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The Use of Orchid Pollinia or Pollinaria for Taxonomic Identification

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Abstract: The structural variation of pollinia and pollinaria in Orchidaceae is discussed. Pollinia and pollinaria are restricted to two (of the five) orchid subfamilies: Orchidoideae and Epidendroideae. The attributes of pollinia and pollinaria of these subfamilies are commented on and discussed. Pollinia and pollinaria also occur in the plant family Apocynaceae, in the subfamilies Asclepiadoideae and Periplocoideae, but these are structurally different from those found in the family Orchidaceae. A number of morphological features of orchid pollinaria are informative taxonomically and ecologically. These features are briefly discussed and examples are given. The recent description of the first unequivocal fossil orchid (*Meliorchis caribea*; Orchidoideae: Goodyerinae) from a pollinarium attached to an insect pollinator is briefly discussed. This example is used to illustrate the use of informative morphological and palynological characters. A fascinating new perspective, the possibility of species identification using DNA sequencing from pollinia or pollinaria attached to pollinators, is also discussed. Suggestions and future avenues of research focusing on orchid pollination are given.

Key words: DNA barcoding, ecology, morphology, Orchidaceae, pollen, pollinia, pollinaria, pollination, taxonomy

Introduction

A Brief Overview of Orchid Floral Features

In its current delimitation (Cameron et al. 1999; Chase et al. 2003) the orchid family encompasses about 24,190 spp. distributed among five subfamilies. The phylogenetic relationships between these subfamilies are becoming fairly well understood, and are summarized in Figure 1. Orchids are remarkable for a number of reasons. Being monocots, they present a 3-merous perianth made up by three sepals and three petals. The median petal is normally bigger, more colored, dotted and/or ornamented. This floral part is widely known as lip or labellum. The ovary is inferior and the androecium (of 1–3 fertile anthers) and gynoecium are fused into a single structure called gynostemium or column. There are three stigmatic lobes (Dressler 1981, 1993; Judd et al. 2008). Although a significant part of the median stigmatic lobe normally becomes non-receptive (sterile), it produces secretions or tissues that are involved in the pollination process (see below). This modified median stigmatic lobe is widely known as rostellum (Dressler 1981, 1993; Judd et al, 2008).

Most orchids characteristically package pollen into discrete units that are removed as a single unit from the flower during the pollination process (Figure 2). In functional terms, this means that the whole pollen content of a flower is removed during a single pollinator visit. These pollen packages are called pollinia (singular: pollinium). We have to make a short digression here: pollinia plus any secretions and/or tissues that aid in the removal of the structure from the flower are collectively known as pollinarium (plural: pollinaria) (Dressler 1981, 1993; Endress 1994). The terms pollinia and pollinaria (singular: pollinarium) are often used interchangeably, but this is incorrect from a morpho-

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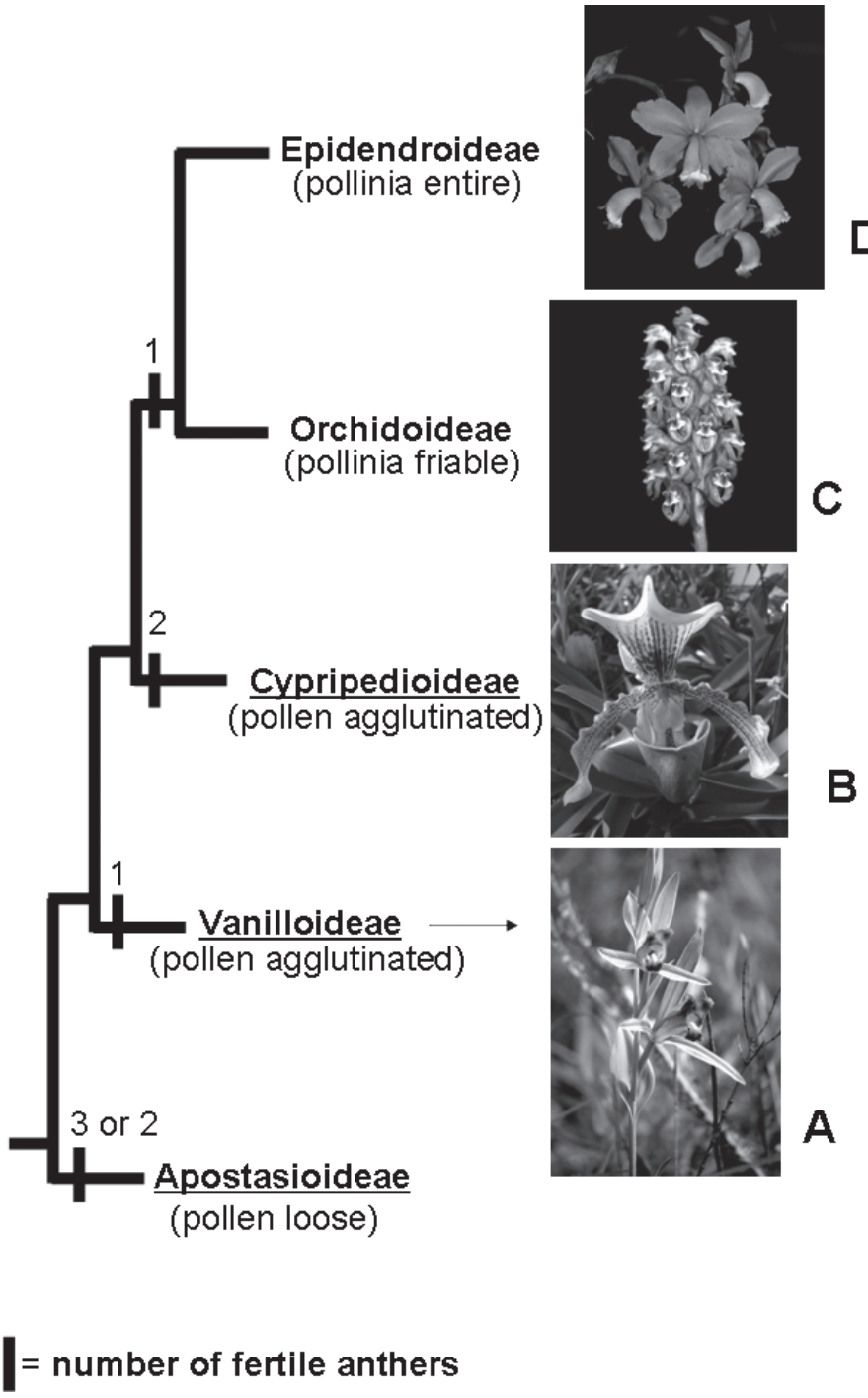

FIGURE 1. Cladogram of Orchidaceae, depicting phylogenetic relationships between subfamilies (modified from Chase et al. 2003) and patterns of pollen aggregation. **A.** *Cleistes libonii* (Vanilloideae), **B.** *Paphiopediulum insigne* (Cypripedioideae), **C.** *Pelexia orobanchoides* (Orchidoideae), **D.** *Cattleya loddigesii* (Epidendroideae).

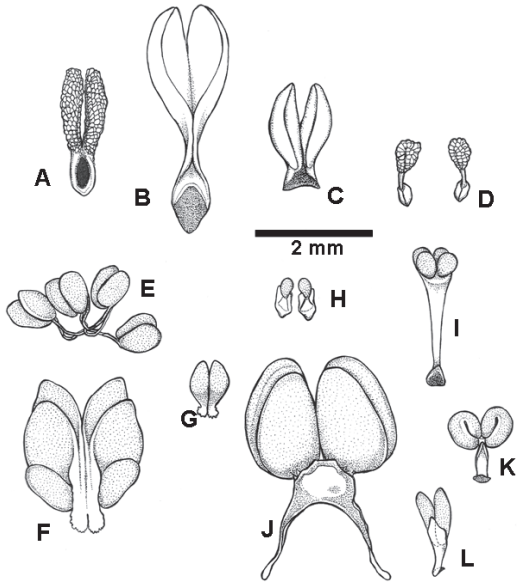

FIGURE 2. Pollinia and pollinaria of twelve sympatric Orchidaceae, occurring in Araucaria forests in Southern Brazil. (A–D) belong to the subfamily Orchidoideae and display friable (divisible) pollinia. (E–L) belong to the subfamily Epidendroideae and display entire pollinia. **A.** *Microchilus austrobrasiliensis* (Goodyerinae). **B.** *Veyretia simplex* (Spiranthinae). **C.** *Cyclopogon diversifolius* (Spiranthinae). **D.** *Habenaria parviflora* (Orchidiinae). **E.** *Isabelia pulchella* (Laeliinae). **F.** *Leptotes unicolor* (Laeliinae). **G.** *Acianthera luteola* (Pleurothallidinae). **H.** *Campylocentrum aromaticum* (Angraeciinae). **I.** *Zygostates dasyrrhiza* (Oncidiinae). **J.** *Brasiliorchis picta* (Maxillariinae). **K.** *Oncidium loefgrenii* (Oncidiinae). **L.** *Oncidium paranaense* (Oncidiinae).

logical perspective. The term pollinia should be restricted to the very pollen-bearing structures of the pollinarium (Dressler 1981, 1993; Endress 1994), and pollinarium to the entire translatory unit or complex involving both fertile (pollinia and pollen content) and sterile (various pollinium stalks) tissues (Dressler 1981, 1993; Endress 1994).

The basic pollinia number for the orchid family as a whole is four, in agreement with the four pollen-sacs usually found in the rest of angiosperms (Dressler 1981, 1993). Within Orchidaceae, however, secondary reductions or increases are common and widespread (Dressler 1981, 1993) (FIGURE 2). It should be noted that pollinia alone cannot be removed from the anther. In the simplest case, pollinia are removed along with rostellar secretions that stick to the pollinator's body surface. During the pollination process, the rostellum is disturbed and a glue-like secretion is released. This floral mechanism is widespread in the neotropical species of the genus *Bulbophyllum* (notice, however, that more morphologically complex situations may also be found in this genus) as well as within the Asian genus *Dendrobium* and close relatives (Dressler 1981, 1993). Pollinia devoid of any additional stalks constitute the so-called ''naked pollinia'' (Dressler 1981, 1993) and are, as explained above, removed with the aid of rostellar secretions.

Most orchids, however, develop a number of additional stalks that are functional during the pollination process. Tail-like, elastic pollinium projections made of viscin and abortive pollen are widespread features. These projections are known as caudicles or caudiculae, which can be very reduced and inconspicuous to the naked eye. Pollinia provided with caudicles and no additional stalks are widespread, for instance, within several genera of the subtribe Pleurothallidinae (e.g., *Dryadella, Specklinia, Acianthera,* etc.) and Laeliinae (e.g., *Cattleya, Isabelia, Leptotes, Pseudolaelia,* most *Sophronitis* spp. and others) (Dressler 1981, 1993) (FIGURE 2E–G). In these orchids the caudiculae are moistened with rostellar secretions that glue onto the pollinator's body surface (Dressler 1981, 1993).

In many Orchidaceae a further step of morphological complexity is achieved when part of the rostellum becomes not only adhesive, but also detachable. This part is often a pad-like structure commonly known as viscidium (plural viscidia) (Dressler 1981, 1993). In these orchids, the viscidium is responsible for gluing the pollinarium to the pollinator's body surface. In the simplest case, the pollinaria consist of pollinia connected by caudicles to a viscidium (FIGURE 2A–D, H–L). There are, however, several variations. In many Orchidaceae, an additional stalk may appear between the pollinia (and caudicles) and the viscidium. These stalks are often elongate and are collectively known as stipes (Greek for ''column'') (FIGURE 2H, I, K, L). The concept of stipes, however, involves at least three different structures. In many cases stipes consist of an epidermal layer of column sterile tissue that becomes detachable (Dressler 1993). This is the commonest kind of stipes, and the name tegula (Greek for ''roof'') has been proposed for such structures (Dressler 1981, 1993). The name hamulus (plural: hamuli) has been proposed for stalks derived from a projection of the rostellum (Dressler 1981, 1993). This kind of stalk is quite restricted and has been reported for a very few taxa (Dressler 1993). Finally, a third kind of stalk (with no formal name so far) was recently described from *Christensonella uncata* (Maxillariinae) (as *Maxillaria uncata*) and related spp. In these orchids, the stalk between the pollinia and the viscidium consists of a long, straight,

TABLE 1. Pollinia from museum accessions from which nrITS and *trn*L-*trn*F sequences could be obtained.

Species	Voucher	Collection year	Preservation	NCBI accession
Catasetum fimbriatum	FLAS 181451 (pollinia from herbarium)	1986	Air dried	EU441210
Catasetum saccatum	Gerlach 1771 (pollinia on insect)	1999	Unknown	EU441204, EU441214*
Coelogyne fimbriata	Leiden cult. 923836 (fresh pollinia)	2004	Air dried	EU441205
	Leiden cult. 30759 (fresh leaves)	1997	Air dried	AF302745
Coelogyne swaniana	Leiden cult. 27645 (fresh pollinia)	2004	Air dried	EU441206
	Leiden cult. 27645 (pollinia silica dried)	1996	Air dried	EU441213*
Paphinia cristata	Gerlach 1285 (pollinia on insect)	1997	Unknown	EU441207, EU441211*
	Whitten s.n. (leaves from herbarium)	1989	Air dried	EU441209

Note: The DNA sequences obtained were subjected to a BLAST search in the NCBI GenBank for species identification. Sequences of the same species obtained from leaves and pollinia (either fresh, silica-dried, from herbarium, or glued to insects) were completely identical. * Indicates sequences from *trn*L-*trn*F; all others are from nrITS.

detachable band-like strand consisting of the clinandrium and the subjacent part of the rostellum (Singer & Koehler 2004). Pollinaria with stipes (of whichever kind) consist of pollinia connected via caudicles to a stipe, which is in turn connected to a viscidium. Therefore, these pollinaria consist of four different parts (Dressler 1981, 1993; Endress 1994) (FIGURE 2H, I, K, L).

In summary, a pollinarium is a complex pollen-translatory unit bearing parts with different tissue origins: pollinia and caudicles derive from the androecium; rostellar secretions or viscidia originate from the gynoecium; and any additional, stipes-like pollinium stalks originate from sterile column tissues (except for the hamulus, which is derived from the rostellum) (Dressler 1981, 1993).

After having introduced the basic concepts of pollinarium morphology, we now proceed to address the following questions: Is the presence of pollinaria a consistent feature within the Orchidaceae? Is the presence of pollinaria restricted to Orchidaceae? Are pollinarium features taxonomically informative? Are pollinarium features ecologically informative? Are pollinarium features evolutionary conserved? Can pollinarium features aid in the identification of orchids?

METHODS

a) Non-molecular Characters

The data discussed here were gathered during 11 years (1997–2008). Fresh flowers and pollinators laden with pollinaria were obtained during fieldwork trips or from plants cultivated at UNICAMP (Universidade Estadual de Campinas, SP), at ESALQ-USP, at the São Paulo Botany Institute (IBt) or in the first author's personal collection. From 1997 to 2005, pollinaria and pollinators were photographed at the Taxonomy Laboratory, Botany Department, UNICAMP using a Nikon SMZ-U binocular stereomicroscope, connected to a Nikon FD-× 35 mm camera. From 2005 onwards, photos were obtained using either a 35 mm Pentax camera or a Sony Cybershot DSC-H7 digital camera. Throughout the text we adopt and follow the orchid classification scheme proposed by Chase et al. (2003) and the morphological terminology suggested by Dressler (1981, 1993).

b) DNA Extraction from Orchid Pollinaria

Pollinia from several different orchid taxa (see Table 1 for more details) were extracted using a non-destructive protocol in which the pollinia (either fresh, silica-dried, from herbarium or glued to insects) were soaked in an extraction buffer (Nucleospin AP1 or Qiagen Plant AP1) for several hours; then the tissue was removed, rinsed with deionized water, and frozen. The remaining mixture was then carried through the extraction using either the Nucleospin Plant Kit (Macherey-Nagel, The Netherlands) or Qiagen DNeasy Plant Kit (QIAGEN, Amsterdam, The Netherlands) following manufacturer's specifications. The only modifications to this protocol were extended incubation times in extraction buffer (one hour at 65 C), and in the final elution buffer (15 minutes); the final DNA concentration was increased by using only 75% of the recommended elution buffer (according to manufacturer's instructions).

The nuclear ribosomal Internal Transcribed Spacer (ITS) region was amplified using the primers SE17 and SE26 from Sun et al. (1994). Reactions were amplified using a MJ Research PTC 200 thermalcycler with an initial denaturation step of 5 minutes at 96 C, then 30 cycles

of 1 minute at 96, 1 minute at 50 C, and 3 minutes at 72 C, followed by an extra elongation step of 7 minutes at 72 C. The chloroplast *trn*L-*trn*F spacer region was amplified using primers E and F (Taberlet et al., 1991) and thermalcycling parameters similar to ITS except a higher annealing temperature (55 C) and a one minute elongation step. All loci were amplified using 1 unit of Promega Taq (Promega Benelux, Leiden, The Netherlands) with buffer, 15–30 mg/ μl of MgCl, 250 mM of each dNTP, 0.25 mg/μl Bovine Serum Albumin (BSA), 250 μM of each primer, and 1 μl of template in a 25 μl reaction. Products of the PCR were cleaned using the Qiaquick PCR Purification kit (QIAGEN). Cleaned PCR products were sequenced in both directions using the ABI Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) following manufacturer's instructions. Sequences were visualized on a PE Applied Biosystems 377 automated DNA sequencer. The resulting chromatograms were assembled and edited using Sequencher 4.1 (Gene Codes Corp.).

All sequences were checked by using a BLAST search on the NCBI website. For DNA from pollinia removed from insects and for which there was no corresponding genus on the NCBI database, the sequences were compared to sequences from leaves or pollinia collected from positively identified voucher specimens (TABLE 1).

DISCUSSION

The Presence of Pollinaria Within and Outside Orchidaceae

It is commonly believed that all orchids have pollinia and pollinaria, but as we indicate below, this is an oversimplification. Darwin (1862) already noticed that pollen cohesiveness varies significantly within and among orchid groups. The diagram depicted in FIGURE 1 indicates the presence/absence of pollinia/pollinaria at the subfamily level, and is quite informative since we can infer or propose patterns of character evolution using a phylogenetic framework. The subfamily Apostasioideae is unequivocally the sister group of the remaining Orchidaceae (Cameron et al. 1999; Chase et al. 2003). This subfamily encompasses only two genera (*Neuwiedia* and *Apostasia*) and contains about seventeen spp. geographically restricted to Tropical Asia and part of Oceania (Chase et al. 2003, Dressler 1993, de Vogel 1969). Remarkably, the flowers of these species exhibit three or two (plus a staminode) distinct, fertile anthers whose loose pollen grains are released in monads or tetrads. The anthers of *Neuwiedia* have longitudinal slits (resulting from longitudinal dehiscence) whereas those of *Apostasia* are poricidal (anthers have a single terminal pore) (Dressler 1993). The flowers of the subfamily Apostasioideae resemble, in many additional features, those of Hypoxidaceae or other related non-orchid monocot families such as Boryaceae and Lanariaceae. All these monocot families have been identified as putative sister-groups of Orchidaceae (Cameron et al. 1999, Chase et al. 2003). A few pollination studies have explored the pollination mechanisms of *Neuwiedia* (Kocyan & Endress 2001, and references therein), and they all indicate that pollen-collecting stingless bees (Apidae: Meliponina) are the main pollinators. We are unaware of any studies on the pollination biology of *Apostasia.* Since the anthers of this genus form a tight tube around the style, it is reasonable to expect that pollen grains are released through ''buzz-pollination,'' a pollination strategy already documented in other angiosperm families with poricidal anthers such as Solanaceae, Melastomataceae, and others (Dressler 1993, Endress 1994). Before leaving the Apostasiods, it should be emphasized that this is the only orchid group in which pollen constitutes the only reward for pollinators (Kocyan & Endress 2001).

The subfamilies Vanilloideae and Cypripedioideae are the successive sister groups of the remaining Orchidaceae (FIGURE 1) (Chase et al. 2003). Although in these two subfamilies the pollen is pasty or agglutinated, it does not form true pollinia (Singer et al. 2006) (FIGURES 1, 3). Some authors (e.g., Szlachetko & Rutkowsky 2000) apply the term ''pollinia'' for the agglutinated pollen of several Vanilloideae and Cypripedioideae, but, as we indicate below, these structures do not constitute pollinia from a functional point of view. This matter becomes particularly clear under the light of the pollination studies available for these two subfamilies. All studies consistently report that pollen is released as smears of monads or tetrads on the body surface of pollinators (Banziger 1996, Banziger et al. 2005, Merhoff 1983, and Pansarin 2003). The subfamily Vanilloideae has a single fertile anther whose pollen is agglutinated and more or less pasty (FIGURE 3A), which is released as smears on the dorsal surface of pollinators (Dressler 1993, Pansarin 2003). The anther in these flowers is hyperimcumbent (''bent'' sensu Dressler 1993) and provided with a thick, though flexible filament (FIGURE 3A). Pollen is released gradually, during successive pollinator visits. Some recent studies indicate that some vanilloids secrete nectar as a pollinator reward (Pansarin 2003). Pollinators enter the flower, and as they

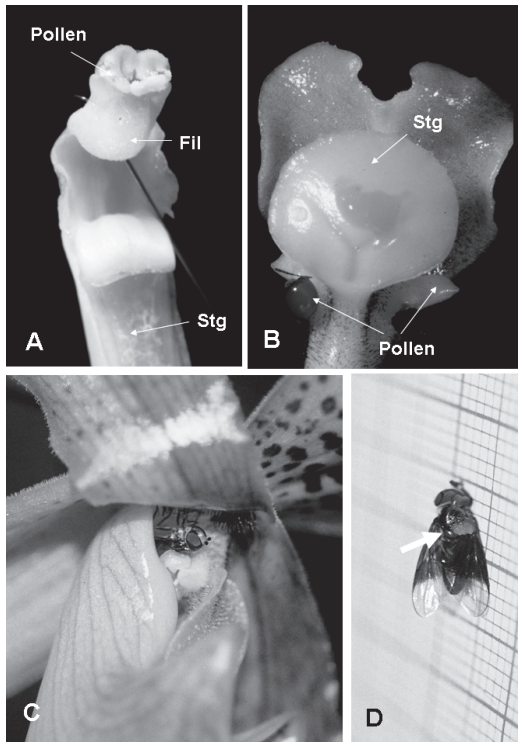

FIGURE 3. Orchidaceae without pollinia. **A.** Column of *Vanilla chamissonis* (Vanilloideae). B)–D) *Paphiopedilum insigne* (Cypripedioideae). **B.** Column. **C.** Syrphid fly leaving the flower by bumping on one of the two anthers. **D.** Syrphid fly with pollen smear (arrow) onto the scutellum. Fil: anther filament. Stg: stigmatic surface.

retreat to leave, they get covered with a dusty layer of pollen..

Orchids of the subfamily Cypripedioideae develop two lateral and fertile anthers (FIGURES 1, 3B) and a median and conspicuous, shield-like staminode (Dressler 1981, 1993). The pollen in these orchids is pasty (genera *Cypripedium* and *Phragmipedium*) or viscous (*Paphiopedilum*) (FIGURE 3B) and does not form true pollinia either (Banziger 1996, Banziger et al. 2005, and Singer et al. 2006). As for the Vanilloideae, available pollination studies indicate that the pollen is released as smears or pollen-pads on the pollinator (Banziger 1996, Banziger et al. 2005, and van der Cingel 1995, 2001) (FIGURE 3C, D). Notice, however, that the pollination biology differs significantly between these two subfamilies. Whereas Vanilloids secrete nectar, Cypripedioids are rewardless and present elaborated trap-flowers. Although there is a significant amount of literature regarding the pollination biology of European and Asiatic taxa (van der Cingel 1995, 2001, and reference therein), very little information is available (at best) for neotropical taxa (van der Pijl & Dodson 1966). In this group of orchids, pollinators, lured to the staminode by floral features such as odor, color, and hairiness, accidentally fall inside the conch-like labellum. In order to leave the flower, insects must pass through one of the two possible passages just below the anthers (FIGURE 3C). In doing so, they become smeared with pollen before leaving the flower (FIGURE 3D). Retrorse hairs prevent passing through the labellum, much in the same way as in the flowers of Aristolochiaceae (Endress 1994). Bees and flies are the primary pollinators of these flowers. Pollination and fruit set are characteristically low, as one would expect for rewardless flowers (Neiland & Wilcock 1998).

The subfamilies Orchidoideae and Epidendroideae are unequivocally sister groups (FIGURE 1) (Cameron et al. 1999, Chase et al. 2003). This clade is characterized by the presence of true pollinia (FIGURES 1, 4, 5). Pollen masses are dislodged as a whole by the pollinators during floral visits (Dressler 1993). However, morphological features of the pollinia and pollinaria of both subfamilies differ substantially (FIGURES 1, 2, 4, 5). This topic will be treated in more detail in the following section.

Are pollinia and pollinaria restricted to Orchidaceae? The answer is no. Pollinaria are also a consistent feature of the family Apocynaceae (Gentianales), in subfamily Asclepiadoideae (formerly Asclepiadaceae, popularly known as milkweeds) (Judd et al. 2008) (FIGURE 6A–C). Notice, however, that the pollinaria of Asclepiadoideae are analog (this is, not homologue) to those present in Orchidaceae. A brilliant developmental explanation of the floral features of Asclepiadoideae (as Asclepiadaceae) can be found in Endress (1994). In milkweeds, the androecium and gynoecium are fused into a gynostegium (vs. a gynostemium or column in the Orchidaceae) (FIGURE 6A) and each flower produces five pollinaria (vs. one—or rarely two—pollinaria in Orchidaceae) (Endress 1994). In Apocynaceae, each pollinarium is composed by two pollinia (each one from adjacent anthers, so each pollinarium bears pollen from two anthers) connected via two translator-arms to a corpusculum (Endress 1994) (FIGURE 6A, B). This corpusculum is not adhesive, like the orchid viscidium, but functions as a clip and clasps onto slender, straight body parts of the pollinator (mouth-parts, hairs, spines, etc.) (FIGURE 6C). When the pollinator leaves the flower, it pulls out the pollinarium (Judd et al. 2008). Pollinaria of Asclepiadoideae invariably attach to the mouthparts or legs of their pollinators. In addi-

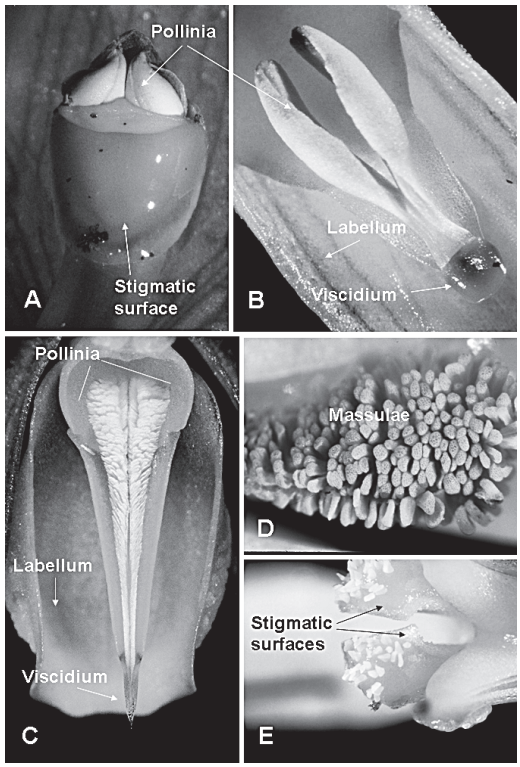

FIGURE 4. Pollinaria in Orchidoideae. **A.** *Geoblasta penicillata* (Chloraeinae): granular pollinia without pollinium stalks. **B.** *Sarcoglottis fasciculata* (Spiranthinae): pollinarium with granular pollinia and terminal viscidium. Examples of massulate pollinia (C–E). **C.** *Aspidogyne conmelinoides* (Goodyerinae). **D.** *Habenaria fastor* (Orchidiinae). **E.** Stigmatic surfaces of *Habenaria pleiophylla* loaded with massulae.

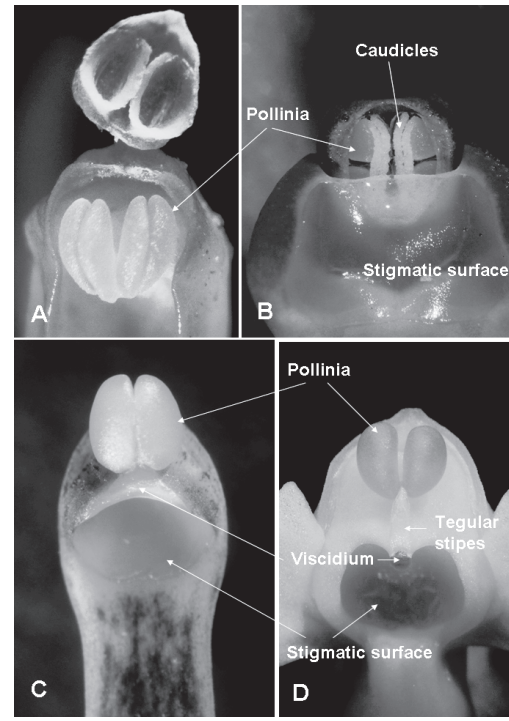

FIGURE 5. Pollinaria in Epidendroideae. **A.** *Liparis nervosa* (Malaxideae): example of ''naked pollinia.'' **B.** *Pseudolaelia corcovadensis* (Laeliinae): pollinarium with long caudicles devoid of viscidium. **C.** *Brasiliorchis marginata* (Maxillariinae): pollinarium with viscidium devoid of stipes. **D.** *Oncidium gracile* (Oncidiinae): pollinarium with tegular stipes.

tion, Apocynaceae of the subfamily Periplocoideae have also evolved pollinarium-like structures. These structures, however, are only superficially similar to pollinaria and consist of a pasty pad of pollen tetrads (not equivalent to true pollinia) that rests on a cup-like or spoon-like translator (FIGURE 6D). This translator has a proximal adhesive gland that glues the unit to the pollinator (Endress 1994, Judd et al. 2008).

In summary, only two (Orchidoideae and Epidendroideae) out of the five orchid subfamilies display true pollinia and pollinaria. However, these two subfamilies are by far the most species-rich orchid clades, accounting for 98% of the species described to date (Chase et al. 2003). In Apostasioideae, the sister-group to the remaining Orchidaceae, the pollen is free and loose. In the other successive sister groups (i.e., subfamilies Vanilloideae and Cypripedioideae) the pollen is somehow agglutinated, but it does not form true pollinia. Pollinaria analog to those of Orchidaceae have also evolved in the Apocynaceae subfamily Asclepiadoideae (bearing true pollinia) and superficially pollinarium-like structures with pasty pollen tetrads held in spoon-like translators occur in Apocynaceae of subfamily Periplocoideae.

Taxonomic Information of Morphological Features of Orchid Pollinaria

As indicated above, the presence of true pollinaria within Orchidaceae is restricted to a clade composed of two subfamilies: Orchidoideae and Epidendroideae. The pollinaria of these two subfamilies, in general, can easily be set apart morphologically (Dressler 1981, 1993; Freudenstein & Rasmussen 1997, 1999; Rasmussen 1982; and Singer et al. 2006) (FIGURES 1, 2, 4, 5).

The pollinia of the subfamily Orchidoideae are always friable (divisible) in some way (FIGURES 1, 2, 4, 5). They could be either granular, soft (FIGURES 2B, C, 4A, B), or divisible in subunits, the so-called massulae (Dressler 1981,

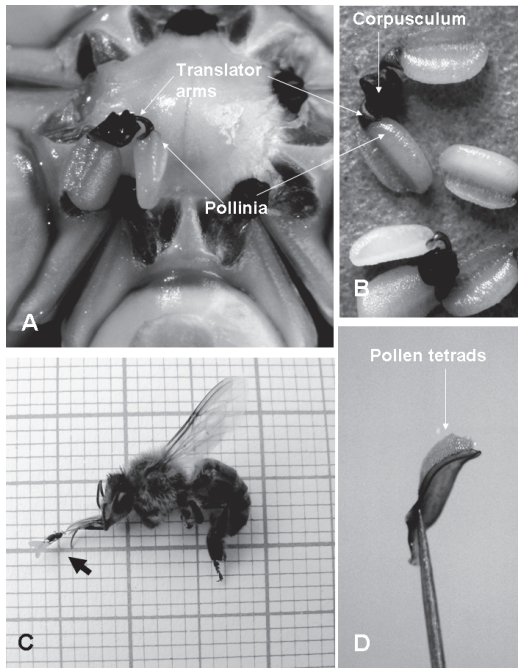

FIGURE 6. Pollinaria and translators in Apocynaceae. (A–B) *Schubertia grandiflora* (Asclepiadoideae). **A.** Gynostegium with a pollinarium removed. **B.** Detail of two pollinaria. **C.** Honey-bee (*Apis mellifera*) with a pollinarium of *Araujia cericifera* (Asclepiadoidae) clasping its galeae. **D.** Spoon-like translator of *Cryptostegia grandiflora* (Periplocoideae).

1993; Freudenstein & Rasmussen 1997, 1999; Rasmussen 1982; and Singer et al. 2006) (FIGURES 2A, D, 4C–E). Functionally, this means that if pollinaria are dislodged in a single visit of a pollinator, the pollen content may be spread onto the stigmatic surfaces of several flowers. It has been speculated that this may promote (to some degree) the chances of cross-pollination (Singer et al. 2006, Singer & Sazima 1999, 2000, 2001). The pollinia of Orchidoideae may lack additional stalks (Chloraeinae) (FIGURE 4A) or be provided with caudicles and viscidia (Orchideae, Cranichideae) (Dressler 1981, 1993; Rasmussen 1982) (FIGURES 2A–D, 4B–E). Granular, soft pollinia are typically found in the tribes Codonorchideae, Diurideae and Cranichideae (except Goodyerinae) (sensu Chase et al. 2003). Sectile (massulate) pollinia are characteristic of Orchideae and subtribe Goodyerinae (sensu Chase et al. 2003).

Conversely, the pollinia of the vast majority of the Epidendroideae are entire, globose and indivisible (FIGURES 1, 2E–L, 5). These are the so-called ''waxy'' or ''hard'' pollinia, which are commonly depicted in textbooks (Dressler 1981, 1993; Endress 1994). Most of these pollinia are globose, clavate or rounded to some degree (Dressler 1993). In Epidendroideae, the pollinia may be naked (FIGURE 5A) or may form a complex structure, with caudicles and stalks such as stipes (either of the tegular or hamular kinds) and viscidia (Dressler 1993) (FIGURES 2E-L, 5B–D).

There are a few Epidendroids with sectile pollinia (e.g., Tropidieae). Notice, however, that sectile pollinaria in Orchidoideae and Epidendroideae are easily distinguishable: whereas orchidoid sectile pollinaria display more or less equally-shaped massulae, those of Epidendroideae bear dimorphic massulae (literally, massulae of two different shapes along the pollinia) (Freudenstein & Rasmussen, 1997, 1999; Ramírez et al. 2007).

Features such as number, texture (friable vs. entire) and shape of pollinarium parts are quite consistent and often diagnostic among orchid taxonomic groups. For instance, most Cranichideae (Orchidoideae) orchids bear pollinaria with four more or less clavate, friable pollinia, caudicles and a terminal viscidium (alternatively, some authors consider them two pairs of bipartite pollinia) (Dressler 1993) (FIGURE 2A–D). Pollinaria of the subtribes Maxillariinae and Zygopetaliinae (Epidendroideae) consistently bear four indivisible pollinia, in two pairs (Dressler 1993, Singer & Koehler 2004) (FIGURES 2J, 5C). However, the viscidia of Zygopetaliinae tend to be lanceolate in shape, a rare condition within Maxillariinae (Dressler 1993). Orchids of the neotropical subtribe Oncidiinae (sensu Chase et al. 2003) may display pollinaria with two (the prevailing condition) (FIGURES 2K, L, 5D) or four (FIGURE 2I) (in the former Ornithocephaliinae) roundish pollinia (Dressler 1993). However, in all cases these pollinaria bear a well-developed tegular stipe and a terminal pad-like to roundish viscidium (Dressler 1993) (FIGURE 2I, K, L). In the neotropical subtribe Stanhopeinae, there are also two pollinia connected to a tegular stipe, but the viscidium in this subtribe is normally lanceolate in shape (Dressler 1993). In addition, the pollinia in Stanhopeinae tend to be laterally flattened (as opposed to normally globose in Oncidiinae) (Dressler 1981, 1993). The viscidium in the subtribe Maxillariinae is frequently horseshoe-shaped; a rare feature in other orchid subtribes (Singer & Koehler 2004) (FIGURE 2J, 5C).

As shown above, a number of morphological pollinarium features are taxonomically informative. It is therefore not surprising that orchid classifications have often relied (among other characters) on pollinarium features for systematic purposes (Lindley 1840; Dressler 1981,

1993; Szlachetko & Rutkowski 2000). Pollinaria alone may not be sufficient for outlining classification schemes, but species identification is possible in some cases. A good practical example is the case of orchid pollination by the neotropical Euglossini bees (Ackerman 1982, Dressler 1982, Roubik & Ackerman 1987, Singer & Sazima 2004, Singer et al. 2006, van der Pijl & Dodson 1966). The males of these bees actively gather aromatic compounds secreted by (among many other angiosperms) orchid flowers of several subtribes. While gathering these fragrances the bees act as the pollinators of these orchids. With the identification and chemical synthesis of the attractive compounds it was possible to study orchid pollination by baiting male bees, which could then be checked for orchid pollinaria (Ackerman 1982, Dressler 1982, Roubik & Ackerman 1987, Singer & Sazima 2004, Singer et al. 2006, van der Pijl & Dodson 1966). This methodology allows the study of the interaction between regional euglossine faunas and orchid floras. As a rule, orchid pollinaria are identified on the basis of morphological features alone. Of course, this work is much facilitated by the existence of preceding floristic studies and collaboration between entomologists and orchid specialists. We have to stress, however, that orchid identification through pollinarium morphological features are by no means restricted to taxa pollinated by Euglossine bees. As explained above, any well-preserved orchid pollinarium could be identified at least to the level of subfamily, tribe and subtribe based on gross morphological features (pollinia texture, number and shape of pollinarium parts). Well-trained specialists with good knowledge of particular orchid floras and some fieldwork experience are often able to identify the pollinaria of cogeneric, sympatric taxa to species level, solely through the use of morphological characters.

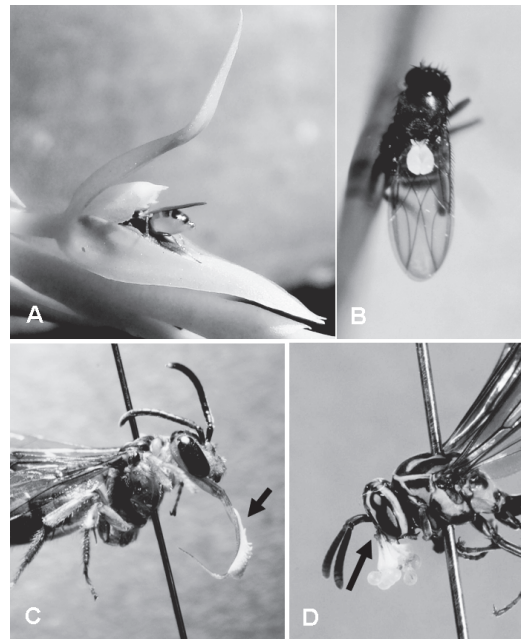

FIGURE 7. Common examples of orchid pollination. (A–B) *Acianthera* (Pleurothallidinae) flowers have an incumbent anther, and a more or less parallel labellum and column. Consequently, they glue their pollinaria on the dorsum (scutellum) of their fly pollinators (B). **A.** *Acianthera glumacea.* **B.** *Megasellia* sp. (Phoridae) with pollinarium of *Acianthera luteola.* **C.** *Microchilus arietina* flowers have an erect anther and parallel column and labellum. Consequently, they glue their pollinaria on the proboscis of pollinators (in this case, an *Osiris* sp. bee). **D.** *Capanemia thereziae* (Oncidiinae) has an incumbent anther, but column and labellum are perpendicular. The pollinaria are glued on the head (just below the antennae) of the pollinators (*Polybia fastidiosuscula* wasps).

Floral Attributes that can be Inferred from Pollinarium Features

The very place of pollinarium adhesion on the pollinator depends on the following variables: 1) orchid and pollinator morphology and 2) pollinator behavior in the flower (the way the pollinator handles the flower) (Ramírez et al. 2007, Singer et al. 2006). We can infer some morphological floral attributes based on the location of a particular pollinarium on the pollinator. This is particularly helpful for identification purposes. Three main situations are found:

1) Pollinaria adhered on the back or dorsum of their pollinators (e.g., several Maxillariinae, several Pleurothallidinae (FIGURE 7A, B) and several *Cattleya* spp., etc.) normally belong to orchids whose flowers have an incumbent anther and parallel column and lip.
2) Pollinaria adhered on the pollinator's mouthparts, proboscises or bills (e.g., many Cranichideae) (FIGURE 7C) normally belong to orchids whose flowers have an erect anther and parallel labelum and column (FIGURE 4B, C).
3) Pollinaria adhered on the pollinator's head (e.g., several *Ophrys* species, several Oncidiinae spp.) (FIGURE 7D) normally belong to orchids whose flowers have an incumbent anther and labellum and column perpendicularly disposed.

Of course, there are exceptions and special cases. The genus *Epidendrum* (Laeliinae) presents a modification of situation 2), where the lateral sides of both column and labellum are fused and thus delineate a narrow, funnel-like

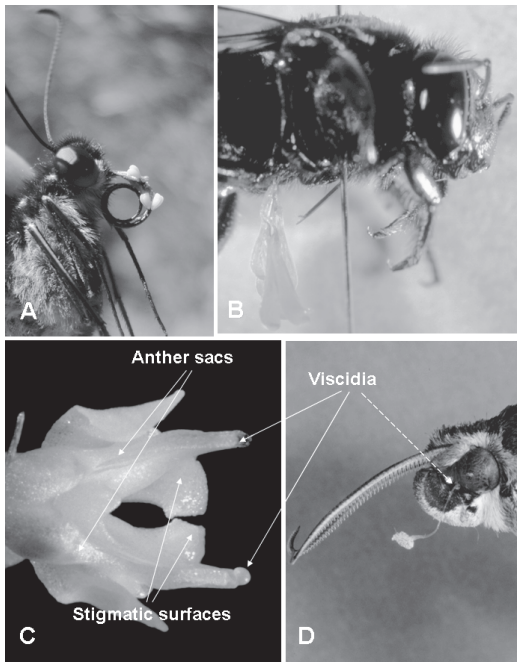

FIGURE 8. Some special cases of orchid pollination. **A.** Swallowtail butterfly with two pollinaria of *Epidendrum fulgens* (Laeliinae) on its proboscis. **B.** Male of *Eufriesea violacea* (Euglossini) with pollinaria of *Cirrhaea saccata* (Stanhopeinae) adhered to its trochanters. **C.** Column of *Habenaria pleiophylla* (Orchidinae) showing its characteristic features. **D.** Male of *Aellopos* sp. (Sphingidae) with pollinarium of *Habenaria pleiophylla* adhered to its eyes.

cavity where lepidopteran pollinators can barely insert their probosces. Consequently, the pollinaria of these orchids adhere to the proboscis of their pollinators (FIGURE 8A). The non-resupinate flowers of *Cirrhaea* and other Stanhopeinae genera deposit their pollinaria on the legs of their male euglossine pollinators (FIG. 8B). During floral ontogeny, the anther in the genus *Habenaria* is profoundly split and two pollinaria are therefore produced (Singer et al. 2007) (FIGURE 8C). Pollinators are Lepidoptera, and the pollinaria adhere to their eyes, the only surface smooth enough (Lepidoptera bodies are covered with scales) to allow pollinaria to attach (Singer et al. 2007) (FIGURE 8D).

Summarizing, the placement of a given pollinarium onto a pollinator may help us infer some features of the flower of origin, and thus may be useful during the taxonomic determination.

As wisely highlighted by Dressler (1993), some structural aspects of pollinarium and stigma are tightly correlated. For instance, the presence of globose, entire pollinia in Epidendroideae is correlated with the presence of sunken, very concave stigmatic cavities (Dressler 1993) (FIGURE 5B–D). This has been interpreted as an adaptation to maximize the arrest of whole pollinia (and consequently, maximize the chances of pollination) (Dressler 1993). Therefore, if we find an insect bearing a pollinarium with globose, entire pollinia, it can be inferred that it belongs to a taxon in the subfamily Epidendroideae, with a markedly concave stigmatic cavity (Dressler 1993).

Conversely, orchids of subfamily Orchidoideae bear friable pollinia and open, flat to slightly convex stigmatic surfaces (FIGURE 4A, E). This has been interpreted as a strategy to increase the chances of multiple-pollination events, since an orchidoid pollinarium releases only a fraction of its pollen content during a pollination event. Consequently, an orchidoid stigmatic surface could, in theory, receive pollen loads from different donors (Singer & Sazima 1999, 2000, 2001; Singer et al. 2006). Therefore, if we find an insect with a friable pollinarium, chances are that it belongs to an orchid of subfamily Orchidoideae, and that it may display a broad, open stigmatic surface.

The Case of *Meliorchis caribea*, the First Unequivocal Orchid Fossil

Recently, the first unequivocal orchid fossil came to light (Ramírez et al. 2007). Other putative fossil Orchidaceae have been described elsewhere, but they consist of compressions (leaves, flowers or fruits) bearing non-unequivocal orchid features. The morphological features preserved in these fossils do not exclude the possibility that they belong to another monocot family.

The holotype of *Meliorchis caribea* consists of a very well preserved pollinarium attached to the mesoscutellum of a worker *Proplebeia dominicana* (Apidae: Meliponina) (FIG. 9). Both pollinarium and bee are entombed in a piece of amber excavated from Miocene amber mines dated 15–20 million years (myr) old, from the Dominican Republic, in the Caribbean Island of Hispaniola (Ramírez et al. 2007). The name *Meliorchis* (the Meliponine pollinated orchid) emphasizes that it was discovered with its pollinator, a stingless bee; and the epithet ''*caribea*'' honors the Caribbean Region, where the specimen was originally found. As commented above, the pollinarium is so well preserved that it shows the essential features to elucidate its systematic position within Orchidaceae. Indeed, the location of the pollinarium on the body of the pollinator allowed the inference of the orchid's pollination mechanism, and also allowed

FIGURE 9. Amber preserved holotype of *Meliorchis caribea* (Orchidoideae: Goodyerinae), the first unequivocal fossil orchid, described from a pollinarium adhered to the mesoscutellum of the extinct stingless bee *Proplebeia dominicana.* The fossil, dated with an age of 15–20 million years, was found in a Miocene amber mine from the Dominican Republic, in the Caribbean island of Hispaniola.

the reconstruction of several floral features absent in the fossil itself. In many ways, the case of *Meliorchis caribea* synthesizes all the information provided above for it shows how pollinarium features can be informative about taxonomy, morphology and ecology.

The fossil pollinarium is sectile and made up of similar looking massulae (Ramírez et al. 2007). These features alone suggest that it belongs to orchid subfamily Orchidoideae (see FIGURES 2A, D, 4C–E). A comparison with pollinarium/pollen features of representative extant genera of Orchidoideae strongly suggested that it belonged to the subtribe Goodyerinae, a hypothesis which received further support through cladistic analyses based on morphological, palynological and ecological features (Ramírez et al. 2007). Therefore, *Meliorchis caribea* was formally described and placed in the subfamily Orchidoideae, within the subtribe Goodyerinae (Ramírez et al. 2007).

Morphological features of the pollinarium (horseshoe-shaped viscidium) and its specific placement on the pollinator (bee's mesoscutellum) (FIGURE 9) indicated that *Meliorchis caribea* had an incumbent anther (a feature absent in extant Goodyerinae), as well as a parallel column and labellum. In essence, its floral mechanism was the same as in many Maxillariinae orchids, which also lay their pollinaria on the scutellum of their pollinators (Singer 2002, Singer & Koehler 2004, Singer et al. 2004, 2006). Because the pollinarium is sectile (and then, friable), it was also inferred that the stigmatic surface had to be open and broad, as usual in extant Orchidoideae (FIGURE 4A, E). In addition, the size of the pollinator and pollinarium allowed estimating the height between labellum and column (Ramírez et al. 2007). The pollination mechanism was found to be similar to those of several Maxillariinae orchids, where insect pollinators walk inside a floral cavity and withdraw the pollinaria when retreating, in order to leave the flower (Ramírez et al. 2007).

In addition, because *M. caribea* unequivocally belongs to Orchidaceae, and is reliably dated (15–20 mya), a calibrated molecular clock analysis allowed scientists to estimate the time of origin of Orchidaceae, which was calculated to the Late Cretaceous, about 76–84 myr ago (Ramírez et al. 2007).

APPLICATION

Identification of Orchid Species using DNA Extracted from Pollinaria Found on Pollinators

Development of the Polymerase Chain Reaction (PCR) and automated DNA sequencing has been revolutionary for the fields of biology in general and systematics, in particular. For centuries, taxonomic affinities among taxa have been a matter of opinion and classifications have often relied on the subjective weight that systematists gave to particular characters. The combination of cladistic methodology and molecular tools allowed the elaboration of large data matrixes that can be run with the help of appropriate computer programs, making the outcome statistically supported and less subjective. The products of these analyses are statistically supported diagrams depicting the most plausible relationships among taxa, based on their recovered molecular data (Judd et al. 2008). Molecular data are not essentially better or less prone to errors than morphological, chemical or anatomical ones (Judd et al. 2008). However, depending on the molecular marker used, hundreds of characters can be recovered for each taxon. Therefore, bigger and more robust data matrixes can be accumulated. Anybody trying to perform non-molecular cladistic analyses for several taxa has faced the problem that, in practice, it is quite difficult to assemble more than 35 characters. This means that only a limited number of taxa can be included in cladistic analyses based on morphological data only.

Researchers around the world are now committed to finding the appropriate DNA segments

that should allow quick species identification in the so-called Consortium of the Barcode of Life (CBOL) initiative (http://barcoding.si.edu). Ideally, DNA barcodes are relatively short and omnipresent within a particular taxonomic group. They mutate relatively fast, which results in significant sequence variation between species, and, in principle, relatively low sequence variation within species. Kress et al. (2005) proposed the nuclear ribosomal Internal Transcribed Spacer regions (nrITS) as a potential DNA barcode for flowering plants.

A potentially revolutionary offset of the CBOL initiative is the possibility of orchid species identifications from DNA extracted from pollinaria or remains of pollinaria glued to pollinators. Widmer et al. (2000) showed that DNA barcoding can be used very well alongside morphology for identification of pollinators of European terrestrial orchid species or species groups belonging to the genera *Dactylorhiza, Himantoglossum, Orchis* and *Ophrys* which left pollinia on bees caught by netting. When nrITS sequences were analysed from these pollinia, it was found that at least half of the orchid-pollinator relationships discovered with this approach had not been reported previously.

PCR products can be obtained not only from freshly collected pollinia glued to pollinators caught not too long ago, but also from pollinia stored in museum collections (Table 1). This means that valuable new information about pollinators now can be obtained for epiphytic orchids as well. This is very good news as the pollination ecology of epiphytic orchids is often very difficult to study in the field due to inaccesible high tree crown substrates and rarity of pollination events.

Not all museum curators are too keen to sacrifice rare specimens in their collections for DNA extraction. Rohland et al. (2004) therefore developed a non-destructive DNA extraction method to overcome reluctance of curators to contribute material for DNA barcoding purposes. In this method, specimen material is not ground but only soaked in an extraction buffer with low amounts of demineralizing reagents. Afterwards, the buffer is processed for DNA extraction, whereas the original specimen can be dried for future morphological and/or molecular studies. Likelihood of DNA retrieval and quality of retrieved DNA was not found to be correlated with the length of time of storage in the museum. On the other hand, when we subjected orchid pollinia to non-destructive DNA extraction, different types of chemical treatments during preparation of the specimens affected DNA quality such as soaking time in the buffer and the number of successive extractions of the same specimen (TABLE 1).

Suggestions for Researchers Dealing with Orchid Pollinaria and Orchid Pollination

The last part of this contribution is intended to suggest a few ideas that, in our view, could be helpful for researchers dealing with orchid pollination.

1) **Assemble photographic databases.** Digital, good quality photos of fresh pollinaria should be taken and organized, preferably taxonomically (subfamily, tribe, subtribe, genus and species). Orchid living collections (especially, if biased to particular floras) are especially well-suited for this purpose. If you are involved in floristic inventories, take such photos in the field. Several, moderately-priced digital cameras are now available and some models already have a reasonable macro function so that there is no real need for buying additional lenses. Each photographer has her/his own preferences regarding light, shot-speed, etc. It is extremely important, however, that photos have a good general focus (pollinarium parts, color and shape must be clear) and high definition (4 MP onwards, just in case magnification is necessary). Photos with high definition and focus will be extremely helpful for further identification. A reference collection with properly identified and mounted pollinaria (some researchers assemble collections with labeled pollinaria mounted on entomological pins) may be, of course, really useful. However, pollinaria turn dark and lose much of their original aspect with age. Therefore, we strongly suggest that researchers make photos of them when they are still ''fresh'' in appearance. Do not forget to make sufficient backups of your digital photos.
2) **Make such databases widely available.** We are living in an extraordinary era of information availability and diffusion of knowledge. The Internet has opened unthinkable opportunities that we would have regarded as impossible a decade or so ago. On the other hand, the production of ''classic'' media such as journals and books continues to be expensive. Many (perhaps all) researchers intend to produce books on the object/s of their research but face the sad shortcomings of lacking the necessary money. In our opinion, both authors and institutions should start to seriously consider the possibility of producing E-books that are nothing but handsome PDF files freely available at the web. Such

publications are extremely cheap to make and they can contain color illustrations, something that is really expensive in printed media. If necessary, these files can be printed, just as any ''classic'' media. If you are concerned about authorship, notice that there are already legal options regarding documents available and distributed via Internet (See, for instance, the Creative Commons Initiative). In addition, E-books (as any PDF file) can be constructed in such a way that they cannot be modified or have parts of their content copied or ''recycled.'' Making such documents available through the Web is the fastest way to make your work available to researchers worldwide, a possibility that can hardly be achieved through conventional ways. An extraordinary and inspiring example of this phenomenon is the Peruvian Portal Siamazonia (*http://www.siamazonia.org.pe/*), where hundreds of well-crafted documents regarding the biodiversity of Peruvian Amazonia are freely available. Similar examples can be found elsewhere.

3) **Become as independent as possible.** Many students accumulate huge amounts of unidentified orchid pollinia and then consult a specialist. This seems reasonable but the sad fact is that specialists are frequently overcrowded with work of their own and lack the necessary time for proper attention. Students dealing with Orchidaceae should seriously consider learning as much as possible about orchid taxonomy. Preferably, they should identify their vouchers by themselves and send problematic or dubious items to specialists only. We hope that if some documents and databases as suggested above become available, identification of orchid pollinia will become much easier for a wide usergroup.

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