank the staff of Parque Estadual Ilhado Cardoso and the people of the “Projeto Parcelas Permanentes” (Biota/FAPESP) for logistic support. We would also like to thank the staff of the Taxonomy Laboratory at Botany Department, Unicamp for allowing the use of photographic material. Professor Victor Becker (Serra Bonita, Bahia) identified all mentioned Lepidoptera. This contribution was made possible through a FAPESP grant (Process 01/08958-1) to the first author, which is greatly acknowledged.

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