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ORIGINAL ARTICLE

Reproductive biology and isolation mechanisms in rupicolous species of the *Acianthera prolifera* complex (Orchidaceae) occurring in southeastern Brazil

Marcos Cabral de Melo · Pedro Paulo Goulart Taucce · Eduardo Leite Borba

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Abstract We studied the floral biology and performed experimental intra- and interspecific pollinations in populations of a complex of four *Acianthera* (Orchidaceae) species occurring in Brazilian *campo rupestre* vegetation (*A. hamosa*, *A. limae*, *A. modestissima*, and *A. prolifera*). All four species flower synchronously, are partially intercompatible, and exhibited some degree of self-sterility. Floral morphology is similar in all the species, with their principal differences associated with size of the floral structures. The four species were visited only by Diptera species of the families Phoridae (*Megaselia* spp.) and Chloropidae, but visits are rare and fruit set is very low. Sympatric species were not pollinated by the same Diptera species. *Acianthera hamosa* and *A. modestissima* have the smallest flowers, and no marked morphological differences between them were observed; they were both pollinated by very similar *Megaselia* species. Both prepollination barriers and postpollination events are important to maintaining the isolation of the species, functioning as overlapping filters that diminish the possibility of gene flow between them. However, putative hybrids between *A. prolifera* and *A. limae* have been found. Conversely, *A. hamosa* and *A. modestissima*, which are recognized only by vegetative characters that show high phenotypic plasticity, seem only to be isolated by geographical barriers, and they may actually constitute a single species or be sister species.

Keywords Mating systems · Myophily · Orchidaceae · Pleurothallidinae · Pollination · Self-incompatibility

Introduction

Orchids demonstrate enormous species richness (approx. 25,000) and wide geographical distribution (Dressler 1993). Part of their high floral diversity is traditionally explained as a consequence of the highly specific plant–pollinator relationships common in the family. As such, great importance has been attributed to pollinator behavior and to floral differences (prepollination barriers) in the reproductive isolation of orchid species and in the consequent diversification of the group (Gill 1989). However, some recent studies have demonstrated that prepollination barriers in orchids may fail even in very specialized pollination systems, making the evolution of postpollination barriers important for maintaining species integrity (Cozzolino et al. 2004; Scopece et al. 2007; Silva-Pereira et al. 2007). However, reports of natural hybridization between orchids are common, even though many of these hybrids are sterile (Scacchi et al. 1990; Borba and Semir 1998a, b; Ellis and Johnson 1999; Azevedo et al. 2006).

Orchids are associated with various groups of pollinators, although insects of the orders Hymenoptera and Diptera predominate (van der Pijl and Dodson 1966). Pollination by Diptera (myophily) occurs in many lineages in the Orchidaceae, and although these cases are widely dispersed, they are better represented in the Neotropical subtribe Pleurothallidinae (approximately 4,000 species) and in the Pantropical genus *Bulbophyllum* (approximately

M. C. de Melo · P. P. G. Taucce · E. L. Borba (✉)
Departamento de Botânica, Laboratório de Sistemática Vegetal, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, Pampulha, Belo Horizonte, Minas Gerais 31270-901, Brazil
e-mail: borba@gmx.net

E. L. Borba
Centro de Ciências Naturais e Humanas, Universidade Federal do ABC, Rua Santa Adélia, 166, Bangu, Santo André, São Paulo 09210-170, Brazil

2,100 species) (Christensen 1994). These two groups together comprise approximately 25% of all orchid species, and their flowers share many convergent characteristics associated with myophily (Dressler 1993). Myophily has been considered by some authors to be an inefficient, promiscuous, and probably unstable pollination system (van der Pijl and Dodson 1966). However, some studies have demonstrated the participation of Diptera in highly specialized systems, including orchids whose flowers show intricate adaptations that can be dependent on the behavior, size, and positioning of the insects to effect pollination (Borba and Semir 1998b, 2001; Blanco and Barboza 2005). These specialized relationships appear to be important in the evolutionary history of these myophilous orchids because they can constitute efficient reproductive barriers between species, drive the evolution of floral morphology, and determine the patterns of reproductive success (Borba and Semir 1998b, 2001; Tremblay et al. 2005; Jersáková et al. 2006). In that sense, studies involving myophilous orchids contradict works that have cast doubt on the existence of specialized plant–pollinator relationships (e.g., Herrera 1988; Waser et al. 1996), and have reinforced the validity of correlations observed between floral characters (odor, color, morphology, rewards, etc.) and groups of pollinators that characterize pollination syndromes (Faegri and van der Pijl 1979).

Myophily appears to be related to the evolution of self-incompatibility in the Orchidaceae. Diptera exhibit behavior that can favor self-pollination, as their visits are generally long and include visits to various flowers on the same inflorescence. As such, genetic barriers to self-fertilization appear to have been selected in myophilous orchids to minimize autogamy and avoid reductions in genetic variability in populations of these species (Borba et al. 2001a, b). This hypothesis is supported in the Pleurothallidinae, in which pollination by Diptera is associated with genetic barriers to autogamy in various lineages of the subtribe (Christensen 1992; Borba et al. 1999, 2001b, in press; Barbosa et al. 2009; Gontijo et al. 2010).

Acianthera hamosa (Barb.Rodr.) Pridgeon & M.W.Chase, *A. limae* (Porto & Brade) Pridgeon & M.W.Chase, *A. modestissima* (Rchb.f. & Warm.) Pridgeon & M.W.Chase, and *A. prolifera* (Herb. ex Lindl.) Pridgeon & M.W.Chase constitute a group of orchids distributed along the mountain chains of southeastern Brazil (Pabst and Dungs 1975), and their flowers have very similar morphology (Fig. 1), apparently associated with sapromyophily (Faegri and van der Pijl 1979). There are records of sympatric populations of the pairs *A. prolifera*/*A. modestissima* and *A. limae*/*A. prolifera*. Although there is no indication of hybridization between the first pair, this seems to occur in sympatric populations of *A. limae* and *A. prolifera* (Melo and Borba in press). *Acianthera hamosa* and *A. modestissima* have flowers that are very similar in size, shape, and odor, and they can be differentiated only by vegetative characters, leading to doubt concerning the existence of any reproductive barriers between these taxa or if they truly represent distinct biological species (Melo and Borba in press).

In this context, the present work sought to respond to the following questions: (a) What are the possible pre- and postpollination reproductive barriers that exist between the species of this complex, and what are their hybridization potentials? (b) Is there pollinator specificity associated with these supposedly myophilous species, and if so, is this specificity correlated with floral characteristics such as color, odor, and/or floral size? (c) Is there any association between myophily and events that avoid self-fertilization in this group, such as genetic barriers? To that end, we examined the reproductive phenology (the degree of overlap between flowering and reproductive periods) in four of these populations, some of which are sympatric (*A. limae*/*A. prolifera* and *A. modestissima*/*A. prolifera*), their floral biology, and the reproductive systems of the four species in terms of their degrees of self-compatibility, intraspecific compatibility (intra- and interpopulational), and interspecific compatibility.

Materials and methods

Study sites and species studied

The four species studied were from five different localities in Minas Gerais State, Brazil: Serra do Caraça in the municipality of Catas Altas (*A. limae* and *A. prolifera*), Serra da Piedade in the municipality of Caeté (*A. modestissima* and *A. prolifera*), Serra do Curral in the municipality of Belo Horizonte (*A. modestissima*), Carrancas (*A. hamosa*), and Serra da Calçada in the municipality of Nova Lima (*A. limae*). These localities have *campo rupestre* vegetation growing on outcrops of quartzite (Serra do Caraça) and on nodulous iron formations (*canga*) (Serra da Piedade, Serra do Curral, and Serra da Calçada) found in the Espinhaço Range, as well as outcrops of sandstone lying outside this mountainous zone (Serra de Carrancas). Voucher specimens were deposited in the BHCB herbarium at the Universidade Federal de Minas Gerais (Table 1). *Campo rupestre* vegetation occurs at altitudes above 800 m, and is characterized by having an open herbaceous structure on sandy and rocky soils, and a shrub and herbaceous structure on isolated rock outcrops of quartzite, sandstone or nodulous iron *canga* (Giulietti and Pirani 1988). *Acianthera modestissima* and *A. prolifera* occur in open areas on rock outcrops of quartzite and *canga*, and can be rupicolous or epiphytic on species of *Vellozia*

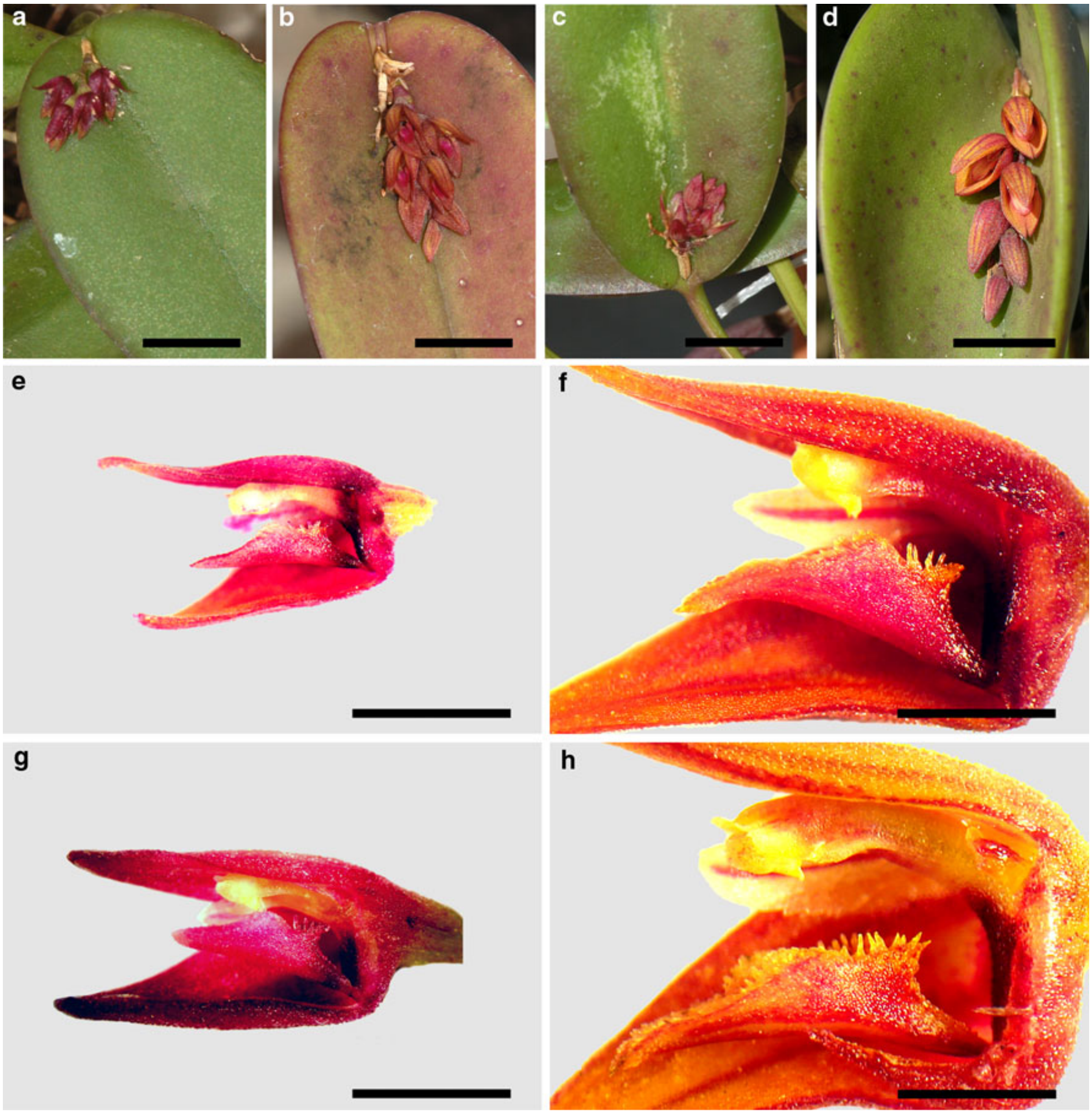

Fig. 1 Inflorescences (**a–d**) and flowers in side view (**e–h**) of *Acianthera* species occurring in Brazilian *campo rupestre*; lateral sepal, lateral petal, and part of distal sepal removed. **a**, **e** *A. hamosa* from Carrancas-MG; **b**, **f** *A. limae* from the Serra do Caraça-MG; **c**, **g** *A. modestissima* from the Serra da Piedade-MG; **d**, **h** *A. prolifera* from the Serra do Caraça-MG. Scale bars = 1 cm (**a–d**) and 2 mm (**e–h**)

(Velloziaceae). On the other hand, *A. hamosa* and *A. limae* occur in the interior of gallery forests in areas of *campo rupestre* vegetation, growing directly on stones or on the bases of the trunks of trees. The individuals of these species were numerous and grew close to one another (1–3 m) in all of the populations studied, commonly forming small clumps of individuals.

The flowers are resupinate and distichous, with an average of three to four flowers per inflorescence (ranging from one to five) in *A. hamosa* and *A. modestissima*, and four to five flowers (ranging from one to seven) in *A. limae* and *A. prolifera*, respectively (Fig. 1, Table 2). Floral morphology is similar in all four species, with their principal differences being associated with the sizes of the floral structures (Table 3, Fig. 1). The sepals are erect, fleshy, with their adaxial faces being papilose and yellowish purple; the lateral sepals are fused, giving the flowers a tubular shape. The lip is fleshy, trilobate, spatulate and with two lateral calli in its median portion, purple or yellow, and articulated with the column. A trapezoidal cavity is formed in the floral interior when viewed laterally, due to erect sepals, petals, lip, and column (Fig. 1e–h). The length and width of the sepals and petals do not directly reflect the size of this floral cavity in any of the species

Table 1 Populations of *Acianthera hamosa*, *A. limae*, *A. modestissima*, and *A. prolifera* studied, and number of individuals used in experimental pollinations (*N*)

Species/population	Name	*N*	Location	Voucher
A. hamosa				
Carrancas-MG	H4	12	21°28′14″S; 44°40′55″W	Melo 08
A. limae				
Serra do Caraça, Catas Altas-MG	L1	25	20°05′36″S; 43°28′29″W	Melo 05
Serra da Calçada, Nova Lima-MG	L5	20	20°05′15″S; 43°59′01″W	Melo 07
A. modestissima				
Serra do Curral, Belo Horizonte-MG	M3	17	19°58′13″S; 43°55′43″W	Melo 06
Serra da Piedade, Caeté-MG	M2	18	19°49′17″S; 43°40′53″W	Melo 03
A. prolifera				
Serra do Caraça, Catas Altas-MG	P1	30	20°05′36″S; 43°28′29″W	Melo 04
Serra da Piedade, Caeté-MG	P2	23	19°49′17″S; 43°40′53″W	Melo 02

Vouchers are deposited in the herbarium BHCB

Table 2 Reproductive phenology, and pollinarium removal and deposition in populations of *A. hamosa*, *A. limae*, *A. modestissima*, and *A. prolifera*

Population	*A. hamosa* H4	*A. limae* L1 (*n* = 42)	*A. modestissima* M2 (*n* = 35)	*A. prolifera*	
				P1 (*n* = 41)	P2 (*n* = 35)
Flowering individuals	–	66.7%	96.7%	95.1%	91.4%
Fruiting individuals	–	17.9%	27.6%	5.1%	25.0%
Inflorescences	–	96	112	178	16
Flowers	–	484	384	775	690
Fruits	–	11	11	2	9
Fruits/inflorescence average (min.–max.)	3.7 (1–5)[c]	5.0 (1–7)	3.4 (1–5)	4.4 (1–7)	4.3 (1–7)
Fruit set	–	2.3%	2.8%	0.3%	1.3%
Pollinarium removal (*N*)[a]	64.5% (62)	30.4% (115)	60.7% (91)	–	–
Pollinarium deposition (*N*)[a]	19.3% (62)	4.3% (115)	15.7% (91)	0.4% (257)	0% (179)
Average daily pollinarium removal (*N*)[b]	26.7% (71)	2.7% (1352)	17.4% (156)	0% (944)	0.3% (1762)

N = sample size. See Table 1 for the names of the populations

[a] Data obtained from withered flowers collected in the populations. Pollinaria of *A. prolifera* flowers are not retained in withered flowers

[b] Data obtained from examination of flowers open at the beginning and end of the day, during periods of observation of flower visitors

[c] Data from individuals growing in a greenhouse

(Table 3). Rather, the dimensions of the floral cavity are directly related to the measurements of the labellum, column, and column foot: the height of the opening corresponds to the space between the lip and column; the width is delimited below by the distance between the calli of the lip; and its depth corresponds to the length of the column.

Acianthera hamosa and *A. modestissima* have the smallest flowers, and no marked morphological differences between them were observed (Fig. 1, Table 3). These two species have inflorescences with an average of from three to four flowers. The floral cavity is relatively small in relation to the other two species, with the opening being approximately 0.9 (0.5–1.2) mm high × 0.5 (0.4–0.6) mm wide, and 2.5 (1.9–2.9) mm long. The flowers of *A. limae* are similar in shape to those of *A. hamosa* and *A. modestissima*, but differ mainly by being larger (Fig. 1f; Table 3). The floral cavity of *A. limae* has an opening approximately 1.3 (1.0–1.5) mm high × 0.8 (0.5–1.0) mm wide, and 3.6 (2.6–4.4) mm long, and is thus characteristically larger than those of *A. hamosa* or *A. modestissima*. There is overlap in the dimensions of the openings and the lengths of the floral cavities of *A. limae* and *A. prolifera*. *Acianthera prolifera* has the largest flowers of the group (Fig. 1h; Table 3). This species has the largest floral cavity, with an opening 2.0 (2.6–1.3) mm high × 1.2 (1.5–1.0) mm wide, and 4.6 (3.3–5.8) mm long.

Table 3 Measures of flower parts of *Acianthera hamosa*, *A. limae*, *A. modestissima*, and *A. prolifera*

Character	*A. hamosa* (*N* = 19)	*A. limae* (*N* = 46)	*A. modestissima* (*N* = 40)	*A. prolifera* (*N* = 44)
Dorsal sepal (length × width)	5.1 ± 0.5 × 1.9 ± 0.2	7.3 ± 0.8 × 2.2 ± 0.2	5.2 ± 0.5 × 2.1 ± 0.2	9.5 ± 0.8 × 3.2 ± 0.4
Petal (length × width)	2.9 ± 0.2 × 1.1 ± 0.1	3.3 ± 0.3 × 1.0 ± 0.2	2.8 ± 0.3 × 1.0 ± 0.1	4.2 ± 0.5 (3.4–5.2) × 1.5 ± 0.3
Fused lateral sepals (length × width)	5.3 ± 0.5 × 2.2 ± 0.3	7.1 ± 0.8 × 2.7 ± 0.3	5.1 ± 0.6 × 2.4 ± 0.3	8.9 ± 0.9 × 3.7 ± 0.4
Lip (length × width)	3.4 ± 0.3 × 1.6 ± 0.2	4.2 ± 0.3 × 2.0 ± 0.2	3.2 ± 0.4 × 1.4 ± 0.2	5.2 ± 0.5 × 2.5 ± 0.3
Column length	2.4 ± 0.2	2.9 ± 0.3	2.3 ± 0.3	3.6 ± 0.3
Column foot length	1.1 ± 0.1	1.2 ± 0.2	1.1 ± 0.2	1.6 ± 0.2
Floral cavity opening (height × width)[a]	0.9 ± 0.1 × 0.5 ± 0.1	1.3 ± 0.1 × 0.8 ± 0.1	0.9 ± 0.1 × 0.4 ± 0.1	2.0 ± 0.3 × 1.2 ± 0.2

Values presented in centimeters as average ± standard deviation. *N* = sample size

[a] For this character, *N* = 15 in all four species; height corresponds to the distance between the lip and column; width is the distance between the lip calli

Phenology

Data on the reproductive phenology of sympatric populations were collected monthly for 1 year: *A. limae* and *A. prolifera* from August 2006 to July 2007, and *A. modestissima* and *A. prolifera* from September 2006 to August 2007. Forty-two individuals of *A. limae* and 41 of *A. prolifera* were marked at Serra do Caraça, and 35 individuals each of *A. prolifera* and *A. modestissima* at Serra da Piedade. During each field visit the numbers of emerging inflorescences were counted, as well as the number of inflorescences with flowers in anthesis, and the number of flowers and developing fruits. Data concerning the average duration of the inflorescences and flowers for each of the species were obtained by observing individuals cultivated in a greenhouse.

Floral biology

Fresh flowers from individuals cultivated in a greenhouse were dissected and inspected using a stereomicroscope to check for the presence of secretions (such as nectar or oils). In a previous anatomical study with these species, Melo et al. (2010) detected the presence of osmophores on the sepals, which secrete nitrogenated compounds, and nectaries on the lip. Stigmatic receptivity was evaluated in each species using hydrogen peroxide (Dafni 1992) on three flowers sampled every 48 h from the beginning until the end of floral anthesis.

The activity of floral visitors was observed in five populations at Serra da Piedade (*A. prolifera* and *A. modestissima*), Serra do Caraça (*A. prolifera* and *A. limae*), and Carrancas (*A. hamosa*). Observations were first undertaken during the hours from 6:00 to 18:00 h, but later adjusted to 8:00 to 17:30 h according to observed floral visitor activity and the perceived intensity of floral odors. The available flowers were marked and examined at the start and at the end of the day to evaluate the percentage of natural removal of pollinaria during that period and to note any pollinator activity outside of the normal observation period. Additionally, abscised flowers were collected and examined using a stereomicroscope to evaluate the proportions of flowers that had pollinaria removed and/or deposited in the stigmatic cavity. Samples of floral visitors were collected with the aid of a suction device, sent to specialists for identification, and deposited in the entomological collection of the Departamento de Zoologia, Universidade Federal de Minas Gerais.

Mating systems

Approximately 20 individuals from each population were collected and cultivated in a greenhouse at the Universidade Federal de Minas Gerais (Table 1). Only individuals from distinct tree trunks or stones were collected to avoid sampling clones through vegetative propagation. The experimental pollinations were performed 6 months after collecting and replanting. Self-, intrapopulational cross-, and intraspecific interpopulational cross-pollinations were performed, and depending on the availability of additional flowers, interspecific bidirectional pollinations were performed between the species, giving priority to crosses between sympatric populations. Only 1- to 3-day-old flowers were used in the pollination experiments, with a maximum of three flowers pollinated per inflorescence. Some of the inflorescences in each population were not manipulated in order to monitor the occurrence of agamospermy and/or spontaneous self-pollination. We carried out a total of 1,690 experimental pollinations, with 1,001 intraspecific and 689 interspecific crossings.

Additional intraspecific cross-pollinations were performed in the field in the *A. modestissima* population at Serra da Piedade to determine if the low fruit set observed in experimental pollinations in this species was due to aborting flowers in the cultivated plants. Although the cultivated plants of *A. hamosa* also demonstrated low fruit formation, this procedure was not repeated in natural populations due to difficulties related to access to their population. In addition to these pollinations, we also performed three additional treatments in the greenhouse with two populations of *A. modestissima* (Serra da Piedade and Serra do Curral), utilizing flowers of different ages or flowers that had been previously manipulated ($n = 20$ for each treatment):

1. Pollination of recently opened flowers from which the pollinaria were removed and then stored (in small open glass flasks at room temperature). After 4 and 6 h these pollinaria were used to pollinate the same flowers from which they were removed to investigate possible alterations in stigmatic receptivity and/or pollen viability after pollen removal.
2. Pollination of older flowers (6–8 days after anthesis) to examine possible variations in stigmatic receptivity over time.
3. Pollination of flowers whose pollinaria were removed 3 days earlier, receiving pollinaria of recently opened flowers, to examine possible alterations in stigmatic receptivity promoted by the removal of the original pollinaria (protandry).

Fruit set and development were monitored until maturation, and the mature and aborted fruits and abscised flowers were fixed in 50% formalin-acetic acid-alcohol (FAA). The viability of samples of at least 200 seeds per fruit was evaluated as judged by their morphological character as seen under an optical microscope (Borba et al. 1999, 2001a; Barbosa et al. 2009). Some fruits formed through interspecific pollination had seeds with embryos that were slightly smaller than those formed by cross-pollination. To determine if these smaller embryos were viable, samples of nonfixed seeds from intra- and interspecific crosses were immersed in 1% solution of 2,3,5-triphenyltetrazolium chloride and maintained for 24 h at room temperature. The embryos of the seeds from fruits formed by interspecific pollination were observed to stain in a very similar manner to those from fruits formed by intraspecific pollination, and thus were considered viable in our counts.

Pollinations were also made in which development was interrupted at regular intervals (after 24 h, and 3, 5, 7, 9, 12, 15, 20, 30, 40, and 50 days) and the resulting flowers and fruits fixed in 50% FAA. Mature and aborted fruits, abscised pollinated flowers, as well as the interrupted flowers were treated with a solution of 8 N NaOH at 60°C for approximately 60 min (fruits) or 30 min (flowers). These fruits and flowers were then washed, stained with aniline blue, and observed by epifluorescence microscopy (modified from Martin 1959) for pollen germination, growth and morphology of the pollen tubes, penetration of the ovules, and embryo and seed development.

Results

Phenology and floral biology

The proportions of flowering individuals was high (67–97%) in all of the species, although only low fruit set was observed during the study period (Table 2). The sympatric species flowered synchronously, with overlapping being observed during essentially their entire flowering periods (Fig. 2). At Serra do Caraça, the flowering peaks of *A. limae* and *A. prolifera* coincided, and the same situation occurred with *A. prolifera* and *A. modestissima* at Serra da Piedade. All of the species flowered in synchrony in the greenhouse, with at least some flowers being present during almost the entire year. The population of *A. limae* at Serra do Caraça flowered for almost the entire study period (except for September and October 2006). The flowering peak of this population occurred between February and March 2006 (Fig. 2). Fruit set was approximately 2.3%, with 18% of flowering individuals having at least one fruit (Table 2). The individuals of *A. modestissima* flowered from November 2006 to April 2007, with a flowering peak between November and December, and reduced flowering between January and April (Fig. 2). Fruit set was 2.8% in this population, with approximately 30% of flowering individuals producing fruits. The two *A. prolifera* populations demonstrated temporal shifts in their flowering peaks: at Serra do Caraça it occurred from January to July 2007, while at Serra da Piedade, peak flowering was concentrated between December 2006 and January 2007 (Fig. 2). Additionally, individuals of *A. prolifera* were observed flowering during all of the months of the study period at Serra do Caraça, with only a small decrease in flowering from August to November 2006. However, only approximately 5% of flowering individuals produced fruits, with general fruit set of 0.3% (Table 2). Total fruit set at Serra da Piedade was 1.3% during the study period, with 25% of flowering individuals producing fruits (Table 2).

Flower opening was diurnal in all of the species, and generally occurred in the morning hours. The flowers accumulate a small quantity of nectar at the base of the labellum that forms a thin shiny layer. Tests using ammonium hydroxide vapor did not indicate the presence of nectar guides formed by pigments that absorb in the ultraviolet spectrum. Tests with hydrogen peroxide indicated

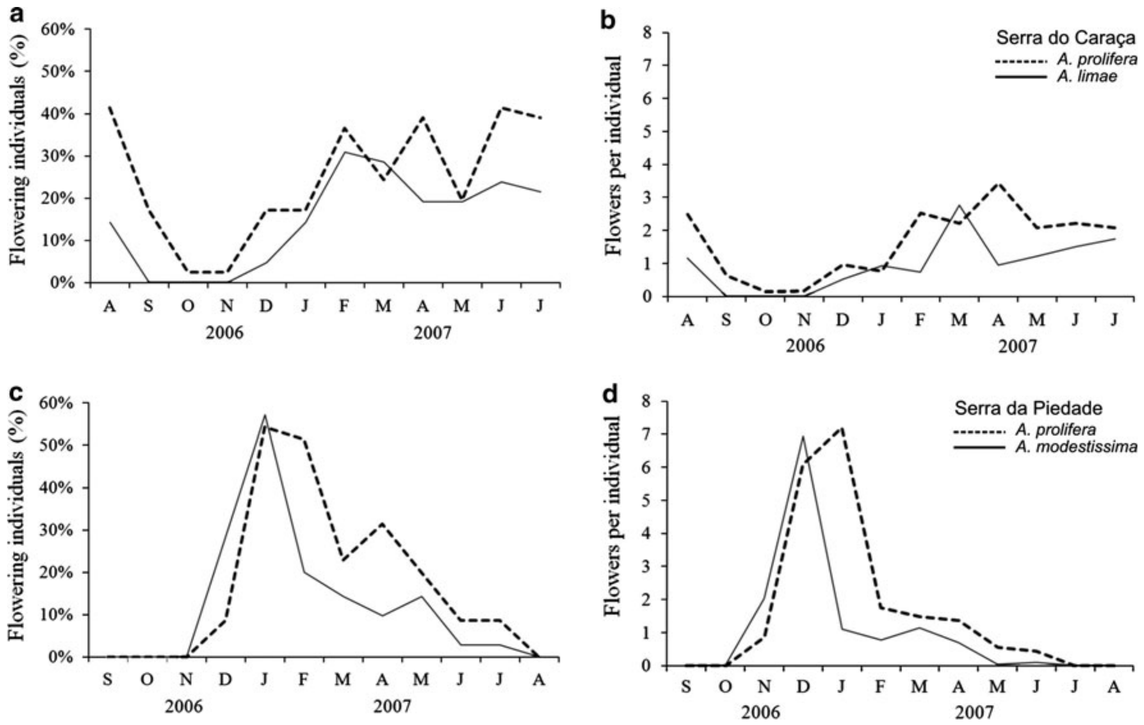

Fig. 2 Percentage of individuals flowering and total number of flowers available per individual in sympatric populations of *Acianthera limae* (42 individuals) and *A. prolifera* (41 individuals) at Serra do Caraça (**a**, **b**) and *A. modestissima* (35 individuals) and *A. prolifera* (35 individuals) at Serra da Piedade (**c**, **d**), followed every month during the period from August 2006 to August 2007

that the stigma was receptive during the entire period after anthesis. The flowers of *A. hamosa* and *A. modestissima* abscise approximately 9 days after anthesis, while those of *A. limae* and *A. prolifera* abscise approximately 15 to 18 days after anthesis, respectively. The flowers of *A. hamosa* and *A. modestissima* have a very slight odor, similar to the smell of decaying plant material, which was only perceptible during the warmest periods of the day (11:00–14:00 h). The odor produced by *A. limae* flowers is similar to that of *A. hamosa* and *A. modestissima*, although more intense, and could be perceived from 09:00 to 17:00 h. The odor produced by *A. prolifera* differed from the other species, being similar to the odor of fresh fish; it was perceived from 08:30 to 17:00 h, although it was more intense during the warmest period of the day (from 11:00 to 15:30 h).

Floral visitors

The four species were visited only by Diptera species of the families Phoridae (*Megaselia* spp.) and Chloropidae (Table 4). The behavior of the visitors was very similar on all four orchid species. After making erratic flights, the insects landed on the substrate or on leaves of a given individual, then reaching the inflorescence by way of a short flight. The visitors then completely penetrated into the narrow floral cavity canal. To penetrate into the cavity, the insect positioned itself in front of the flower and then walked on the labellum, which moved downward due to the weight of the insect and allowed it to pass through the narrow floral opening. The fly then introduced almost its entire body into the floral cavity, to the point at which its pressure on the labellum diminished; this floral structure then returned to its original position and pushed the insect against the column. While the fly attempted to back out of the flower its scutellum touched the rostellum and removed the pollinaria together with the anther cap; the cap then fell away as the insect exited the flower. The visits always lasted less than 30 s. After leaving the flower carrying the pollinaria, the insect quickly visited other flowers of the same inflorescence or of other inflorescences near the same individual. No visits by female flies demonstrating ovipositioning behavior on the flowers were observed in these species.

Only visits by males and females of *Megaselia* (Diptera: Phoridae) were observed in the populations of *A. hamosa* and *A. modestissima* (Table 4). The visits occurred only on sunny days (generally preceded by rainy days), and always during the warmest period of the day (>30°C), between 11:00 and 15:00 h. No pollinarium was removed outside of the observation times in either of the species. In flowers of *Acianthera modestissima*, 19 visits of flies of *Megaselia* sp. 1 were observed, with three removals and one deposition of pollinarium in the stigmatic cavity, during 81 h of

Table 4 Floral visitors and effective pollinators of *A. hamosa*, *A. limae*, *A. modestissima*, and *A. prolifera*

Species/pop.	Total hours of observation	Visitor (length × width)	Number of visits ([a])	Pollinator/visitor
A. hamosa	45	*Megaselia* sp. 4 (1.2 × 0.6)	5 (100%; 2)	P
A. limae	273.5	Chloropidae sp. 1 (2.7 × 1.2)	10 (12%; 0)	V
		Chloropidae sp. 2 (2.4 × 0.9)	36 (42%; 0)	V
		Megaselia sp. 2 (1.7 × 0.7)	3 (3%; 2)	P
		Megaselia sp. 3 (2.4 × 1.1)	37 (43%; 0)	V
A. modestissima	81.5	*Megaselia* sp. 1 (1.2 × 0.64)	19 (100%; 4)	P
A. prolifera				
P1	112.5	Chloropidae sp. 2 (2.1 × 0.9)	4 (100%; 0)	V
P2	159	Chloropidae sp. 3 (2.4 × 0.9)	9 (90%; 0)	V
		Megaselia sp. 1 (1.3 × 0.9)	1 (10%; 0)	V

Body measurements of insects are shown in millimeters. See Table 1 for the names of the populations

P effective pollinator, based on efficiency of pollinarium removal; *V* visitor

[a] Percentage of visits by species of floral visitor; number of pollinarium removals observed

observation (Table 4; Fig. 3c, d). Pollinarium removal and its subsequent deposition in another flower of the same inflorescence (geitonogamy) was observed in this species. At the end of each day of observation, approximately 17% of the flowers that had been marked at the start of the day had their pollinarium removed. Approximately 60% of abscised flowers ($n = 91$), had their pollinarium removed, and 16% had pollinarium deposited in their stigmatic cavity (Table 4). Five visits were observed to flowers of *A. hamosa* (with two pollinarium removal) by *Megaselia* sp. 4 during 45 h of observation (Table 4; Fig. 3a). Approximately 27% ($n = 71$) of marked flowers had their pollinarium removed by the end of the day. In abscised flowers, 65% ($n = 62$) had their pollinarium removed, and 19% had pollinarium deposited in the stigmatic cavity (Table 2).

The flowers of *A. limae* at Serra do Caraça were visited by females of two morphospecies of *Megaselia* (Phoridae) and two of Chloropidae. A total of 46 (54%) visits by species of Chloropidae and 40 visits (46%) by species of *Megaselia* were observed in 273.5 h of observation (Table 4). Pollinaria were observed to be removed only by individuals of *Megaselia* sp. 2 (Fig. 3b). Individuals of the two species of Chloropidae and *Megaselia* sp. 3 attempted to enter the flowers of *A. limae* but could not pass the calli of the labellum that narrow the opening. Visits to the flowers of *A. limae* occurred throughout most of the day (09:00–17:00 h), with peaks of activity between 10:00–12:00 h and 14:00–17:00 h. Visits were most frequent during humid days with cloudy and rainy weather. The removal rate of pollinaria per day was approximately 2.7% ($n = 1{,}352$). Among the abscised flowers that were analyzed ($n = 115$), approximately 30% had their pollinarium removed, but only 4% had pollinarium deposited in the stigmatic cavity (Table 2).

No pollination event was observed during floral visits to the two populations of *A. prolifera* (Table 4). Four visits by individual females of Chloropidae sp. 2 were observed at Serra do Caraça (112.5 h of observation), all of them occurring when the plants were grouped together for observation. After 159 h of observation of the population at Serra da Piedade we recorded nine visits by males and females of Chloropidae sp. 3. Additionally, a visit by a single female of *Megaselia* sp. 1 was also observed at Serra da Piedade. All of the floral visitors in both localities completely entered the flowers and remained at the base of the labellum for approximately 5 min without removing the pollinarium. The percentage of pollinarium removal during the day was 0% in the population at Serra do Caraça ($n = 944$) and 0.3% at Serra da Piedade ($n = 1{,}762$). The abscised flowers of this species did not furnish any information about pollinarium removal as that structure is not retained. Only 0.4% of these flowers had pollinarium deposited at Serra do Caraça ($n = 257$) and 0% at Serra da Piedade ($n = 179$) (Table 2).

Mating systems

Intraspecific pollinations

Spontaneous fruit set was not observed in any of the species. Pollinated flowers that did not give rise to fruits lasted slightly shorter than unpollinated flowers, abscising after 12–14 days in *A. prolifera*, 7–11 days in *A. limae*, and within 5–8 days in *A. modestissima* and *A. hamosa*. Many of the self-pollinated fruits aborted precociously, reaching a maximum after 2 months, while the fruits resulting from cross-pollinations matured in 2.5–3 months. Most of the fruits produced from self-pollinations that did not abort had reduced numbers of seeds (Fig. 4i) that remained attached

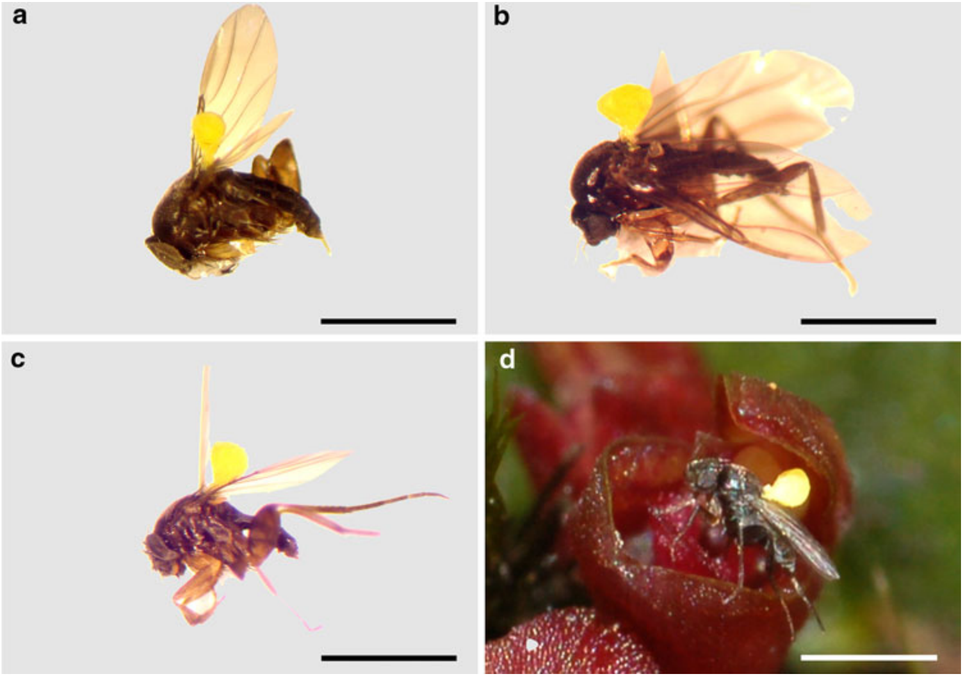

Fig. 3 Floral visitors of *Acianthera* species occurring in Brazilian *campo rupestre*. **a** *Megaselia* sp. 4 (Phoridae), pollinator of *A. hamosa* at Carrancas-MG; **b** *Megaselia* sp. 2, pollinator of *A. limae* at Serra do Caraça-MG; **c**, **d** *Megaselia* sp. 1, pollinator of *A. modestissima* at Serra da Piedade–MG. Notice the pollinarium attached to the scutellum of the insects. Scale bars = 1 mm

to their placentas. It was therefore necessary to scrape the seeds off with a scalpel in order to evaluate their viability.

The proportions of pollinations resulting in fruits were most similar between *A. limae* and *A. prolifera*, and between *A. modestissima* and *A. hamosa* (Table 5). In the first two species the fruit set rates per population varied from 20% to 50% by self-pollination (initial tests), from 35% to 42% in intrapopulational cross-pollinations, and from 29% to 53% in intraspecific interpopulational cross-pollinations. Fruit set was very low in all of the pollination experiments undertaken with *A. modestissima* and *A. hamosa*, varying from 0% to 8% for self-pollination in the populations, from 0% to 12% in intrapopulational cross-pollinations, and from 8% to 18% in intraspecific interpopulational cross-pollinations.

Fruit set in self-pollination experiments was less than that seen in intra- and interpopulational cross-pollinations in the four species (with the exception of the *A. prolifera* population at Serra do Caraça), although the percentages were always generally similar. Approximately 50–100% of the fruits formed in self-pollination experiments fell without opening and had reduced numbers of seeds in relation to cross-pollination experiments (Fig. 4i). Fruit set in intraspecific interpopulational pollinations was greater than intrapopulational pollinations only in populations of *A. prolifera* and *A. modestissima* at Serra da Piedade, although their values were very close. Fruit set in intraspecific cross-pollinations undertaken under field conditions in *A. modestissima* (Serra da Piedade) was 5% ($n = 20$), being similar to the fruit set observed in the pollinations performed in greenhouse. No fruit was formed in pollination experiments that used flowers of different ages or flowers that had been previously manipulated in *A. modestissima*.

Interspecific crossings

The species presented different degrees of intercompatibility, with variations according to the crossing directions and the populations involved (Table 5). In general, species that share the same groups of pollinators (*A. limae*, *A. hamosa*, and *A. modestissima*) demonstrated low fruit set or total incompatibility among themselves. In the pollination experiments involving *A. limae* and *A. modestissima*, only one fruit was formed when *A. limae* (L5) received pollen from *A. modestissima* from Serra da Piedade (M2), and no fruit was formed when pollination was performed with pollen from *A. modestissima* from Serra do Curral (M3). On the other hand, *A. limae* (L1) demonstrated high fruit set (23% and 32%) when pollen from both populations of *A. modestissima* (M2 and M3) was used. Only one fruit was formed in the interspecific crossings between *A. modestissima* and the populations of *A. limae*, when *A. modestissima* (M3) received pollen from *A. limae* (L1). In crossings between *A. modestissima* and *A. prolifera* fruit set was always similar to that seen in intraspecific pollinations, with no differences between the degrees of interspecific compatibility between their populations.

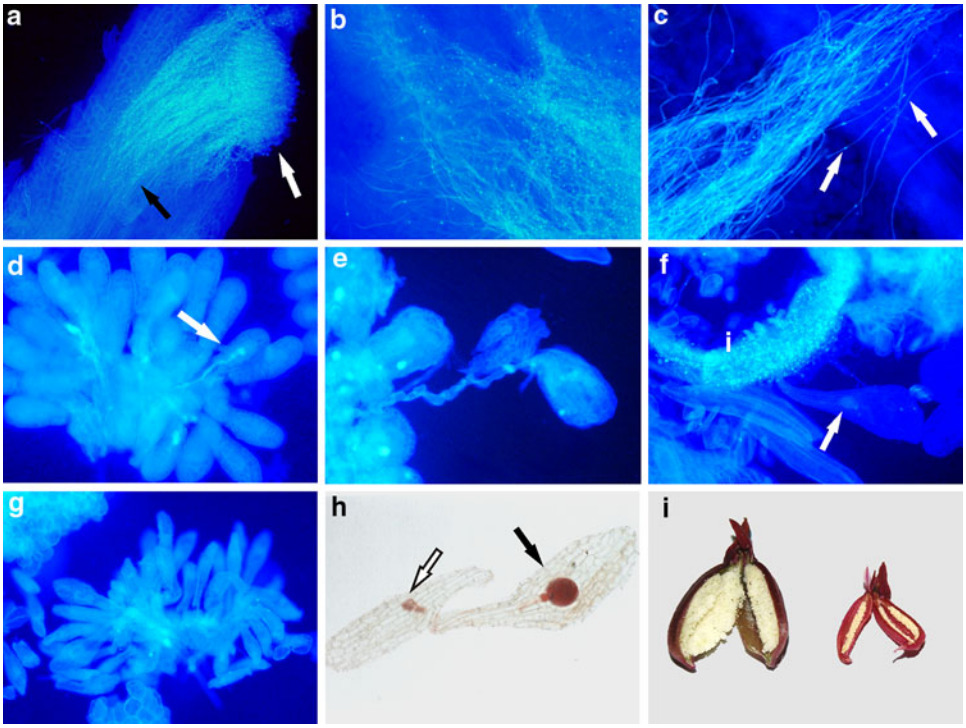

Fig. 4 Pollen tube growth by fluorescence microscopy and seeds and fruits developed in experimental pollinations in *Acianthera* species. **a** Pollinarium (white arrow) in the stigmatic cavity with normal pollen tubes growing up to the middle region of the column (black arrow), nearly 6 days after cross-pollination (*A. limae*). **b**, **c** Bundles of pollen tubes entering the ovary, reaching the placenta 12–15 days after cross-pollination (*A. limae*); notice the pugs of callose (arrows) deposited at regular intervals. **d**, **e** Ovules penetrated by pollen tubes (arrow) after self- (*A. limae*; **d**) and cross-pollination (*A. prolifera*; **e**). **f** Bundle of pollen tubes in a 50 days fruit from cross-pollination of *A. limae*; notice a well-developed seed (arrow). **g** 50 days fruit from self-pollination (*A. limae*); notice that the seeds are less developed compared with **f**, and embryos are rudimentary or absent. **h** Well-developed seed with viable embryo (black arrow) and seed with rudimentary embryo (white arrow), in cross-pollination in *A. prolifera*. **i** Fruits from a same inflorescence of *A. prolifera*: left, cross-pollination, with large amounts of normal seeds; right, self-pollination, with a small amount of seeds, which are undeveloped and not dispersing

Pollen tubes growth

Only approximately half of the flowers analyzed (abscised and interrupted) in all of the treatments showed germinated pollen grains. Interspecific pollinations that did not give rise to fruits were associated with the absence of germinated pollen grains. However, when the grains did germinate, their pollen tubes demonstrated rectilinear growth, homogeneous diameter, and deposition of callose plugs at regular interval, without any observed differences between the various experimental intra- and interspecific treatments (Fig. 4a–f).

Due to the low fruit set observed in *A. hamosa* and *A. modestissima*, complete development of the pollen tubes (until their penetration of the ovules and the posterior development of seeds) could only be monitored in *A. limae* and *A. prolifera*. Germination of pollen grains in these species after intraspecific cross-pollination was observed to initiate 3 days after pollination. The extension of the pollen tubes was restricted to the stigmatic cavity during this period, but these tubes were already directing themselves towards the style canal. At 5 days, a large number of pollen tubes had penetrated the style canal and reached the median region of the column (Fig. 4a). By 7–9 days, the tubes had reached the base of the column, and by 15 days they had penetrated the ovary and extended to the placental region (Fig. 4b, c). At approximately 20 days, the bundles of tubes had grown within the ovary and between the developing ovules, decreasing in diameter as they progressed. After approximately 30 (*A. limae*) and 40 days (*A. prolifera*) the first pollen tubes had penetrated the ovules (Fig. 4e), so that after 50 days it was possible to observe developing seeds (and some already fully formed) (Fig. 4f). At 60 and 70 days, the fruits were full of mature seeds. A very similar pattern was seen in fruits formed after self-pollination, with no differences in terms of the quantity, growth velocity or morphological aspects of the pollen tubes in relation to those resulting from cross-pollination. In the same way, after between 30 and 40 days, large quantities of tubes were observed penetrating the ovules in self-pollinated flowers (Fig. 4d). These penetrated ovules, however, did not develop into seeds (Fig. 4g), and after

Table 5 Percentage of fruit set in experimental pollinations in *A. hamosa*, *A. limae*, *A. modestissima*, and *A. prolifera*

Species/population	Self		Intrapop.	Interpop.	Interspecific						
	Start fruiting	Reach maturity[a]			×P1	×P2	×L1	×L5	×M2	×M3	×H4
A. hamosa											
H4	0 (20–7)	0	4.3 (46–09)	–	–	–	–	–	0 (09–04)	0 (13–05)	–
A. limae											
L1	26.8 (56–18)	13.3	37.8 (81–21)	28.6 (28–15)	17.5 (40–15)	25.0 (28–08)	–	–	23.1 (26–11)	32.3 (31–14)	–
L5	20.0 (50–20)	50.0	35.7 (42–14)	32.1 (28–12)	42.3 (26–11)	12.1 (34–14)	–	–	5.0 (20–10)	0.0 (21–11)	–
A. modestissima											
M3	0 (28–10)	0	0 (68–12)	7.9 (38–10)	4.8 (21–08)	4.0 (25–08)	0 (20–09)	0 (19–12)	–	–	15.4 (13–07)
M2	8.1 (69–02)	50.0	12.2 (49–10)	18.4 (38–15)	4.5 (22–08)	23.1 (39–8)	6.9 (29–08)	0 (20–9)	–	–	10.0 (10–04)
A. prolifera											
P1	46.2 (78–22)	16.7	41.7 (96–28)	35.5 (31–15)	–	–	47.0 (36–13)	16.0 (25–08)	35.0 (20–10)	52.4 (21–05)	–
P2	28.8 (59–19)	58.8	36.4 (66–14)	53.3 (30–10)	–	–	39.1 (23–07)	8.9 (34–12)	51.3 (39–11)	48.0 (25–04)	–

In parentheses: number of pollinations and number of individuals used. *Self* self-pollination, *intrapop.* intrapopulational cross-pollination, *interpop.* interpopulational cross-pollination. See Table 1 for the names of the populations

[a] Percentage of fruits that start fruiting and reach maturity

50 days the fruit aborted before opening and few seeds could be observed inside them (Fig. 4i).

Seed viability

Fruits from intra- and interpopulational cross-pollinations produced large quantities of seeds with high viability (varying from 89% to 98%) (Fig. 5a), which was similar to the results observed with interspecific pollinations (Fig. 5b). However, the fruits resulting from self-pollination generally had fewer seeds and they remained attached to the placenta (Fig. 4i); seed viability varied greatly, although most had viability less than 50% (Fig. 5a).

Discussion

Floral biology and reproductive phenology

This study found that at least three of the four *Acianthera* species studied are pollinated by species of Phoridae, plus a high number of flower visits by Chloropidae. These results together with other studies place these groups of Diptera as the most important species of flower visitors for this genus in Brazilian *campo rupestre* (Borba and Semir 2001) and other vegetation formations (Singer and Cocucci 1999; Santos-Filho 2007). Usually, pollination by flies in Orchidaceae has been associated with only a few families of Diptera, generally Mycetophilidae, Sciaridae, Syrphidae, and Drosophilidae (for a revision, see Christensen 1994). However, more recent studies have demonstrated that other families, such as Chloropidae, Milichiidae, and Phoridae, are important orchid pollinators (Borba and Semir 1998b; Verola 2002; Albores-Ortiz and Sosa 2006) as they are for other plant groups (Disney 1994; Sakai 2002).

The presence of nectar may be associated with a low degree of specificity in attracting floral visitors in *A. limae* and *A. prolifera* (Melo et al. 2010), which are both visited by Chloropidae and Phoridae in a system similar to that observed in the nectariferous *A. teres* and *A. ochreata* (Borba and Semir 2001). Although this system is not valid for *A. hamosa* and *A. modestissima*, which also produce nectar, these latter two species seem to have a specialized relationship with *Megaselia* spp. Additionally, the presence of nectar was not associated with high fruit set, in a pattern different from that proposed by recent workers (Tremblay et al. 2005; Peter and Johnson 2009). In some species of *Acianthera* whose flowers also contain nectar and attract species of Chloropidae and Phoridae, the pollination systems were interpreted as partial deceit, as the females do not oviposit in the flowers (Borba and Semir 2001). Systems of complete deceit generally exhibit very specialized relationships between the plants and their pollinator groups

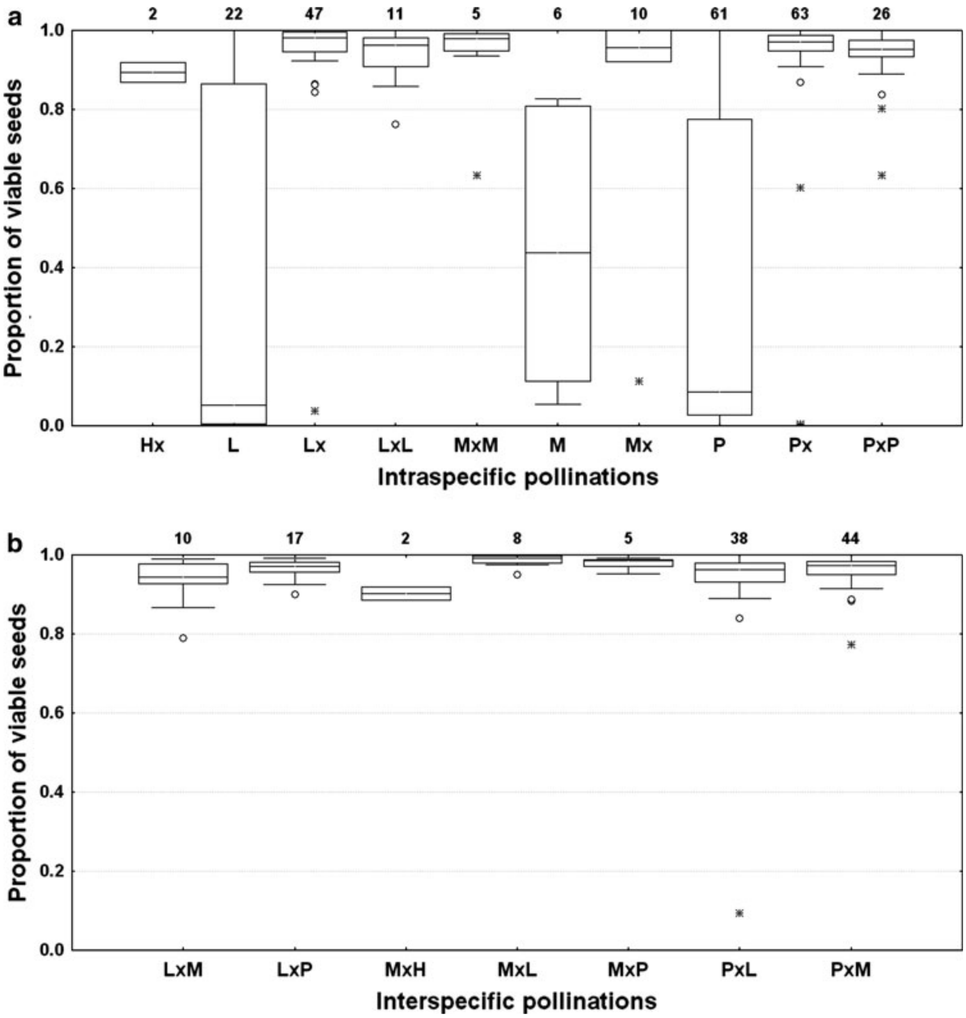

Fig. 5 Box plots of seed viability per fruit in experimental intra- (**a**) and interspecific (**b**) pollinations in *Acianthera* species (H = *A. hamosa*, L = *A. limae*, M = *A. modestissima*, P = *A. prolifera*). L, M, P = self-pollination; Hx, Lx, Mx, Px = intrapopulation cross-pollination; LxL, MxM, PxP = interpopulation pollination; the second letter indicates the pollen donor in interspecific crosses. The number of fruits sampled is shown on the upper axis. The box encloses 50% of the data, and the central line marks the median. Inner and outer whiskers indicate the interquartile range. Circles indicate outlying values, and asterisks indicate extreme outliers

(monophyly), as is observed in *A. johannensis* and *A. fabiobarrosii* (Borba and Semir 2001) and in other orchids (Jersáková et al. 2006). Sapromyophily has been associated with species that have flowers that produce disagreeable odors (e.g., of feces, fungus or decomposing material), dark purple colors, no nectar, and that are pollinated by deceit (van der Pijl and Dodson 1966; Faegri and van der Pijl 1979; Proctor et al. 1996; Jersáková et al. 2006). According to some authors, sapromyophilous species may offer nectar in their flowers, but the insects are attracted as a result of their reproductive instincts and in search of food, as in some species of Apocynaceae and in *Aristolochia* (Proctor et al. 1996). The presence of nectar in sapromyophilous flowers may be associated with peculiarities of some pollination mechanisms and contribute to maintenance of the pollinator in the flowers for more time, as is seen in species of *Bulbophyllum* (Borba and Semir 1998b).

Acianthera hamosa and *A. modestissima* exhibit relatively high pollinaria removal percentages (>60%) as compared with other myophilous species (Tremblay et al. 2005), but only one-third of the pollinaria that are removed are actually deposited on stigmas, resulting in low fruit set. Low pollinaria deposition percentages have been observed in several orchids, and may be associated with low efficiency, low abundance, inconstancy, and promiscuity of the pollinator (Proctor et al. 1996; Tremblay et al. 2005). Conversely, the low fruit set observed in *A. hamosa* and *A. modestissima* may also be associated with the mating system of these species. The low fruit set of these two species in pollination experiments suggests the existence of some mechanism that limits their fruit set, independent of pollen availability. Both of these species occur on nutrient-poor soils and experience significant water stress, and can therefore be considered to be subject to strong limitations in available resources. Limitations imposed by nutrient levels and pollination agents have been indicated as the principal causes of the low fruit set observed in most orchid species (Tremblay et al. 2005).

The low fruit set observed in the field in *A. limae* and *A. prolifera* (approximately 15 and 30 times less than the fruit set in the experiments, respectively) seems to be related to the presence of floral specializations to a restricted group of pollinators and/or the low abundance of their pollinators at the study localities. In the majority of the observed insect visits to *A. limae*, and in all of those to *A. prolifera*, the Diptera visitors were incapable of removing the pollinaria. This low efficiency of pollinaria removal seems to be common in orchids, with the flower being visited by diverse insects that are not effective pollinators (Tremblay et al. 2005). Borba and Semir (2001) observed the same phenomenon in the five species of *Acianthera* they studied, with most floral visitor species being unable to efficiently remove the pollinaria. This may be the result of floral morphology evolution (i.e., mechanical barriers) against insect species with short scutellum that generally clean themselves after removing a pollinaria, or that have promiscuous behaviors that would favor hybridization. According to Tremblay et al. (2005), there are many reports of orchids showing large variation in fruit set in different years and among populations in different regions that could be associated with fluctuations of the populations of their pollinators. This apparent instability does not necessarily have long-term negative effects on these species of orchids, however, especially among those that are perennial and that can reproduce vegetatively, as the species we studied. Sexual reproduction events, even being rare and just by chance, can produce highly productive fruits (in terms of number and quality of seeds) that can significantly contribute to maintaining their populations (Neiland and Wilcock 1998).

Our study showed that the similar floral morphology in the allopatric species (*A. hamosa*, *A. limae*, and *A. modestissima*) is associated with the same group of pollinators (*Megaselia* spp.; Phoridae). Because *A. prolifera* shows great floral similarity (odor, color, and size) to *A. johannensis* and *A. fabiobarrosii* that are pollinated by the same species of Chloropidae (Borba and Semir 2001), we suggest that these insects may be its principal pollinators. As such, we hypothesize that adaptive radiation may have occurred between the sympatric *A. prolifera* and the species pollinated by Phoridae, as observed in the species studied by Borba and co-workers (Borba and Semir 2001; Borba et al. 2001b, 2002). Interestingly, convergence and radiation also appear to have occurred among these two groups of *Acianthera* spp. occurring in *campo rupestre*. *Acianthera ochreata* and *A. teres* have flowers with morphology and odor very similar to *A. hamosa*, *A. limae*, and *A. modestissima* (Melo and Borba in press), and the former two species are also pollinated by species of *Megaselia* (Borba and Semir 2001). These observations are pertinent to the hypothesis of the existence of convergence in floral characters that is associated with pollinators, and which is the basis for the pollination syndromes (Faegri and van der Pijl 1979). This phenomenon has been demonstrated in species of *Acianthera*, and our results indicate that this trend may be common in the genus (Borba and Semir 2001; Borba et al. 2002). These ideas have been questioned by researchers who defend the view that generalized systems should be the rule for most flowers (Herrera 1988; Waser et al. 1996). However, these criticisms have been parried by a series of studies that have demonstrated the existence of specialized pollination systems that are associated with phenomena of convergence of floral characteristics (Ollerton et al. 2003; Jurgens 2006).

Mating systems

All of the species exhibited some degree of self-sterility, but this does not seem to be associated with a typical incompatibility system, as no significant alterations were observed in the fruit set in self-pollination experiments or incompatibility reactions in the stigma, style or ovary (de Nettancourt 1997). The lack of development of the ovules that were penetrated by pollen tubes in situations of self-pollination may be associated with a late-acting self-incompatibility system (LSI) (Sage et al. 1999) or the occurrence of strong inbreeding depression (Seavey and Bawa 1986). In principal, the presence of large variations in the percentages of viable seeds in fruits generated by self-pollination, even among fruits produced by the same individual, would seem to indicate the presence of strong inbreeding depression in these plants. However, the presence of strong inbreeding depression between populations that have historically been exposed to endogamy would not be expected, as this would result in the purge of the genetic load of these populations (Charlesworth et al. 1990). LSI has been associated with various families (Seavey and Bawa 1986; Lipow and Wyatt 1999; Sage et al. 1999; Gibbs et al. 2004; Bianchi et al. 2005; Sage et al. 2006), but there are presently no reports of this phenomenon in species of Orchidaceae or related families.

Borba et al. (2001a) encountered similar results to ours in *A. johannensis*, *A. ochreata*, and *A. teres*, which demonstrated similar fruit set in self-pollination and cross-pollination events, with low seed viability by self-pollination. These authors also reported difficulties in interpreting low seed viability as being due to the effects of inbreeding depression or of possible LSI, but raised the possibility of the existence of another system associated with distinct reactions in the ovaries of these species. Self-incompatibility is considered an exception in the Orchidaceae, but has been demonstrated in diverse genera of Pleurothallidinae (Christensen 1992; Borba et al. 2001a; Tremblay et al. 2005; Barbosa et al. 2009; Gontijo et al. 2010) and seems to be a generalized characteristic in the subtribe, being present in various distinct lineages (Borba et al. in press). Self-incompatibility in Pleurothallidinae may be selected for as it contributes to maintaining high levels of genetic variability in populations of these myophilous orchids (Borba et al. 2001b) in response to the behavior of its pollinator, which tends to favor self-pollination (Borba and Semir 2001).

Reproductive barriers

Our results indicate that both prepollination barriers and postpollination events are important to maintaining the isolation of the species examined in this study, although the action and relevance of each were different among each pair of species. In spite of *A. modestissima* and *A. prolifera* being sympatric and flowering synchronously, their integrities appear to be maintained through the degree of specificity of their pollinators, which is favored by large differences in the sizes of their flowers. Such pollinator specificities are the principal elements that guarantee reproductive isolation in other myophilous and sympatric species of *Acianthera* and *Bulbophyllum* (Borba and Semir 1998b, 2001), and of various other orchids (van der Pijl and Dodson 1966; Dressler 1993). However, some studies have demonstrated the importance of additional barriers against hybridization in cases where ethological isolation fails or does not exist (e.g., Borba and Semir 1999; Silva-Pereira et al. 2007). On the other hand, *A. limae* and *A. prolifera* are also sympatric, but have weak ethological and mechanical barriers, which could allow gene flow between them. These two species have overlapping measurements in their floral cavities that may determine a weak mechanical isolation between them, and hybridization may eventually occur (Melo and Borba in press). *Acianthera hamosa*, *A. limae*, and *A. modestissima* share the same group of pollinators, but either geographical or mechanical barriers make hybridization between them difficult. There are no apparent mechanical barriers between the flowers of *A. hamosa* and *A. modestissima*, but these species are geographically isolated. The diminutive size of their flowers seems to erect mechanical barriers between these two species and *A. limae*, and especially *A. prolifera*.

Prezygotic genetic barriers were also observed in these species, acting at the level of pollen grain germination. The absence of germination and/or irregular pollen tubes in interspecific crosses are generally due to incompatible interactions between the pollen and the pistils of different species (Hodnett et al. 2005; Ruane and Donohue 2007; Silva-Pereira et al. 2007). The evolution of these barriers may be associated with factors such as the levels of prepollination isolation, phylogenetic similarity, genetic incongruity or other aspects of the evolutive history of the plants (Grant 1981; Moyle et al. 2004). These genetic barriers were observed between sympatric populations (*A. limae*—L1, receiving pollen from *A. prolifera*—P1) and between species that share the same group of pollinators (*A. limae* and *A. modestissima*), but were absent in populations with ethological/mechanical isolation (*A. prolifera* and *A. modestissima*) or ecological isolation (*A. prolifera*—P1, receiving pollen from *A. limae*). This is congruent with studies that have demonstrated the evolution of genetic barriers between some species of orchids that are not reproductively isolated by prepollination barriers (Cozzolino and Widmer 2005; Scopece et al. 2007).

It is important to note that other postpollination phenomena may be important in maintaining the integrity of these species, such as their capacity to adapt or the reproductive success of the hybrids. Very specialized habitats, for example, can be the only barriers against hybridization among some species of orchids (e.g., Smidt et al. 2006). Ecological barriers appear to exist between *A. prolifera* and *A. limae*, although they are only partially efficient: *A. prolifera* (P1) has a very restricted habitat (rock surfaces exposed to direct sunlight) that seems to constitute an effective ecological barrier against possible hybridization with *A. limae* (L1), while *A. limae* (L1) occurs in more heterogeneous environments (forest edges and interiors) that are probably more favorable to hybrid establishment.

The existence of different barriers appears to be important to the reproductive isolation of these species, functioning as overlapping filters that diminish the possibility of genetic flux between them (Grant 1981; Ellis and Johnson 1999), as was observed for species of *Bulbophyllum* (Borba and Semir 1998a, b, 1999; Borba et al. 1999). The degree of efficiency of these filters will determine the potential for hybridization between the species and the possible direction of the interspecific genetic flux. Our understanding of these factors allows us to infer that the populations with the greatest potential for hybridization are *A. limae* (L1) and *A. prolifera* (P1), as any lapse in the specificity of their pollinators could lead to hybridization with unidirectional genetic flux (in the direction of *A. limae*—L1). These observations are coherent with the existence of presumed hybrids and/or introgressing individuals only in localities where *A. limae* (L1) occurs (Melo and Borba in press). On the other hand, *A. hamosa* and *A. modestissima* are intercompatible, share the same group of pollinators, and have very similar flowers, and for these reasons they seem only to be isolated because of the existence of geographical barriers between them (they occur on distinct geological formations). Consequently, these two taxa may actually constitute a single species or be sister species (Melo and Borba in press). Studies with molecular markers will be useful for examining the efficiency of the reproductive barriers that exist between these species, and the relationships that exist between them.

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