PHYLOGENETIC RELATIONSHIPS IN NARTHECIACEAE (DIOSCOREALES), WITH FOCUS ON POLLEN AND ORBICULE MORPHOLOGY

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ABSTRACT. — A molecular phylogenetic analysis of Nartheciaceae is presented, with nine species of the family's five genera. The main phylogenetic findings are: (a) *Nietneria* and *Narthecium* are placed in a clade sister to *Lophiola*; (b) sister to the *Lophiola-Narthecium-Nietneria* clade is a clade formed by *Aletris* and the monospecific *Metanarthecium*; (c) the inclusion of *Metanarthecium luteo-viride* in *Aletris,* as proposed by several authors, is well supported. The pollen and orbicule morphology of representatives of five genera is described. The results underline a close relationship between *Nietneria, Narthecium*, and *Lophiola* and confirm the previously reported observations of *Metanarthecium* pollen and the types of sexine ornamentation in *Aletris*. Pollen grains of *Nietneria* are monosulcate with a microreticulate sexine, confirming a close relationship with *Lophiola* and *Narthecium.* Spherical smooth-surfaced orbicules were observed in all genera of Nartheciaceae and the presence of a circular perforation on the orbicule surface is potentially synapomorphic for the family.

KEY WORDS. — Dioscoreales, Nartheciaceae, *Nietneria*, molecular phylogeny, orbicules, pollen morphology.

INTRODUCTION

The family Nartheciaceae Fr. ex Bjurzon comprises five genera (CADDICK *et al.* 2002a). Nartheciaceae are perennial herbs with short tuberculate or creeping rhizomes, erect stems and terminal spikes or racemes. They occur mostly in wet habitats, such as swamps and bogs (TAMURA 1998). *Aletris* L. (ca. 33 species) is the largest genus of Nartheciaceae, with representatives in Eastern Asia and North America (TAMURA 1998). *Narthecium* Huds. (ca. eight species) has a disjunct distribution and occurs in temperate regions of North America, Europe and Asia, but is absent from China (TAMURA 1998). *Lophiola* Ker Gawl. (one species), easily recognizable by its whitish woolly inflorescence, grows in acid, pine barren bogs from New Jersey to Florida and Nova Scotia. In some treatments more than one *Lophiola* species are recognized based on morphological differences between specimens from different populations (ROBERTSON 1976, DAHLGREN *et al.* 1985, TAMURA 1998). This morphological variation may be due to ecological conditions (ROBERTSON 1976, ZAVADA *et al.* 1983, AMBROSE 1985). *Lophiola* was previously linked with

Lachnanthes Elliot and placed in Haemodoraceae (GEERINCK 1969, HUTCHINSON 1973, ORNDUFF 1979). However, this position proved to be incorrect (SIMPSON 1981, SIMPSON & DICKISON 1981, ZAVADA *et al.* 1983) and based on several morphological characters *Lophiola* was transferred to Nartheciaceae (AMBROSE 1980, 1985, GOLDBLATT 1995, ZOMLEFER 1997, 1999), a placement later confirmed by the molecular analyses of CADDICK *et al.* (2000, 2002b), FUSE & TAMURA (2000) and TAMURA *et al.* (2004). *Nietneria* Klotzsch & R.Schomb. is restricted to savannas from highlands of Venezuela and Guyana. Two *Nietneria* species are delimited (FRAME 2004, MAAS & WESTRA 2005), but some authors recognize only one species (DAHLGREN *et al.* 1985, TAMURA 1998). The fifth genus of Nartheciaceae, *Metanarthecium* Maxim., contains a single species, *M. luteo-viride,* which is endemic to Japan where it grows in subalpine meadows. Recently, some authors included *M. luteo-viride* in *Aletris*, on the basis of morphological similarities and a shared basic chromosome number, x = 13 (TAMURA 1998, ZOMLEFER 1999). This was supported by the combined molecular and morphological analysis of CADDICK *et al.* (2002b), which grouped *Metanarthecium* with *Aletris farinosa* with high bootstrap support. However, the molecular analysis of FUSE & TAMURA (2000) and TAMURA *et al.* (2004) placed *Metanarthecium* sister to *Narthecium* and *Lophiola*, with no bootstrap support. TAMURA *et al.* (2004) presented evidence for the inclusion of *Isidrogalvia* Ruiz & Pav. (Tofieldiaceae) in Nartheciaceae. In their combined *matK-rbcL* strict consensus tree *Isidrogalvia schomburgkiana* was sister to *Narthecium* with maximum bootstrap support. *Isidrogalvia*, which is indigenous to South America, consists of six species (CRUDEN 1991, CRUDEN & DORR 1992). However, a recent study with *matK* data on a broad Tofieldiaceae sampling, including three *Isidrogalvia* species, confirmed the placement of *Isidrogalvia* in Tofieldiaceae with maximal bootstrap support (AZUMA & TOBE 2005).

The circumscription and affinities of the family have been subject to change. Most authors previously included the genera of Nartheciaceae in a broadly defined Melanthiaceae (TAMURA 1998). As a result, *Narthecium, Aletris, Metanarthecium, Lophiola* and *Nietneria* were linked with *Tofieldia, Pleea, Isidrogalvia, Harperocallis, Japanolirion* and the saprophytic genus *Petrosavia* (AMBROSE 1980, TAMURA 1998). DAHLGREN *et al.* (1985) also included *Heloniopsis, Helonias* and *Ypsilandra* together with the five Nartheciaceae genera of the present treatment in a tribe Narthecieae, in the family Melanthiaceae. Several recent molecular analyses independently indicated that Nartheciaceae are related to Burmanniaceae and Dioscoreaceae, and therefore, the family should be included in Dioscoreales (APG I 1998, CADDICK *et al.* 2000, CHASE *et al.* 2000, FUSE & TAMURA 2000, CADDICK *et al.* 2002b, APG II 2003, TAMURA *et al.* 2004, CHASE *et al.* 2006, GIVNISH *et al.* 2006, MERCKX *et al.* 2006).

Some of the studies mentioned above also give an indication of intergeneric relationships in Nartheciaceae. In the analyses of CADDICK *et al.* (2002b), FUSE & TAMURA (2000) and TAMURA *et al.* (2004), *Lophiola* and *Narthecium* form a clade with high bootstrap support, although in these studies only one species from each genus was sampled. In the combined molecular (*rbcL*, *atpB* and 18S rDNA) and morphological study of CADDICK *et al.* (2002b) the *Lophiola-Narthecium* clade was sister to a strongly supported *Aletris farinosa-Metanarthecium luteo-viride* clade. This contradicted the analyses of FUSE & TAMURA (2000) based on *matK* sequence data. Their results placed *Metanarthecium* as sister to *Lophiola* and *Narthecium* instead of *Aletris. Nietneria* was not incorporated in any molecular study.

Although Dioscoreales are clearly defined in recent molecular studies, their morphology is heterogeneous. Recent morphological research in Dioscoreales focused mainly on microsporogenesis and pollen morphology (CADDICK *et al.* 1998, SCHOLS *et al.* 2001, 2003). These morphological features provided taxonomically useful characters, particularly at the genus level. SCHOLS *et al.* (2001, 2003) showed that pollen characters are valuable for delimiting sections within *Dioscorea* (Dioscoreaceae) and are, moreover, highly congruent with molecular data (SCHOLS *et al.* 2005). CADDICK *et al.* (1998) studied microsporogenesis

and pollen morphology in Dioscoreales and allied taxa. They concluded that 'both microsporogenesis and pollen morphology provide characters which are taxonomically useful at the genus level, especially in Dioscoreaceae and its close allies, but probably less informative at higher taxonomic levels, although this has yet to be tested in morphological and combined morphological-molecular analyses' (p. 329). Since these and previous studies contained an incomplete sampling of Nartheciaceae, we examined the pollen morphology of representatives of five genera of Nartheciaceae. Previous pollen morphological observations of Nartheciaceae were published by ZAVADA *et al.* (1983), TAKAHASHI & KAWANO (1989) and CADDICK *et al.* (1998). According to their results the pollen grains of *Lophiola, Narthecium, Metanarthecium* and *Aletris* are monosulcate and sexine ornamentation varies from microreticulate in *Narthecium* and *Lophiola,* reticulate in *Metanarthecium* to perforate, reticulate and gemmate in *Aletris.* Pollen grains of *Nietneria* have not been examined before.

We also investigated the presence of orbicules on the inner anther locule wall of Nartheciaceae. Orbicules are small sporopollenin bodies that are usually produced by secretory tapeta. Since secretory tapeta are present in all three families of Dioscoreales, orbicules likely occur in Burmanniaceae and Nartheciaceae (FURNESS & RUDALL 1998, SCHOLS 2004). The function of orbicules remains obscure. Although they occur throughout the angiosperms and their presence often offers systematically valuable information, though data on their distribution is incomplete (HUYSMANS *et al.* 1998, 2000).

The objective of this study was to investigate intergeneric relationships in Nartheciaceae using plastid *atpB-rbcL* spacer, *trnL* intron and *trnL-trnF* intergenic spacer and nuclear 18S rDNA sequences from nine selected taxa. Both maximum parsimony and Bayesian analyses were used for phylogenetic reconstruction. Additionally, pollen and orbicules of 11 representatives of Nartheciaceae were observed using scanning electron microscopy and the results are discussed within the present phylogenetic framework.

MATERIALS AND METHODS

We inferred the intergeneric relationships of Nartheciaceae using sequences from the plastid *atpB-rbcL* spacer, the *trnL* intron and *trnL-trnF* intergenic spacer region. We selected these particular noncoding DNA regions because they evolve relatively rapidly and are, therefore, effective in resolving phylogenetic questions at the genus level (e.g. MANEN & NATALI 1996, CHANDERBALI *et al.* 2001). In addition the nuclear 18S rDNA region was sequenced for all samples. This generally conservative DNA region has proven to be very useful for phylogeny inference in Dioscoreales (CADDICK *et al.* 2002b, MERCKX *et al.* 2006). Our sampling includes species of all genera of Nartheciaceae. *Lophiola* specimens from two different populations, one from Florida and one from Nova Scotia, are present. In order to distinguish these specimens, we labelled the specimen from Nova Scotia as *L. americana.* According to most authors *L. americana* is a synonym of *L. aurea* (ROBERTSON 1976, ZAVADA *et al.* 1983, AMBROSE 1985)*.* Since Dioscoreaceae and Burmanniaceae are most likely sister to Nartheciaceae (CADDICK *et al.* 2002a, MERCKX *et al.* 2006), we selected species of *Dioscorea* and *Burmannia* as outgroups.

MOLECULAR TECHNIQUES

Total genomic DNA was extracted from silica-dried material using a modified CTAB protocol (DOYLE & DOYLE 1987, CHASE & HILLS 1991). Problematic samples were extracted with the DNeasy Plant Mini Kit (QIAGEN, Venlo, the Netherlands) or the Puregene DNA Extraction Kit (Gentra Systems, Minneapolis, USA). Some extractions were additionally purified with QIAquick columns (QIAGEN) following the PCR clean-up instructions. A number of additional DNA extractions were purchased from the Royal Botanic Gardens Kew DNA Bank. Table 1 lists the samples used in this study.

All amplifications were performed with a GeneAmp 9700 PCR systems (Applied Biosystems, Foster City, USA). Each PCR reaction (25 µl) contained 5 µL MilliQ H_2O, 2.5 µL 10x PCR Buffer (Genecraft, Lüdinghausen, Germany), 5 µL forward primer (2.2 µM), 5 µL reverse primer (2.2 µM), 2.5 µL dNTP (10 mM), 0.2 µL BioTerm Taq polymerase (5U µL^{-1}) (Genecraft) and 5 µL template DNA (1/10 dilution).

The plastid *atpB-rbcL* spacer region was amplified with the primers *atpB*-1 and *rbcL*-1 from CHIANG *et al.* (1998). For some samples, however, we modified

Table 1. GenBank accession numbers and voucher data for taxa included in the DNA analyses.

Taxon	Accession number atpB-rbcL spacer	Accession nunmber trnL-trnF spacer	Accession number trnL intron	Accession number 18S rDNA	Voucher
Nartheciaceae					
Aletris farinosa L.	EU186229	EU186242	EU186254	EU186221	Chase 105 (NCU) RBG Kew DNA Bank (MWC105)
Aletris foliosa (Maxim.) Bureau & Franch.	EU186230	EU186244	EU186255	EU186222	K. Cameron RBG Kew DNA Bank (MWC1931)
Aletris lutea Small	EU186231	EU186243	EU186256	DQ786092	J. Anderson 35 (LV)
Lophiola americana Wood	EU186224	EU186237	EU186249	EU186218	J. Anderson 36 (LV)
Lophiola aurea Ker Gawl.	EU186225	EU186236	EU186248	DQ786091	R. Newell 23/8 (LV)
Metanarthecium luteo-viride Maxim.	EU186228	EU186241	EU186253	AF309410	Inoue RBG Kew DNA Bank (MWC630)
Narthecium californicum Baker	EU186227	EU186240	EU186252	EU186220	Janeway & Castro 7607 (LV)
Narthecium ossifragum (L.) Huds.	EU186226	EU186239	EU186251	AF309411	A. Jacquemart 46-9 (LV)
Nietneria paniculata Steyerm.	EU186234	EU186238	EU186250	EU186219	O. Hokche & P.J.M. Maas 849 (U)
Dioscoreaceae					
Dioscorea tokoro Makino ex Myabe	EU186233	EU186246	EU186258	DQ786088	V. Merckx 1 (LV)
Dioscorea communis (L.) Caddick & Wilkin	EU186232	EU186245	EU186257	EU186223	V. Merckx 2 (LV)
Burmanniaceae					
Burmannia longifolia Becc.	EU186235	EU186247	EU186259	AF309398	K. Cameron s.n. (NCU) RBG Kew DNA Bank (MWC1930)

these primers (VMATPB: 5'-ACATCTAGTACTGGTC-CAATAA-3' and VMRBCL: 5'-AACACCAGGTTTG-AATCCAA-3'). The thermal cycling protocol comprised 30 cycles of 30s at 94°C, 45s at 51°C and 45s at 72°C, followed by 7 minutes extension at 72°C.

All PCR products were sequenced directly after cleaning with QIAquick purification columns (QIA-GEN). Sequencing reactions were run on an ABI 310 automated sequencer (Applied Biosystems). Assembling and editing of sequences were done using the Staden Package (STADEN *et al.* 1998). Sequences for each genetic region were aligned separately with CLUSTALX (THOMSON *et al.* 1997). The alignment was adjusted manually using MACCLADE 4.04 for Mac OS X (MADDISON & MADDISON 2001). Sequences are available from GenBank (accessions EU186218-EU186256), and the aligned Nexus data files are available from the corresponding author.

PHYLOGENETIC ANALYSES

Maximum parsimony analyses on the three datasets were performed with PAUP* version 4.0b10 (SWOFFORD 2002) using the branch and bound search algorithm with the MulTrees option on. Bootstrap analyses were performed with 10.000 replicates using the heuristic search option, TBR and MulTrees on, with 1.000 stepwise-addition replicates per bootstrap replicate and holding 50 trees at each step.

We used MRBAYES 3.1.2 (HUELSENBECK & RON-QUIST 2001, RONQUIST & HUELSENBECK 2003) for Bayesian inference of phylogenies from all datasets. Models of evolution were compared on our data using the Akaike information criterion (AIC) as implemented by MODELTEST 3.06 (POSADA & CRANDALL 1998, 2001). MODELTEST selected the TVM+G model of evolution for the *atpB-rbcL* spacer dataset, HKY+G for the *trnL* intron data, and TrN+I for the *trnL-trnF* intergenic spacer and 18S rDNA data. Because the models TVM and TrN are not implemented in MRBAYES, the GTR model was used instead. In the combined Bayesian analysis, a mixed-model approach was used. The combined data were partitioned and the same models of evolution were used on the partitions as selected for the single analyses. For each analysis four chains (one cold, three heated) were started from random trees and run for 2×10^6 generations. Every 100 generations a tree was saved. Five thousand (25%) of the resulting 20 001 trees were discarded (burn-in) before the majority rule tree was computed using PAUP* 4.0b10.

POLLEN AND ORBICULE MORPHOLOGY

For all observations, dried material from BR and U was used (Table 2).

Mature flowers were hydrated in Agepon, a wetting agent, and dehydrated through an acetone series. Because pollen of Nartheciaceae is relatively thin-walled and therefore not resistant to acetolysis, specimens were subjected to critical point drying (CPD). Following CPD, with a Balzers CPD 030 apparatus, anthers were removed and pollen grains were mounted on a stub with carbon strip tape and coated with gold using a SPI-Module Sputter Coater (SPI Supplies). Specimens were examined on a JEOL JSM 5800 scanning electron microscope.

For each species, the longest axis (LA), the shortest axis (SA), perforation size, perforation density and orbicule diameter were measured on digital SEM images using CARNOY 2.1 for Mac OS X (SCHOLS *et al.* 2002). Terminology follows PUNT *et al.* (1994).

Table 2. Voucher information for the species used for pollen morphological observations.

| Species	Voucher
Aletris farinosa L.	C. Bell 4164 (BR)
Aletris lutea Small	Anonymous 58 (BR)
Aletris pauciflora (Klotzsch) Hand.-Mazz.	F. Billiet & J. Leonard 6546 (BR)
Lophiola americana Wood	S. Leonard & K. Moore (BR)
Lophiola aurea Ker Gawl.	H. Ahles 56603 (BR)
Metanarthecium luteo-viride Maxim.	M. Togasi 1493 (BR)
Narthecium asiaticum Maxim.	K. Okamoto 776 (BR)
Narthecium californicum Baker	T. Howell (BR)
Narthecium reverchonii Celak.	J. Leonard 5164 (BR)
Narthecium scardicum Kôzanin	P. Frost-Olsen 13746 (BR)
Nietneria corymbosa Klotzsch & R.Schomb.	R. Liesner 21129 (U)

Table 3. Separated and combined molecular data set sizes, phylogenetic utility of the characters and tree statistics.

|	Number of characters	Constant characters	Parsimony uninformative characters	Parsimony informative characters	Number of most parsimonious trees	Length of shortest tree
atpB-rbcL spacer	944	741	113	90	3	250
trnL intron	659	522	68	69	13	154
trnL-trnF spacer	520	328	141	51	1	213
18S rDNA	1699	1594	39	66	1	130
Combined data	3822	3185	361	276	1	755

RESULTS

PHYLOGENETIC ANALYSES

Dataset statistics and the number of most parsimonious trees are given in Table 3. Topologies produced by the maximum parsimony and Bayesian analyses of the separated and combined molecular data are highly congruent (Figs. 1, 2). In what follows branch support is considered 'strong' when more than 85% bootstrap support (BS) and 95% Bayesian posterior probability (BPP) was obtained. 'Moderate' is used for support higher than 75% BS and 85% BPP. Branch support is 'weak' when under 75% BS and 85% BPP. The monophyly of Nartheciaceae is strongly supported in all analyses. Several well-supported clades can be recognized within the family: a moderately to strongly supported *Narthecium-Nietneria-Lophiola* clade consisting of a strongly
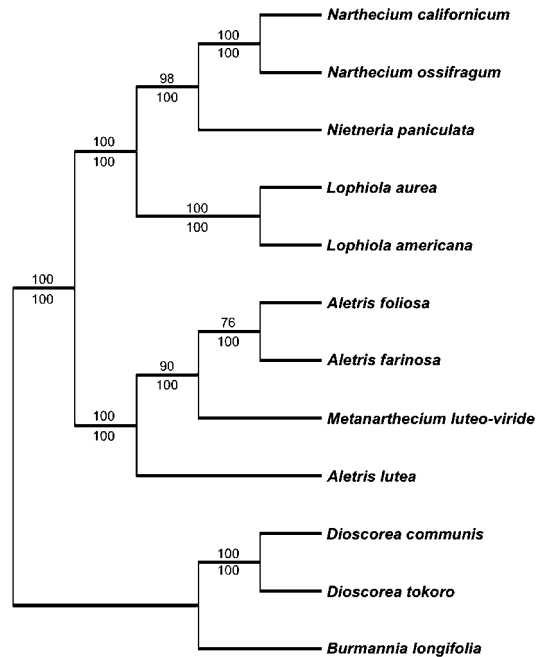
Fig. 2. The single most parsimonious tree obtained from the Maximum Parsimony analysis of the combined molecular data (length = 619 steps; CI = 0.952; RI = 0.918). Bayesian analysis delivered the same topology. Numbers above branches = bootstrap support values; numbers below branches = Bayesian posterior probabilities.
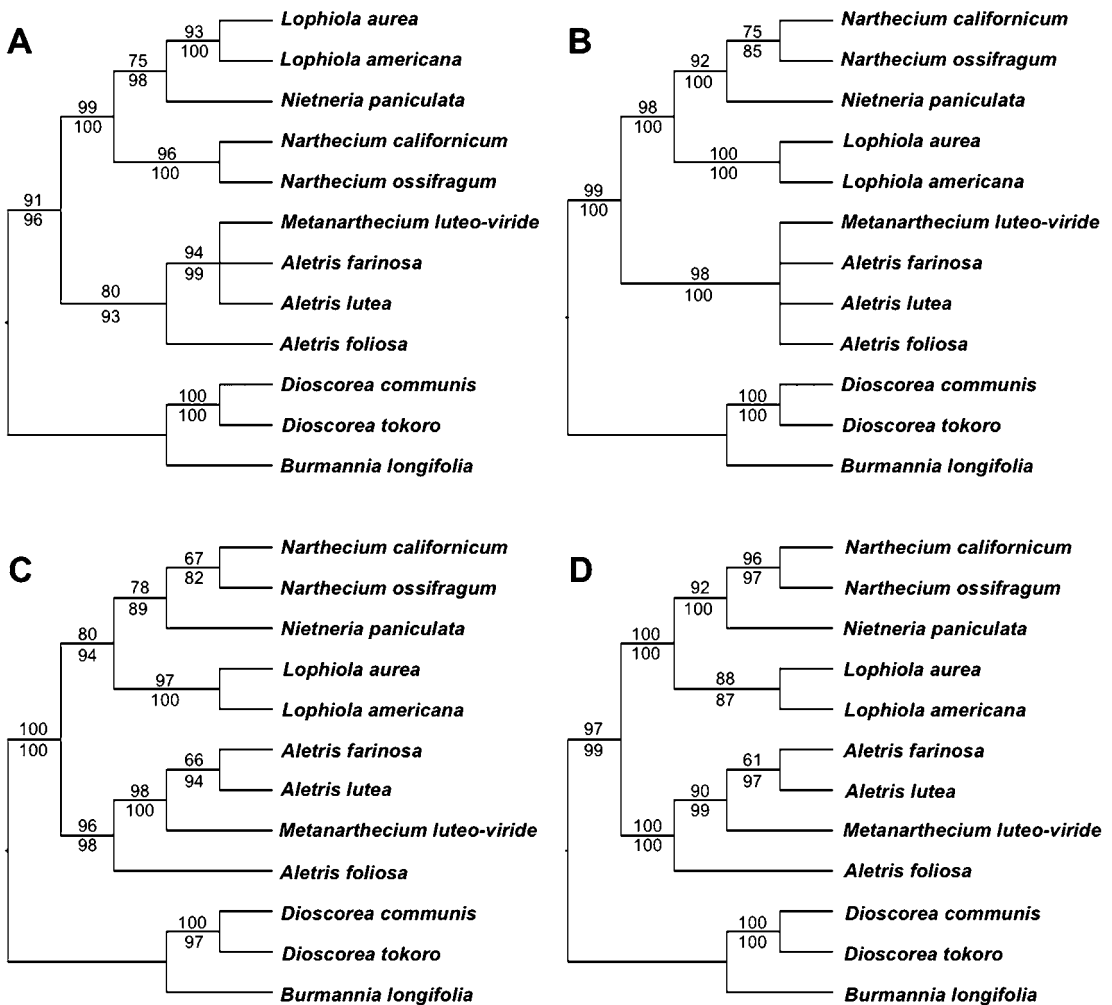
Fig. 1. Comparison of the consensus trees found with Maximum Parsimony and Bayesian analyses on the different datasets. A. Strict consensus tree of three most parsimonious trees from the *atpB-rbcL* spacer dataset (length = 250; CI = 0.940; RI = 0.912). B. Strict consensus of 13 optimal trees from the analysis on the *trnL* intron dataset (length = 154; CI = 0.942; RI = 0.920). C. The single most parsimonious tree found in the analysis on the *trnL-trnF* intergenic spacer sequences (length = 213; CI = 0.972; RI = 0.937). D. The single most parsimonious tree from the analysis of the 18S rDNA data (length = 130; CI = 0.900; RI = 0.920). Numbers above branches = bootstrap support values; numbers below branches = Bayesian posterior probabilities.

supported *Lophiola* clade and a weakly to strongly supported *Narthecium* clade. *Nietneria paniculata* is sister to *Lophiola* in the analysis based on *atpB-rbcL* data, but is sister to *Narthecium* in the other analyses with moderate to strong support.

An *Aletris* clade, including *Metanarthecium,* is present in all topologies with support ranging from moderate in the *atpB-rbcL* spacer analysis to strong in the other analyses. In this clade *Aletris foliosa* is sister to the other *Aletris-Metanarthecium* species, except in the *trnL* intron analysis where it is part of a polytomy. The two other *Aletris* species sampled, *A. farinosa* and *A. lutea,* group together in the *trnL-trnF* intergenic spacer and 18S rDNA topology with weak bootstrap support. Support for this clade is moderate in the combined analysis.

POLLEN MORPHOLOGY (FIGS. 3-10)

Pollen characters are summarized in Table 4. Pollen grains of all specimens are monosulcate. The range of the longest axis (LA) varies from 17.61 µm in *Lophiola aurea* to 32.90 µm in *Aletris pauciflora*. Measurements of the shortest equatorial axis (SA) range from 8.98 µm in *Metanarthecium luteo-viride* to 12.78 µm in *Aletris pauciflora.* SA values might not be as reliable as LA values because the grains tend to collapse inwards along the shortest axis. Sexine ornamentation is microreticulate in *Narthecium* (Figs. 3, 6, 10), *Lophiola* (Fig. 8) and *Nietneria* (Fig. 4), reticulate in *Metanarthecium* (Fig. 9) and perforate (Fig. 5) and gemmate (Fig. 7) in the *Aletris* species examined.

ORBICULES (FIGS. 11-16)

All species examined, except *Aletris farinosa* and *Aletris lutea* where no orbicules were observed*,* possess smooth-surfaced orbicules in the anther locule, ranging in size from 0.48 µm in *Aletris pauciflora* to 1.03 µm in *Nietneria corymbosa* (Table 4)*.* All orbicules are spherical except in *Lophiola aurea,* in which they are more or less doughnut-shaped (Fig. 11). A circular perforation on the orbicule surface occurred in all specimens.

Table 4. Summary of pollen and orbicule characters for all species studied. Abbreviations: #Ap., number of apertures; Micro Ret., micro reticulate; LA, longest equatorial axis; SA, shortest equatorial axis; Perfsize, perforation size; Perf/µm2, mean number of perforations per square micrometer; L, diameter of orbicules.

Species	#Ap.	Ornamentation	LA (µm)[a]	SA (µm)[a]	Perfsize (µm)[a]	Perf²/µm²	L (µm)[a]
Narthecium californicum	1	Micro Ret.	17.6-(19.62)-20.3	8.8-(9.42)-11.0	0.1-(0.33)-0.4	4.85	0.8-(0.93)-1.1
Narthecium asiaticum	1	Micro Ret.	15.3-(18.28)-20.2	7.7-(9.81)-10.5	0.2-(0.41)-0.5	4.80	0.6-(0.84)-0.9
Narthecium reverchonii	1	Micro Ret.	19.8-(22.63)-23.6	9.9-(12.04)-13.9	0.2-(0.37)-0.7	4.30	0.8-(1.02)-1.2
Narthecium scardicum	1	Micro Ret.	21.0-(22.22)-24.2	10.3-(12.54)-14.1	0.2-(0.45)-0.7	2.85	0.7-(0.93)-1.1
Aletris farinosa	1	Perforate	21.7-(23.34)-24.8	8.2-(10.44)-12.7	0.2-(0.51)-0.7	1.38	N.A.
Aletris pauciflora	1	Gemmate	25.5-(32.90)-34.8	10.1-(12.78)-15.6	N.A.	N.A.	0.4-(0.48)-0.5
Aletris lutea	1	Perforate	23.4-(26.84)-29.0	7.8-(10.69)-13.4	0.1-(0.34)-0.5	1.30	N.A.
Lophiola aurea	1	Micro Ret.	16.1-(17.61)-19.8	10.2-(12.39)-14.8	0.1-(0.22)-0.3	6.40	0.8-(1.02)-1.1
Lophiola americana	1	Micro Ret.	16.3-(19.10)-23.7	8.0-(10.58)-11.9	0.1-(0.29)-0.3	5.00	0.6-(0.81)-1.1
Metanarthecium luteo-viride	1	Reticulate	21.2-(22.89)-24.0	6.7-(8.98)-9.7	0.8-(1.39)-2.3	0.76	0.6-(0.74)-0.8
Nietneria corymbosa	1	Micro Ret.	17.1-(19.50)-23.6	9.8-(12.92)-14.8	0.2-(0.38)-0.4	2.97	0.8-(1.03)-1.2

[a] Given as x-(y)-z, with x = minimum, y = mean, z = maximum; N.A.: not applicable.
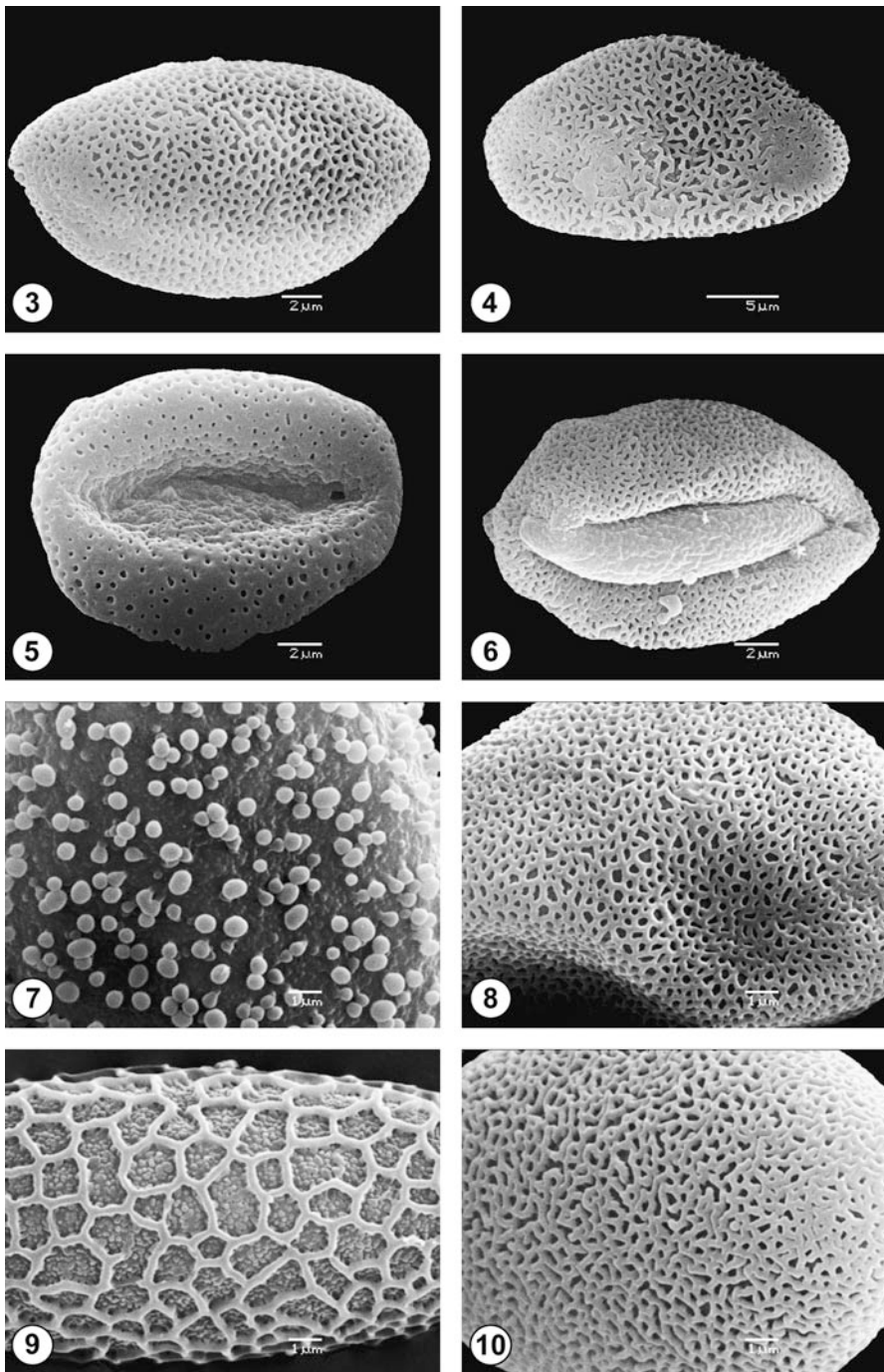
Figs. 3-10. Pollen grains of Nartheciaceae (SEM). 3. *Narthecium scardicum*, equatorial view, microreticulate sexine. 4. *Nietneria corymbosa*, equatorial view, microreticulate sexine. 5. *Aletris farinosa,* distal polar view, perforate sexine. 6. *Narthecium reverchonii*, distal polar view, micro-reticulate sexine. 7. *Aletris pauciflora*, detail of gemmate sexine. 8. *Lophiola aurea*, microreticulate sexine. 9. *Metanarthecium luteo-viride*, reticulate sexine. 10. *Narthecium reverchonii*, detail of microreticulate sexine.
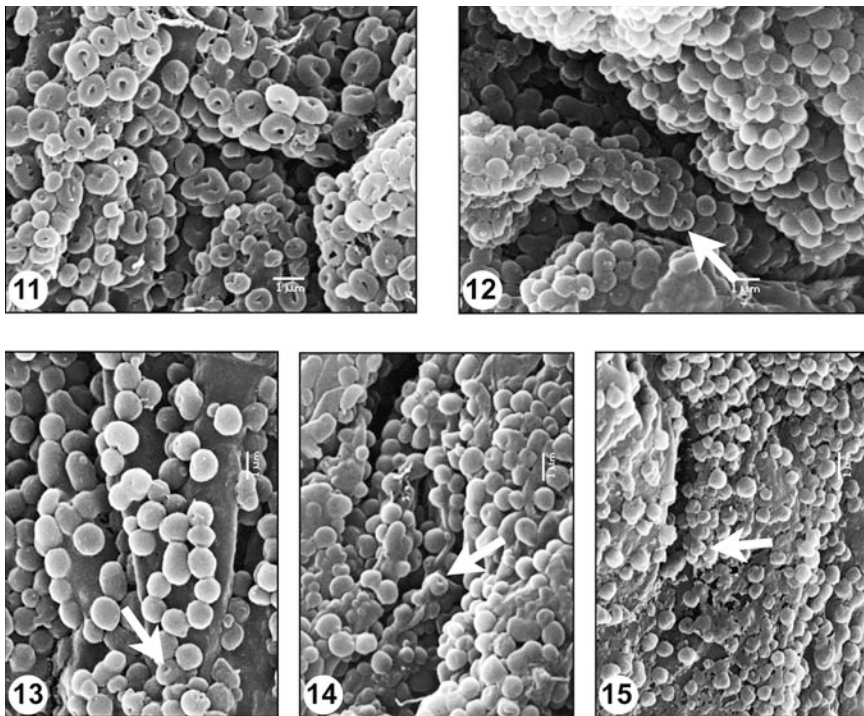
Figs. 11-15. Orbicules on the inner locule wall, note central perforations (arrows) (SEM). 11. *Lophiola aurea*, doughnut-shaped orbicules. 12. *Lophiola americana*. 13. *Nietneria corymbosa.* 14. *Narthecium reverchonii*. 15. *Metanarthecium luteo-viride.*

DISCUSSION

MONOPHYLY OF NARTHECIACEAE

As in all previous molecular surveys (CAD-DICK *et al.* 2000, FUSE & TAMURA 2000, CADDICK *et al.* 2002b, TAMURA *et al.* 2004, CHASE *et al.* 2006, GIVNISH *et al.* 2006, MERCKX *et al.* 2006) our analyses support the monophyly of Nartheci-aceae. Although well defined on the basis of molecular analyses, there are only a few morpho-logical synapomorphies known for Nartheci-aceae. Besides the absence of calcium oxalate raphides, campylotropous ovules, hypogynous flowers and the lack of integument cuticles (CAD-DICK *et al.* 2002b) our study shows that the pres-ence of spherical smooth-surfaced orbicules with a circular perforation is a potential synapomorphy for Nartheciaceae. Orbicules occur throughout Dioscoreales in several *Dioscorea* species (SCHOLS *et al.* 2001, 2003) and in *Trichopus* (SCHOLS 2004) but none have a perforation on the orbicule surface similar to the perforation of Nartheciaceae. Despite a secretory tapetum, orbicules are absent in *Tacca* and Burmanniaceae (SCHOLS 2004). As for *Stenomeris*, the remaining Dioscoreaceae genus, there are no data about orbicules.

INTERGENERIC RELATIONSHIPS

Narthecium-Nietneria

Inclusion of *Nietneria corymbosa* in Narthe-ciaceae is well supported. Similarly to our study this Neotropical genus was also found related to *Narthecium* in the phenetic morphological study of AMBROSE (1980) (see also DAHLGREN *et al.* 1985)*.* A position close to *Narthecium* and *Lophiola* is

further suggested by pollen morphological data. Pollen of *Nietneria corymbosa* is very similar in size and sexine ornamentation to species of *Narthecium* and *Lophiola* examined (Figs. 3, 4, 8). Orbicule morphology in *Nietneria* resembles that of other Nartheciaceae having a smooth surface with a circular perforation.

Lophiola and Narthecium-Nietneria

Previous molecular analyses (FUSE & TAMURA 2000, CADDICK *et al.* 2002b, TAMURA *et al.* 2004), without *Nietneria,* retained a highly supported *Lophiola-Narthecium* clade. This clade, with *Nietneria* included, is also found in our analyses. The close affinity between *Lophiola, Nietneria*, and *Narthecium* is also supported by pollen grain morphology. The pollen grains of *Lophiola aurea, L. americana, Nietneria corymbosa, Narthecium reverchonii, N. scardicum, N. asiaticum, N. californicum* (this study), and *N. americanum* (TAKAHASHI & KAWANO 1989) are very similar in size and sexine ornamentation and are difficult to distinguish from each other. The size of the perforations is somewhat smaller in *Lophiola* compared to *Narthecium* and *Nietneria*, with the consequence that the number of perforations per square micrometer is higher in *Lophiola* than in *Narthecium* and *Nietneria.* The unusual doughnut-shaped orbicules in *Lophiola aurea* (Fig. 11) contrast with the spherical orbicules observed in *Lophiola americana* (Fig. 12). The difference in orbicule shape between *L. aurea* and *L. americana* is, however, not a convincing indication for the delineation of two distinct species in *Lophiola*. Intraspecific variation of orbicules is occasionally observed and it is possible that the doughnut-shaped orbicules in *L. aurea* just represent another stage of the orbicule generation (HUYSMANS *et al.* 1998). Since only one *Lophiola* specimen from each population was sampled, we cannot draw straightforward conclusions on the existence of two *Lophiola* species from the molecular results. The few differences between the sequences of *L. aurea* and *L. americana* could be explained by intraspecific variation, since all DNA regions used in this study, except 18S rDNA, are rather fast-evolving. An in-depth com-parative morphological study coupled with a molecular survey with several specimens from each population could clarify the taxonomy in *Lophiola.*

Aletris and Metanarthecium

An *Aletris-Metanarthecium* clade sister to the *Lophiola-Narthecium* clade is present in all topologies, and the support for this clade varies from moderate to strong. This clade corresponds to the strongly supported *Aletris farinosa-Metanarthecium luteo-viride* clade in the analysis of CADDICK *et al.* (2002b). These results support the inclusion of the monospecific genus *Metanarthecium* in *Aletris* as suggested by TAMURA (1998) and ZOMLEFER (1997, 1999). A broader sampling of *Aletris* species, especially from Asia is, however, necessary to assign the position of *Metanarthecium* in *Aletris.* We were not able to obtain material from Japanese or Chinese *Aletris* species, probably the closest relatives of *M. luteo-viride,* and in our current combined topology *Metanarthecium* is sister to a clade with two North American *Aletris* species: *A. lutea* and *A. farinosa*.

In *Aletris*, pollen sexine ornamentation is highly variable. We observed both perforate and gemmate sexine ornamentation and TAKAHASHI & KAWANO (1989) also found some species with a reticulate sexine. American *Aletris* species have a perforate sexine ornamentation while the Asian (and Malayan) species have pollen grains with a reticulate or gemmate sexine ornamentation. The sexine ornamentation of *M. luteo-viride* is reticulate (TAKAHASHI & KAWANO 1989, this study) and corresponds to the *Aletris* species of the same region. The lumina of *Metanarthecium* are, however, much larger and as a result the pollen grains of *Metanarthecium* can be easily distinguished from the reticulate *Aletris* species.

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