

MATING SYSTEMS IN THE PLEUROTHALLIDINAE (ORCHIDACEAE): EVOLUTIONARY AND SYSTEMATIC IMPLICATIONS

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ABSTRACT. We developed a project addressing the determination of the reproductive system through experimental pollinations of species in the major genera representing all major lineages of Pleurothallidinae in order to determine occurrence of self-incompatibility in the subtribe, in which group it has possibly appeared for the first time, and how many times it has evolved. Additionally we surveyed the floral biology of species of *Octomeria*, a genus with morphological characters typical of bee-pollinated flowers that was previously regarded as mellitophilous. At the moment, all but one of the species studied in selected large genera of the major lineages (*Acianthera*, *Anathallis*, *Masdevallia*, *Octomeria*, *Specklinia*, and *Stelis*) are self-incompatible. The species studied may possess complete, strong or partial self-incompatibility. We found two different sites where self-incompatibility reactions occur, the stigma and the stylar channel, and both sites were not found in the same genus except for *Anathallis*. In *Anathallis*, the two groups that differ morphologically (formerly *Pleurothallis* subgen. *Specklinia* sect. *Muscosae* and sect. *Acuminatae*) exhibit different sites of reaction. Flowers of *Octomeria* species produce nectar and are pollinated by both male and female Sciaridae flies, and the populations have high genetic variability similar to that found in *Acianthera* species. Our results indicate that self-incompatibility is a generalized widespread feature of the myophilous clade of the Pleurothallidinae and possibly evolved early in the subtribe, enabling the maintenance of high levels of genetic variability in the populations of each species in spite of pollinator behavior. Moreover, although sapromyophily (pollination by female flies by deceit) is much more common in the group, myophily *s.s.* (pollination by anthophilous flies seeking nectar) apparently evolved first from the plesiomorphic mellitophilous condition. In addition to the morphological synapomorphy uniting the members of the former circumscription of the subtribe (an articulation between the ovary and pedicel), our results also point to the occurrence of two biological synapomorphies in the group — self-incompatibility and myophily. Based on these differences, we suggest the splitting of Pleurothallidinae as presently recognized into two subtribes: one composed of the large, self-incompatible, myophilous clade and the other of the small, self-compatible, ornithophilous clade.

RESUMEN. Desarrollamos un proyecto dirigido a determinar, a través de polinización experimental, la auto-incompatibilidad de los géneros representativos de los linajes más importantes de Pleurothallidinae, buscando el grupo donde posiblemente apareció por primera vez y cuántas veces ha ocurrido desde entonces. Adicionalmente estudiamos la biología floral de algunas especies de *Octomeria*, un género con características típicas de plantas con flores polinizadas por abejas, que previamente han sido conocidas como melitófilas. Hasta el momento, todas excepto una, de las especies estudiadas de los géneros seleccionados (*Acianthera*, *Anathallis*, *Masdevallia*, *Octomeria*, *Specklinia*, and *Stelis*) son auto-incompatibles, pudiendo ser fuerte o parcialmente auto-incompatibles. Se encontró dos sitios donde las reacciones de auto-incompatibilidad podrían ocurrir, siendo éstas el estigma y el canal del estilo. Los dos sitios de auto-incompatibilidad no fueron encontrados dentro de las especies del mismo género, a excepción de *Anathallis*. En este género, los dos grupos que difieren morfológicamente (anteriormente *Pleurothallis* subgen. *Specklinia* sect. *Muscosae* and sect. *Acuminatae*) presentan diferentes sitios de auto-incompatibilidad. Las flores de las especies de *Octomeria*, producen néctar y son polinizadas tanto por machos y hembras de moscas Sciaridae y sus poblaciones tienen alta variabilidad genética, similar a la encontrada en las especies de *Acianthera*. Nuestros resultados indican que la auto-incompatibilidad es una característica generalizada y ampliamente distribuida del clado miofílo de Pleurothallidinae y que posiblemente evolucionó tempranamente

en la subtribu, permitiendo el mantenimiento de un alto nivel de variabilidad genética en las poblaciones de cada especie a pesar del comportamiento de su polinizador. Más aún, a pesar que la sapromiofilia es mucho más común en el grupo, la miofilia s.s. (polinización por moscas antófilas que buscan néctar) aparentemente se desarrolló a partir de una condición melitófila plesimórfica. Adicionalmente a la morfología sinapomórfica que une a los miembros de la subtribu (una articulación entre el ovario y el pedicelo) nuestros resultados apuntan a la presencia de dos sinapomorfias biológicas dentro del grupo, la auto-incompatibilidad y la miofilia. Basados en estas diferencias, sugerimos la división de Pleurothallidinae, como está actualmente reconocida, en dos subtribus: una compuesta por el gran clado de especies auto-incompatibles y miófilas; la segunda, compuesta por el clado de especies auto-compatibles y ornitófilas.

KEY WORDS: Orchidaceae, Pleurothallidinae, reproductive system, pollination, self-incompatibility

The high variation in floral morphology displayed by orchids is mostly attributed to the striking adaptations of these plants to attract pollinators, which range from insects to birds. The morphological adaptations found in Orchidaceae favoring cross-pollination and often highly specific plant-pollinator relationships are considered largely responsible for the great numbers of species in the family (Dodson, 1962). Most orchid species are self-compatible, but self-fertilization is avoided usually by preventing self-pollination. In this family, self-pollination is generally avoided by pre-pollination barriers, which may be morphological, mechanical or ethological (van der Pijl & Dodson, 1966; Dressler, 1981; Borba & Semir, 1999; Singer & Cocucci, 1999). Apparently this mating system helps guarantee the maintenance of moderate to high levels of genetic variability within the populations. However, breeding systems alternative to the more common allogamy by pre-pollination barriers are found scattered throughout the family, such as agamospermy, self-pollination, and self-incompatibility (Tremblay *et al.*, 2005). In contrast to agamospermy and autonomous self-pollination, which are more common in Orchidoideae, self-incompatibility is more frequent in Epidendroideae, mainly in Cymbidieae, Vandeeae, and Dendrobieae (Catling & Catling, 1981; Catling, 1982; Tremblay *et al.*, 2005). Genetic barriers (i.e., self-incompatibility) have been found to be responsible for low levels of inbreeding in some species of orchids (Agnew, 1986; Johansen, 1990; Christensen, 1992; Dressler, 1993; Pedersen, 1995). Usually they are associated with species pollinated by insects that remain for a long time in flowers, a behavior that may promote autogamy or geitonogamy (Christensen, 1992; Pedersen, 1995, Borba & Semir, 1998, 1999, 2001; Singer & Cocucci, 1999; Borba *et al.*, 2001a).

The main pollinators of Orchidaceae are members of Hymenoptera, although a wide variety of other pollinator groups is known (van der Pijl and Dodson, 1966; Proctor *et al.*, 1996). Most researchers in floral biology of orchids have devoted time mainly to exploring more complex mechanisms of pollination, such as relationships with male euglossine bees, pseudocopulation mechanisms, and more species-specific relationships. Relatively little attention has been given to the reproductive biology of orchid species pollinated by flies, probably because dipterans have been considered promiscuous and inefficient pollinators (Christensen, 1994; Proctor *et al.*, 1996). However, more recently some studies have demonstrated high specificity between some orchid species and flies, mainly in the Pantropical *Bulbophyllum* (Dendrobieae) and the Neotropical Pleurothallidinae (Epidendreeae; Borba & Semir, 1998, 2001; Tan *et al.*, 2002, 2006). These two unrelated taxa are the main groups of myophilous orchids (Christensen, 1994). Myophily also occurs in many other unrelated lineages within the family, indicating that pollination by dipterans is a characteristic that has appeared several times in orchid phylogeny (van der Pijl & Dodson, 1966; Dressler, 1993; Christensen, 1994).

Pleurothallidinae comprise about 4100 species in 37 genera, encompassing 20% of the species of the family, and is the largest orchid group pollinated by Diptera. The species are characterized morphologically by the absence of pseudobulbs and the presence of an articulation between the ovary and the pedicel. An exception is seen in the clade composed by three small genera from Central America (*Dilomilis*, *Neocogniauxia*, and *Tomzanonia*), currently included in the subtribu (Pridgeon & Chase, 2001; Pridgeon *et al.*, 2001, 2005; (Fig. 1). In recent molecular phylogenetic

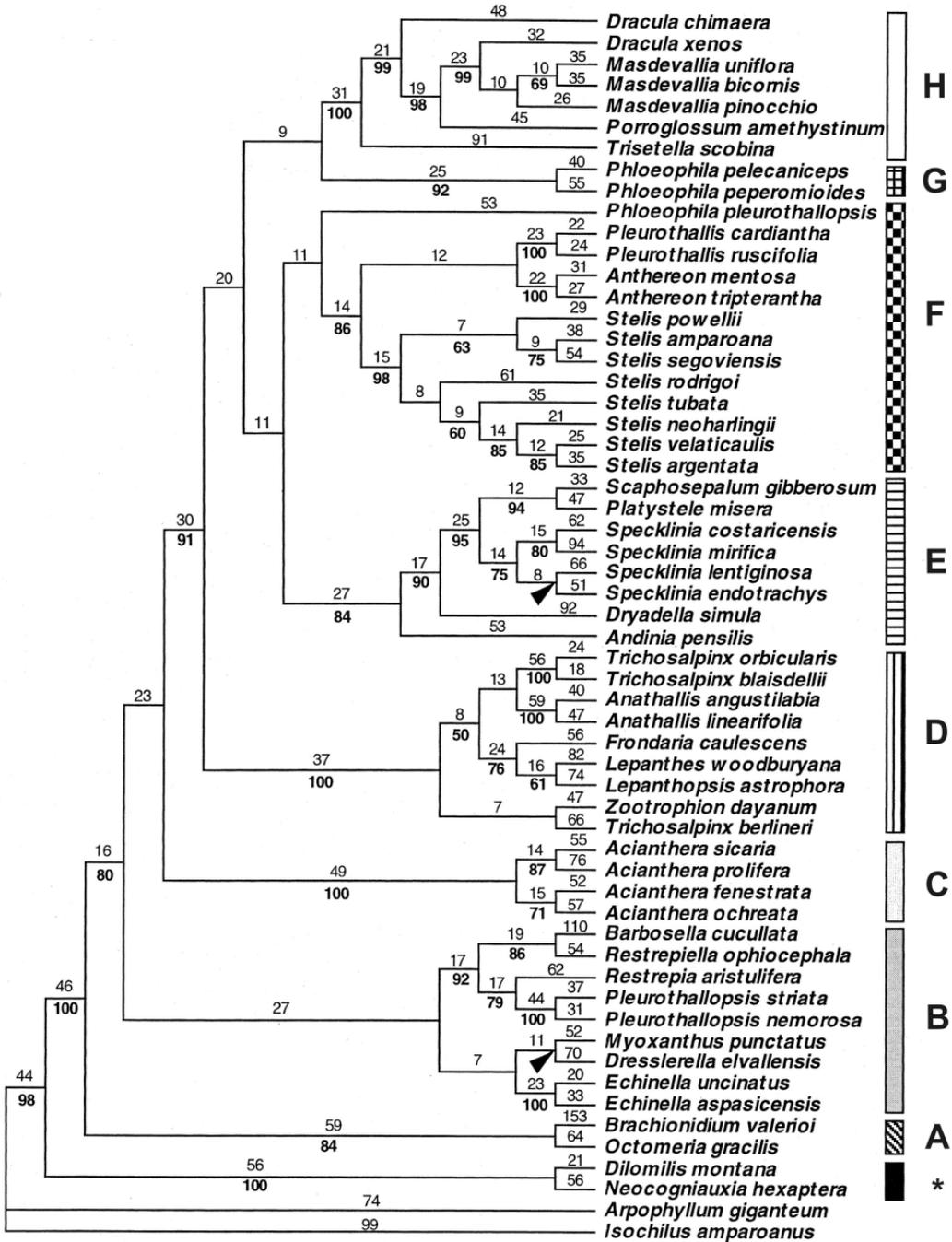


FIGURE 1. Phylogeny of the Pleurothallidinae. The single most-parsimonious, successively weighted, gap-coded *matK/trnL*/ITS DNA data set of Pridgeon *et al.* (2001) but with revised epithets. Number above branches are Fitch lengths, and those below branches are equally weighted bootstrap percentages >50%. Letters indicate the main clades according to Pridgeon *et al.* (2001); * indicates the clade included in the recent circumscription of the subtribe that previously belonged to Laeliinae. Outgroups are represented by *Arpophyllum giganteum* and *Isochilus amparoanus*. Reproduced from Pridgeon & Chase (2001) with permission.

analyses, in spite of being a monophyletic and easily recognizable group, its original circumscription was enlarged to include that small clade (a total of only eight species) of bird-pollinated, self-compatible species that previously were placed in Laeliinae (Pridgeon *et al.*, 2001, 2005). In the combined analysis (*matK* and *trnL-F* cpDNA and ITS nrDNA) of this recent phylogenetic analysis, the clade consisting of *Octomeria* and *Brachionidium* is the sister group to the rest of the members in the older delimitation of the subtribe (*sensu* Luer, 1986; Dressler, 1993). In *trnL-F* and ITS separate analyses, *Octomeria* alone is the sister group of the remaining Pleurothallidinae (*sensu* Luer, 1986). The whole larger subtribe is sister to Laeliinae. Laeliinae are primarily self-compatible and mellitophilous, but show a radiation to several bird- and lepidoptera-pollinated groups and a few self-incompatible species (Borba and Braga, 2003; van den Berg *et al.*, 2005, 2009).

Flowers of Pleurothallidinae species have a morphology, odor, and coloration typical of plants pollinated by Diptera, such as a return to radially and presence of mobile appendages, unpleasant smell, and dull purplish and yellow colors with spots and lines (van der Pijl & Dodson, 1966; Fig. 2). Because almost all species of Pleurothallidinae are pollinated by species of Diptera, myophily is considered by some authors to be a plesiomorphic syndrome in the subtribe (van der Pijl & Dodson, 1966). However, the species of at least two of the three genera of the clade recently included in the subtribe are apparently pollinated by hummingbirds (Ackerman, 1995; Pridgeon *et al.*, 2005). Another important exception is the genus *Octomeria*, which is represented by approximately 150 species with a Neotropical distribution, concentrated mainly in northern South America and southeastern Brazil. Floral morphology is highly consistent in the genus, and it is typical in morphology, odor, and coloration typical of plants pollinated by bees (Fig. 2F-I) (van der Pijl & Dodson, 1966; Fenster *et al.*, 2004). These facts led us to question when myophily arose in the subtribe before becoming a widely occurring characteristic in the group.

Mating systems have been investigated in representatives of only three genera of Pleurothallidinae: one species of *Stelis* (Christensen, 1992), three of *Lepanthes* (Tremblay *et al.*, 2005), and

five of *Acianthera* (Borba *et al.*, 2001a; previously included in *Pleurothallis* subgen. *Acianthera*; Pridgeon and Chase, 2001; Borba, 2003), which are all self-incompatible (Tables 1, 2). All five species of *Acianthera* studied by Borba *et al.* (2001a) had reaction-site and pollen-tube morphology similar to those observed in species with homomorphic gametophytic self-incompatibility (de Nettancourt, 1977; Dafni & Calder, 1987; Murfett *et al.*, 1996; Richards, 1996). They found some features that may indicate a distinct incompatibility system, but only diallelic crossing and embryology studies can define which incompatibility system (gametophytic or sporophytic) is shown by those species (Richards, 1996; Lipow & Wyatt, 2000). *Dilomilis*, however, is self-compatible (Ackerman, 1995). This is a plesiomorphic characteristic in Laeliinae (Borba & Braga, 2003); however, self-incompatibility has evolved in some species of this group, e.g., some *Epidendrum* species (Adams & Goss, 1976; Pansarin, 2003). The distribution of self-incompatibility in remaining Epidendreae indicates that it is also a derived character in Pleurothallidinae.

Mating system and pollinator behavior are key factors determining the genetic variability of a population. The behavior of flies on flowers favors self-pollination, as they make long visits to numerous flowers on the same inflorescence or individual (Borba & Semir, 1998, 2001). Thus, because flies exhibit a behavior that enables self-pollination and orchids are typically self-compatible, we could expect that populations of myophilous orchids should have low genetic variability. However, several populations of the five *Acianthera* species studied by Borba *et al.* (2001b) showed high levels of genetic variability, much higher than previously reported for other Orchidaceae (e.g., Scacchi & De Angelis, 1989; Schlegel *et al.*, 1989; Scacchi *et al.*, 1990; Corrias *et al.*, 1991; Klier *et al.*, 1991; Case, 1994) and near the maximum known for plant species (Hamrick & Godt, 1990). Borba *et al.* (2001a) suggested that both self-incompatibility and inbreeding depression are responsible for the maintenance of high levels of genetic variability found in these species and that it may have arisen in these groups in response to pollinator behavior. A similar correlation has been also observed in *Bulbophyllum* species (Azevedo *et al.*, 2007).



FIGURE 2. Flowers of representative species of Pleurothallidinae studied. A. *Acianthera limae*; B. *A. saurocephala*; C. *Anathallis microphyta*; D. *A. sclerophylla*; E. *Masdevallia infracta*; F. *Octomeria campos-portoi*; G. *O. crassifolia*, with pollinator *Bradysia* sp. (Sciaridae) on the dorsal sepal; note the pollinarium attached to the insect's thorax; H. *O. grandiflora*; I. *O. wawrae*; J. *Specklinia pristeoglossa*; K. *Stelis* aff. *peliochyla*; L. *Stelis* sp.; M. *S.* aff. *hypnicola*; N. *Acianthera prolifera*; O-P. Mature fruits of experimentally self- (left) and cross-pollinated (right) flowers of *Acianthera prolifera*; note the least amount of seeds in the self-pollinated fruit.

Facing this scenario found so far in Pleurothallidinae, we hypothesize that self-incompatibility arose in the subtribe in response to a selection pressure caused by the change from a group that promotes cross-pollination (bees) and maintains moderate levels of heterozygosity to another group that enables a higher frequency of self-pollination (flies), which would lead

to the reduction of heterozygosity in populations. As a result of the evolution of self-incompatibility in the group, populations were able to maintain high levels of genetic variability. This hypothetical evolutionary scenario could be weakened if we found basal mellitophilous species (before the advent of myophily) with self-incompatibility or myophilous

TABLE 1. List of taxa examined of representative genera of the main clades of Pleurothallidinae (according to Pridgeon *et al.*, 2001; see Fig. 1). All sampled species in this work are native to Brazil. n= sample size.

Species	Indiv. (n)	Locality	Reference
<i>Acianthera</i> Scheidw. (clade C)			
<i>A. adamantinensis</i> (Brade) F.Barros	17	10, 13	Borba <i>et al.</i> , 2001b
<i>A. aveniformis</i> (Hoehne) C.N.Gonç. & Waechter	08	8	this study
<i>A. fabriarrosii</i> (Borba & Semir) F.Barros & F.Pinheiro	13	13	Borba <i>et al.</i> , 2001b
<i>A. hamosa</i> (Barb.Rodr.) Pridgeon & M.W.Chase	12	8	this study
<i>A. johannensis</i> (Barb.Rodr.) Pridgeon & M.W.Chase	146	8, 15, 22, 26, 28	Borba <i>et al.</i> , 2001b
<i>A. limae</i> (Porto & Brade) Pridgeon & M.W.Chase	45	9, 24	this study
<i>A. modestissima</i> (Rchb.f. & Warm.) Pridgeon & M.W.Chase	35	3, 5	this study
<i>A. ochreate</i> (Lindl.) Pridgeon & M.W.Chase	39	8, 20,	Borba <i>et al.</i> , 2001b
<i>A. prolifera</i> (Herb. ex Lindl.) Pridgeon & M.W.Chase	53	5, 9	this study
<i>A. saurocephala</i> (Lodd.) Pridgeon & M.W.Chase	06	8	this study
<i>A. teres</i> (Lindl.) Borba	86	4, 5, 10, 25, 27	Borba <i>et al.</i> , 2001b
<i>Anathallis</i> Barb.Rodr. (clade D)			
<i>A. heterophylla</i> Barb.Rodr.	9	8	this study
<i>A. microphyta</i> (Barb.Rodr.) C.O.Azevedo & Van den Berg	8	17	this study
<i>A. rubens</i> (Lindl.) Pridgeon & M.W.Chase	4	9	this study
<i>A. sclerophylla</i> (Lindl.) Pridgeon & M.W.Chase	8	5, 9	this study
<i>Lepanthes</i> Sw. (clade D)			
<i>L. rubripetala</i> Stimson	--	Central America	Tremblay <i>et al.</i> , 2005
<i>L. rupestris</i> Stimson	--	Central America	Tremblay <i>et al.</i> , 2005
<i>L. woodburyana</i> Stimson	--	Central America	Tremblay <i>et al.</i> , 2005
<i>Masdevallia</i> Ruiz & Pav. (clade H)			
<i>M. infracta</i> Lindl.	7	9	this study
<i>Octomeria</i> R.Br. (clade A)			
<i>O. alexandri</i> Schltr.	05	21	this study
<i>O. crassifolia</i> Lindl.	27	1, 5, 9, 14	this study
<i>O. grandiflora</i> Lindl.	26	2, 9, 18, 23	this study
<i>O. praestans</i> Barb. Rodr.	15	8	this study
<i>Specklinia</i> Lindl. (clade E)			
<i>S. pristeoglossa</i> (Rchb.f. & Warm.) Luer	15	12, 19	this study
<i>Specklinia</i> sp.	02	23	this study
<i>Stelis</i> Sw. (clade F)			
<i>S. argentata</i> Lindl.	--	Ecuador	Christensen, 1992
<i>S. aff. hypnicola</i> (Lindl.) Pridgeon & M.W.Chase	16	27	this study
<i>S. aff. petiochyla</i> Barb. Rodr.	11	9	this study
<i>Stelis</i> sp.	11	5, 9	this study

Localities: 1. Aiuruoca-MG; 2. Araponga-MG; 3. Belo Horizonte-MG; 4. Brumadinho-MG; 5. Caeté-MG; 6. Caldas-MG; 7. Camocim de São Félix-PE; 8. Carrancas-MG; 9. Catas Altas-MG; 10. Diamantina-MG; 11. Ecuador; 12. Funilândia-MG; 13. Grão Mogol-MG; 14. Itamarandiba-MG; 15. Itutinga-MG; 16. Jacobina-BA; 17. Joaquim Felício-MG; 18. Mariana-MG; 19. Matozinhos-MG; 20. Morro do Chapéu-BA; 21. Mucugê-BA; 22. Nazareno-MG; 23. Nova Friburgo-RJ; 24. Nova Lima-MG; 25. Ouro Preto-MG; 26. Santa Rita do Ibitipoca-MG; 27. Santana do Riacho-MG; 28. São João Del Rei-MG. Acronyms for Brazilian states: BA – Bahia; MG - Minas Gerais; PE - Pernambuco; RJ - Rio de Janeiro.

TABLE 2. Percentage of fruit set and seed viability in experimental self- and cross-pollinations and site of incompatibility reaction in species of representative genera of the main clades of Pleurothallidinae (according to Pridgeon *et al.*, 2001; see O).

Species	Fruit set % (n)			Seed viability % (min.-max.)			Self-incompatibility	Site of incompatibility reaction
	Self-pollin.	Intrapop. cross-pollin.	Interpop. cross-pollin.	Self-pollin.	Intrapop. cross-pollin.	Interpop. cross-pollin.		
<i>Acianthera</i> (clade C)								
<i>A. adamantinensis</i>	4.2 (24)	80.8 (26)	77.8 (18)	29	93 (76-97)	93 (78-98)	strong	stylar channel (base of the column)
<i>A. aveniformes</i>	0 (26)	23 (26)	--	--	--	--	complete	--
<i>A. fabiobarrosii</i>	5.3 (19)	78.6 (14)	--	33	92 (77-98)	--	strong	stylar channel (base of the column)
<i>A. hamosa</i>	0 (20)	4.3 (46)	--	0	90 (87-92)	--	complete	stylar channel (base of the column)
<i>A. johannensis</i>	12.4 (209)	59.0 (222)	66.0 (412)	20 (0-62)	90 (4-100)	90 (0-100)	partial	stylar channel (base of the column)
<i>A. lima</i>	6.6 (106)	37.1 (124)	30.3 (56)	16 (0-100)	97 (4-100)	95 (76-100)	strong	stylar channel (base of the column)
<i>A. modestissima</i>	3.3 (90)	5.1 (117)	13.1 (76)	20 (5-99)	95 (11-100)	96 (63-100)	partial	stylar channel (base of the column)
<i>A. ochreatea</i>	12.4 (89)	39.4 (132)	44.3 (176)	28 (0-87)	94 (46-100)	91 (0-100)	partial	stylar channel (base of the column)
<i>A. prolifera</i>	11.7 (137)	39.5 (162)	44.3 (61)	33 (0-100)	95 (0-100)	95 (63-100)	partial	stylar channel (base of the column)
<i>A. saurocephala</i>	0 (42)	81 (118)	--	0	82 (52-98)	--	complete	beginning of the stylar channel (distal part of the column)
<i>A. teres</i>	12.6 (159)	44.3 (167)	52.9 (240)	13 (0-86)	89 (42-99)	90 (54-99)	partial	stylar channel (base of the column)
<i>Anathallis</i> (clade D)								
<i>A. heterophylla</i>	0 (20)	33 (33)	--	0	98 (97-99)	--	complete	stigma (absence or low pollen germination)
<i>A. microphyta</i>	0 (20)	62 (29)	--	0	93 (81-99)	--	complete	stylar channel (base of the column)
<i>A. rubens</i>	0 (19)	50 (20)	--	0	95 (72-100)	--	complete	stigma (absence or low pollen germination)
<i>A. sclerophylla</i>	4 (96)	26.5 (49)	39.1 (23)	49 (44-53)	97 (94-99)	96(89-99)	strong	stigma (absence or low pollen germination)
<i>Lepanthes</i> (clade D)								
<i>L. rubripetala</i>	0.0 (11)	30.0 (44)	--	--	--	--	complete	--
<i>L. rupestris</i>	0.0 (25)	31.0 (78)	--	--	--	--	complete	--
<i>L. woodburyana</i>	0.0 (50)	60.9 (92)	--	--	--	--	complete	--
<i>Masdevallia</i> (clade H)								
<i>M. infracta</i>	32 (37)	48 (23-5)	--	73 (58-87)	93 (66-99)	--	half the population with complete self-incompatibility, the remaining self-compatible	stigma (absence or low pollen germination)
<i>Octomeria</i> (clade A)								
<i>O. alexandrii</i>	20 (5)	42 (12)	--	--	74 (35-96)	--	partial	--
<i>O. crassifolia</i>	0	88 (68)	82 (60)	0	91 (60-98)	94 (81-98)	complete	stigma (absence or low pollen germination)
<i>O. grandiflora</i>	3 (31)	84 (84)	97 (17)	65	91 (69-99)	--	strong	stigma (absence or low pollen germination)
<i>O. praestans</i>	0 (108)	84 (157)	--	0	77 (7-99)	--	complete	stigma (absence or low pollen germination)
<i>Specklinia</i> (clade E)								
<i>S. pristeoglossa</i>	7 (86)	31 (84)	24 (9)	28 (9-46)	91 (74-97)	91 (87-93)	strong	stigma (absence or low pollen germination)
<i>Specklinia</i> sp.	0 (20)	75 (8)	--	0	98 (96-99)	--	complete	stigma (absence or low pollen germination)
<i>Stelis</i> (clade F)								
<i>S. argentata</i>	1.9 (54)	29.3 (185)	--	--	--	--	strong	--
<i>S. aff. hypnicola</i>	6.9 (68)	43.2 (44)	--	2 (1-3)	91 (83-95)	--	strong	mainly stigma (absence or low pollen germination)
<i>S. aff. petiochyla</i>	0 (21)	31 (29)	--	0	93 (85-97)	--	complete	stigma (absence of pollen germination)
<i>Stelis</i> sp.	0 (39)	44 (32)	60 (5)	0	93 (84-99)	93	complete	stigma (absence of pollen germination)
<i>Zootrophion</i> (clade D)								
<i>Z. atropurpureum</i>	55(9)	58 (12)	--	70 (3-98)	98 (94-100)	--	self-compatible	normal development

self-compatible species with high genetic variability.

We developed a project addressing the determination of the reproductive system through experimental pollinations of species in the major genera representing all of the major lineages of Pleurothallidinae in order to determine: 1) the extent of occurrence of self-incompatibility in the subtribe; 2) in which group it has possibly appeared for the first time; and 3) how many times it has evolved in the subtribe. Additionally, we surveyed the floral biology of species of *Octomeria* in order to determine if myophily arose at the base of the clade that corresponds to the older circumscription of the subtribe or if it arose later in phylogeny.

Materials and methods

Mating systems — We sampled 22 species in eight genera belonging to seven of the main clades of Pleurothallidinae as defined by Pridgeon *et al.* (2001; Fig. 1, Table 1): *Acianthera* (six species), *Anathallis* (four spp.), *Masdevallia* (one), *Myoxanthus* (one), *Octomeria* (four), *Specklinia* (two), *Stelis* (three), and *Zootrophion* (one). It was not possible to sample clade G, because we could not obtain individuals of *Phloeophila* species, the only genus of this lineage. Studies with the *Myoxanthus* species sampled (clade B) are still in progress and are not presented here. Thus,

we present the results of six of the eight main clades of Pleurothallidinae. Vouchers were deposited in the herbarium BHCN (Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais).

The populations studied were located in areas of *campo rupestre* vegetation and forests in southeastern (Minas Gerais and Rio de Janeiro states) and northeastern (Bahia state) Brazil, with the individuals occurring as epiphytes in semideciduous, gallery, and Atlantic rain forests or as rupicolous on rock outcrops. Experimental pollinations were performed on cultivated plants. Individuals were collected for acclimatization and maintained for a minimum of six months in a greenhouse at the Universidade Federal de Minas Gerais. Three types of pollination experiments were performed, depending on the availability of flowers in each population. All populations were subjected to self-pollination and intrapopulation cross-pollination experiments with a tentative number of at least 20 pollinations per treatment. Interspecific crosses were performed in some species with a multipopulation sampling. We tried to use about 15 to 20 individuals per population and at least two populations per species. Sometimes the numbers were lower, but in some cases they were as high as 160 pollinations per treatment, 30 individuals per population, and six populations per species. Additionally, some marked emasculated or not emasculated flowers were followed in order to verify the possible occurrence of diplosporic agamospermy or spontaneous self-pollination. All flowers used in the pollination experiments were checked on a daily basis to verify the formation and development of fruits.

Additional self- and cross-pollinations were performed and subsequently harvested at regular intervals after pollination (ranging from two to 15 days, depending on the flower longevity of the species) in order to observe pollen-tube growth. These flowers (and senescent flowers and mature fruits of the earlier described treatments) were fixed in 50% FAA and subsequently treated with a solution of 10N NaOH at 60 C for approximately 25 min. (or 50 min. in the case of fruits). The material was then washed in distilled water and stained with Aniline Blue for examination by epifluorescence microscopy (modified from Martin, 1959).

The fruits were collected at the start of their dehiscence and fixed in 50% FAA. Approximately

300 seeds from each fruit were examined by optical microscopy and classified as viable or non-viable on a morphological basis only, according to the morphology and relative size of the embryo: seeds with well-developed embryos were considered viable, and seeds with no embryo or a rudimentary embryo were considered inviable (Fig. 3A; Borba *et al.*, 2001a).

Floral biology of Octomeria species—Field observations were made in populations of *O. crassifolia* and *O. grandiflora* in the Serra do Caraça Mountains (municipality of Catas Altas, Minas Gerais state, 20°5'36"S, 43°28'29"W) to determine their floral visitors. Observations of *O. grandiflora* were made on January and February 2007 from 07:00 to 18:00, totaling 88 hours of observations. Observations of floral visitor activities in *O. crassifolia* were made on January 2007, between 06:00 and 12:00, totaling 63 hours of observation. Floral visitors were captured and sent to specialists for identification. Here we present only a summary description of the pollination of the species with the identity of pollinators, because a detailed description of the reproductive biology of the species is being published elsewhere (Barbosa *et al.*, 2009).

Results

Fruit set and seed viability in experimental pollinations — Flowers that did not produce fruits abscised within 5-10 days, except for *Acianthera prolifera* and *Anathallis microphyta*, flowers of which abscised after 12-14 and 21 days, respectively. Fruits terminated development in approximately 60-90 days (*Anathallis heterophylla*, and *Acianthera*, *Octomeria*, *Specklinia*, *Stelis* species), 110-130 days (other *Anathallis* species) or 150 days (*Anathallis sclerophylla* and *Masdevallia infracta*). No emasculated or unpollinated flower of any species developed fruits.

Fruit set in experimental intra-population cross-pollinations was variable among species, ranging from 5 to 88% (Table 2). Fruit set in inter-population cross-pollinations was similar to that found in intra-population crossings, the latter being usually slightly lower or more rarely higher (e.g., *Acianthera limae*). Except for one species, fruit set in self-pollinations was always lower than in cross-pollination experiments, ranging from 0 to 55%, but rarely above 20% (only

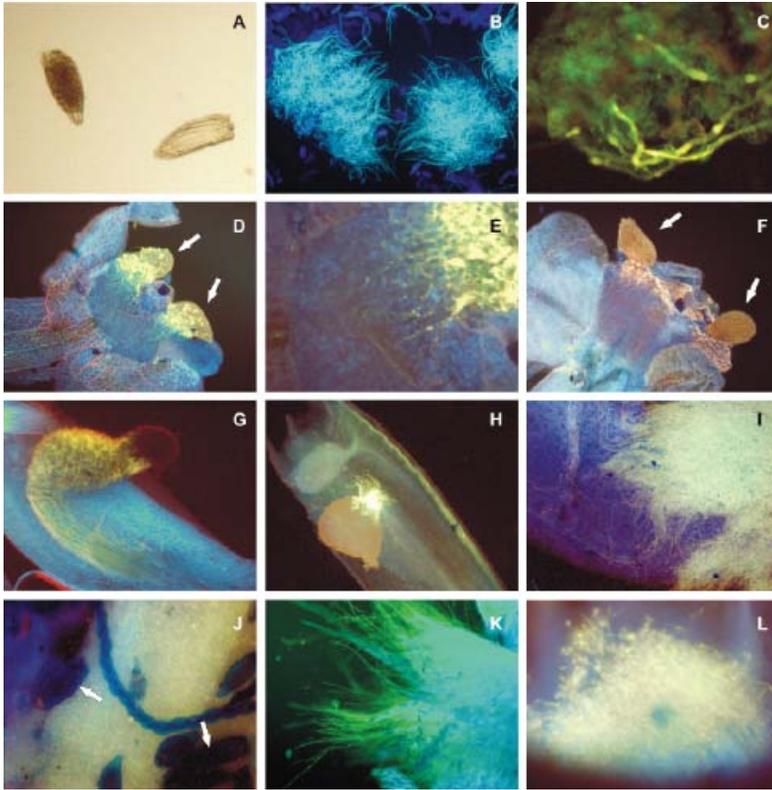


FIGURE 3. A. Normal (left; considered viable) and embryo-lacking (right; considered inviable) seeds of *Anathallis rubens* (see Materials and Methods for explanation); B-L. Pollen germination and tube growth in fluorescence microscopy. B-C. Cross- (B) and self-pollinated (C) flowers of *Octomeria crassifolia*, after three and six days, respectively. Note several pollen tubes with normal development in (B) and the rare pollen tubes in (C), the latter with irregular growth and irregular deposition of callose on the pollen tube walls; D-F. Cross- (D, E) and self-pollinated (F) flowers of *Stelis* aff. *peliochyla*, after four and six days, respectively. Note the pollinaria with germinated (D) and ungerminated (F) pollen grains (arrows); in (E) detail of pollen tubes of (D); G-H. Cross- (G) and self-pollinated (H) flowers of *Specklinia pristeoglossa*, after six and four days, respectively; note the few pollen tubes formed in (H); I. Self-pollinated flower of a self-compatible individual of *Masdevallia infracta*, with normal pollen tubes reaching the base of the column; J. Pollen tubes in a mature fruit from a cross-pollinated flower of *Masdevallia infracta*; note the well-developed mature seeds (arrows); K-L. Cross- (K) and self-pollinated (L) flowers of *Acianthera saurocephala*, after six and eight days, respectively. Note several pollen tubes with normal development in (K) and the pollen tubes in (L) with irregular growth and high deposition of callose.

two species). Ten species did not set any fruits in self-pollinations, and fruit set in this treatment was lower than 10% in other six species. Fruit size and time of development in cross-pollination fruits were usually higher than in self-pollinated fruits. The latter also had fewer seeds, and sometimes they failed to open, mainly in *Acianthera* species (Fig. 2N-P). However, for *Zootrophion atropurpureum*, fruit set in both self- and cross-pollinations was nearly 55%. In this species, they were also similar in size, time of development, and seed content in both treatments.

Although *Masdevallia infracta* had a high average fruit set in self-pollination, all fruits were developed in only four of the seven individuals sampled. These individuals had fruiting ranging from 17% ($n=6$) to 100% ($n=1$, and 2); the individual that had the largest number of self-pollinated fruits ($n=9$) had 67%. The other three individuals did not set any fruits with self-pollination ($n=2$, 2, and 9), but they did in cross-pollination (one of them with 100% fruit set in four cross-pollinations), indicating that they are not sterile. Bidirectional crossings between one pair of self-

compatible and self-incompatible individuals ($n=12$) set fruit only when the self-incompatible individual was the pollen donor ($n=4$). Another pair of similar individuals had 100% fruit set, independently of direction of crossing. Average seed viability was always high in cross-pollinated fruits, usually greater than 80% (except for *Octomeria alexandri*, 74%, and *O. praestans*, 77%; Table 2). However, seed viability in some individual fruits was possibly as low as 7% in this treatment. Conversely, average seed viability in self-pollinated fruits was usually never greater than 70% (except for *Masdevallia infracta*, 73%), and always lower than in cross-pollinated capsules (including *Z. atropurpureum*). Again, seed viability in some individual fruits in this treatment was as high as 100% but more frequently lower than 20%. We found no evidence for polyembryony in any capsule examined of any species.

Pollen germination and pollen-tube growth—In experimental cross-pollination, germination of pollen grains began at the end of the first day up to the third day after pollination, depending on the species. In this pollination treatment, practically all pollen grains germinated, and the pollen tubes demonstrated uniform growth, with callose plugs deposited regularly along their lengths (Fig. 3B, D, E, G, K). These tubes grew down through the style canal and penetrated the ovary on about the fifth to eighth day after pollination, except for *Anathallis microphyta* and *Acianthera* species, for which it occurred on the 11th day. Penetration of the ovules occurred from 20 days after pollination. In self-pollinated flowers that developed fruits, pollen-tube growth occurred in a manner similar to that observed in cases of cross-pollination.

We found two general patterns for pollen and pollen-tube behavior in self-pollinated flowers that did not develop fruits (Table 2). In *Masdevallia infracta* and all *Octomeria*, *Stelis*, *Specklinia*, and *Anathallis* (except *A. microphyta*) species, pollen grains in the majority of self-pollinated flowers did not germinate (Fig. 3C, F). However, in some flowers, the few pollen grains that did initiate germination demonstrated irregular growth and acquired a twisted appearance when they reached the initial portion of the style with irregular deposition of callose on the pollen tube walls and at the extremity of the pollen tube itself (Fig. 3H). These tubes rarely reached the base of the column and never

penetrated the ovary. In all *Acianthera* species and *Anathallis microphyta*, pollen-tube growth occurred in a way similar to that observed in cross-pollination until approximately seven days, when the tubes had penetrated halfway through the column. However, from that point onward, pollen tubes began to take on an abnormal aspect, with irregular trajectory, variation in diameter, and excessive deposition of callose. At almost 15 days, these flowers had pollen tubes with abnormal characteristics that had reached the base of the column but never penetrated the ovary. *Acianthera saurocephala* showed a slight variation of this pattern, the pollen tubes assuming abnormal appearance as above in the beginning of the styler channel (distal part of the column; Fig. 3L), but pollen tubes never reached the base of the column. In individuals of *Masdevallia infracta* that set fruit in self-pollinations, pollen tubes had normal development, similar to that found in cross-pollinated flowers (Fig. 3I, J).

Pollen tubes in fruits with a high percentage of normal seeds developed normally (Fig. 3J). However, fruits with a high percentage of empty seeds (lacking embryos) usually showed normal pollen tube growth only in the column; they became irregular when entering the ovary, filled with callose, and formed a tangled mass around the ovules. The last behavior was more frequent in fruits developed from self-pollinations, mainly in *Acianthera* species, which showed higher fruit set in this treatment than the other species.

Floral biology of Octomeria species—*Octomeria crassifolia* was pollinated exclusively by both males and females in similar proportions of four species of *Bradysia* (Diptera, Sciaridae; Fig. 1G). During 63 hours of observation, a total of 92 visits were recorded and 31 pollinarium removals and six pollinarium depositions were observed. The visits occurred more frequently early in the morning, between 06:00 and 08:00. Pollinators of *O. grandiflora* were rarely observed in flowers of *O. crassifolia*. However, pollination did not occur because of their large size. Males and females of a single species of *Pseudosciara* (Diptera, Sciaridae) pollinated flowers of *O. grandiflora*; 19 visits occurred in 88 hours of observation. Unlike those observed in *O. crassifolia*, visits occurred mainly in the afternoon between 14:00 and 16:00. In both species, the visits lasted usually less than a minute. Three visits by the

pollinators of *O. crassifolia* were observed on the flowers of *O. grandiflora*, but no pollinarium was removed due to the small size of these insects.

The pollinators of the two species demonstrated similar behavior. Generally, the insect landed on the external part of the dorsal sepal and then moved to the labellum, which shifts slightly downward. The insect continued moving toward the base of the labellum, feeding on the nectar produced on the disk. When the insect attempted to leave, the pollinarium became affixed to the dorsal region of its thorax (the scutellum) and was removed from the flower. In the population of *O. crassifolia*, 64.3% of the flowers had their pollinarium removed, and in 35.7% of the cases pollinia were successfully deposited. Deposition of pollinarium occurred in 8% of the flowers of *O. grandiflora*.

Discussion

Absence of fruit set in unpollinated or emasculated flowers and absence of polyembryony indicate that none of the studied species is capable of developing fruits by autonomous self-pollination or agamospermy; thus, a pollination is required for the formation of fruits in these species. Absence or low fruit set in self-pollination versus moderate to high fruit set in cross-pollinations indicates occurrence of complete or strong self-incompatibility in the majority of the species studied (see Table 2). Complete or strong self-incompatibility was also found in the majority of Pleurothallidinae species studied so far (Christensen, 1992; Borba *et al.*, 2001a; Tremblay *et al.*, 2005; see Table 2).

However, in some species, as observed in a previous study in *Acianthera* (Borba *et al.*, 2001a), fruit set in self-pollination was about the half that observed in cross-pollination experiments. As emphasized by Borba *et al.* (2001a), strict self-incompatibility and self-compatibility are extremes of a continuum between which there is often no clear-cut difference, and relatively few species fit exactly in these extremes. The distinction between the two conditions is arbitrary, however, and various authors have used different indices to determine whether a particular species is self-compatible or self-incompatible (e.g., Bawa 1974, 1979; Zapata and Arroyo, 1978; Sobrevilla & Arroyo, 1982; Jaimes & Ramirez, 1999). Thus, some

species in this study (e.g., *Acianthera prolifera*) could be considered self-incompatible or self-compatible depending on which method is employed. Some of these indices have combined seed viability with fruit set, which we think is improper, because seed viability is probably related to inbreeding depression. If that practice is used for these species, they would be considered clearly self-incompatible on account of the low seed viability in fruits from self-pollinated flowers. The pollen tube reaction and the homogeneous timing of the reaction in the stylar canal or absence of pollen germination lead us to suggest the occurrence of partial self-incompatibility in these species.

Conversely, *Zootrophion atropurpureum* can be clearly considered self-compatible, in spite of the reduced seed viability in fruits from self-pollination when compared to crosspollination. But *Masdevallia infracta*, which could be considered self-compatible if based simply on total fruit set, probably has a more complex mating system. Based on the individual analysis, half the population of *Masdevallia infracta* exhibit strong self-incompatibility (with the reaction site on the stigma because of little or no pollen germination), and half the population is composed of self-compatible individuals, as we could see by the bundle of normal pollen tubes in some self-pollinated flowers. Bidirectional crossings between self-compatible and self-incompatible individuals indicate that self-compatibility in these individuals may be caused by silencing of the gene responsible for the stylar self-incompatibility factor. Thus, the species, or at least this population, may have a mixed mating system, which may ensure both reproductive success and genetic variability. A similar situation was observed in *Asclepias exaltata* L., with a few self-compatible individuals occurring in a self-incompatible population (Lipow & Wyatt, 2000). This may also explain the occurrence of some fruit set in self-pollinations of those species considered as having strong or partial self-incompatibility. Unfortunately, the sample size of *Z. atropurpureum* was low, and so we cannot know whether the same occurs in this species/population or if it is in fact self-compatible. We will try to increase the number of experimental pollinations and individuals sampled to answer this question.

The species showed two different self-incompatibility reactions, one typical of gametophytic self-incompatibility (pollen tubes becoming irregular

in their trajectory, with variation in diameter and excessive deposition of callose) and the other typical of sporophytic self-incompatibility (absence of pollen grain germination; Richards, 1996). In addition to one *Anathallis* species, the reaction typical of gametophytic self-incompatibility was observed only in all *Acianthera* species. There are currently no published studies involving diallelic crosses in Orchidaceae, although they are fundamental to understanding control of self-incompatibility in the group. We need more studies to determine if this picture represents either two incompatibility systems (gametophytic and sporophytic) or two sites of the same reaction. Intermediate situations, as found in species such as *Acianthera saurocephala* and *Stelis aff. hypnicola*, indicate that the latter scenario is more plausible.

All *Anathallis* species showed strong self-incompatibility, but we found the two different sites where self-incompatibility reactions occur, the stylar channel and the stigma, which are related to the two vegetative morphological groups, formerly *Pleurothallis* subgen. *Specklinia* sect. *Muscosae* and *P.* subgen. *Acuminatia* (Luer, 1986, 1999). This division was based principally on their growth habit and the leaf-to-stem proportions of the plants. Despite differences in morphology, the new circumscription of the genus eliminated this division, and both groups were combined to form the core of the genus *Anathallis* (Pridgeon & Chase, 2001). This is the only genus in which both sites of incompatibility were found. In this same clade, there is a report of strong self-incompatibility in three *Lepanthes* species by Tremblay *et al.* (2005). In *Anathallis microphyta*, formerly included in *Pleurothallis* subgen. *Specklinia* sect. *Muscosae* and recently transferred by Luer (2006) to *Panmorphia*, germination of the pollen grains in self-pollinated flowers is normal, and the incompatibility reaction occurs when the pollen tubes reach the base of the column. On the other hand, in the three species formerly belonging to *Pleurothallis* subgen. *Acuminatia*, the pollen grains fail to germinate or pollen germination is low and the pollen tubes do not penetrate the stylar channel. We believe that in order to reach a better understanding of the distinct infrageneric relationships and the evolution of the self-incompatibility mechanisms in the genus, additional studies examining the mating systems and molecular

phylogenetic analysis are necessary, both using larger samples of species belonging to the two morphological groups.

Experiments in progress by our group indicate that self-incompatibility can also be assigned to other species of *Pleurothallidinae*, such as *Octomeria campos-portoi*, *O. diaphana*, *O. wawrae*, *Myoxanthus exasperatus*, *M. punctatus*, and additional *Acianthera* and *Specklinia* species. However, the results were not included here because of the still low sample size. Occurrence of self-incompatibility in *Myoxanthus* species is particularly important to our study, since they represent a clade not sampled in this study (see Fig. 1).

Our results indicate that self-incompatibility is a generalized, widespread characteristic of the myophilous clade of the subtribe, in spite of the occurrence of self-compatibility in *Zootrophion*, possible by reversal.

Several populations of two of these *Octomeria* species (*O. grandiflora* and *O. crassifolia*) have been scored for genetic variability (Barbosa *et al.*, unpublished), and all of them show high genetic variability, measured by using ISSR markers. The values we found are higher than the values found in other orchid species, including self-compatible *Laeliinae*, and above the average values for outcrossing plants in general. This picture is similar to that previously found in 22 populations of five *Acianthera* species by using allozymes. Self-incompatibility and inbreeding depression apparently are factors responsible for maintaining high levels of genetic variability in these populations (Borba *et al.*, 2001a, 2001b).

The phylogenetic position of *Octomeria* indicates that self-incompatibility and myophily arose at the beginning of the line that gave rise to the core group of the subtribe, corresponding to the older circumscription of this group (Luer, 1986; Dressler, 1993). As such, self-incompatibility and myophily may be considered as biological synapomorphies of this large myophilous clade. The concomitant occurrence of myophily and self-incompatibility in all of the clades studied indicates two hypothetical routes for the evolution of these two characteristics in the subtribe. In the first, the evolution of self-incompatibility would have permitted the establishment of myophily in the group. In the second, self-incompatibility would have

arisen as a result of selection pressures that arose by the change of pollinators, from an insect group with a behavior favoring cross-pollination to a group favoring autogamy, which would lead to a reduction in heterozygosity in the populations. At the present time, our results cannot distinguish which of the two characteristics was first established in the group.

Sapromyophily is apparently widely distributed and represents the predominant pollination mechanism in the subtribe (van der Pijl & Dodson, 1966; Christensen, 1994). Some characteristics of *Octomeria* flowers, such a yellow perianth, nectar guides on the calli, nectar reward at the base of the lip, and agreeable citriny odor are found in myophilous species but are also common in mellitophilous species. Because of these and other characteristics such as a zygomorphic perianth and purple lip, *Octomeria* flowers were falsely regarded as mellitophilous (van der Pijl & Dodson, 1966). Thus, we suggest that myophily *sensu stricto* (pollination by anthophilous flies seeking for nectar) may be a plesiomorphic characteristic in the myophilous clade of the subtribe in spite of the most common occurrence of sapromyophily (pollination by female flies by deceit) and may represent a transition from rewarding melittophily to deceptive sapromyophily. Adaptive radiation from melittophily to myophily is not uncommon in orchids, and the application of pollination syndromes can be especially conflicting in these cases (e.g. Pansarin, 2008).

In the older circumscription of the subtribe, the articulation between the pedicel and the ovary was considered an important diagnostic characteristic for the group (Luer, 1986; Dressler, 1993). With the inclusion of the small clade containing the genera *Dilomilis*, *Neocogniauxia*, and *Tomzania* (which do not have this characteristic), no morphological synapomorphy is recognized for the group (Pridgeon *et al.*, 2001). For this reason, and the occurrence of self-compatibility and ornithophily in some species of this clade, we suggest that the older circumscription of the subtribe should be maintained with self-incompatibility and myophily as biological synapomorphies and the articulation as a morphological synapomorphy for Pleurothallidinae. As such, the establishment of a subtribe comprising the genera *Dilomilis*, *Neocogniauxia*, and *Tomzania* should be considered, even taking into consideration the reduced size of the group. "Taxonomic inflation"

should not be the main argument for its inclusion in the subtribe (see Pridgeon *et al.*, 2001). As these two groups are sister to each other, such taxonomic rearrangement is still supported by the results found by Pridgeon *et al.* (2001).

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