

## **Isolation mechanisms between two sympatric *Sophronitis* (Orchidaceae) species endemic to Northeastern Brazil**

V. Silva-Pereira<sup>1</sup>, E. de Camargo Smidt<sup>1</sup>, E. Leite Borba<sup>2</sup>

<sup>1</sup>Departamento de Ciências Biológicas, Laboratório de Sistemática Molecular de Plantas, Universidade Estadual de Feira de Santana, Feira de Santana, Bahia, Brazil

<sup>2</sup>Instituto de Ciências Biológicas, Departamento de Botânica, Laboratório de Sistemática, Universidade Federal de Minas Gerais, Pampulha, Belo Horizonte, Minas Gerais, Brazil

Received 3 January 2007; Accepted 12 June 2007; Published online 26 October 2007  
© Springer-Verlag 2007

**Abstract.** *Sophronitis sincorana* and *S. pfisteri* (Orchidaceae) are endemic to the *campo rupestre* vegetation, northeastern Brazil, occurring sympatrically and flowering synchronously. In this work we studied the reproductive biology of both species and investigated reproductive barriers between them. Both species are partially self-compatible, pollinator dependent, and are pollinated by males and worker bees of *Bombus brevivillus*. Pollinaria are deposited on the anterior portion of the mesonotum of these insects in *S. pfisteri*, and on the scutellum in *S. sincorana*. The flowers of both species are deceptive, as they have a dry cuniculus. Morphological differences in the labellum and the gynostemium impede inter-specific pollination of *S. pfisteri* with pollen from *S. sincorana*. Very low fruiting results from inter-specific crosses of *S. sincorana* with pollen from *S. pfisteri*. Although viable seeds can be produced from experimental inter-specific crosses, no natural hybrids were found. Species integrity appears to be maintained by additive unidirectional reproductive barriers between them.

**Keywords:** *Bombus brevivillus*; *campo rupestre*; Chapada Diamantina; Orchidaceae; pollination; reproductive biology; reproductive isolation; *Sophronitis pfisteri*; *Sophronitis sincorana*

### **Introduction**

A well known characteristic of the family Orchidaceae is its very high potential for forming inter-specific hybrids. Production of artificial hybrids is extremely common, with hybrids arising from crosses between related species, species of different genera, or even between different sub-tribes (Dressler 1981; Arditti 1992). The occurrence of natural hybrids in this family is also relatively common in natural environments, and is well documented in the literature (Dressler 1993, Tremblay et al. 2005; Cozzolino et al. 2006). However, the natural coexistence of related species with the maintenance of species integrity is only

possible if there are efficient mechanisms in place that deter inter-specific genetic flow (Dressler 1981; Levin 2000).

The principal mechanisms of reproductive isolation in the family Orchidaceae act at the pre-pollination phase, and involve ecological or ethological factors (Dodson 1962; van der Pijl and Dodson 1966; Dressler 1981; Gill 1989; Borba and Semir 1999). Among the diverse mechanisms described for this family, the principal mechanical barrier to the transfer of pollinaria between sympatric species arises from the differences in floral structure over an almost limitless range of forms and sizes (Dodson 1962; Thien and Marcks 1972; Borba and Semir 1999, 2001). Various visual and chemical attractors that reduce the sharing of pollinators between plant species are also related to the specificity observed in the flower-pollinator interactions (Gill 1989).

Reproductive inter-incompatibility between closely related species, although rare, is known to occur independently in unrelated genera in Orchidaceae, such as *Cryptostylis* (Stoutamire 1975), *Oncidium* (Sanford 1964), *Bulbophyllum* (Borba et al. 1999) and *Disa* (Johnson et al. 1998). Karyotype differences can also act as efficient post-zygotic reproductive barriers between closely related species that share the same pollinators in the subtribe Orchidinae (Cozzolino et al. 2004).

The Laeliinae (Epidendroideae, Orchidaceae) comprises approximately 40 genera and 1450 species distributed throughout the Neotropical zone (Dressler 1993; van den Berg et al. 2000, 2005). This subtribe contains some of the most important genera of ornamental plants, such as *Cattleya*, *Laelia*, and *Sophronitis*. Due to extensive collecting, as well as the destruction of their native habitats, a number of species within this group are considered to be threatened with extinction (IUCN 1996). A number of different groups of pollinators have been reported within the Laeliinae, especially insects within the orders Hymenoptera and Lepidoptera (Dodson 1962; van der Pijl and Dodson 1966). The first group is responsible for pollination in a majority of the genera, and

is a large part responsible for the origin and diversity of this subtribe. Studies of the phylogeny of the subtribe and of pollination in the putatively less derived genera suggest that pollination by large bees is the plesiomorphic condition (Matias et al. 1996, van den Berg et al. 2000; Borba and Braga 2003).

The genus *Sophronitis* comprises approximately 40 species distributed throughout South America. The majority of these species have a restricted distribution, principally the rupicolous species found in the Brazilian states of Minas Gerais and Bahia and in the southeastern and northeastern regions of that country. This genus was recently expanded with molecular phylogenetic studies (van den Berg and Chase 2000; van den Berg et al. 2000), and currently comprises species of *Sophronitis* (s. str.) as well as some Brazilian species previously included within *Laelia* (van den Berg 2005). Chiron and Castro (2005) have divided the genus *Sophronitis* in several minor genera, based on morphological affinities. However, in this paper we are following the classification by van den Berg (2005), presented in the Genera Orchidacearum series. Studies of floral biology and pollination in the genus *Sophronitis* are limited to observations of unidentified species of hummingbirds visiting *Sophronitis milleri* (van der Pijl and Dodson 1966), *S. coccinea* (Manuel et al. 1996), and *S. mantiqueirae* (Buzato et al. 2000).

The two species of the genus *Sophronitis* examined in the present study have their distribution limited to the *campo rupestre* vegetation in the Serra do Sincorá Range, part of the larger Chapada Diamantina Mountain Range in northeastern Brazil. *Sophronitis sincorana* (Schltr.) Van den Berg and M.W. Chase and *S. pfisteri* (Pabst and Senghas) Van den Berg and M.W. Chase occur sympatrically and flower synchronously, but apparently do not produce natural hybrids. We attempted to discover what mechanisms might be acting to maintain the genetic integrity of these two taxa, especially in light of the fact that genetic barriers are not well developed in this family, and especially not in this genus. As such, the present work sought to examine these two

species more closely in order to describe their floral biology as well as their pollination and reproductive systems, and to identify the mechanisms involved in their reproductive isolation.

### Materials and methods

**Study area.** The study was carried out in populations located in the Parque Nacional da Chapada Diamantina, Bahia state, northeastern Brazil, located in the Espinhaço Mountain Range. The exact locations of the populations are being omitted due to the threat of unauthorized collecting. This mountain range is about 1000 km long and between 50 and 100 km wide, and comprises a large number of component mountain chains with an average altitude of approximately 1000 m. The Espinhaço Range is delimited on the south side by the Serra de Ouro Branco (21°25'S) in central Minas Gerais State, and on the north by the municipality of Juazeiro (10°00'S), Bahia state, where it is called Chapada Diamantina (Giulietti and Pirani 1988; Giulietti et al. 1997). The Espinhaço Range is considered the center of origin and diversity of a number of taxa, with discontinuities between it, the Andes, the *restingas* (shoreline areas) of eastern Brazil, and especially the northern portion of South America (Giulietti and Pirani 1988; Harley 1988; Giulietti et al. 1997). The *campos rupestres* vegetation, typical of areas above 800–900 m, is essentially restricted to the large Brazilian mountain ranges of Espinhaço, Serra da Canastra, and Serra Geral (Harley 1988; Giulietti et al. 1997). An herbaceous layer of monocotyledons predominates in this vegetation type, with scattered dicotyledonous shrubs and sub-shrubs. Giulietti and Pirani (1988) have suggested that the discontinuity between mountain ranges (as well as between the discontinuous rocky outcrops within those ranges) has contributed to the great diversity and high degree of endemism found in the *campos rupestres* vegetation. The climate there corresponds to type Cwa in Köppen's (1948) classification (high altitude tropical with mild summers, with a well defined dry season from May until September, and a wet season between October and March).

**Species.** *Sophronitis sincorana* and *S. pfisteri* are endemic to the Serra do Sincorá (Chapada Diamantina, northeastern Brazil), in the municipality of Mucugê, where they frequently occur in sympatry and flower in synchrony, from October to January.

The individuals of *S. sincorana* are rupicolous or epiphytic herbs, growing on rocks and on *Vellozia* (Velloziaceae) species, always near the rocky slopes of the Serra do Sincorá. They have short inflorescences, not rising from a spathe, and somewhat large flowers with lamellae on the lip. In addition to sexual reproduction, *S. sincorana* individuals reproduce vegetatively, forming groups of clones on the rocks. This species was described in 1917, and remained almost unknown until 1969, when it was described by Augusto Ruschi as *Cattleya grosvenori*, also for the Chapada Diamantina. According to Withner (1990), *S. sincorana*, previously included in *Laelia* sect. *Hadrolaelia*, is a species frequently used for hybridization in horticulture due to its small, but robust, habit and its large pink flowers. The restricted geographic range distribution and over-collection by orchid enthusiasts have caused this species to be threatened with extinction.

*Sophronitis pfisteri* is the only species belonging to the complex included previously in the section *Parviflorae* of *Laelia* with pink flowers in the Chapada Diamantina (Withner 1990). At lower elevations there is a different species, *S. bahiensis* (Schltr.) Van den Berg and M.W. Chase, that also belongs to section *Parviflorae*, but it has either yellow or orange flowers and a different flowering time. *Sophronitis pfisteri* grows on rocks, mainly in small cracks where organic material accumulates. It is distributed along the rocky slopes of the Serra do Sincorá, extending to inner parts of the mountain range, above 1200 m. The individuals have inflorescences longer than the leaves, arising from a sheath, with flowers somewhat smaller than those of *S. sincorana* and with the mid lobe of the lip bent backward. *Sophronitis pfisteri* is rarely used for hybridization in horticulture, but is very ornamental and is frequently collected in the study area. *Sophronitis sincorana* and *S. pfisteri* were transferred to the genera *Hadrolaelia* and *Hoffmannsegella*, respectively, by Castro and Chiron (2002), a position followed by some orchidologists and most orchid growers, but not adopted by us.

**Reproductive biology.** The behaviour of pollinators visiting *Sophronitis sincorana* was investigated from November 19 to 23, 2002, and from November 18 to 24, 2003, for a total of 170 hours of observations. Observations on *S. pfisteri* were carried out from November 18 to 24, 2003, for a total of 90 hours of observations. Observations were carried out between 06:00 and 18:00 h for both

species. In order to check for the possible removal of pollinaria outside of the direct observation period, flowers were marked at the end of the day and examined early the next morning. Floral visitors were collected on the flowers and later identified by specialists for each group. The presence of nectaries was checked by dissecting the flowers. Osmophores were identified by staining with neutral red (Vogel 1990). The presence of pigments that absorb in the ultraviolet spectrum was tested by staining in an ammonium hydroxide atmosphere (Scogin et al. 1977). The number of fruits that developed in open pollination was determined by direct counting of swollen ovaries until fruit dehiscence. Both pollinaria removal from anthers and deposition on stigmas were counted from withered flowers collected randomly in *S. sincorana* population, and used as an estimation of pollinator visit and pollination frequencies, respectively. The number of pollinaria of *S. pfisteri* that was removed or deposited was not evaluated due to the small number of individuals and flowers available, and to the fact that many of these plants were used in experiments on reproductive biology.

Breeding system for both species was determined through experimental pollinations (self- and cross-pollinations; 15 flowers for each treatment and each species). Floral buds ( $n = 15$ ) were bagged to exclude pollinators in tests of spontaneous self-pollination and agamospermy. Interspecific compatibility was evaluated through bidirectional crossings, using 15 flowers of each species receiving pollen from the other species. Approximately 400–600 seeds per fruit were scored for viability (presence of a well-developed embryo), as described by Borba et al. (2001). Results were analyzed using one-way ANOVA with further Tukey multiple comparisons of means, using BioEstat 3.0 software (Ayres et al. 2003). Additional pollinations were carried out and the flowers were fixed in 50% FAA six, 10, 16, 24, 32 and 165 days after these experimental pollinations. The column (gynostemium) of those flowers were softened in 10N NaOH at 60 °C for 70 min., washed in distilled water and then stained with aniline blue, squashed and observed by epifluorescence microscopy (modified from Martin 1959) to examine pollen tube growth and ovule/seed morphology.

## Results

**Flower morphology.** *Sophronitis sincorana* and *S. pfisteri* have similar overall flower morphol-

ogy, but differ mainly in size of the flower parts and number of flowers (Table 1, Fig. 1A–C). The inflorescences of *S. sincorana* have a short peduncle (up to 100 mm), bearing one or occasionally two or three open flowers at one time. *Sophronitis pfisteri* has long peduncle (up to 300 mm) with five to 10 flowers, but with only two or three of them opened at one time. Flowers of both species have patent, oblong-lanceolate, and magenta petals and sepals. The lip is trilobed, the disc (median-central region of the lip) is white with five keels and the lateral lobes envelop the gynostemium. The terminal lobe is wide and has crisped margins. In *S. sincorana* it is straight, while in *S. pfisteri* it is reflexed. Both species have a tubular cavity originating from adnation of the lip base to the column (cuniculus), but this varies greatly in length between the two species. This cavity is similar to a nectar cavity, although nectar production was not observed at any time during the day.

Osmophores were observed in both species in the disc (a position that coincides with keels) and on the lateral lobes of the lip, which externally covers the column. The odor is sweet and weak, and appears to be similar in the two species examined. It is emitted between 8:00 and 14:00 h, but is most intense between 10:00 and 12:00 h, as perceived by the authors. Exposure to ammonium hydroxide indicated that the mid region of the lip in both species absorbs ultraviolet light.

**Floral visitors.** *Sophronitis sincorana* and *S. pfisteri* are visited and pollinated by bees of the family Apidae – workers and males of *Bombus* (*Fervidobombus*) *brevivillus* Franklin, 1913. These insects are of medium size (ca. 1.0 cm×0.5 cm) and rapid flight, with generalist habits of pollen and nectar collecting, and are the most bees most frequently observed in the study area.

All visitations of *Bombus brevivillus* observed to the flowers of *Sophronitis sincorana* and *S. pfisteri* occurred between 11:00 and 13:00 h, and visits were very rare. Only two visits were observed in *S. sincorana*, and four in *S. pfisteri*, pollinarium removal occurring in all of them. The bees visited the flowers rapidly

**Table 1.** Comparative flower morphology of *Sophronitis sincorana* and *S. pfisteri* at Chapada Diamantina, Northeastern Brazil. Values presented as mean  $\pm$  standard deviation (minimum-maximum)

| Character           | <i>S. sincorana</i> (n = 12)  | <i>S. pfisteri</i> (n = 10)  |
|---------------------|---|--|
| Peduncle (length)   | 75 $\pm$ 14.7 (50–100) mm   | 44 $\pm$ 11.9 (300–600) mm   |
| Flower colour       | Sepals and petals magenta, lip magenta with white disc                                  | Sepals and petals magenta, lip magenta with white disc                               |
| Number of flowers   | 1–3   | 5–12   |
| Sepal shape         | Oblanceolate  | Lanceolate   |
| Dorsal sepal size   | 50 $\pm$ 8.3 (34–65) $\times$ 15 $\pm$ 1.1 (12–19) mm                                   | 18.5 $\pm$ 0.97 (17–20) $\times$ 4.95 $\pm$ 0.3 (4–6) mm                             |
| Lateral sepals size | 50 $\pm$ 8.4 (34–65) $\times$ 14.3 $\pm$ 1.84 (10–18) mm                                | 17 $\pm$ 1.8 (15–20) $\times$ 4.96 $\pm$ 0.34 (4–6) mm                               |
| Petal shape         | Oblanceolate  | Lanceolate   |
| Petal size          | 51 $\pm$ 8.5 (34–66) $\times$ 27.7 $\pm$ 4.5 (19–36) mm                                 | 18.6 $\pm$ 1.35 (17–20) $\times$ 5.05 $\pm$ 0.44 (4–6) mm                            |
| Lip margin          | Undulate to crispate  | Crispate   |
| Lip size            | 46.5 $\pm$ 6.7 (34–57) $\times$ 38.7 $\pm$ 7.5 (20–50) mm                               | 14.6 $\pm$ 2.8 (11–18) $\times$ 10.5 $\pm$ 0.53 (10–11) mm                           |
| Lip base            | 21.3 $\pm$ 4.8 (11–30) mm   | 11.4 $\pm$ 1.26 (10–13) mm   |
| Lip terminal lobe   | 20.7 $\pm$ 5 (11–32) mm   | 6.4 $\pm$ 1.35 (5–8) mm  |
| Column size         | 22.9 $\pm$ 4.6 (19–32) $\times$ 11.2 $\pm$ 5.8 (5–18),<br>4.9 $\pm$ 0.89 (4–7) mm thick | 9.6 $\pm$ 1.17 (8–11) $\times$ 4.9 $\pm$ 1.2 (3–6),<br>4.0 $\pm$ 0.67 (3–5) mm thick |
| Cunuculus           | 12 $\pm$ 3 (7–19) mm  | 9.3 $\pm$ 1.3 (8–12) mm  |

(ca. three seconds), and then flew away from the plant for long distances. No sequential visits to either flowers of the same or the other species were observed. For both species, the bees rapidly approached the flowers and used the terminal lobe of the labellum as a landing platform, holding on to the edges of the petal with their first two pairs of legs.

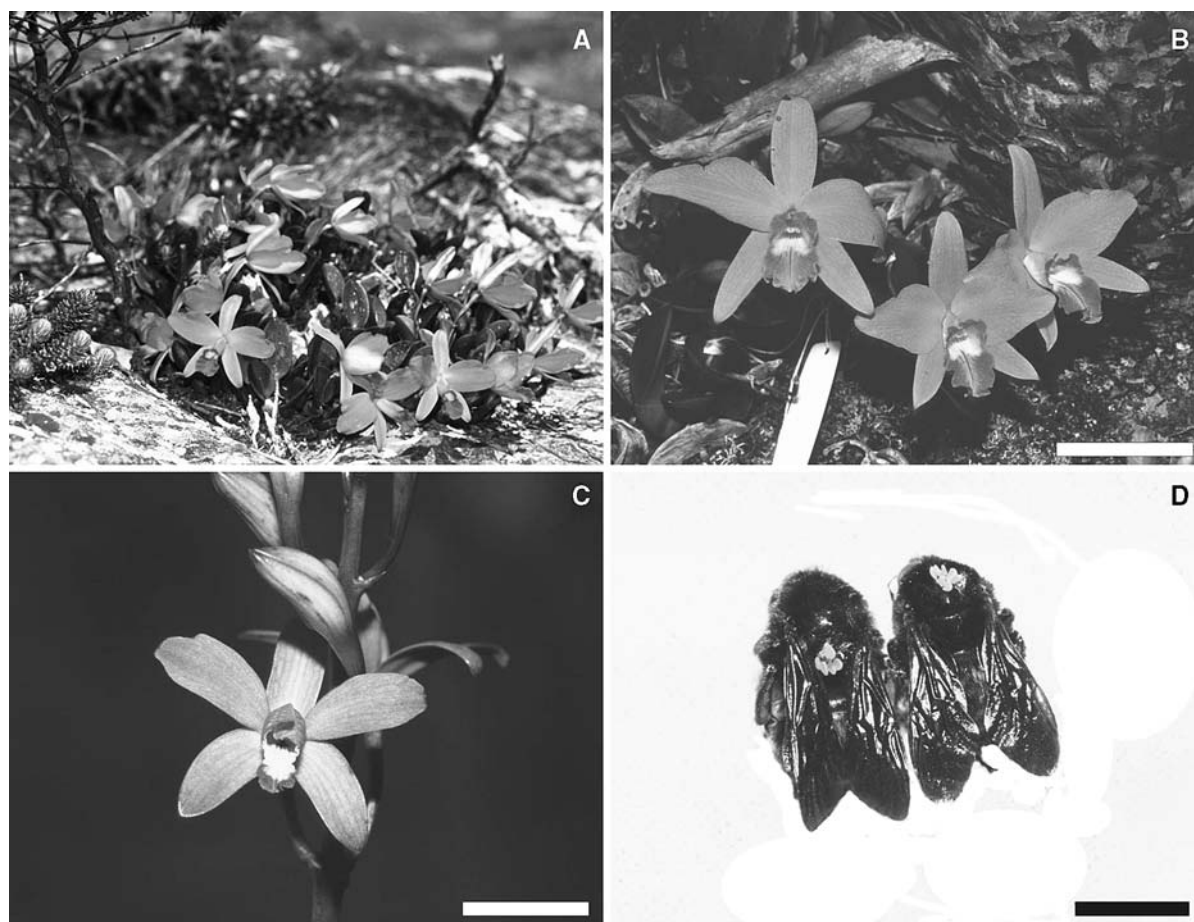
*Sophronitis sincorana* has relatively large flowers, so that the weight of the bee does not bend the labellum and the insect must force its way into the floral tube formed by the labellum. The bee fully enters the chamber formed by the floral parts but, not finding nectar inside, will then backup, taking with it the pollinarium that has been attached to its scutellum (Figs. 1D, 2B).

*Sophronitis pfisteri* has smaller flowers, so the weight of the bee will bend the labellum downward, exposing part of the gynostemium and partially opens the cavity formed by the lateral lobes and the gynostemium. The visiting bee then forces its head and part of its thorax

between the gynostemium and the labellum to further open the cavity. Then it inserts its glossa into the cavity in an attempt to collect nectar (Fig. 2A). When the insect withdraws from the floral chamber it lifts the anther cap and releases the pollinarium, which adheres to the anterior portion of its mesonotum (Fig. 1D).

Pollinaria removal from *Sophronitis sincorana* was 32.2%, while pollinaria deposition on the stigma was 9.8% (n = 153 flowers). Fruit formation under natural conditions was approximately 4%.

**Mating systems.** Fruits were not formed by spontaneous self-pollination or by apomixis in either *S. sincorana* or *S. pfisteri*. The fruits of *S. sincorana* mature approximately five months after pollination, while the fruits of *S. pfisteri* mature approximately three months after pollination. Both species demonstrated low fruit formation rates when self-pollinated in comparison with intra-specific cross-pollinations (Table 2). The fruits formed by self-pollination also demonstrated a very low percentage of



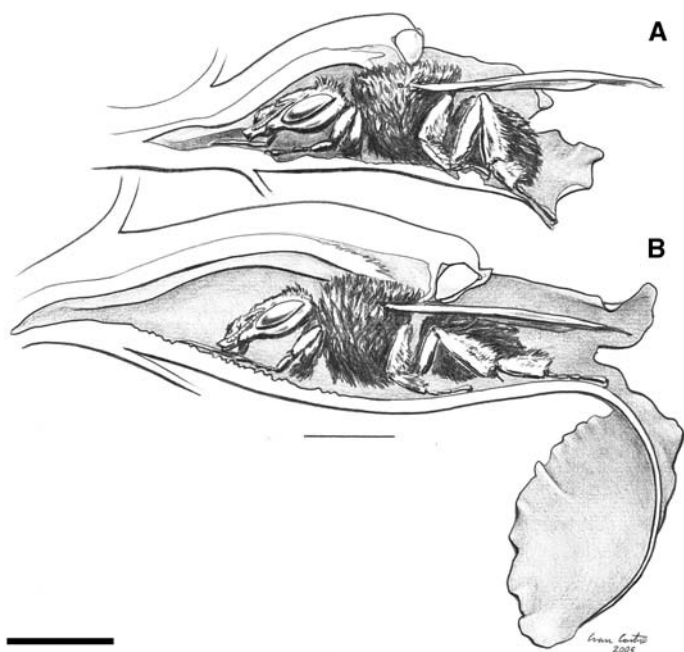
**Fig. 1.** A, B *Sophronitis sincorana*. C Flower of *S. pfisteri*. D *Bombus brevivillus* with pollinarium of *S. sincorana* (left) and *S. pfisteri* (right) adhered to the scutellum and mesonotum, respectively. Scale bars = 5 (B), 2 (C) and 0.5 (D) cm

seeds with developed embryos, and significant differences in their average embryonic viability were measured by the Tukey test (for *S. sincorana*  $q = 7.95$ ,  $p < 0.01$ , and for *S. pfisteri*  $q = 13.85$ ,  $p < 0.01$ ) (Table 2). The spontaneous abortion of fruits was high in experimental self-pollination in both *S. sincorana* (53.4%) and *S. pfisteri* (66.7%).

Fruit production rates using *S. pfisteri* as the female parent were similar for both inter-specific and intra-specific crosses although the percentage of viable seeds was significantly lower for the former (Table 2). Using *S. sincorana* as the female parent resulted in considerably fewer fruits for inter-specific than for intra-specific crosses. The percentage of

viable seeds was also less when *S. sincorana* was the female parent. No significant differences were observed between the average levels of viable seeds resulting from reciprocal inter-specific crosses.

Epifluorescence microscopic analysis revealed small morphological differences between the pollen tubes of the species studied. The pollen tubes of *S. sincorana* were thicker than those of *S. pfisteri*, although similar pollen tube growth inside the stigmatic chamber was observed in both species under all experimental treatments. The pollen tubes were thin-walled, with regular and directional growth, without any apparent thickenings along the stylar canal. The pollen tubes reached the end of the



**Fig. 2.** Flowers of *Sophronitis pfisteri* **A** and *S. sincorana* **B** in longitudinal section with *Bombus brevivillus* inside the chamber formed by the lip lateral lobes and the column inserting its tongue into the cuniculus trying to collect nectar. Scale bar = 0.5 cm

stigmatic cavity after six days of growth, having grown approximately two millimeters in the interior of the chamber of *S. pfisteri* and approximately four millimeters in the interior of the chamber of *S. sincorana*. After approximately 16 days, the pollen tubes in *S. sincorana* had passed through the entire stylar canal and had reached the ovary, where the ovules were still immature. Pollen tubes in *S. pfisteri* reached the ovary after 24 days. After 32 days the pollen tubes were observed around the placenta, although the ovules were still immature at this time. At this phase the walls of the pollen tubes in self-pollinated flowers began to show irregular and sinuous growth. In fruits of 165 days it was possible to observe penetration of the ovules, but an intense thickening gave the pollen tubes a wrinkled appearance in cases of self-pollination.

In the inter-specific crosses using *S. sincorana* as the female parent, the pollen tubes began to demonstrate irregular and sinuous growth near the placenta after 32 days. Seeds were visible in the interior of the fruits after

165 days, and the pollen tubes that remained had a slightly wrinkled aspect due to intense thickening.

### Discussion

Our current knowledge of the phylogeny of the genus *Sophronitis* (sensu van den Berg and Chase 2000, van den Berg 2005) and its pollination mechanisms suggest that it represents a more inclusive phylogenetic grouping composed of species probably pollinated by hummingbirds within a more diverse group of species pollinated by bees (van der Pijl and Dodson 1966, van den Berg et al. 2000). However, this is probably the first paper that presents real data on reproductive biology of any species in the genus. *Sophronitis sincorana* and *S. pfisteri* demonstrate a type of pollination widely distributed within the family Orchidaceae known as “gullet flower” (Dressler 1981), which apparently evolved a number of times within the family (van der Pijl and Dodson 1966, Thien and Marcks 1972, Smidt

**Table 2.** Fruit set and average seed viability per fruit in experimental crosses of *Sophronitis sincorana* and *S. pfisteri* at Chapada Diamantina, Northeastern Brazil. Sample size in parentheses

| Treatment       | <i>S. sincorana</i>   |   | <i>S. pfisteri</i>    |  |
|-----------------|-----------------------|---|-----------------------|--|
|                 | Fruit set %           | Seed viability %                          | Fruit set %           | Seed viability %                         |
| Not manipulated | 0 ( <i>n</i> = 15)    | –   | 0 ( <i>n</i> = 15)    | –  |
| Selfing         | 46.6 ( <i>n</i> = 15) | 8.5 ± 4.3 ( <i>n</i> = 7) <sup>1</sup>    | 33.3 ( <i>n</i> = 15) | 9.3 ± 7.5 ( <i>n</i> = 5) <sup>2</sup>   |
| Crossing        | 80 ( <i>n</i> = 15)   | 47.3 ± 17.8 ( <i>n</i> = 12) <sup>1</sup> | 60 ( <i>n</i> = 15)   | 73.5 ± 13.4 ( <i>n</i> = 9) <sup>2</sup> |
| Interspecific   | 26.6 ( <i>n</i> = 15) | 26.0 ± 19.7 ( <i>n</i> = 4)               | 53.3 ( <i>n</i> = 15) | 47.6 ± 21.0 ( <i>n</i> = 8)              |

Significantly different means by Tukey test (<sup>1</sup> *q* = 7.95, *p* < 0.01; <sup>2</sup> *q* = 13.85, *p* < 0.01)

et al. 2006). The labellum, column, and sometimes the petals of “gullet flowers” form a chamber into which the pollinator must insert its body when seeking possible floral rewards, and the pollinarium adheres to the dorsal region of the bee when it leaves the flower. In the present study, we observed small variations of this mechanism related to the size of the floral parts, in which *S. sincorana* has larger flowers and a labellum too large to be bent downward by the pollinator’s weight only; in *S. pfisteri* the labellum is bent by the animal’s weight, opening the floral chamber.

The low frequency of pollinaria deposition and fruiting in *S. sincorana* is commonly observed in other orchid species that use the deceptive mechanism of attracting pollinators, seen in the two species studied, as well as other species of Laeliinae (Janzen et al. 1980; Montalvo and Ackerman 1987; Borba and Semir 1998, 2001; Borba and Braga 2003; Smidt et al. 2006). The low visitation frequency in natural populations is compensated by a high degree of allogamy (Nilsson 1992; Tremblay 1992; Cozzolino and Widmer 2005; Tremblay et al. 2005; Jerkásová et al. 2006).

Although the genus *Bombus* demonstrates low diversity in Neotropical regions – having only one subgenus *Fervidobombus*, with approximately 20 species (Cameron and Williams 2003) – the members of this group are important pollinators of the Orchidaceae there. Numerous studies have identified these bees as being the principal pollinators of species of *Cleistes*, *Pseudolaelia*, *Cattleya*, *Sophronitis*, *Pelexia*, *Sobralia*, *Oncidium*, and

*Odontoglossum*, genera that demonstrate a wide variety of pollination mechanisms, colors and odors, and that inhabit diverse ecosystems such as the Atlantic Coastal Forest, altitudinal forests, and *campo rupestre* vegetation (van der Pijl and Dodson 1966; Singer and Sazima 1999; Borba and Braga 2003; Pansarin 2003, Smidt et al. 2006).

Self-incompatibility and complete self-compatibility represent two extremes in plant reproduction, with gradations being more common (Bawa 1974; Schemske and Lande 1985). The two species of *Sophronitis* studied here can be considered partially self-compatible, for although the pollen tubes of both species show slight abnormalities in self-pollination regimes, fruits are formed with seeds containing embryos, although only in small numbers. Systems of self-incompatibility have been described in only a few species of Orchidaceae (e.g. Dafni and Calder 1987; Borba et al. 2001), with details similar to those described for homomorphic gametophytic self-incompatibility occurring in the stylar canal of other angiosperms (Richards 1997). Thickening of the pollen tubes when these penetrate the ovary was observed in *S. sincorana* and *S. pfisteri*, as was also described in *Bulbophyllum weddellii* by Borba et al. (1999). However, self-incompatibility has been reported only in a few cases for other species of Laeliinae (Matias et al. 1996; Borba and Braga 2003). This partial self-incompatibility associated with pollination mechanism by deceiving is probably responsible for the maintenance of the high levels of genetic and morphological variability observed



in populations of *S. sincorana* (Borba et al. in press).

The levels of inbreeding depression observed in the two species of *Sophronitis* was characterized by high levels of spontaneous fruit abortions in self-pollinated flowers and by high embryo mortality in the flowers that developed fruits to maturity. These are common symptoms of inbreeding depression (Stebbins 1974; Schemske and Lande 1985). In spite of this, the populations of *S. sincorana* examined demonstrated high levels of selfing (Borba et al. in press). This is a common pattern seen within the Orchidaceae, with many reports of low levels of seed formation with viable embryos after self-pollination in non-related groups such as *Spiranthes* (Catling 1982), *Acianthera* (Borba et al. 2001), and Laeliinae, including genera such as *Sophronitis* (Stort and Galdino 1984), *Constantia* (Matias et al. 1996), *Pseudolaelia* (Borba and Braga 2003), and *Cattleya* (Smidt et al. 2006).

Although the family Orchidaceae is widely known for its hybridization under experimental (Arditti 1992) as well as natural conditions (e.g. Borba and Semir 1998; Cozzolino et al. 2006), the synchrony of flowering events and the sharing of pollinators between the two *Sophronitis* species did not result in hybrid formation. Many authors have pointed to pre-pollination barriers, especially differences in flower morphology and pollinator behavior, as the principal factors responsible for the isolation of closely related species in orchids (e.g. Dodson 1962; van der Pijl and Dodson 1966; Thien and Marcks 1972; Gill 1989; Dressler 1981; Steiner et al. 1994; Borba and Semir 1999, 2001). The differences in the length of the gynostemium and the size of the floral parts in *S. sincorana* and *S. pfisteri* insure that the pollinaria will be placed on different parts of the pollinator's body, making the transfer of inter-specific pollen less likely – and this probably represents one of the principal reproductive barriers between these two species. We suggest this partial barrier prevents the deposition of pollen from *S. sincorana* onto *S. pfisteri* because of the

more distal placement of the pollinaria of *S. sincorana* on the scutellum of the bee and the short gynostemium of *S. pfisteri*. However, pollen deposition is apparently not avoided in the inverse direction, due to the long gynostemium found in *S. sincorana*. Unidirectional morphological barriers were also described in sympatric species of *Bulbophyllum* (Borba and Semir 1999).

Our study also detected a partial inter-incompatibility that acts through the abnormal development of the pollen tubes in the interior of the ovaries, thus constituting a complementary post-pollination mechanism of reproductive isolation in these two species. This barrier apparently acts most strongly in the opposite direction from the morphological floral barrier discussed earlier, resulting in less fruiting and lower seed viability when pollen of *S. pfisteri* is transferred to *S. sincorana*. Karyotype differences usually act as efficient post-zygotic reproductive barrier, and it has been cited between closely related species that share the same pollinators in the subtribe Orchidinae (Cozzolino et al. 2004). Unfortunately, chromosome numbers are apparently not known for these *Sophronitis* species.

Thus, due to successive partial barriers, a system of filters is erected that prevents hybridization between these two closely related species. Additional post-fertilization barriers may exist, however, related to low seed viability and to the adaptation and reproductive success of the hybrids, that would further help to maintain species integrity, as seen in some species of *Bulbophyllum* (Borba and Semir 1998, 1999; Borba et al. 1999; Silva et al. 1999) and other groups of orchids (Johnson et al. 1998; Cozzolino et al. 2006).

The authors thank the staff of the Parque Municipal de Mucugê - Projeto Sempre-Viva for support during field work, and Cássio van den Berg, Mario Blanco and one anonymous reviewer for improvements. This work was supported by a grant from Fundo Nacional do Meio Ambiente to ELB (FNMA #75/2001). ELB is supported by a grant (PQ2) from CNPq.

## References

- Arditti J (1992) Fundamentals of orchid biology. Wiley, New York
- Ayres M, Ayres M Jr, Ayres DL, Santos AS (2003) BioEstat 3.0: aplicações estatísticas nas áreas das ciências biológicas e médicas. Sociedade Civil Mamirauá and MCT-CNPq, Belém
- Bawa KS (1974) Breeding systems of tree species of a lowland tropical community. *Evolution* 28: 85–92
- Borba EL, Braga PIS (2003) Biologia reprodutiva de *Pseudolaelia corcovadensis* (Orchidaceae): melitofilia e autocompatibilidade em uma *Laeliinae* basal. *Rev Brasil Bot* 26: 541–549
- Borba EL, Semir J (1998) *Bulbophyllum xipoense* (Orchidaceae), a new natural hybrid from the Brazilian “campos rupestres”: description and biology. *Lindleyana* 13: 113–120
- Borba EL, Semir J (1999) Temporal variation in pollinarium size after its removal in species of *Bulbophyllum*: a different mechanism preventing self-pollination in Orchidaceae. *Pl Syst Evol* 217: 197–204
- Borba EL, Semir J (2001) Pollinator specificity and convergence in fly-pollinated *Pleurothallis* (Orchidaceae) species: a multiple population approach. *Ann Bot* 88: 75–88
- Borba EL, Shepherd GJ, Semir J (1999) Reproductive systems and crossing potential in three species of *Bulbophyllum* (Orchidaceae) occurring in Brazilian ‘campo rupestre’ vegetation. *Pl Syst Evol* 217: 205–214
- Borba EL, Shepherd GJ, Semir J (2001) Self-incompatibility, inbreeding depression and crossing potential in five *Pleurothallis* (Orchidaceae) species. *Ann Bot* 88: 89–99
- Borba EL, Funch R, Ribeiro PL, Smidt EC, Silva-Pereira V (2007) Demography, genetic and morphological variability of the endangered *Sophronitis sincorana* (Orchidaceae) in the Chapada Diamantina, Brazil. *Pl Syst Evol* (in press). DOI: 10.1007/s00606-007-0555-9
- Buzato S, Sazima M, Sazima I (2000) Hummingbird-pollinated floras at three Atlantic Forest sites. *Biotropica* 32: 824–841
- Cameron AS, Williams PH (2003) Phylogeny of bumble bees in the New World subgenus *Fervidobombus* (Hymenoptera: Apidae): congruence of molecular and morphological data. *Molec Phylogenet Evol* 28: 552–563
- Chiron GR, Castro VP (2002) Révision des espèces brésiliennes du genre *Laelia* Lindley. *Richardiana* 2: 4–28
- Catling PM (1982) Breeding systems of northeastern North America *Spiranthes* (Orchidaceae). *Canad J Bot* 60: 3017–3039
- Cozzolino S, Widmer A (2005) Orchid diversity: an evolutionary consequence of deception? *Trends Ecol Evol* 20: 487–494
- Cozzolino S, D’Emerico S, Widmer A (2004) Evidence for reproductive isolate selection in Mediterranean orchids: karyotype differences compensate for the lack of pollinator specificity. *Proc Roy Soc London Ser. B Biol Sci* 271 (Suppl. 5): S259–S262
- Cozzolino S, Nardella AM, Impagliazzo S, Widmer A, Lexer C (2006) Hybridization and conservation of Mediterranean orchids: should we protect the orchid hybrids or the orchid hybrid zones? *Biol Conserv* 129: 14–23
- Dafni A, Calder DM (1987) Pollination by deceit and floral mimicry in *Thelymitra antennifera* (Orchidaceae). *Pl Syst Evol* 158:11–22
- Dodson CH (1962) The importance of pollination in the evolution of the orchids of tropical America. *Amer Orchid Soc Bull* 31: 525–534, 641–649, 731–735
- Dressler RL (1981) The orchids: natural history and classification. Harvard University Press, Cambridge
- Dressler RL (1993) Phylogeny and classification of the orchid family. Dioscorides Press, Portland
- Gill DE (1989) Fruiting failure, pollinator inefficiency, and speciation in orchids. In: Otte D, Endler JA (eds) Speciation and its consequences. Sinauer Associates, Sunderland, pp 433–457
- Giulietti AM, Pirani JR (1988) Patterns of geographic distribution of some plant species from the Espinhaço Range, Minas Gerais and Bahia, Brazil. In: Heyer WR, Vanzolini PE (eds) Proceedings of a workshop on neotropical distribution patterns. Academia Brasileira de Ciências, Rio de Janeiro, pp 39–69
- Giulietti AM, Pirani JR, Harley RM (1997) Espinhaço range region. Eastern Brazil. In: Davis SD, Heywood VH, Herrera-Macbride O, Villa-Lobos J, Hamilton AC (eds) Centres of plant diversity: a guide and strategy for their conservation. Vol 3, The Americas. World Wildlife Foundation and IUCN, Cambridge, pp 397–404

- Harley RM (1988) Evolution and distribution of *Eriope* (Labiatae) and its relatives in Brazil. In: Heyer WR, Vanzolini PE (eds) Proceedings of a workshop on neotropical distribution patterns. Academia Brasileira de Ciências, Rio de Janeiro, pp 71–120
- IUCN/SSC Orchid Specialist Group. (1996) Orchids – Status survey and conservation action plan. IUCN, Gland Switzerland and Cambridge
- Janzen DHP, Devries P, Gladstone DE, Higgins M L, Lewinsohn M (1980) Self- and cross-pollination of *Encyclia cordigera* (Orchidaceae) in Santa Rosa National Park, Costa Rica. *Biotropica* 12: 72–74
- Jersáková J, Johnson SD, Kindlmann P (2006) Mechanisms and evolution of deceptive pollination in orchids. *Biol Rev* 81: 219–235
- Johnson SD, Steiner KE, Whitehead VB, Vogelpoel L (1998) Pollination ecology and maintenance of species integrity in co-occurring *Disa racemosa* L.f. and *Disa venosa* Sw. (Orchidaceae) in South Africa. *Ann Missouri Bot Gard* 85: 231–241
- Köppen W (1948) *Climatologia com un estudio de los climas de la Tierra* (Transl. by Peres PRH). Fondo de Cultura Económica, México
- Levin DA (2000) *The origin, expansion, and demise of plant species*. Oxford University Press, New York
- Manuel R, Warren R, Miller D (1996) *Sophranitis coccinea*: a pollination study. *Orchids* 612–616
- Martin FW (1959) Staining and observing pollen tubes in the style by means of fluorescence. *Stain Tech* 34: 125–128
- Matias LQ, Braga PIS, Freire AG (1996) Biologia reprodutiva de *Constantia cipoensis* Porto & Brade (Orchidaceae), endêmica da Serra do Cipó, Minas Gerais. *Rev Brasil Bot* 19: 119–125
- Montalvo AM, Ackerman JD (1987) Limitations to fruit production in *Ionopsis utricularioides* (Orchidaceae). *Biotropica* 19: 24–31
- Nilsson LA (1992) Orchid pollination biology. *Trends Ecol Evol* 7: 255–259
- Pansarin E (2003) Biologia floral de *Cleistes macrantha* (Barb. Rodr.) Schltr. (Orchidaceae: Vanilloideae: Pogoniinae). *Rev Brasil Bot* 26: 73–80
- Richards AJ (1997) *Plant breeding systems*, 2nd edn. Chapman & Hall, London
- Sanford WW (1964) Sexual compatibility relationship in *Oncidium* and related genera. *Amer Orchid Soc Bull* 33: 1035–1048
- Schemske DW, Lande R (1985) The evolution of self-fertilization and inbreeding depression in plants. II. Empirical observations. *Evolution* 39: 41–52
- Scogin R, Young DA, Jones CE (1977) Anthochlor pigments and pollination biology: II. The ultraviolet patterns of *Coreopsis gigantea* (Asteraceae). *Bull Torrey Bot Club* 104: 155–159
- Silva UF, Borba EL, Semir J, Marsaioli AJ (1999) A simple solid injection device for the analyses of *Bulbophyllum* (Orchidaceae) volatiles. *Phytochemistry* 50: 31–34
- Singer RB, Sazima M (1999) The pollination mechanism in the “*Pelexia* alliance” (Orchidaceae: Spiranthinae). *Bot J Linn Soc* 131: 249–262
- Smidt EC, Silva-Pereira V, Borba EL (2006) Reproductive biology of two *Cattleya* (Orchidaceae) species endemic to Northeastern Brazil. *Pl Spec Biol* 21: 85–91
- Stebbins GL (1974) *Flowering plants: evolution above the species level*. Harvard University Press, Cambridge
- Steiner KE, Whitehead VB, Johnson SD (1994) Floral and pollinator divergence in two sexually deceptive South African orchids. *Amer J Bot* 81: 185–194
- Stort MNS, Galdino GL (1984) Self- and cross-pollination in some species of the genus *Laelia* Lindl. (Orchidaceae). *Rev Bras Gen* 7: 671–676
- Stoutamire WP (1975) Australian terrestrial orchids, thynnid wasps and pseudocopulation. *Amer Orchid Soc Bull* 43:13–18
- Thien LB, Marcks BG (1972) The floral biology of *Arethusa bulbosa*, *Calopogon tuberosus*, and *Pogonia ophioglossoides* (Orchidaceae). *Canad J Bot* 50: 2319–2325
- Tremblay RL (1992) Trends in the pollination ecology of the Orchidaceae: evolution and systematics. *Canad J Bot* 70: 642–650
- Tremblay RL, Ackerman JD, Zimmerman JK, Calvo RN (2005) Variation in sexual reproduction in orchids and its evolutionary consequences: a spasmodic journey to diversification. *Biol J Linn Soc* 84: 1–54
- van den Berg C (2005) *Sophranitis*. In: Pridgeon AM, Cribb PJ, Chase MW, Rasmussen FN (eds) *Genera Orchidacearum*, vol. 4. Oxford University Press, Oxford, pp 313–316
- van den Berg C, Chase MW (2000) Nomenclatural notes on Laeliinae – I. *Lindleyana* 15: 115–119

- van den Berg C, Higgins WE, Dressler RL, Whitten WM, Soto Arenas MA, Culhan A, Chase MW (2000) A phylogenetic analysis of Laeliinae (Orchidaceae) based on sequence data from internal transcribed spacers (ITS) of nuclear ribosomal DNA. *Lindleyana* 15: 96–114
- van den Berg C, Goldman DH, Freudenstein JV, Pridgeon AM, Cameron KM, Chase MW (2005) An overview of the phylogenetic relationships within Epidendroideae inferred from multiple DNA regions and recircumscription of Epidendreae and Arethuseae (Orchidaceae). *Amer J Bot* 92: 613–624
- van der Pijl L, Dodson CH (1966) *Orchid flowers: their pollination and evolution*. University of Miami Press, Coral Gables
- Vogel S (1990) *The role of scent glands in pollination* (Transl. by Bhatti JS). Smithsonian Institution, Washington
- Withner CL (1990) *The Cattleyas and their relatives. II. The Laelias*. Timber Press, Portland