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Pollen Evolution in Yams (*Dioscorea*: Dioscoreaceae)

PETER SCHOLS,^{1,3} PAUL WILKIN,² CAROL A. FURNESS,² SUZY HUYSMANS,¹ and ERIK SMETS¹

¹Laboratory of Plant Systematics, Institute of Botany and Microbiology, K.U.Leuven, Kasteelpark Arenberg 31, B-3001 Leuven, Belgium;

²Royal Botanic Gardens, Kew, Richmond, Surrey, TW9 3AB, U.K.;

³Author for correspondence (peter.schols@bio.kuleuven.ac.be)

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ABSTRACT. Pollen character evolution in yams (*Dioscorea*: Dioscoreaceae) was investigated in relation to the phylogeny obtained from a recent combined analysis of *rbcl* and *matK* gene sequences. The following characters were evaluated: pollen size, aperture number, sexine ornamentation, perforation density, and orbicule presence or absence. Continuous characters were coded using the gap weighting method. Each character was optimized using MacClade onto a tree selected at random from analyses based on molecular data of Wilkin et al. The results indicate that in *Dioscorea* pollen size decreases in the more derived clades. The latter observation may be related to the evolution of annually replaced tubers. Aperture number increases from one in the monosulcate *Stenophora* clade (sister to the rest of *Dioscorea*) to two in other *Dioscorea* clades. This may be related to the presence of simultaneous microsporogenesis in *Dioscorea*. A perforate sexine is plesiomorphic in *Dioscorea* and a striate pattern has evolved more than once and is particularly characteristic in the Malagasy clade. Sexine ornamentation in the *Stenophora* clade is diverse. The endemic allotetraploid *D. pyrenaica* is characterized by large, monosulcate, gemmate pollen. The *Dioscorea* B clade is characterized by pollen with small, dense perforations. Orbicules occur in all *Dioscorea* species, but are absent in most of the outgroup taxa.

Su (1987), Caddick et al. (1998), and Xifreda (2000) were the first to suggest the possible taxonomic significance of pollen morphology within Dioscoreaceae, a family of about 450 species growing in humid tropical and subtropical areas. Their work indicated that *Dioscorea* L., the yam genus to which 90% of all Dioscoreaceae belong, was palynologically diverse. Following these preliminary studies, Schols et al. (2001, 2003) examined pollen of 96 *Dioscorea* species with light microscopy (LM) and scanning (SEM) and transmission electron microscopy (TEM). They described pollen of *Dioscorea* as monosulcate or disulcate, with a mainly perforate, perforate to microreticulate or striate sexine. They also found aperture number and sexine ornamentation to be frequently consistent within samples from each section. Pollen characters indicated support for the relationship between some existing infrageneric taxa, for example *D.* sect. *Asterotricha* and *D.* sect. *Enantiophyllum* Uline originally proposed by Burkill (1960), and between *D.* sect. *Botrysicyos* (Hochst.) Uline and *D.* sect. *Lasiophyton* Uline.

Concurrently, Wilkin et al. (2005) expanded the Dioscoreaceae *rbcl* data set of Caddick et al. (2002) and added *matK* sequences to it. Using these data, they presented the first infrageneric phylogenetic hypothesis for *Dioscorea*. The general tree topology of this combined molecular analysis is as follows. The outgroups, selected on the basis of the earlier analysis of Dioscoreales by Caddick et al. (2002), are representatives of the heteromycotrophic family Burmanniaceae and the Dioscoreaceae with hermaphrodite flowers (*Stenomerris* Planch., *Trichopus* Gaertn, and *Tacca* J.R. & G.Forst). The exclusively rhizomatous *D.* sect. *Stenophora* Uline is sister to the rest of *Dioscorea*, followed by two New

World clades, one of which includes *Dioscorea longirhiza* Caddick & Wilkin, formerly in the genus *Nanarepenta* Matuda. Next, there is a southern and montane African clade consisting of *D. sylvatica* Eckl. and related species, and a European clade comprising former *Tamus* L. species and *D. pyrenaica* Bubani & Bordère ex Gren., although support for the latter clade in particular is weak. The B clade of *Dioscorea* contains almost all Old World species, with the exception of *Rajania* (*Dioscorea*) *cordata* L. Two strongly supported subclades are nested within it, one of which comprises endemic Malagasy taxa. The other is the right-twining *D.* sect. *Enantiophyllum*.

This molecular phylogenetic framework enables us to trace the evolution of pollen morphology within *Dioscorea*. Pollen characters have been assessed in a phylogenetic context previously by several workers, including Rudall et al. (1997), Furness and Rudall (2000, 2001), Doyle et al. (2000), Cameron et al. (2001), and Sauquet and Le Thomas (2003). Most of these studies dealt with pollen evolution within a family or at higher levels. Schols et al. (2001, 2003) found significant variation in pollen morphology of *Dioscorea*. However, studies of the evolution of pollen characters within a genus are comparatively rare even for large genera. Thus our work on *Dioscorea* may indicate the potential for using such methods in the systematics of large genera.

MATERIALS AND METHODS

Pollen Characters. A complete list of *Dioscorea* and *Tacca* specimens for which pollen and anthers were examined is given in Appendix 1. Descriptions of the methods used are given in Schols et al. (2001, 2003). Measurements of pollen perforation density and perforation size were carried out on digital SEM images using

Carnoy 2.1 for Mac OS X (Schols et al. 2002). Terminology follows the international glossary (Punt et al. 1998). All *Dioscorea* pollen data are based on observations by Schols et al. (2001, 2003). Data for *Rajania cordata* are from Raz et al. (2001). For the outgroup taxa, data for *Tacca* are based on observations by the first author (P. Schols, unpubl. data). Data for other Dioscoreaceae with hermaphrodite flowers are from Caddick et al. (1998), and data for Burmanniaceae are from Chakrapani and Raj (1971), Rübsamen (1986), and Caddick et al. (1998).

Three continuous characters (pollen size, perforation density, and perforation size) were coded using Thiele's gap weighting method as implemented by MorphoCode (Schols et al. 2004), with $n = 10$, where n is the number of allowed character states in the Thiele formula (Thiele 1993). MorphoCode offers the user the possibility to import data as tab-separated text, choose the number of character states, perform all necessary calculations and export the newly coded data to a Nexus file. MorphoCode and its source are available free on the MorphoCode website: www.kuleuven.ac.be/bio/sys/mc under the GNU GPL. To compensate for the high weight (10) all continuous characters receive as a result of the gap weighting method, the continuous characters were assigned a weight of 0.1 ($= 1/10$), in order to give them a maximum length equal to the other characters. Non-continuous data were treated as unordered multistate characters (aperture number, ornamentation type, presence or absence of orbicules) and were assigned the default weight of 1.

Character Optimization. Since Wilkin et al. (2005) have obtained a stable overall topology, we felt confident optimising pollen characters onto the trees at this stage of our research. Characters were optimized on one of the 15868 most parsimonious trees chosen at random from the combined *rbcl* and *matK* analysis of Wilkin et al. (2005) using DELTRAN in MacClade 4 (Maddison and Maddison 2001). Adding the pollen characters to the matrix of *rbcl* and *matK* sequences and running a new analysis produced a strict consensus tree of identical topology to that presented by Wilkin et al. (2005) and had only a minor influence on the support indices (results not shown here). Therefore, we decided to optimise the pollen characters on the molecular tree. It should be remembered that the sampling of taxa in Wilkin et al. (2005) is biased towards Old World taxa, particularly Thailand and Madagascar. Due to coding the continuous characters with Thiele's gap weighting method, they have a large number of states (see Schols et al. 2004 for an explanation of the gap-weighting method).

RESULTS

Four trees are presented each showing a single pollen character (Figs. 1–4). Perforation size (not shown) was also optimized but lacked congruence with the phylogeny.

DISCUSSION

Pollen Size (Figs. 1, Fig. 5A). Pollen size is a rather homoplasious character, as may be expected (Fig. 1). Among the outgroup taxa, *Tacca* is distinguished by large pollen (about 50 μm). Within *Dioscorea*, *D. niponica* in the Stenophora clade has large pollen, although most *Stenophora* and New World species sampled are of intermediate size. Large pollen characterizes the southern/montane African clade and *D. pyrenaica* from the European clade. The former includes taxa with large perennial tubers (e.g., *D. elephantipes*). Su (1987) proposed that *Dioscorea* species with perennial tubers or rhizomes have larger pollen grains and this correlation was shown to be statistically significant by Schols et al. (2001, 2003). The reasons for this are

not known, although resource allocation in yams could be a useful area for future work. *Dioscorea pyrenaica*, an endemic from the Pyrenees, was recently shown to be an allotetraploid, which may correlate with large pollen size (Segarra-Moragues and Catalán in press). Within the B clade, there are fewer taxa with large pollen, for example *D. pseudonitens* in the Birmanica clade. In the Malagasy clade, *D. fandra*, which is sister to the rest of the more diversified subclade, has large pollen. The pollen grains of the rest of the more diversified subclade are smaller, as are those of the only member of the *D. arcuatinervis*–*D. bemarivensis* clade sampled, *D. arcuatinervis*. This suggests that evolution has mainly acted to reduce pollen size in Madagascar. Large pollen is absent from the rest of the B clade, which have pollen of less than 30 μm (Fig. 5A). It is difficult to find reasons for this, although it may be correlated with possession of annually replaced tubers in the B clade and in turn the ability to colonise a wider range of habitats (Burkill 1960). The cost of the flexibility afforded by an annually replaced tuber may be less resources available for reproduction.

Aperture Number (Fig. 2, Fig. 5A). The outgroup taxa and the Stenophora clade have a single pollen aperture, with two apertures characterizing the majority of *Dioscorea* species (Fig. 2). In the outgroup, *Burmannia madagascariensis* and *B. longifolia* are ulcerate while the monoecious Dioscoreaceae are monosulcate, with the exception of *Trichopus sempervirens*, which is 4- or 5-pantoporate (Caddick et al. 1998). Most *Dioscorea* species are disulcate and disulcate pollen clearly evolved only once in *Dioscorea* (Figs. 2, Fig. 5A). Exceptions are the Stenophora clade and *D. pyrenaica* (Fig. 2). The Stenophora clade is exclusively monosulcate (Fig. 5A) and *D. birmanica*, a disulcate species placed in section *Stenophora* by Prain and Burkill (1936), is a member of the B-clade. Schols et al. (2003) previously suggested it was misplaced in section *Stenophora* on the basis of pollen morphology. Interestingly, the monosulcate *D. pyrenaica* is a polyploid (see above: Pollen Size), which may explain the occurrence of atypical pollen here. In a few species (e.g., *D. fandra* and *D. hexagona*) the majority of the pollen is disulcate with smaller, collapsed and wrinkled monosulcate grains forming a low percentage (below 5%: Schols et al. 2001, 2003). Possibly this monosulcate pollen is sterile although this requires further testing.

Evolution of pollen aperture number from low to higher numbers has been suggested previously for several groups, for example, Alismatales (Chanda et al. 1988), monocotyledons (Zavada 1983), and eudicots (Van Campo 1976). Experimental evidence demonstrates that increased aperture number may confer a selective advantage in *Viola* species (Dajoz et al. 1991) because it increases the number of prospective germination sites, thus facilitating contact between at least

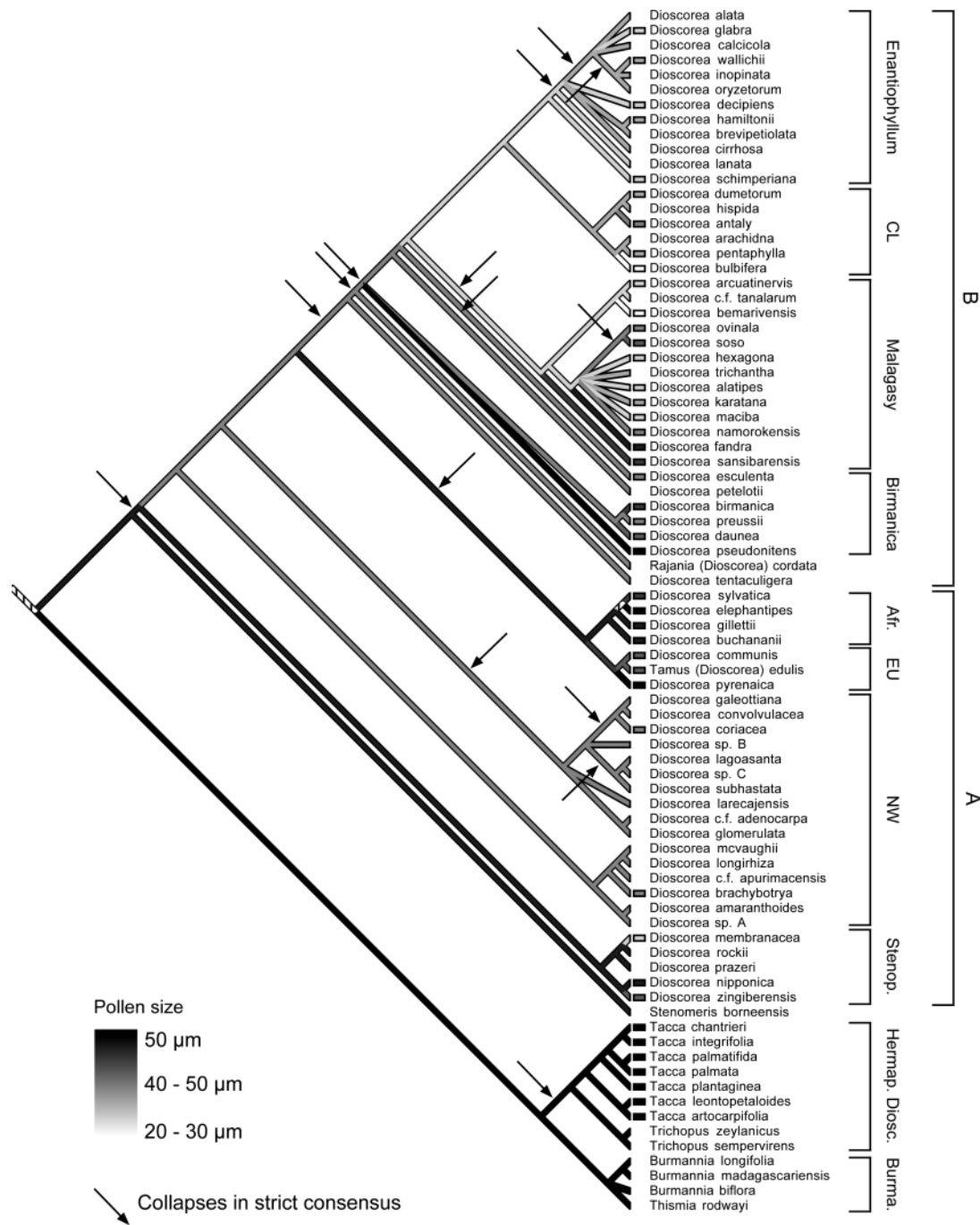


FIG. 1. Pollen size (length of longest axis) optimised onto one of the 15868 most parsimonious trees of Wilkin et al. (2005). As a result of the gap-weighting (Schols et al. 2004) pollen size is represented on a continuous scale, with darker branches representing larger pollen grains. Branches labeled with an arrow collapse in the strict consensus.

one aperture and the stigmatic surface. The increase in aperture number from one to three may be a critical factor in eudicot success (Furness and Rudall, 2004). It has been proposed that pollen aperture number and pattern is related to microsporogenesis (e.g., Black-

more and Crane 1998) and a correlation between simultaneous microsporogenesis and trichotomosulcate apertures has been demonstrated in Asparagales (Rudall et al. 1997). Based on experimental work on *Nicotiana*, Ressayre et al. (2002) proposed that simulta-

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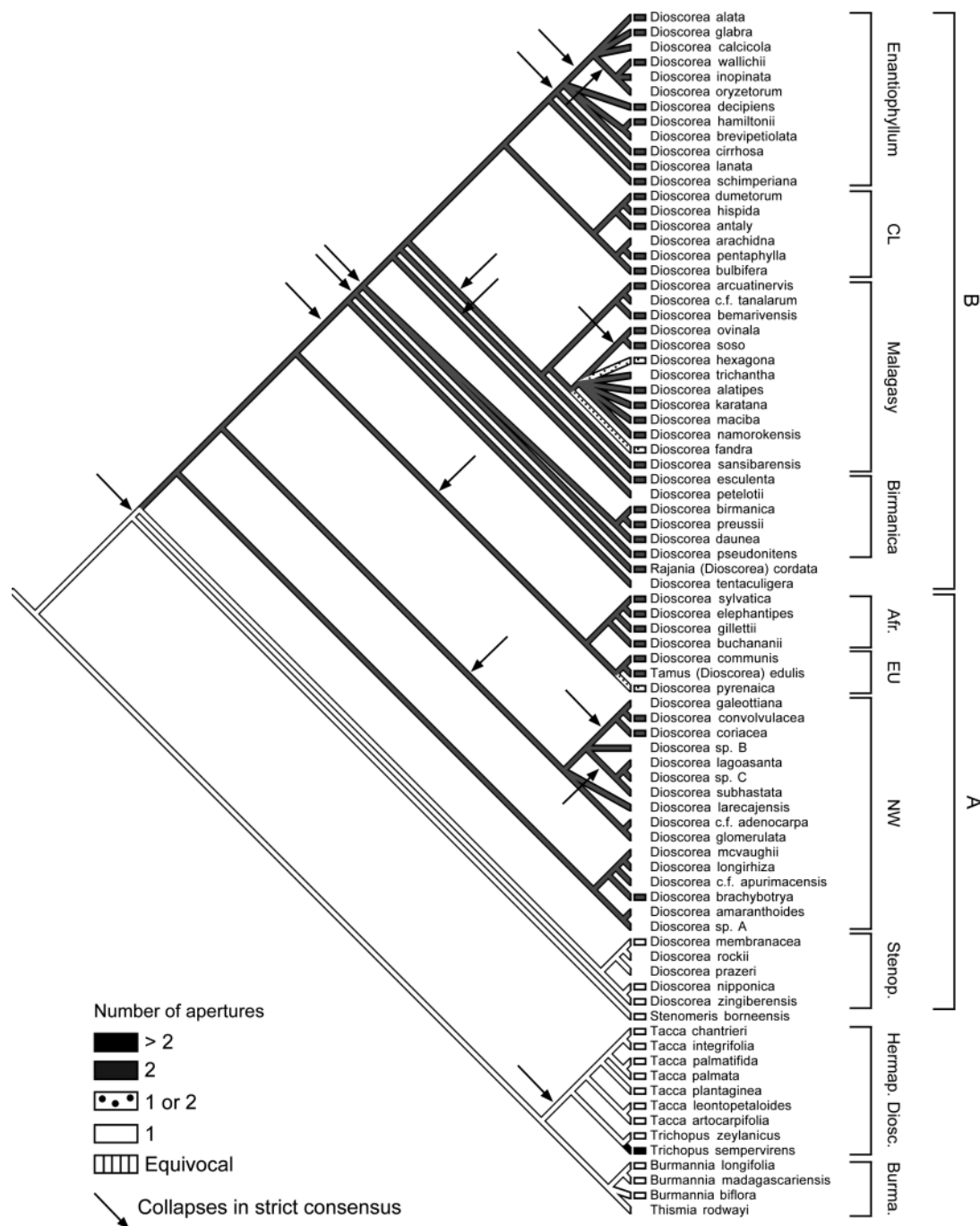


FIG. 2. Aperture number optimised onto one of the 15868 most parsimonious trees of Wilkin et al. (2005). Branches labeled with an arrow collapse in the strict consensus.

neous cytokinesis allows variation in the interaction between nuclei, which is impossible in successive microsporogenesis, thus leading to heteromorphic pollen. Changes in aperture number are thus potentially easy to achieve. Microsporogenesis is successive in most monocotyledons, although the simultaneous type has

arisen several times independently within the group (Furness and Rudall 1999, 2000). Within Dioscoreales, Nartheciaceae and Burmanniaceae have successive microsporogenesis and Dioscoreaceae are simultaneous (Caddick et al. 1998; Furness and Rudall 1999, 2000). The shift from successive to simultaneous microspo-



FIG. 3. Sexine ornamentation optimised onto one of the 15868 most parsimonious trees of Wilkin et al. (2005). Branches labeled with an arrow collapse in the strict consensus.

rogenesis precedes the shift from monosulcate to disulcate pollen (Fig. 2) and may have facilitated the switch to two apertures. The existence of several species with heteromorphic pollen (one or two apertures) also indicates that this change was relatively easy and is in agreement with Ressayre's (2002) hypothesis. In

Dioscorea pollen the change to two apertures appears to be irreversible, probably becoming fixed by a selective advantage conferred by more than one potential germination site. However, some monocots with successive microsporogenesis have multiple apertures. Some Alismatales (Chanda et al. 1988) and the Bur-

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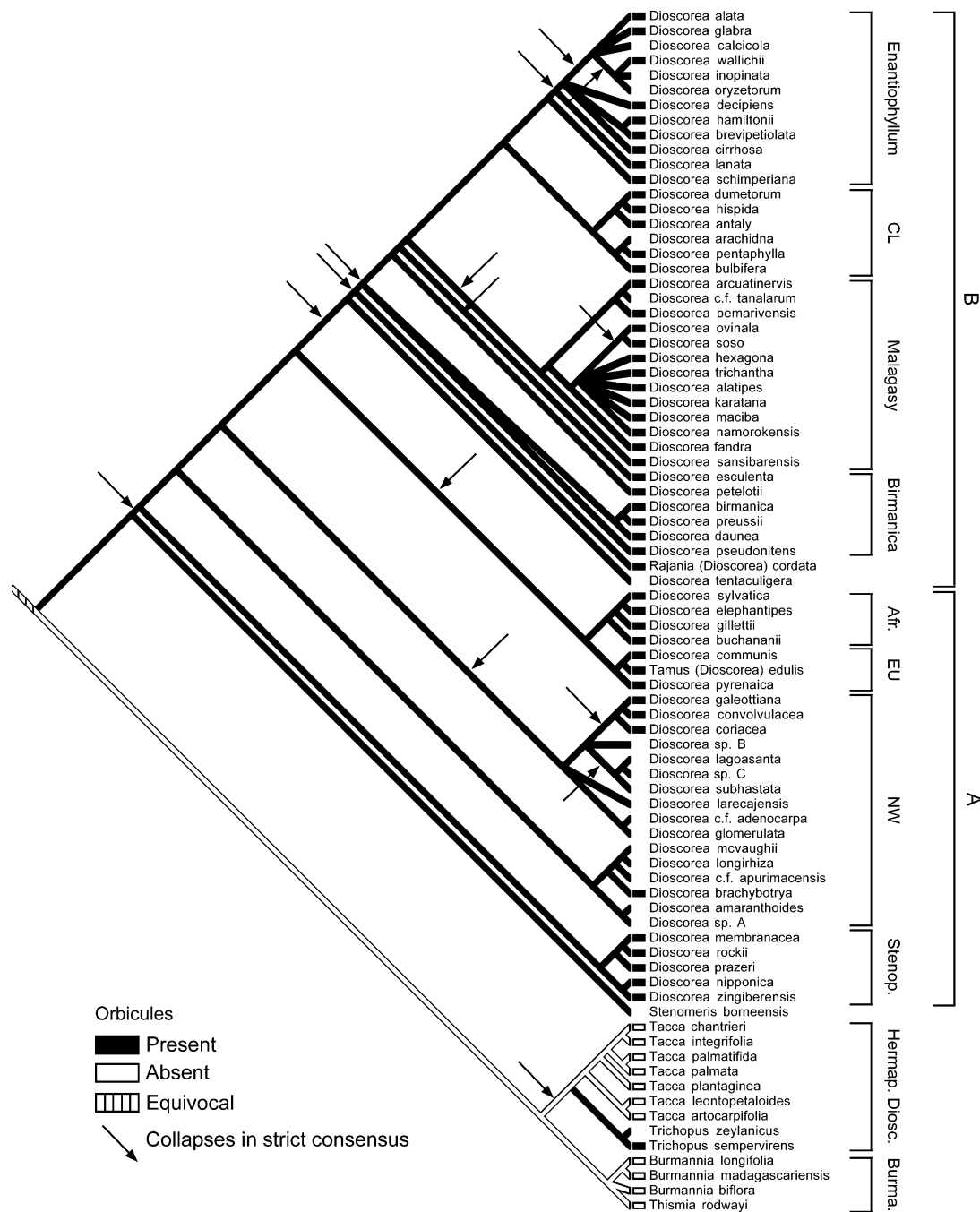


FIG. 4. Orbicule presence optimised onto one of the 15868 most parsimonious trees of Wilkin et al. (2005). Branches labeled with an arrow collapse in the strict consensus.

manniaceae genera *Dictyostegia* and *Thismia* (Chakrapani and Raj 1971; Rübsamen 1986), and Pontederiaceae (Commelinales) are successive and disulcate (Simpson 1987; Ressayre 2001).

Sexine Ornamentation (Figs. 3, 5B–G). A perforate sexine is plesiomorphic and occurs throughout the outgroup and in the majority of *Dioscorea* species sampled

(Fig. 3). Perforation size and density vary (see below: Perforation Density). The perforate sexine type includes perforate to microreticulate (e.g., *D. antaly*) since both ornamentation types are observed in the same grain (Schols et al. 2003).

Striate ornamentation, which is relatively uncommon in the monocotyledons (Van der Ham 1998), is

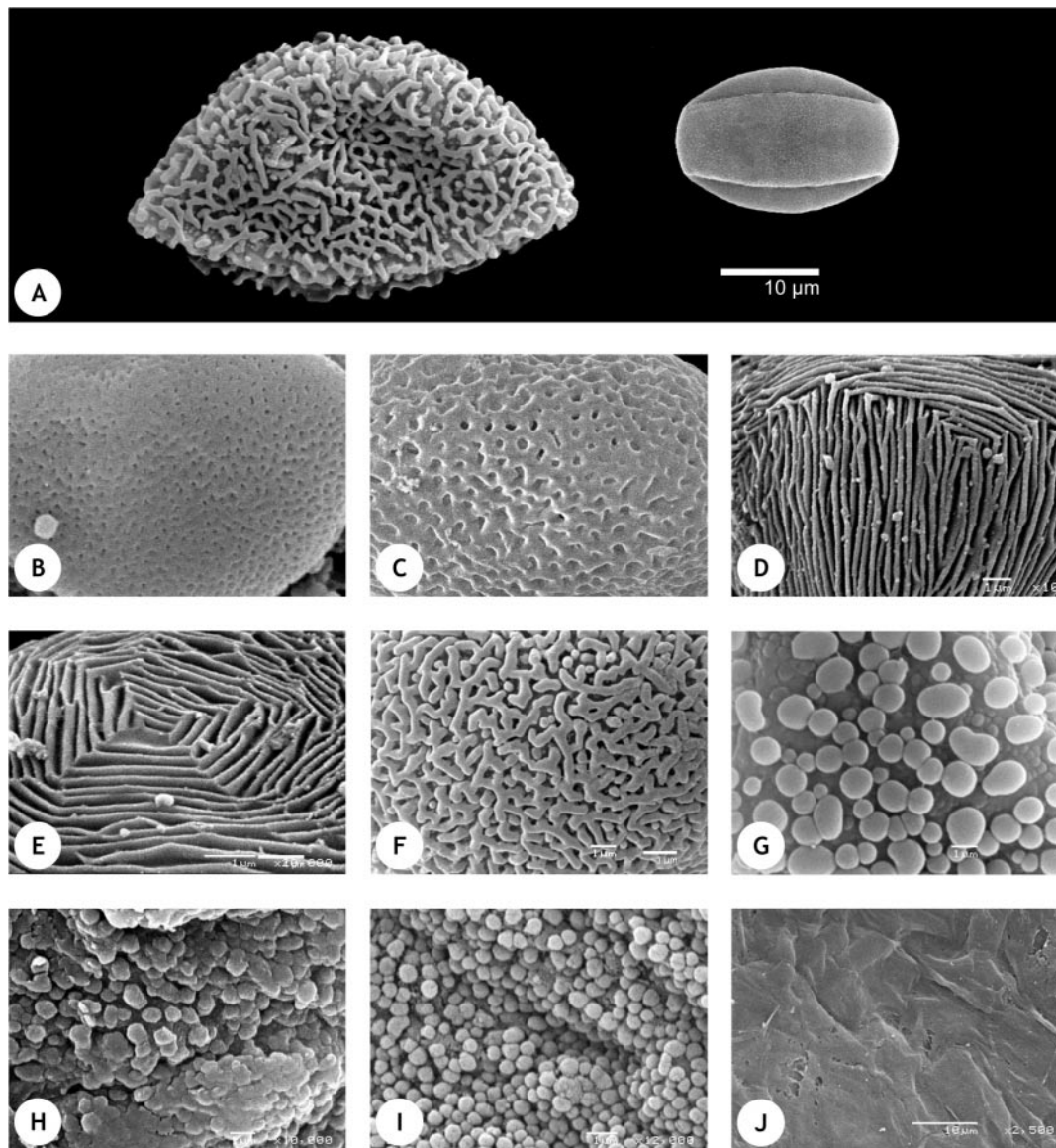


FIG. 5. Pollen characters (SEM). A. Left: *D. nipponica* (Stenophora), monosulcate pollen grain with longest axis of 45 μ m. Right: *D. hamiltonii* (Enantiophyllum), disulcate pollen grain with longest axis of 20 μ m. B. *D. bulbifera* (CL), perforate sexine with small perforations. C. *D. membranacea* (Stenophora) perforate sexine. D. *D. karatana*, striate sexine. E. *D. bemarivensis* (Malagasy), striate sexine with striations arranged in concentric polygons. F. *D. nipponica* (Stenophora), rugulate sexine. G. *D. pyrenaica* (EU), gemmate sexine. H. *D. glabra* (Enantiophyllum), spherical to elliptical orbicules. I. *D. sylvatica* (Afr.), spherical orbicules. J. *Tacca plantaginea*, tapetal remnants at anthesis without orbicules.

found in *D. zingiberensis* in the Stenophora clade, in *D. gillettii* and in almost all Malagasy species sampled, except *D. antaly* in the CL clade (Figs. 3, 5D). A striate sexine also occurs in some New World yams and in some *Rajania* species (Xifreda 2000; Raz et al. 2001; Schols et al. 2001, 2003). These taxa are not included in our analysis due to absence of molecular data. It appears that the striate pattern has arisen independently several times within *Dioscorea*. The fact that the striae in Stenophora and the African clade are almost

twice as wide as those in the Malagasy clade supports this (Schols et al. 2003). There appears to be a single origin for the striate pattern in the Malagasy clade, which is divided into two well-supported sub-clades (Wilkin et al., 2005). In the first, a gemmate sexine has evolved in *D. alatipes* with a reversal to the perforate condition in *D. ovinala*. The second sub-clade containing *D. arcuatineris* and related species is characterised by striae arranged in concentric polygons (Fig. 5E).

A rugulate sexine occurs in *D. nipponica* in the Ste-

nophora clade (Fig. 5F). All three *Stenophora* species sampled have a different ornamentation type (striate, rugulate and perforate), indicating a high level of diversity in this clade. Other *Stenophora* species examined by Schols et al. (2001, 2003) and not included in the analysis also have a variety of sculpturing types. Possibly sexine ornamentation is less fixed here. The New World *Rajania* (*Dioscorea*) *cordata* also has rugulate ornamentation, as do some New World *Dioscorea* species not included in the analysis (Xifreda 2000; Schols et al. 2003), although the rugulate pattern in these taxa does not look like that of *R. cordata* and is therefore unlikely to be homologous.

Gemmae have evolved twice, in the Malagasy *D. alatipes* (see above) and European *D. pyrenaica*, a polyploid with atypical pollen discussed previously (Fig. 5G). The gemmae differ in mean diameter, those of *D. alatipes* are about 0.4 μm and those of *D. pyrenaica* about 1.2 μm , supporting their independent origin. *Dioscorea pyrenaica* is ant pollinated and the gemmate ornamentation here could possibly be a related adaptation (Garcia et al. 1995; Caddick et al. 1998).

Perforation Density (Figs. 5B–C). High perforation density (more than 8 perforations per μm^2) is restricted to the *Dioscorea* B clade, in *D. dumetorum*, *D. bulbifera*, *D. pentaphylla* and the Enantiophyllum clade. Pollen here tends to be smaller (see Pollen Size) with small, dense perforations. A low perforation density (less than 5 perforations per μm^2) however occurs in *D. antalya*. Most other perforate *Dioscorea* species have moderate (between 5 and 8 perforations per μm^2) densities. No tree is shown for this character as there are not enough observations.

Orbicules (Figs. 4, 5H–J). Orbicules—small sporopollenin bodies found in the anther locule—occur throughout *Dioscorea* (Figs. 4, 5H, I). Orbicules in *Dioscorea* are mostly spherical but are sometimes elliptical, irregularly shaped or spiny. They are found on the tangential and radial walls of the tapetal cells (Schols et al. 2001, 2003). Orbicules are associated with secretory tapeta (Huysmans et al. 1998) which occur throughout Dioscoreales (Furness and Rudall 1998). Orbicules are however absent from the outgroup taxa *Tacca* (Fig. 5J; P. Schols, pers. obs.) and Burmanniaceae, which is unusual for taxa with secretory tapeta, although they are present in *Trichopus sempervirens* (L. R. Caddick, pers. comm.).

This study shows that pollen characters can contribute to the systematics of large genera. Most of the palynological characters discussed in this paper show at least some congruence with the plastid gene phylogeny. For example, there is a trend to smaller pollen grains in more derived *Dioscorea* clades and a shift from monosulcate pollen in the basal *Stenophora* clade to disulcate pollen grains in the remainder of the genus. Orbicules occur in almost every *Dioscorea* spe-

cies but are absent from all representatives of *Tacca*. In future, further sampling of pollen, especially New World taxa, will help to give a complete picture of the diversity and relationships in *Dioscorea*. Studies of pollen development, especially pollen apertures, may help to understand the shift from monosulcate to disulcate apertures in *Dioscorea* between the *Stenophora* clade and the rest of the genus.

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APPENDIX 1

Specimens examined.

- D. alatipes* Burkill & H. Perrier—Madagascar, Phillipson 3208 (K). *D. antaly* Jum. & H. Perrier—Madagascar, Wilkin et al. 1103 (K). *D. arcuatineris* Hochr.—Madagascar, Caddick et al. 309 (K). *D. bema-rivensis* Jum. & H. Perrier—Madagascar, Phillipson 3027 (K). *D. bir-manica* Prain & Burkill—Burma, Lacey 6184 (K). *D. brachybotrya* Poepp.—Chile, Comber 462 (K). *D. buehneri* Benth.—Zimbabwe, Biegel 2893 (K). *D. bulbifera* L.—Thailand, Wilkin 864 (K). *D. cirrhosa* Lour.—China, Shiu Ying Hu 9970 (K). *D. coriacea* Humb. & Bonpl. ex Willd.—Ecuador, Fleming 143 (K). *D. daunea* Prain & Burkill—Thailand, Middleton & Parnell 1468 (K). *D. debilis* Uline ex Knuth—Brazil, Harley et al. 20871 (K). *D. decipiens* Hook. f.—Thailand, Wilkin 860 (K). *D. dumetorum* (Kunth) Pax—Malawi, Wilkin & Tawakali 786 (K). *D. elephantipes* Engelm.—S. Africa, Archibald 8014 (K). *D. es-culenta* Burkill—Mollucas, Beguin 2093 (K). *D. fandra* H. Perrier—Madagascar, Caddick et al. 324 (K). *D. galeottiana* Kunth—Mexico, Diaz Vilchis 1228 (BR). *D. gillettii* Milne-Redh.—Ethiopia, Friis, Gilbert & Rasmussen 943 (BR). *D. glabra* Roxb.—Thailand, Wilkin 892 (K). *D. hamiltonii* Hook. f.—Thailand, Wilkin et al. 886 (K). *D. hex-agona* Baker—Madagascar, Wilkin et al. M960 (K). *D. karatana* Wilkin—Madagascar, Wilkin et al. M947 (K). *D. maciba* Jum. & H. Perrier—Madagascar, Wilkin et al. M964 (K). *D. membranacea* Pierre—Thailand, Middleton & Parnell 1468 (K). *D. namorokensis* Wilkin—Madagascar, Wilkin et al. 1118 (K). *D. nipponica* Makino—Togasi 1214 (BR). *D. ovalata* Baker—Madagascar, Wilkin et al. 1115 (K). *D. pentaphylla* L.—Thailand, Boulanger 1109 (K). *D. preussii* Pax—Nigeria, Pilz 1801 (K). *D. proteiformis* H. Perrier—Madagascar, McPherson et al. 14203 (K). *D. pseudo-nitens* Prain & Burkill—Thailand, Garrett 781 (K). *D. pyrenaica* Bub. & Bordere ex Gren.—Spain, Sandwith 4745 (K). *D. sambiranensis* R. Knuth ssp. *ambrensis* H. Perrier—Madagascar, Perrier de la Bathie 17551 (K). *D. sansibarensis* Pax—Tanzania, Faden et al. 96/12 (K). *D. schimperiana* Hochst.—Malawi, Wilkin & Tawakali 763 (K). *D. soso* Jum. & H. Perrier—Madagascar, Wilkin et al. 1105 (K). *D. sylvaica* Eckl.—Zimbabwe, Chase 7939 (K). *D. wallichii* Hook.f.—Thailand, Wilkin 1078 (K). *D. zingiberensis* C.H.Wright—China, Wilson 2921 (K).
- Tacca artocarpifolia* Seem.—Madagascar, Caddick 305 (K). *Tacca chantrieri* André—Chase 175 (K). *Tacca integrifolia* Ker Gawl.—Thailand, Boyce 1074 (K). *Tacca leontopetaloides* Kuntze—Thailand, Wilkin et al. 817 (K). *Tacca palmata* Blume—Malaysia (cult.), Boyce 1982 (K). *Tacca palmatifida* Baker—van Balgooy 3586 (K). *Tacca plantaginea* Drenth—PS-TA1 (LV)