

Exine micromorphology and ultrastructure in Neottieae (Epidendroideae, Orchidaceae)

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Received: 14 June 2013 / Accepted: 12 August 2013 / Published online: 24 August 2013
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Abstract The diverse epidendroid orchid tribe Neottieae is characterized by multiple transitions between autotrophy and mycoheterotrophy, allogamous and autogamous mating systems, pollen released as tetrads or monads, and pollen exine tectate or semitectate. We use transmission and scanning electron microscopy on pollen of ten species of Neottieae to investigate whether the differences in pollen aggregation and exine micromorphology and ultrastructure reflect phylogenetic relationships, or whether this variation is subject to ecological constraints. Our results showed that differences in exine micromorphology are mostly concordant with phylogenetic relationships in Neottieae, i.e. an ascending tendency of pollen ornamentation from tectate (*Cephalanthera*) to semitectate (e.g. *Neottia*). In contrast,

pollen aggregation, when plotted on the most recent phylogeny, shows repeated transitions between monads and tetrads that could be related to ecological constraints. Tetrads are present in species that are nectar rewarding, whereas monads are common in deceptive species. *Cephalanthera* is characterized by recalcitrant pollen, including the frequent occurrence of collapsed pollen. In this genus, the observed shifts from allogamous to autogamous or cleistogamous mating systems could help to reduce pollen damage caused by exposure to dry habitats.

Keywords Mating system · Monads · Neottieae · Pollenkitt · Tetrads · Ulcerate pollen

Electronic supplementary material The online version of this article (doi:10.1007/s00606-013-0899-2) contains supplementary material, which is available to authorized users.

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Introduction

Differences in pollen morphology between contrasting pollination mechanisms such as entomophily and anemophily are often functionally understandable and phylogenetically traceable, at least in some plant groups (Osborn et al. 1991). In contrast, the influence of less obvious pollination transitions (i.e. switches among different pollinator classes) on pollen morphology are less readily traced onto an evolutionary background because of the difficulty in dissecting the effects of phylogenetic constraints on pollen development and the ecological pressures exercised by pollinators/mating system (Ferguson 1990; Hesse 2000). For example, Wang et al. (2003) found that in the entomophilous genus *Pedicularis* (Scrophulariaceae), the type of pollinator is correlated with corolla shape but not with pollen structure.

Among entomophilous flowering plants, orchids are widely cited as a group where flower morphology has been deeply shaped by the intricate (and often specialized)

relationships with their pollinators (van der Pijl and Dodson 1966). Orchids are also known for their high diversity in pollen morphology, especially with respect to packaging of pollen in pollinia, pollen-wall structure and pollen-surface sculpturing (Schill and Pfeiffer 1977; Burns-Balogh 1983; Burns-Balogh and Hesse 1988; Freudenstein and Rasmussen 1997). A remaining question is whether changes in pollinators and mating systems could influence pollen morphology in orchids, even though pollen grains are not directly attached to the body of the pollinator in most orchids. In most epidendroids and orchidoids, the entire pollen mass (pollinium) is connected to a mucilaginous stalk (caudicle or stipe) formed by the breakdown of the tapetum (e.g. Rudall et al. 2013); the caudicle terminates on an adhesive disc termed a viscidium, which is responsible for attachment of the pollinarium to the pollinator (Dressler 1993).

Despite this relative distance between pollinator and pollen, some correlations have been proposed between pollen characters, especially exine micromorphology, and evolutionary trends have been suggested (Burns-Balogh 1982, 1983; Burns-Balogh and Bernhardt 1985; Burns-Balogh et al. 1987; Dressler 1993; Freudenstein and Rasmussen 1999). In Disinae and Coryciinae, pollen exine sculpture patterns were found to produce phylogenetic signal at the generic and subtribal level (Chesselet and Linder 1993). Similarly, Orchidinae—the subtribe that includes *Anacamptis*, *Himantoglossum*, *Neotinea*, *Ophrys*, *Orchis* and *Serapias*—showed correspondence between the two reconstructed main clades (Bateman et al. 2003) and pollen micromorphology (psilate to rugulate or scabrate pollen versus psilate to reticulate; Barone Lumaga et al. 2006).

Tribe Neottieae consists of seven morphologically diverse orchid genera: *Aphyllorchis* Blume, *Cephalanthera* Rich., *Epipactis* Zinn, *Limodorum* Boehm., *Neottia* Guett. (including *Listera* R. Brown), *Palmorchis* Barb. Rodr. and *Thaia* Schweinfurth (Bateman et al. 2005). Most genera are from temperate and subtropical regions of the northern hemisphere, except *Palmorchis*, which occurs in tropical Central and South America, and *Thaia*, a monotypic genus from Thailand. The tribe is circumscribed by several characters such as the terrestrial and rhizomathous growth habit, only incompletely fused gynostemium with the anther either erect or subincumbent, and with soft fragile pollinia that were subsequently viewed as plesiomorphic within Orchidaceae (Bateman et al. 2005). In older taxonomic treatments, the tribe was placed either in subfamily Orchidoideae (e.g. Dressler 1993) or in its own subfamily Neottioideae (Dressler and Dodson 1960), but more recent molecular phylogenetic reconstructions now place Neottieae within the broader subfamily Epidendroideae, as sister to all other epidendroids (Freudenstein et al. 2004; Bateman et al. 2005; Roy et al. 2009; Xiang et al. 2012).

Neottieae is characterized by frequent transitions from allogamous to autogamous mating systems and by an unusually high proportion of partially or completely mycoheterotrophic species (Dressler 1993). For example, all *Limodorum* and *Aphyllorchis* species are mycoheterotrophic, but relatively few *Neottia* and *Cephalanthera* species, and a few aberrant individuals within *Epipactis* (Bateman et al. 2005). Burns-Balogh (1983) interpreted changes in the pollen surface of Neottieae (then classified as subfamily Neottioideae) as the result of transitions from a semitectate to a tectate-perforate condition. Other authors (e.g. Hesse et al. 1989) also proposed that the trend of increasing specialization in angiosperm pollen morphology is associated with various biological factors that influence exine stratification and ornamentation.

Our investigation aims to test these hypotheses by re-evaluating pollen exine micromorphology and pollen-wall ultrastructure in Neottieae in the molecular phylogenetic context, using both new observations and a literature review. One goal is to determine whether variation in pollen micromorphology and ultrastructure strictly reflect phylogenetic relationships among species or are significantly correlated with the recurrent shifts between different pollinator groups, from allogamy to autogamy and from autotrophy to obligate mycoheterotrophy. Pollen micromorphology and ultrastructure of Neottieae have been investigated previously (Ackerman and Williams 1980; Burns-Balogh 1983; Schill and Pfeiffer 1977; Hesse et al. 1989) but these comparative studies were performed at a family scale, on acetolyzed material and without the advantage of a well-resolved phylogenetic context.

Materials and methods

Plant material

Ten species were investigated, representing five Eurasian genera of Neottieae (*Aphyllorchis*, *Cephalanthera*, *Epipactis*, *Limodorum*, *Neottia*; Table 1). Pollinia were collected in the field by inserting small sticks into the flowers to attract viscidia. For *Cephalanthera* species, entire anthers were collected, as this genus possesses loose pollinia. Classification of examined species follows Bateman et al. (2005). Pollen terminology is presented according to Punt et al. (2007). Abbreviations: P/E = Polar/Equatorial ratio.

Scanning electron microscopy (SEM)

SEM observations were performed for all species listed in Table 1. Pollinia of mature flowers were either air dried or stabilized in ethanol 50 %, dehydrated in an ethanol series, critical point-dried in liquid CO₂. Specimens were coated

Table 1 Species examined and comparison of pollen characters

Species	Exine micromorphology	Pollen dispersal	Pollinator/visitor	Reproductive strategy	Habitat
<i>Palmorchis</i> ^a	Psilate ^a	Loose tetrads ^a	Parasitic bees (<i>Osiris</i>) ^c	-	Mixed lowland, riverine, cloud forests ^c
<i>Cephalanthera damasonium</i> (Mill.) Druce	Foveolate to perforate	Monads	-	Autogamy, apomixes, no reward? ^c	Woodland and scrubland ^d
<i>Cephalanthera longifolia</i> (L.) Fritsch	Perforate to reticulate	Monads	Bees ^b	Floral mimesis ^b , apomixes ^c , no reward?	Dry to damp, shady or semi-shady ^c
<i>Cephalanthera rubra</i> (L.) Rich.	Perforate to reticulate	Monads	Solitary bees ^b	Floral mimesis ^b , no reward?	Dry to damp, shady or semi-shady ^c
<i>Epipactis helleborine</i> (L.) Crantz autogamous	Reticulate	Tetrads	-	Nectar feeders ^{b, c}	Broad-leaves and mixed woodland, dense or open ^d
<i>Epipactis helleborine</i> (L.) Crantz allogamous	Reticulate	Tetrads	Wasps ^{b, c}	Pheromone-alarm mimicry ^c	Broad-leaves and mixed woodland, dense or open ^d
<i>Epipactis purpurata</i> Sm.	Reticulate	Tetrads	Wasps ^{b, c}	Pheromone-alarm mimicry ^c	Shady woodland ^d
<i>Neottia nidus-avis</i> (L.) Rich.	Reticulate	Tetrads	Flies? ^b	Allogamy, nectar feeders ^b	Shady woodland ^d
<i>Neottia (Listera) ovata</i> (L.) Bluff & Fingerh.	Reticulate	Tetrads	Ichneumonid wasps, solitary bees	Nectar feeders ^b	Woodland, scrubland and open grassland ^d
<i>Neottia (Listera) cordata</i> (L.) Rich.	Reticulate ^f	Tetrads ^f	Fungus gnats, wasps ^g	Sapromyophily?, nectar feeders ^g	Coniferous woodland ^g
<i>Limodorum abortivum</i> (L.) Sw.	Reticulate	Monads	<i>Anthidium</i> , <i>Bombus</i> ^b , solitary bees ^c	Allogamy, nectar feeders, floral mimesis ^{b, c} , autogamy ^c	Woodland and scrubland ^d
<i>Aphyllorchis caudata</i> Rolfe ex Downie	Reticulate	Monads	-	-	Broad-leaves woodland ^c
<i>Aphyllorchis pallida</i> Blume	Reticulate	Loose tetrads	-	-	Broad-leaves woodland ^c

Data on pollen structure from this paper, except for *Palmorchis* from Hesse et al. (1989)^a. Information for pollinator, reproductive strategy and habitat from Burns-Balogh et al. (1987)^b, Bateman et al. (2005)^c, van der Cingel (1995)^d, Brodmann et al. (2008)^e, Ackerman and Williams 1980^f, Ackerman and Mesler (1979)^g

En-dash indicates data not available

with approximately 30 nm of gold before SEM examination, either at CISME, Università degli Studi di Napoli “Federico II” using a FEI-Quanta 200 ESEM at an accelerating voltage of 25 kV, or at the Department of Biology of Ludwig-Maximilians-Universität, München, using a LEO 438 SEM at an accelerating voltage of 15 kV.

Transmission electron microscopy (TEM)

The following species were examined using TEM: *Cephalanthera longifolia*, *Cephalanthera rubra*, *Neottia nidus-avis*, *Neottia (Listera) ovata*, *Limodorum abortivum*, *Epipactis helleborine*. Pollinia from mature flowers were fixed in 2.5 % (v/v) glutaraldehyde in 0.1 M phosphate buffer at pH 7.2 for 2 days at 4 °C, post-fixed in 1 % (w/v) OsO₄ for 2 h at 4 °C, and dehydrated through an ethanol series. They were then embedded in Spurr’s resin. Ultrathin sections were stained for 12 min in uranyl acetate, post-stained for 8 min in lead citrate and observed using a EM 208S Philips transmission electron

microscope at an accelerating voltage of 80 kV at CISME, Università degli Studi di Napoli “Federico II”.

Results

Cephalanthera damasonium

Pollen is released as small monads (10–25 µm in diameter), each roughly spheroidal when fully expanded (Fig. 1). The aperture condition is ulcerate. Exine sculpturing is from foveolate to reticulate (Fig. 1). The pollen is tectate-perforate, the tectum covering more than 50 % of the pollen-grain surface.

Cephalanthera longifolia (Fig. 2c–f)

Pollen is released as monads, each 10–25 µm in diameter, subspheroidal (Fig. 1) with a boat-like shape in dry

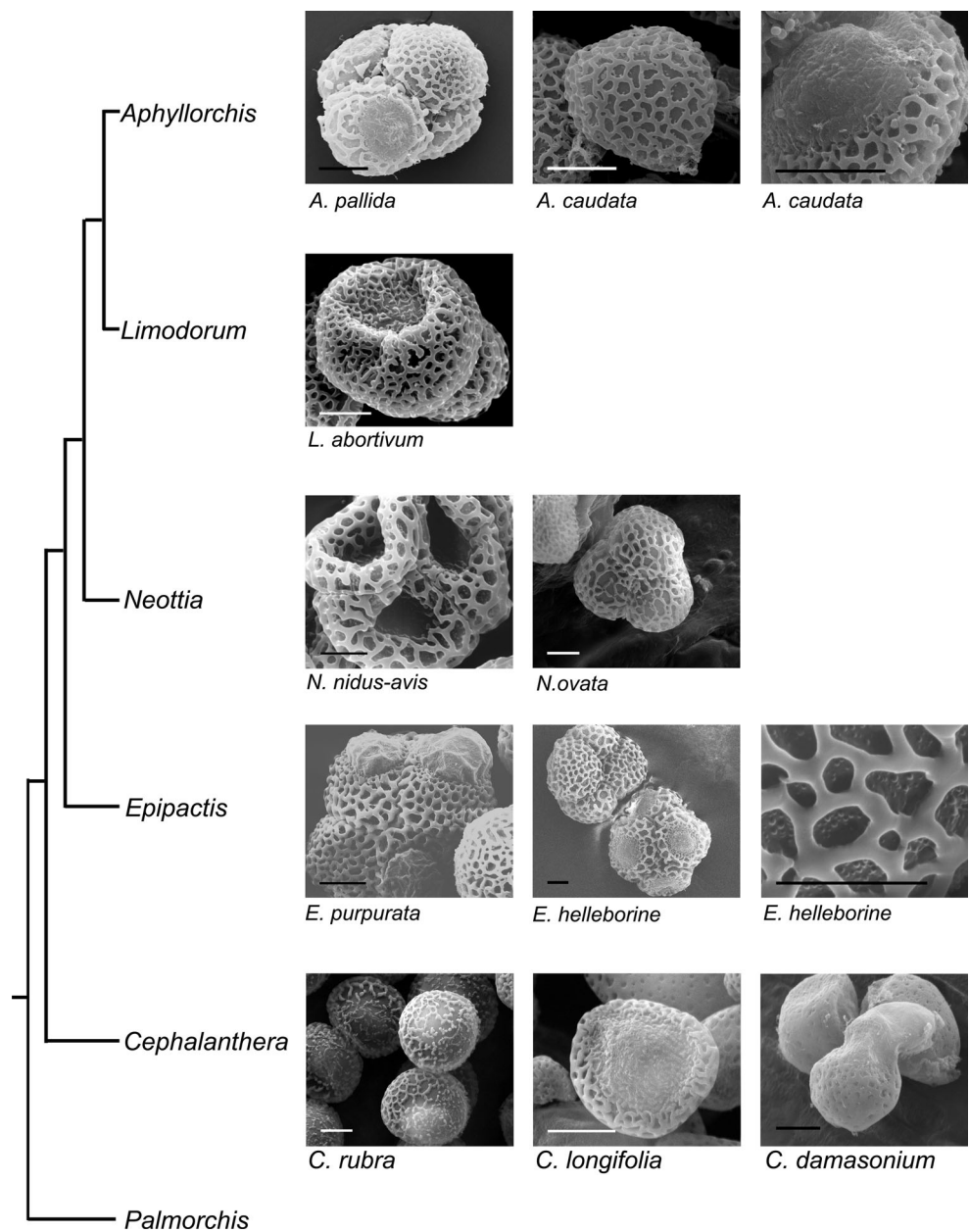
condition. Fully expanded grains are subspheroidal (P/E ratio 0.8); whereas the P/E ratio is 0.4 in collapsed grains. The aperture, ca 15 μm wide, is ulcerate. Exine sculpturing is reticulate (Fig. 1). The pollen ranges from tectate to semitectate, the tectum covering <50 % of the pollen-grain surface. Under TEM, the monads show a pseudo-vacuole underlying a thin cytoplasm at the distal pole (Fig. 2c, d, f); the cytoplasm is rich in rough endoplasmic reticulum (RER). Pollen is dispersed at the two-cell state. The proximal region has a thick tectum, columellate infratectum, distinct and continuous foot layer; lamellate endexine and thin intine (inner pecto-cellulosic wall). The presence of pollenkit marks the free spaces of the infratectum (Fig. 2c, e). The distal region has a discontinuous tectum;

in the apertural region, there is a thick (ca 1 μm) bi-layered intine (Fig. 2c, f). In several (usually collapsed) grains, the distal area shows detachment of the ectexine and thickened intine (Fig. 2d).

Cephalanthera rubra (Fig. 2a, b)

Pollen is released as monads (ca 25 μm in diameter), and is subspheroidal when fully expanded (Figs 1,2a), or boat-shaped in dry condition. The aperture is ulcerate. The pollen ranges from tectate to semitectate; exine sculpturing is reticulate (Figs 1,2a, b). Pollen collected from older flowers shows increased occurrence of collapsed grains (Fig. 2b). Cytoplasm and exine as in *C. longifolia*.

Fig. 1 Diagram of phylogenetic relationships in Neottieae based on Xiang et al. (2012, modified) with SEM pollen images and exine sculpturing arranged accordingly. Scale bars 10 μm



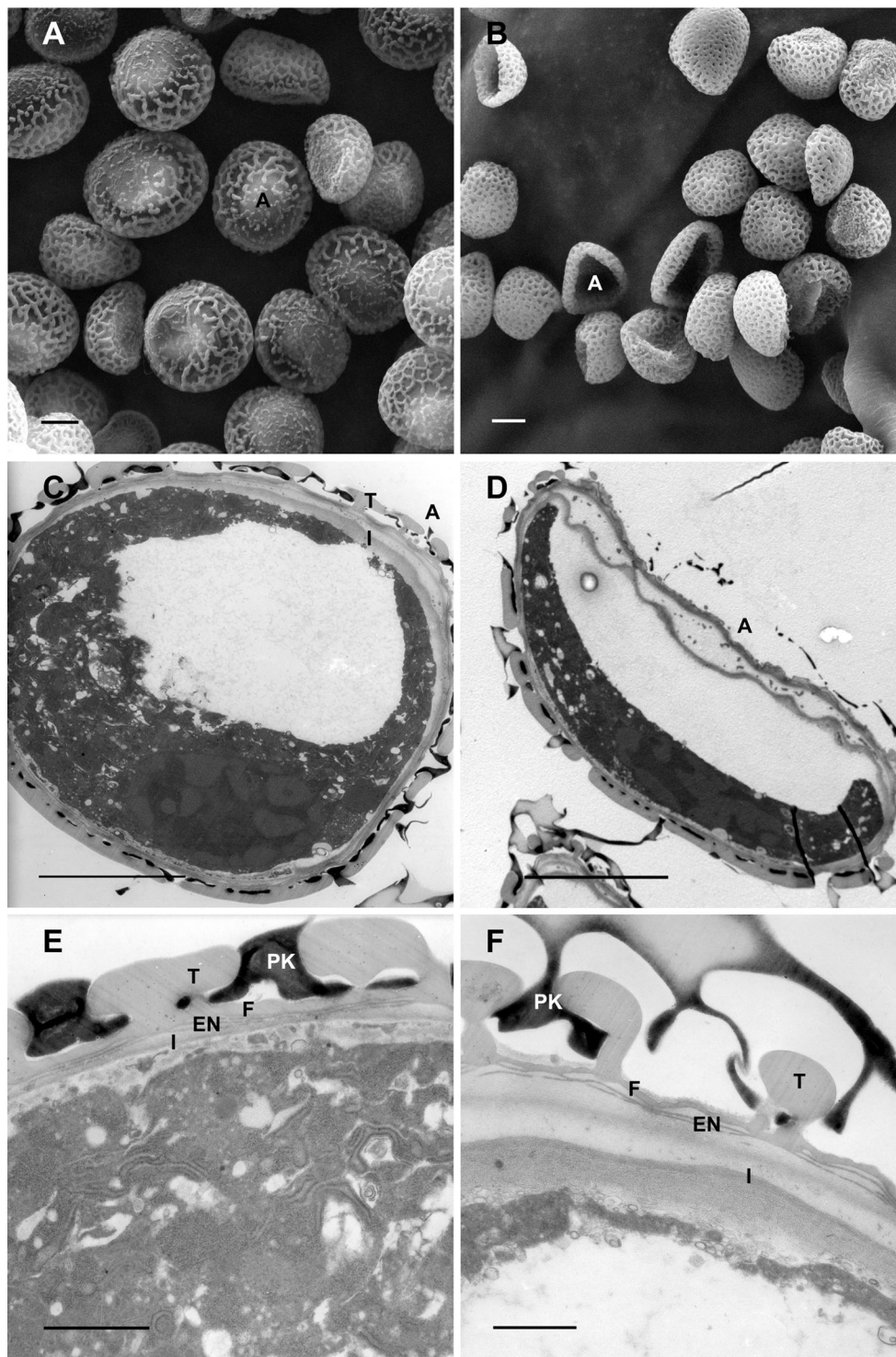


Fig. 2 *Cephalanthera rubra* (a, b), *C. longifolia* (c–f). **a** SEM of monads: distal pole showing ulcus. **b** SEM of collapsed monads. **c** TEM of monad showing spheroidal shape, thick intine in aperture region. **d** TEM of collapsed monad. **e** TEM of proximal region.

f TEM of distal region, with thick intine marking the aperture. *Scale bars A, B 10 μm, C, D 5 μm, E, F 1 μm. A* aperture, *EN* endexine, *F* foot layer, *I* intine, *PK* pollenkitt, *T* tectum

Epipactis purpurata

Pollen is released as acalymmate tetrads, in which the ectexine of each monad is well differentiated but does not form a continuous envelope around the entire tetrad (Punt et al. 2007). The tetrads are each ca 45 μm in diameter. The aperture is ulcerate. The pollen is semitectate and the exine sculpturing is reticulate.

Epipactis helleborine (Fig. 3)

As in *E. purpurea*, pollen is released as acalymmate tetrads, each ca 45 μm in diameter. The aperture is ulcerate. The pollen is semitectate; the exine sculpturing is reticulate. Under TEM, rod-like exine elements shorter than 1 μm (termed micropila) are visible through the reticulum (Fig. 3a). The reticulum differs between allogamous and autogamous plants of *E. helleborine* with respect to the thickness of the ridges that separate the lumina (termed muri; Punt et al. 2007). In allogamous plants, the muri are relatively thin (ca 1 μm), compared with those of autogamous plants (ca 1.6 μm). The pollen is semitectate. Under TEM, the tectum appears discontinuous; the columellae are irregular in shape and length (some related to micropila); the foot layer is distinct and continuous. In the apertural region, the exine is reduced, the endexine is compressed and fragmented, and the intine is thickened (up to 2 μm) and channeled (Fig. 3b). In proximal regions, the tectum is reduced, columellae are irregular in shape and length, the foot layer is continuous, the endexine is lamellate and uncompressed, and the intine is ca 1 μm thick and channeled (Fig. 3b, c, d). The cytoplasm contains several tiny vesicles. Pollen is released at the two-cell stage.

Neottia nidus-avis (Fig. 4a, b)

Pollen is released as acalymmate tetrads, each ca 50 μm in diameter. The aperture is ulcerate. Exine sculpturing is reticulate. Under SEM, rod-like exine elements shorter than 1 μm (micropila) are visible through the reticulum (Fig. 4a). The pollen is semitectate. TEM observations show a discontinuous tectum; columellae are irregular in shape and length (some resembling micropila) adjacent to the thin foot layer. The endexine is lamellate, and the intine is bi-layered (Fig. 4b). In the apertural region, the ectexine is reduced to small, rounded elements of sporopollenin, the endexine lamellae are compressed and the intine is bi-layered. The proximal faces have a granulated exine, a lamellate endexine and a thin intine. The exine sporadically binds together opposite members of a tetrad. Occasional remnants of cytotoxic channels are present. The cytoplasm is rich in RER and small vesicles (0.5–1 μm).

Neottia (Listera) ovata (Fig. 4c, d)

Pollen is released as acalymmate tetrads, each ca 43 μm in diameter. The aperture is ulcerate. Exine sculpturing is reticulate; micropila are visible under SEM (Fig. 1). The pollen is semitectate. Under TEM, the tectum appears reduced; the columellae are irregular in shape and length, immersed in a thick layer of granular material or connected to a thin foot layer that is almost indistinguishable from endexine. The endexine is lamellate and expanded; the intine is bi-layered. The aperture has a highly reduced exine, reduced or absent granular material, compressed endexine lamellae, and bi-layered intine (Fig. 4c). The proximal sides have a granulated exine. Occasional remnants of cytotoxic channels are present (Fig. 4c, d). The exine sporadically binds together opposite members of the tetrad. The cytoplasm is rich in RER and small vesicles. Pollen is released at the two-cell stage.

Limodorum abortivum (Fig. 5)

Pollen is released as monads, and is subspheroidal when expanded (P/E ratio 0.8). Monads are ca 30 μm in diameter. The aperture is ulcerate. Exine sculpturing is reticulate; micropila are not visible (Fig. 5a). The pollen is semitectate. Under TEM, the tectum is reduced, the columellae are irregular in shape, a foot layer is barely discernible, the endexine is lamellate, and the intine is channeled (Fig. 5b, c). The apertural area is marked by an extremely reduced or absent ectexine, compressed endexine, and thick, channeled intine (Fig. 5d). The cytoplasm contains several small vesicles. Pollen is released at the two-cell stage.

Aphyllorchis pallida (Fig. 1)

Pollen is released as loose tetrads, each ca 36 μm in diameter. The aperture is ulcerate. The pollen is semitectate. Exine sculpturing is reticulate, and micropila are visible through the reticulum.

Aphyllorchis caudata (Fig. 1)

Pollen is released as monads, each ca 28 μm in diameter. The aperture is ulcerate. The pollen is semitectate. Exine sculpturing is reticulate; micropila are not visible.

Discussion

Our SEM analyses show a diverse range of exine micro-morphology in Neottieae, ranging from tectate to semitectate (perforate-reticulate) in *Cephalanthera* species and

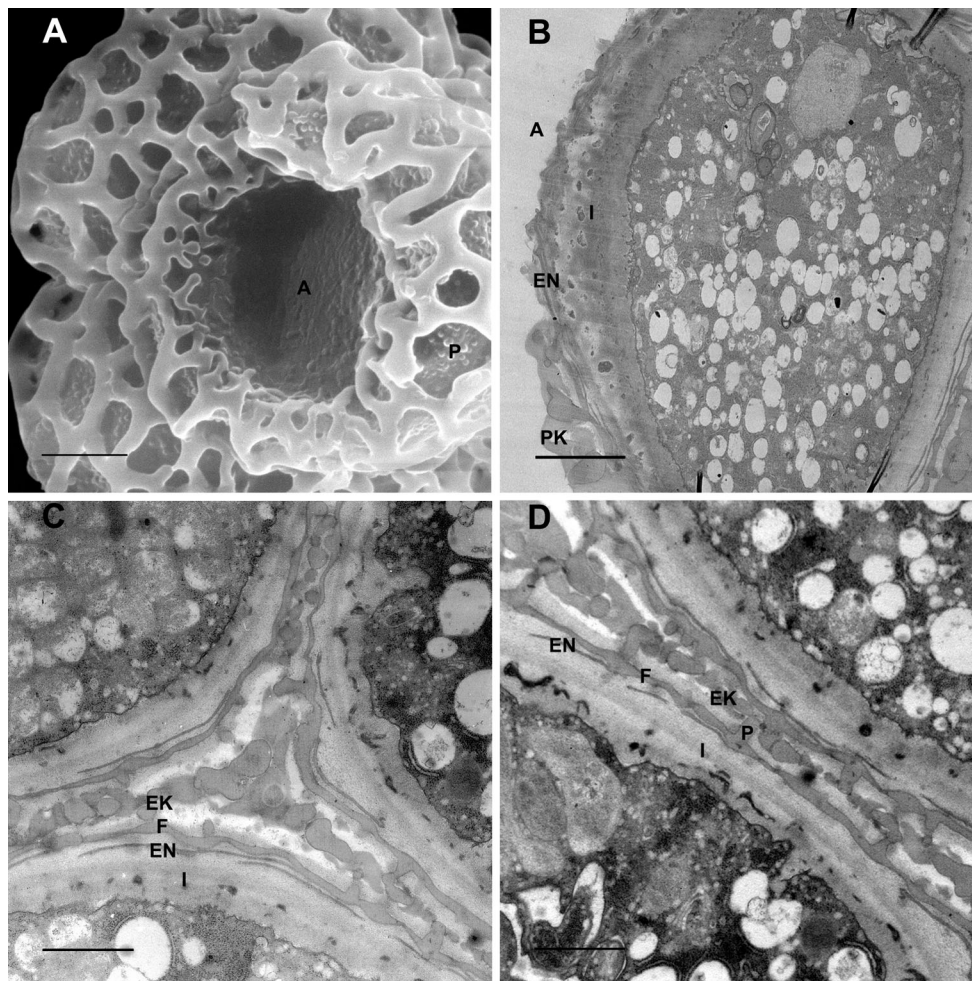


Fig. 3 *Epipactis helleborine*. **a** SEM of tetrad showing aperture region and reticulate exine; micropila visible through the reticulum. **b** TEM of aperture region with reduced exine, fragmented endexine, thickened channeled intine. Cytoplasm shows several small vesicles. **c** TEM of proximal poles with bi-layered channeled intine, scanty

lamellate endexine irregular foot layer and granular ektexine. **d** TEM of proximal faces with bi-layered channeled intine, scanty lamellate endexine, irregular foot layer. Scale bars A 5 μm , B 2 μm , C, D 1 μm . A aperture, EK ektexine, EN endexine, F foot layer, I intine, P micropila, PK pollen kitt, T tectum

semitectate (reticulate) in the clade that includes *Aphyllorchis*, *Epipactis*, *Limodorum* and *Neottia* (Table 1). Hesse et al. (1989) reported tectate (psilate) pollen in an unidentified species of *Palmorchis*, a tropical American genus that was placed as sister to all other Neottieae in molecular analyses (e.g. Freudenstein et al. 2004; Xiang et al. 2012). Thus, if this topology is confirmed, it appears that tectate pollen represents the ancestral condition in Neottieae (Fig. 1), though unfortunately the mycoheterotrophic genus *Thaia* is palynologically and molecularly sparsely studied. Moreover, our TEM observations demonstrate a distinct foot layer in *Cephalanthera* and *Epipactis* species (Figs. 2, 3), whereas a reduced foot layer characterizes the more derived genera *Neottia* and *Limodorum* (Figs. 4, 5), supporting a putative trend toward exine reduction. No data are at the moment disposable regarding *Aphyllorchis* exine ultrastructure. Our results are in direct

contrast with the evolutionary pattern from semitectate to tectate exine proposed by Burns-Balogh (1983) for Neottieae (as Neottioideae), but in agreement with a trend from tectate pollen in the basal *Cephalanthera* to semitectate pollen in advanced “*Listera*” and *Neottia* suggested by Ackerman and Williams (1980).

Orchid pollen shows diverse types of aggregation from a sticky smear of monads in most Cypridodioideae to various forms of pollinia with monads or tetrads in more derived orchid clades (Harder and Johnson, 2008). However, based on our observations and those of Hesse et al. (1989) on *Palmorchis*, pollen aggregation in Neottieae shows a relatively chaotic distribution of these character states, involving recurrent reversals between tetrads and monads (Fig. 1, Table 1).

One question relates to a possible correlation between tectum reduction and the presence of minute rod-like exine

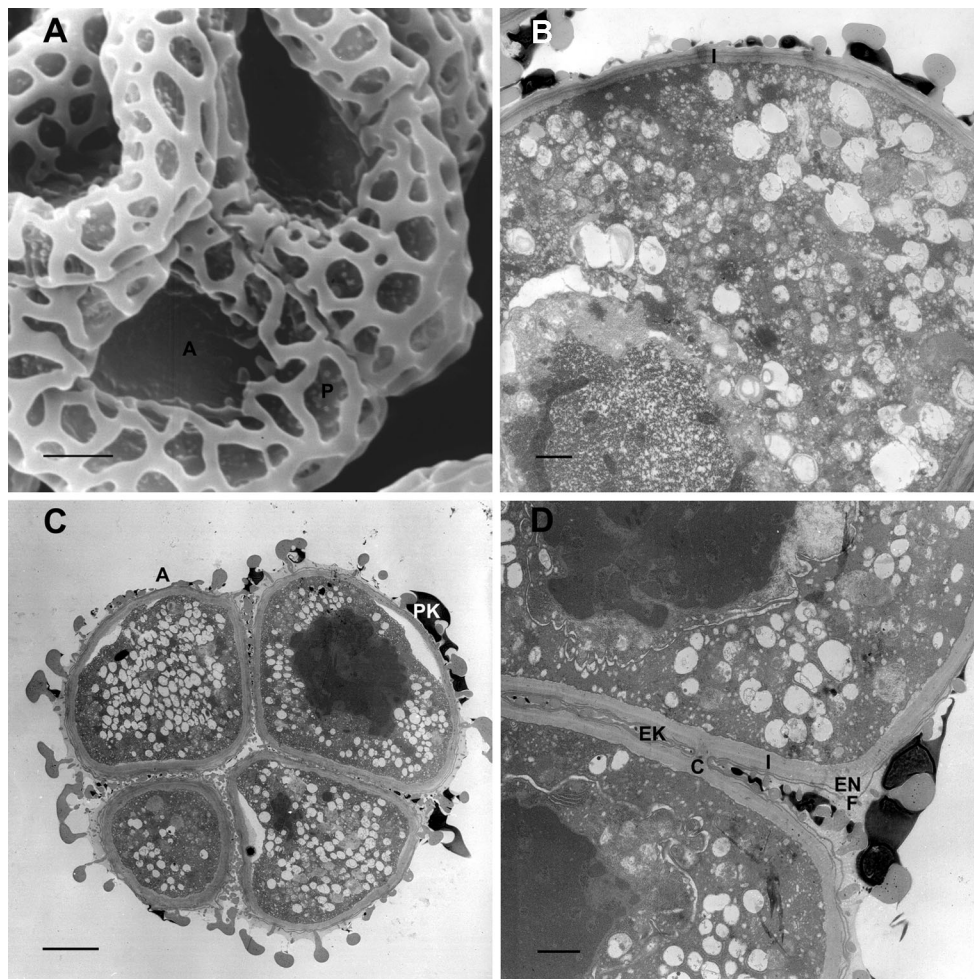


Fig. 4 *Neottia nidus-avis* (a, b), *N. ovata* (c, d). **a** SEM of tetrad showing reticulate exine, micropila visible through the reticulum. **b** TEM of aperture region with reduced exine. **c** TEM of tetrad showing uniformly thick, channeled intine, light granular material

including sporopollenin elements in non-apertural region. **d** TEM detail of tetrad showing residues of cytomictic channel. *Scale bars* A, C 5 μ m, B, D 1 μ m. A aperture, EK ektexine, EN endexine, F foot layer, I intine, P micropila, PK pollenkitt, C cytomictic channel

elements (columellae and micropila) on the one hand, and the recurrent shift between monads and tetrads on the other. Although relatively rarely observed, reversals from tetrads to monads are known (Walker and Doyle 1975; Freudenstein 1999; Doyle et al. 2004). In *Cephalanthera*, which is characterized by monads, micropila are apparently absent and the exine is tectate (Figs. 1, 2). In contrast, in *Epipactis*, which disperses pollen as tetrads, the noticeable reduction of the tectum on the proximal faces exposes micropila and columellae (Figs. 1, 3), perhaps increasing the coherence of the tetrads, in combination with the intine. A role of intine in keeping tetrads together was also suggested by Pacini and Hesse (2002). Columellae and micropila are also present in *Neottia nidus-avis* and *Neottia (Listera) ovata*, both of which have tetrads. In these relatively derived species, the ektexine of the proximal faces is reduced to granulate elements; the sporopollenin components of the opposite members of the

tetrad are sometimes merged together, and occasional remnants of cytomictic channels are present (Figs. 1, 4). Both of these factors could help to bind the elements of the tetrad, only a marginal role being played by the intine. At this regard, the calymmate tetrads present in *Neottia (Listera) cordata*, as showed by Ackerman and Williams (1980) in their comparative study, could be explained as a further step in the process of sporopollenin components merging. In support of this hypothesis, pollen is released as monads in *Aphyllorchis caudata*, which lacks micropila, whereas pollen is released as loose tetrads in *A. pallida*, which is micropilate (Fig. 1). In contrast, in *Limodorum abortivum*, which has monads, micropila are not visible under SEM observation, and TEM images show a very reduced ektexine (Figs. 1, 5).

At least for tetrads, the advantages of pollen aggregation are often thought to depend on the prevailing conditions for pollination and/or mating (Harder and Johnson 2008). We

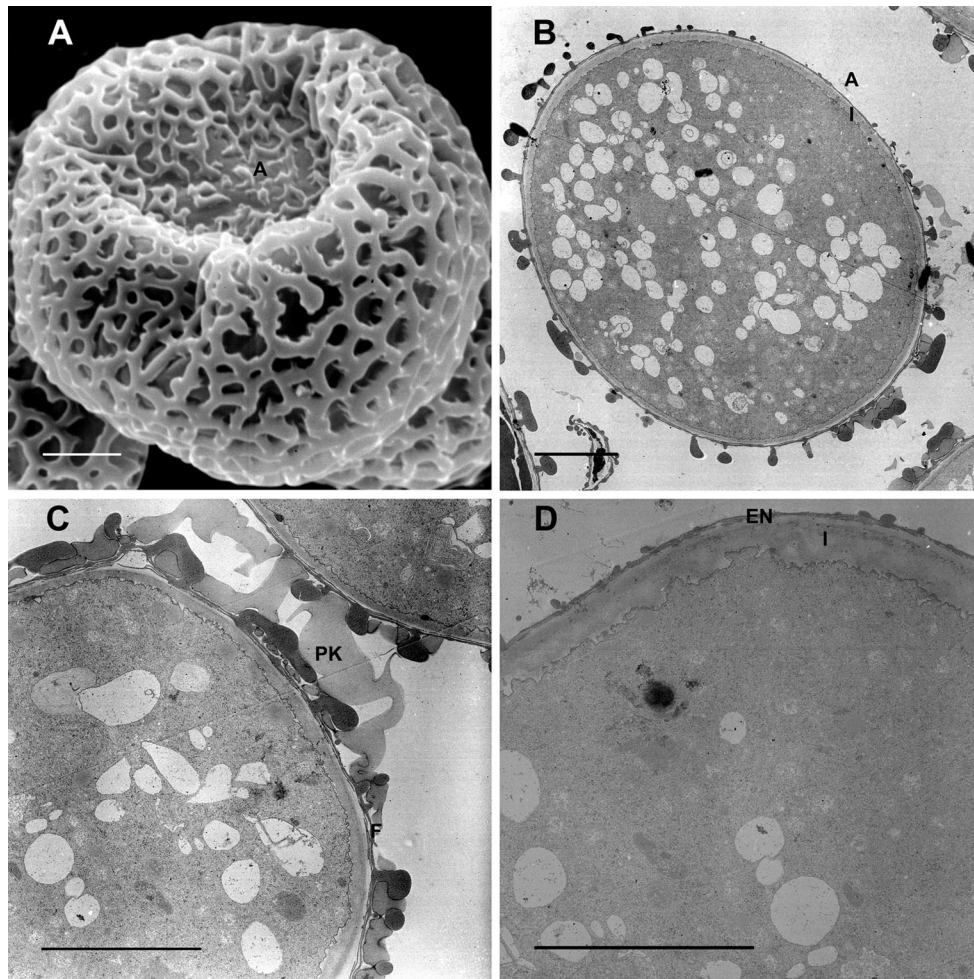


Fig. 5 *Limodorum abortivum*. **a** SEM of monad: distal view showing aperture region and reticulate exine ornamentation. **b** TEM showing monad with several vesicles in the cytoplasm. **c** TEM showing proximal pole, exine with thin foot layer, scanty endexine lamellae.

d TEM of aperture region, showing reduced exine, fragmented endexine, and lightly thickened intine. Scale bars 5 μm . A aperture, EN endexine, F foot layer, I intine, PK pollenkitt

found no obvious link between pollen aggregation and pollination strategy in Neottieae.

Aggregated pollen could be beneficial if it enhances the pollen-transfer efficiency (Harder and Johnson 2008). We found that rewarding neottiid genera are mostly characterized by tetrads. The notable exception is *Limodorum*, which is basically autogamous; loose pollen grains can fall onto the subjacent stigma. *Epipactis*, which disperses pollen as tetrads, is characterized by a mechanism termed pheromone-alarm mimicry (Brodmann et al. 2008), which relies on a few specialized pollinators (wasps). Under the tetrad-pollination hypothesis, *Epipactis* would therefore be expected to have a relatively high pollen-transfer efficiency. In contrast, the related but generalized food-deceptive species of *Cephalanthera* (with monads) would be expected to experience a higher level of pollen loss. However, there is no apparent correlation between pollen aggregation and shifts in mating system observed among

related species. In particular, autogamous lineages are common within genera with either tetrads (*Epipactis*) or monads (*Cephalanthera*).

Different types of pollen aperture presentation can be observed in Neottieae, from a weak reticulum to an ulcerate aperture. As a result, Ackerman and Williams (1980) reported that aperture position is difficult to define in Neottieae, citing the presence of both ulcerates and inaperturate pollen. Similarly, Zavada (1983) reported that pollen in Neottieae can be ulcerate, porate or inaperturate. Our TEM images show the presence of bi-layered and eventually channeled intine, mildly or distinctly thickened in the aperture area. The endexine is visible between the intine and the foot layer as slightly spaced or fragmented lamellae near the aperture and as scanty lamellae in other regions.

The pollen aperture usually acts as the (exclusive) germination site (Hesse et al. 2009). Structurally, the apertures

are typically specialized areas where the exine is thinner and the intine is thickened, channeled or in multiple layers (Furness and Rudall 2003). However, pollen grains of some species lack a distinct aperture, though inaperturate pollen can be either omniaperturate, in which the intine is uniformly thickened and often channeled, or functionally monoaperturate, in which the intine has localized thickenings (Furness and Rudall 1999). Our observations show that the pollen of Neottieae represents a functionally monoaperturate type, because localized “cryptic” apertures are present. Inaperturate pollen could increase germination efficiency (Furness and Rudall 1999) and typically occurs in environments where pollen is not subject to severe desiccation and, thus, may remain viable also with a reduced exine (Furness and Rudall 1999). This ecological constraint appears to hold true for Neottieae, which generally do not occur in either dry or seasonally very dry habitats.

Overall, our results indicate that pollen micromorphology is not influenced by shifts in mating system (allogamy to autogamy) or transitions from autotrophy to heterotrophy in Neottieae. Indeed, in comparing allogamous and autogamous *E. helleborine*, only an increment of tectum coverage was observed in the autogamous lineage. Similarly, no significant difference in exine micromorphology was found between the photosynthetic *Neottia ovata* and the mycoheterotrophic *N. nidus-avis*.

In *Cephalanthera longifolia* and *C. rubra*, pollen grains readily collapse; only careful critical-point drying allows the maintenance of a subspheroidal shape, and the proportion of collapsed monads increases with flower age (Fig. 2a, b). Under TEM, the distal apertural regions are marked by slightly uncompressed endexine lamellae, a thick bi-layered intine and reduced ectexine (Fig. 2c, f). The cytoplasm is marked by a pseudo-vacuole that anticipates the cytoplasm collapse that is observed in *C. longifolia* (Fig. 2d) when the aperture is complete. The presence of this pseudo-vacuole in *C. longifolia* and *C. rubra* suggests that it was not an artefact resulting from suboptimal fixation quality.

Certain types of recalcitrant or desiccation-sensitive pollen die rapidly after anther opening or after pollen dispersal because they lack homeostatic mechanisms for maintaining a constant water content (Franchi et al. 2011). The monads of *Cephalanthera* show some characteristics typical of recalcitrant pollen, such as absence of furrows and rapid water loss, yielding frequent cup-shaped grains. Exine reduction at the broad aperture (Figs. 1, 2c, d, f) also results in loss of pollenkit and renders the monads more unstable. Pollenkit is a sticky material produced by the tapetum; it typically increases resistance to desiccation and conveys sporophytic incompatibility substances derived from the tapetum (Blackmore et al. 2007). As recalcitrant

pollen only survives for a few hours after anther opening, we speculate that in *Cephalanthera*, which can occur in relatively dry habitats, the shift from an allogamous to an autogamous or even cleistogamous mating system could be related to this structural constraint (Table 1), resulting in reduction of water loss and damage induced by air exposure after anther opening.

Acknowledgments We thank Richard Bateman for useful comments on the manuscript. We also thank two anonymous reviewers for their comments and suggestions that significantly improved the revised MS.

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