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 & Dickson 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, Helionas and Ypsilandra together with the five Nartheciaceae genera of the present treatment in a tribe Nartheciaceae, 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, Metanartheicum and Alotis are monosulcate and sexine ornamentation varies from micoreticulate in Narthecium and Lophiola, reticulate in Metanartheicum to perforate, reticulate and gemmate in Alotis. 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, Chandrabali et al. 2001). In addition the nuclear 18S rDNA sequence 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 (Genta 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 H₂O, 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 Chang et al. (1998). For some samples, however, we modified
Table 1. GenBank accession numbers and voucher data for taxa included in the DNA analyses.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Accession number atpB-rbcL spacer</th>
<th>Accession number trnL-trnF spacer</th>
<th>Accession number trnL intron</th>
<th>Accession number 18S rDNA</th>
<th>Voucher</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nartheciaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Aletris farinosa</em> L.</td>
<td>EU186229</td>
<td>EU186242</td>
<td>EU186254</td>
<td>EU186221</td>
<td>Chase 105 (NCU) RBG Kew DNA Bank (MWC105)</td>
</tr>
<tr>
<td><em>Aletris foliosa</em> (Maxim.) Bureau &amp; Franch.</td>
<td>EU186230</td>
<td>EU186244</td>
<td>EU186255</td>
<td>EU186222</td>
<td>K. Cameron RBG Kew DNA Bank (MWC1931)</td>
</tr>
<tr>
<td><em>Aletris lutea</em> Small</td>
<td>EU186231</td>
<td>EU186243</td>
<td>EU186256</td>
<td>DQ786092</td>
<td>J. Anderson 35 (LV)</td>
</tr>
<tr>
<td><em>Lophiola americana</em> Wood</td>
<td>EU186224</td>
<td>EU186237</td>
<td>EU186249</td>
<td>EU186218</td>
<td>J. Anderson 36 (LV)</td>
</tr>
<tr>
<td><em>Lophiola aurea</em> Ker Gawl.</td>
<td>EU186225</td>
<td>EU186236</td>
<td>EU186248</td>
<td>DQ786091</td>
<td>R. Newell 23/8 (LV)</td>
</tr>
<tr>
<td><em>Metanarthecium luteo-viride</em> Maxim.</td>
<td>EU186228</td>
<td>EU186241</td>
<td>EU186253</td>
<td>AF309410</td>
<td>Inoue RBG Kew DNA Bank (MWC630)</td>
</tr>
<tr>
<td><em>Narthecium californicum</em> Baker</td>
<td>EU186227</td>
<td>EU186240</td>
<td>EU186252</td>
<td>EU186220</td>
<td>Janeway &amp; Castro 7607 (LV)</td>
</tr>
<tr>
<td><em>Narthecium ossifragum</em> (L.) Huds.</td>
<td>EU186226</td>
<td>EU186239</td>
<td>EU186251</td>
<td>AF309411</td>
<td>A. Jacquemart 46-9 (LV)</td>
</tr>
<tr>
<td><em>Nietneria paniculata</em> Steyerm.</td>
<td>EU186234</td>
<td>EU186238</td>
<td>EU186250</td>
<td>EU186219</td>
<td>O. Hokche &amp; P.J.M. Maas 849 (U)</td>
</tr>
<tr>
<td>Dioscoreaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Dioscorea tokoro</em> Makino ex Myabe</td>
<td>EU186233</td>
<td>EU186246</td>
<td>EU186258</td>
<td>DQ786088</td>
<td>V. Merckx 1 (LV)</td>
</tr>
<tr>
<td><em>Dioscorea communis</em> (L.) Caddick &amp; Wilkin</td>
<td>EU186232</td>
<td>EU186245</td>
<td>EU186257</td>
<td>EU186223</td>
<td>V. Merckx 2 (LV)</td>
</tr>
<tr>
<td>Burmanniaceae</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Burmannia longifolia</em> Becc.</td>
<td>EU186235</td>
<td>EU186247</td>
<td>EU186259</td>
<td>AF309398</td>
<td>K. Cameron s.n. (NCU) RBG Kew DNA Bank (MWC1930)</td>
</tr>
</tbody>
</table>
these primers (VMATPB: 5'-ACATCTAGTACTGGTCCAA3-3' and VMRBCL: 5'-AACCCAGTTTAGTAACTCAA-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 CLUSTAL (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.

PHYLGENETIC 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 & Ronquist 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 MODELEST 3.06 (Posada & Crandall 1998, 2001). MODELEST selected the TVM+G model of evolution for the apB-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 × 10⁶ 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 ORICULE 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 Nartheciacae 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 oricule 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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Voucher</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aletris farinosa L.</td>
<td>C. Bell 4164 (BR)</td>
</tr>
<tr>
<td>Aletris lutea Small</td>
<td>Anonymous 58 (BR)</td>
</tr>
<tr>
<td>Aletris pauciflora (Klotzsch) Hand.-Mazz.</td>
<td>F. Biliot &amp; J. Leonard 6546 (BR)</td>
</tr>
<tr>
<td>Lophiota americana Wood</td>
<td>S. Leonard &amp; K. Moore (BR)</td>
</tr>
<tr>
<td>Lophiota aurea Ker Gawl.</td>
<td>H. Ahles 56603 (BR)</td>
</tr>
<tr>
<td>Metanarthecium luteo-viride Maxim.</td>
<td>M. Togasi 1493 (BR)</td>
</tr>
<tr>
<td>Narthecium asiaticum Maxim.</td>
<td>K. Okamoto 776 (BR)</td>
</tr>
<tr>
<td>Narthecium californicum Baker</td>
<td>T. Howell (BR)</td>
</tr>
<tr>
<td>Narthecium reverchonii Celak.</td>
<td>J. Leonard 5164 (BR)</td>
</tr>
<tr>
<td>Narthecium scaradicum Közanin</td>
<td>P. Frost-Olsen 13746 (BR)</td>
</tr>
<tr>
<td>Nieteria corymbosa Klotzsch &amp; R.Schomb.</td>
<td>R. Liesner 21129 (U)</td>
</tr>
</tbody>
</table>
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

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

<table>
<thead>
<tr>
<th>Dataset</th>
<th>Number of characters</th>
<th>Constant characters</th>
<th>Parsimony informative characters</th>
<th>Parsimony uninformative characters</th>
<th>Number of most parsimonious trees</th>
<th>Length of shortest tree</th>
</tr>
</thead>
<tbody>
<tr>
<td>atpB-rbcL spacer</td>
<td>944</td>
<td>741</td>
<td>522</td>
<td>113</td>
<td>3</td>
<td>250</td>
</tr>
<tr>
<td>trnL intron</td>
<td>659</td>
<td>520</td>
<td>328</td>
<td>69</td>
<td>13</td>
<td>154</td>
</tr>
<tr>
<td>trnL-trnF spacer</td>
<td>944</td>
<td>741</td>
<td>522</td>
<td>113</td>
<td>3</td>
<td>250</td>
</tr>
<tr>
<td>18S rDNA</td>
<td>1699</td>
<td>1594</td>
<td>154</td>
<td>90</td>
<td>69</td>
<td>153</td>
</tr>
<tr>
<td>Combined data</td>
<td>3822</td>
<td>3185</td>
<td>213</td>
<td>90</td>
<td>69</td>
<td>276</td>
</tr>
</tbody>
</table>

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. Niereneria 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 species 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 Niereneria (Fig. 4), reticulate in Metanarthecium (Fig. 9) and perforate (Fig. 5) and gemmate (Fig. 7) in the Aletris species examined.

**ORICULES (FIGS. 11-16)**

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

<table>
<thead>
<tr>
<th>Species</th>
<th>LA (μm)</th>
<th>SA (μm)</th>
<th># Ap</th>
<th>Ornamentation</th>
<th>Perf/μm</th>
<th>Perf/min.</th>
<th>Perf/vμm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Narthecium californicum</td>
<td>20.3-20.7</td>
<td>10.0-10.7</td>
<td>1</td>
<td>Micro Ret.</td>
<td>4.85</td>
<td>0.8-0.93</td>
<td>0.03-0.4</td>
</tr>
<tr>
<td>Narthecium occidentale</td>
<td>20.2-20.6</td>
<td>10.0-10.7</td>
<td>1</td>
<td>Micro Ret.</td>
<td>4.80</td>
<td>0.6-0.84</td>
<td>0.02-0.2</td>
</tr>
<tr>
<td>Narthecium schizocarpum</td>
<td>19.8-20.0</td>
<td>10.0-10.7</td>
<td>1</td>
<td>Micro Ret.</td>
<td>4.30</td>
<td>0.7-0.93</td>
<td>0.03-0.1</td>
</tr>
<tr>
<td>Narthecium pauciflora</td>
<td>20.8-21.2</td>
<td>10.0-10.7</td>
<td>1</td>
<td>Micro Ret.</td>
<td>4.25</td>
<td>0.4-0.84</td>
<td>0.03-0.1</td>
</tr>
<tr>
<td>Aletris atrata</td>
<td>20.2-20.6</td>
<td>10.0-10.7</td>
<td>1</td>
<td>Macro Ret.</td>
<td>4.85</td>
<td>0.4-0.84</td>
<td>0.03-0.1</td>
</tr>
</tbody>
</table>

*Given as x-y-z, with x = minimum, y = mean, z = maximum; N.A.: not applicable.
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 Nartheciaceae. Although well defined on the basis of molecular analyses, there are only a few morphological synapomorphies known for Nartheciaceae. 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 presence 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 Taccu 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 Nartheciaceae 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 comparative 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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