A Plastid Gene Phylogeny of the Yam Genus, *Dioscorea*: Roots, Fruits and Madagascar

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ABSTRACT. Following recent phylogenetic studies of the families and genera of Dioscoreales, the identification of monophyletic infrageneric taxa in the pantropical genus *Dioscorea* is a priority. A phylogenetic analysis based on sequence data from the plastid genes *rbcL* and *matK* is presented, using 67 species of *Dioscorea* and covering all the main Old World and selected New World lineages. The analysis used 14 outgroup taxa, including *Trichopus* Gaertn., *Tacca* J.R. & G. Forster, *Stenomeris* Planch., *Burmannia* L. and *Thismia* Griff. The main findings are: a) that a clade of rhizomatous taxa is sister to the rest of *Dioscorea*; b) the main Old World groups (such as the right-twining *D.* sect. *Enantiophyllum*) are monophyletic and c) there are two distinct lineages among the endemic Malagasy taxa. The consequences of the results for infrageneric classification of *Dioscorea* is considered, in particular the possibility of greatly simplifying the classifications of Knuth and Burkill. The results are also used to present novel hypotheses of character evolution in selected underground storage organ, inflorescence, fruit and seed characters and to discuss the origins of diversity in *Dioscorea*.

Dioscoreales are one of the most critical taxa in monocot systematics (Chase 2004 and references therein). Since the revolution in that field stimulated by the works of Huber (1969) and Dahlgren et al. (1985), the composition and relationships of the order have been the subject of an increasing level of study. In particular, the recent research of Caddick et al. (2002a; 2002b), using morphological and sequence data (from three genes), has demonstrated conclusively that the order comprises three families: Nartheciaceae, Burmanniaceae, and Dioscoreaceae. All are relatively small families (Kubitzki 1998). The largest of these in terms of number of species, Dioscoreaceae, comprise four genera (Caddick 2002a, 2002b). Three are monoecious and contain relatively few species. *Tacca* J.R. & G. Forst. (at least ten species) has a unilocular ovary with many ovules; unlike most taxa in the family it does not climb but is a herb with a short stem. *Stenomeris* Planch. (two species) is a climber with a trilocular ovary with many ovules; it forms a three-winged, dehiscent capsule at least 25 cm long. Its flowers have an urceolate torus, with the stamens inserted towards the torus mouth and reflexed into it. The third monoecious genus, *Trichopus* Gaertn. (two species), possesses an ovary with two ovules in each of three locules, with up to five ovules aborting during the development of an irregularly dehiscent to indehiscent fruit. Its hypanthium is relatively small and not urceolate. *Dioscorea* L., the only dioecious genus, comprises ca. 450 species and has a

trilocular ovary with two ovules per locule like *Trichopus*, but it lacks the complex arrangement of expanded stamen connectives and the ''umbrella-like'' stigma of that genus. With the generic and familial limits now better understood, the main systematic challenge in terms of biodiversity in Dioscoreales is *Dioscorea*. *Dioscorea* is also by far the most geographically widespread taxon, being almost ubiquitous in tropical and subtropical regions, with a few species being found in temperate areas. Species of *Dioscorea* are of significant importance as food (mainly in the form of their starchy tubers or ''yams'') and pharmaceuticals (e.g., corticosteroids and the contraceptive pill; Coursey 1967).

Dioscorea has presented a challenge to systematists for many years due to its great morphological diversity, dioecy, and small flowers. The first taxonomic treatments of significant numbers of species were those of Kunth (1850) and Uline (1898). The last complete monograph was published by Knuth (1924). Using a typically narrow ''*Pflanzenreich*'' species concept, he recognized ca. 600 species and divided them into four subgenera based on seed wing position, and then into 60 sections. However, when studied by a contemporary systematist, it is clear that many of Knuth's infrageneric taxa are clearly para- or even polyphyletic, for example the Old World compound-leafed species studied by Wilkin and Caddick (2000) and Wilkin (1999). Knuth divided these taxa into three sections and 51 species based on geography. The research cited

above showed that there are approximately 18 species that can be be divided into two groups with clear morphological differences between them. Knuth even managed to place a single species in different sections under two different names (*D. cochleari-apiculata* De Wild. in sect. *Botryosicyos* (Hochst.) Uline and the synonymous *D. stolzii* R. Knuth. in sect. *Lasiophyton* Uline). Therefore, a reappraisal of Knuth's classification is needed.

The taxonomic ideas of Knuth were to some extent refined and improved by Burkill (Prain and Burkill 1936, 1938; Burkill 1939, 1951, 1952, 1960; Burkill and Perrier de la Bâthie 1950). He had a thorough knowledge of the tropical African and Asian species of *Dioscorea* and based his classification on an intimate knowledge of their morphology and ecology derived from many years of study of herbarium specimens and living plants. However, his knowledge of species from China, Madagascar, and the New World was restricted to herbarium material. In his infrageneric classification of the Old World taxa, Burkill avoided the rank of subgenus, and instead divided some 220 species into 23 sections. Like Knuth, he emphasized seed characters, but he added underground organ morphology and development and male inflorescence morphology to the character set used. This resulted in a classification more complex than that of Knuth, but one with a tendency to separate taxa using what now appear to be autapomorphies. Since 1960, the genus has been the subject of piecemeal floristic studies (e.g., Miège 1968; Milne-Redhead 1975; Tellez and Schubert 1994; N'Kounkou 1993; Miège and Sebsebe 1998; Ding and Gilbert 2000). The only complete taxonomic treatment was that of Huber (1998), in which the Knuth/Burkill system of classification was recapitulated, with all of the dioecious taxa of Dioscoreaceae included in subfamily Dioscoreoideae as ''genera and genus-equivalent sections'' (Huber's terminology). Although this idea was novel, the flaws inherrent in the treatments of Knuth and Burkill meant that many of the taxa used were not systematically sound, and the decision to raise them all to generic rank was subjective.

Our assessment of these studies of *Dioscorea* indicated that the systematics of the genus needed complete revision, and that the revision should use cladistic analysis of DNA sequence data for a significant number of species of the genus. The aim was to discover phylogenetic patterns of relationship and, in particular, to produce monophyletic infrageneric entities. Recently published examples of similar systematic research on large genera include Manos et al. (2001), Goldblatt et al. (2002), Perret et al. (2003), and Chase et al. (2003). As in Chase et al. (2003), it was hoped that this approach could also be helpful in beginning a new phase of searching for wild relatives of the main cultivated yams and medicine providers, and thus as-

sist plant breeders, pathologists, phytochemists, and other applied biologists. A partial dataset for plastid *rbcL* already existed from those taxa sampled by Caddick et al. (2002b), and *matK* was selected to provide further resolution following its successful use in other studies of monocot taxa (e.g., Fuse and Tamura 2000; Gravendeel et al. 2001; Ge et al. 2002). The main systematic hypotheses to be evaluated were the monophyly of *Dioscorea* and Knuth and Burkill's sections of *Dioscorea*, towards the aim of constructing a classification based on the principle of monophyly.

The generation of a phylogenetic tree for *Dioscorea* based on sequence data, and therefore independent of morphology, also allowed morphological character evolution to be explored. Similar studies have been undertaken, by, for example Cameron et al. (2001) for pollen morphology in Malphigiaceae and Allen et al. (2003) and Lamb Frye and Kron (2003) for selected macromorphological characters in *Erythronium* L. and Polygonaceae, respectively. Tuber morphology, stem twining direction, dioecy, and fruit/seed wing shape are among the most important characters in the systematics of *Dioscorea*. A broadly sampled tree also allows biogeographical hypotheses to be evaluated, such as Burkill's (1960) suggestion that there are two independent lineages in Madagascar; a group with deeply buried tubers and seeds winged at the base only and another with shallowly buried tubers at the end of long roots and seeds winged all round the margin. Overall, this study represents the first step in understanding the diversity of *Dioscorea* in a phylogenetic context.

MATERIALS AND METHODS

Taxon Sampling. This study sampled 50 paleotropical species of *Dioscorea*, covering all of the main lineages evident from the infrageneric classifications of Knuth (1924) and Prain and Burkill (1960) and from the authors' own study of morphology (Table 1). Material from Thailand was particularly important in achieving this aim; the Thai flora contains almost all of the Asian species groups. There is also a sampling bias towards Madagascar. Both of these areas have high numbers of *Dioscorea* species per unit area: approximately one species per 12,000 km² in Thailand and one per 14,500 km² in Madagascar, while there is one species per 53,000 km² in Zambia and one per 50,000 km² in Malesia (Malaysia, Indonesia, the Philippines and New Guinea). Seventeen Neotropical species were included. Although it would be desirable to include more taxa from this region, the topology obtained was congruent with that of previous studies sampling more New World species (Raz, unpublished data; Bharathan et al. 2001). To significantly improve the sampling and support of the trees would mean two or three years more work, especially field collection. A total of 14 outgroup taxa were used from *Tacca*, *Trichopus*, and *Stenomeris* in Dioscoreaceae and *Burmannia* L. and *Thismia* Griff. in Burmanniaceae. Although they clearly nest within *Dioscorea* (Caddick et al. 2002b), some taxa formerly placed in *Tamus* L. and *Rajania* L. lack names in *Dioscorea* and are referred to by their published name with *Dioscorea* in parenthesis. New combinations will be made in due course.

DNA Extraction, Amplification, and Sequencing. DNA was extracted from c. 0.5–1.5 g fresh, 0.1–0.2g silica-gel dried leaves (Chase and Hills 1991), or 0.1–0.2 g leaves from herbarium sheets using a 2X CTAB method modified from Doyle and Doyle (1987).

TABLE 1. Sources of tDNA for taxa sequenced for *rbc*L and *ma*tK., including voucher specimen details and GenBank accession numbers for sequences.

Burmannia biflora L., *Chase 157* (NCU), USA, AY956483 (*matK*), AF206742 (*rbcL*). *Burmannia longifolia* Becc., *Johns et al. 9157* (K), Indonesia (Papua), AY956484 (*matK*), AF307484 (*rbcL*). *Burmannia madagascariensis* Mart. & Zucc., *Caddick et al. 312* (K), Madagascar, AY956485 (*matK*), AF307486 (*rbcL*)

Dioscorea c.f. *adenocarpa* Mart. ex Griseb., *Wood 9178* (K), Bolivia, AY950686 (*matK*). *Dioscorea alata* Lour., *Tamura & Fuse 10019* (Osaka City Univ. Botanic Garden), AB040208 (*matK*). *Dioscorea alata* Lour., *Wilkin 1090* (K), Thailand, AY667098 (*rbcL*). *Dioscorea alatipes* Burkill & H. Perr., *Caddick et al. 334* (K), Madagascar, AY950687 (*matK*). *Dioscorea alatipes* Burkill & H. Perr., *Caddick et al. 333* (K), Madagascar, AY667099 (*rbcL*). *Dioscorea amaranthoides* C. Presl, *Wood 12383* (K), Bolivia, AY950688 (*matK*). *Dioscorea antaly* Jum. & H. Perr., *Wilkin et al. 1103* (K), Madagascar, AY667100 (*rbcL*). *Dioscorea antaly* Jum. & H. Perr., *Wilkin et al. 1104* (K), Madagascar, AY956476 (*matK*). *Dioscorea* c.f. *apurimacensis* R. Knuth, *Lewis 3210* (LOJA), Ecuador, AY956477 (*matK*), AY667101 (*rbcL*). *Dioscorea arachidna* Prain & Burkill, *Wilkin et al. 862* (K), Thailand, AY956478 (*matK*), AF307468 (*rbcL*). *Dioscorea arcuatinervis* Hochr., *Caddick et al. 310* (K), Madagascar, AY956479 (*matK*), AY667102 (*rbcL*). *Dioscorea bemarivensis* Jum. & H. Perr., *Caddick 340* (K), Madagascar, AY667103 (*rbcL*). *Dioscorea bemarivensis* Jum. & H. Perr., *Wilkin et al. 1124* (K), Madagascar, AY956480 (*matK*). *Dioscorea birmanica* Prain & Burkill, *Thapyai et al. 409* (BKF), Thailand, AY956481 (*matK*), AY667104 (*rbcL*). *Dioscorea brachybotrya* Poepp., *Rudall 1/97* (K), Chile, AY956482 (*matK*), AF307469 (*rbcL*). *Dioscorea brevipetiolata* Prain & Burkill, *Wilkin et al. T964* (K), Thailand, AY956486 (*matK*), AY667105 (*rbcL*). *Dioscorea buchananii* Benth., *Bingham et al. 10290* (K), Zambia, AY956487 (*matK*), AY904790 (*rbcL*). *Dioscorea bulbifera* L., *Foster et al. 12* (K), Madagascar (RBG Kew cult. 1998–533), AY956488 (*matK*), AY904791 (*rbcL*). *Dioscorea calcicola* Prain & Burkill, *Wilkin et al. 814* (K), Thailand, AY956489 (*matK*), AY904791 (*rbcL*). *Dioscorea cirrhosa* Lour., *Thapyai 375* (BKF), Thailand, AY904792 (*rbcL*). *Dioscorea communis* (L.) Caddick & Wilkin, *Chase 536* (K), UK, AY956490 (*matK*), AF307474 (*rbcL*). *Dioscorea convolvulacea* Schltdl. & Cham., *Chase 197* (NCU), Trinidad, AY956491 (*matK*), AJ235805 (*rbcL*). *Dioscorea coriacea* Humb. & Bonpl. ex Willd., *Lewis 3220* (LOJA), Ecuador, AY956492 (*matK*). *Dioscorea daunea* Prain & Burkill, *Thapyai et al. 518* (BKF), Thailand, AY956493 (*matK*), AY904793 (*rbcL*). *Dioscorea decipiens* Hook. f., *Wilkin et al. 861* (K), Thailand, AY956494 (*matK*). *Dioscorea decipiens* Hook. f., *Wilkin 860* (K), Thailand, AF307454 (*rbcL*). *Dioscorea dumetorum* (Kunth) Pax, *Wilkin 761* (K), Malawi (RBG Kew cult. 1994–1455), AY956495 (*matK*), AF307464 (*rbcL*). *Dioscorea elephantipes* (L'Hér.) Engl., *UCI Arb. 6773* (NCU), Cultivated, AY956496 (*matK*), AF307461 (*rbcL*). *Dioscorea esculenta* (Lour.) Burkill, *Wilkin et al. 995* (K), Thailand, AY956497 (*matK*), AY904794 (*rbcL*). *Dioscorea fandra* Jum. & H. Perr, *Caddick et al. 324* (K), Madagasar, AY956498 (*matK*), AY904795 (*rbcL*). *Dioscorea galeottiana* Kunth, *Tellez 13090* (MEXU), Mexico, AY956499 (*matK*), AY904796 (*rbcL*). *Dioscorea gillettii* Milne-Redh., *Vollesen 61* (K), Kenya, AY956500 (*matK*), AY904797 (*rbcL*). *Dioscorea glabra* Roxb., *Wilkin et al. 874* (K), Thailand, AY956501(*matK*), AF307456 (*rbcL*). *Dioscorea glomerulata* Hauman, *Wood 8065* (K), Bolivia, AY957586 (*matK*). *Dioscorea hamiltonii* Prain & Burkill, *Wilkin et al. 886* (K), Thailand, AY957587 (*matK*), AF307465 (*rbcL*). *Dioscorea hexagona* Jum. & H. Perr., *Wilkin et al. M960* (K), Madagascar, AY957588 (*matK*), AY904798 (*rbcL*). *Dioscorea hispida* Dennst., *Wilkin et al. 855* (K), Thailand, AY957589 (*matK*), AF307463 (*rbcL*). *Dioscorea inopinata* Prain & Burkill, *Thapyai et al. 513* (BKF), Thailand, AY957590 (*matK*), AY904799 (*rbcL*). *Dioscorea karatana* Wilkin, *Wilkin et al. 947A* (K), Madagascar (RBG Kew cult. 1999–2988), AY957591(*matK*). *Dioscorea lagoa-santa* Uline ex R. Knuth, *Wood 8182* (K), Bolivia, AY957592(*matK*). *Dioscorea lanata* Balf. f., *Miller 10423* (E), Socotra, AY957593 (*matK*), AF307458 (*rbcL*). *Dioscorea larecajensis* Uline ex R. Knuth, *Wood 9960* (K), Bolivia, AY957594 (*matK*). *Dioscorea longirhiza* Caddick & Wilkin, *Tellez 13081* (MEXU), Mexico, AY957595 (*matK*), AF307473 (*rbcL*). *Dioscorea maciba* Jum. & H. Perr., *Caddick et al. 318* (K), Madagascar, AY957596 (*matK*), AY904800 (*rbcL*). *Dioscorea mcvaughii* B.G. Schub., *Tellez 13080* (MEXU), Mexico, AY957597(*matK*), AF307460 (*rbcL*). *Dioscorea membranacea* Pierre, *Wilkin et al. 878* (K), Thailand, AY957598 (*matK*), AF307467 (*rbcL*). *Dioscorea namorokensis* Wilkin, *Wilkin et al. 1123* (K), Madagascar, AY957599 (*matK*). *Dioscorea nipponica* Makino, *Chase 6225* (K), RBG Kew cult. 1969–19664, AY957600 (*matK*), AF307455 (*rbcL*). *Dioscorea ovinala* Baker, *Wilkin et al. 1108* (K), Madagascar, AY972481 (*matK*). *Dioscorea oryzetorum* Prain & Burkill, *Thapyai 464* (BKF), Thailand, AY972482 (*matK*), AY904801 (*rbcL*). *Dioscorea pentaphylla* L., *Wilkin et al. 888* (K), Thailand, AY972483 (*matK*), AF307470 (*rbcL*). *Dioscorea petelotii* Prain & Burkill, *Wilkin et al. 1023* (K), Thailand, AY973484 (*matK*), AY904802 (*rbcL*). *Dioscorea prazeri* Prain & Burkill, *Wilkin et al. 1075* (K), Thailand, AY973485 (*matK*), AY904803 (*rbcL*). *Dioscorea preussii* Pax, *Davis 3019* (K), Cameroon, AY973486 (*matK*). *Dioscorea pseudo-nitens* Prain & Burkill, *Thapyai et al. 452* (BKF), Thailand, AY973487 (*matK*). *Dioscorea pyrenaica* Bubani & Bordère ex Gren., *Sandwith 4745* (K), Spain, AF307471 (*rbcL*). *Dioscorea rockii* Prain & Burkill, *Wilkin et al. 1036* (K), Thailand, AY972488 (*matK*), AY939882 (*rbcL*). *Dioscorea sansibarensis* Pax, *Wilkin et al. 973* (K), Madagascar (RBG Kew cult. 1998–525), AY972489 (*matK*), AY939883 (*rbcL*). *Dioscorea schimperiana* Hochst. ex Kunth, *Wilkin et al. 762* (K), Malawi (RBG Kew cult. 1995–1450), AY972490 (*matK*), AF307466 (*rbcL*). *Dioscorea soso* Jum. & H. Perr., *Wilkin et al. 1102* (K), Madagascar, AY939884 (*matK*). *Dioscorea soso* Jum. & H. Perr., *Wilkin et al. 1106* (K), Madagascar, AY972491 (*matK*). *Dioscorea subhastata* Vell., *Wood 9093* (K), Bolivia, AY972492 (*matK*). *Dioscorea sylvatica* Ecklon, *Chase 6184* (K), RBG Kew Cult. 1994–796, AY972826 (*matK*), AF307462 (*rbcL*). *Dioscorea* c.f. *tanalarum* H. Perr., *Caddick et al. 302* (K), Madagascar, AY972827 (*matK*), AY939885 (*rbcL*). *Dioscorea tentaculigera* Prain & Burkill, *Thapyai 436* (BKF), Thailand, AY972828 (*matK*), AY939886 (*rbcL*). *Dioscorea trichantha* Baker, *Wilkin et al. 1153* (K), Madagascar, AY972829 (*matK*), AY939887 (*rbcL*). *Dioscorea wallichii* Hook. f., *Wilkin et al. 1072* (K), Thailand, AY973830 (*matK*), AY939888 (*rbcL*). *Dioscorea zingiberensis* C.H. Wright, *TCMK 244* (K), China, AY973831 (*matK*), AY939889 (*rbcL*). *Dioscorea* sp. A, *Wood & Guzman 17511* (K), Bolivia, AY973832 (*matK*). *Dioscorea* sp. B, *Lewis 3177* (LOJA), Ecuador, AY973833 (*matK*). *Dioscorea* sp. C, *Wood 8032* (K), Bolivia, AY973834 (*matK*). *Rajania* (*Dioscorea*) *cordata* L., *Axelrod 8407* (UPR), Cuba, AY973835 (*matK*), AF307472 (*rbcL*)

Stenomeris borneensis Oliv., *BRUN 19173* (K), Brunei, AF307475 (*rbcL*). *Stenomeris borneensis* Oliv., *BRUN 19174* (K), Brunei, AY973836 (*matK*)

Tacca artocarpifolia Seem., *Caddick 305* (K), Madagascar, AF307481 (*rbcL*). *Tacca chantrieri* André, *Chase 175* (NCU), Cultivated, AY973837 (*matK*), AJ286561 (*rbcL*). *Tacca integrifolia* Ker Gawl., *Boyce 1074* (K), Thailand, AY973838 (*matK*), AF307478 (*rbcL*). *Tacca leontopetaloides* (L.) O. Kuntze, *Wilkin et al. 817* (K), Thailand, AY973839 (*matK*), AF307480 (*rbcL*). *Tacca palmata* Blume, *Boyce 1082* (K), RBG Kew cult. 1996–1257, AY973840 (*matK*), AF307479 (*rbcL*). *Tacca palmatifida* Baker, *Chase 1377* (K), Cult. Bogor XI.B.VI.134, AY973841 (*matK*), AY939890 (*rbcL*). *Tacca plantaginea* (Hance) Drenth, *Leiden B.G. 920520* (L), Cult., AY973842 (*matK*), AF307483 (*rbcL*)

TABLE 1. Continued.

Tamus (*Dioscorea*) *edulis* Lowe, *Chase 3425* (K), Canary Is., AY973843 (*matK*), AY939891 (*rbcL*)
Thismia rodwayi F. Muell., *Garnock-Jones 2218* (WELTU), New Zealand, AY939892 (*rbcL*)
Trichopus sempervirens (H. Perr.) Caddick & Wilkin, *Caddick 304* (K), Madagascar, AF307476 (*rbcL*). *Trichopus sempervirens* (H. Perr.) Caddick & Wilkin, *Wilkin 948* (K), Madagascar, AF973844 (*matK*). *Trichopus zeylanicus* Gaertn., *Caddick 346* (K), Sri Lanka, AF307477(*rbcL*). *Trichopus zeylanicus* Gaertn., *Chase 16354* (K), Sri Lanka, AY973845 (*matK*)

DNA was precipitated in 2.5 vol. ethanol or 2/3 vol. isopropanol for herbarium dried specimens. DNA samples were purified on CsCl$_2$/ethidium bromide gradients (1.55 g/ml) and stored at $-20°$C. For problematic samples, a 150 µl aliquot of DNA was cleaned and concentrated using a 'Wizard' mini-column, following manufacturer's instructions (Promega, Crawley, West Sussex, UK).

Amplification of *rbcL* was undertaken in two halves using the primers 1F, 724R, 636F, and 1360R as described in Caddick et al. (2002b). These four primers were also used for sequencing.

Amplification of *matK* was carried out in the same manner as Salazar et al. (2003), using the 50 µl reaction method with PCR master mix. In addition to the -19F and 2R primers used to amplify the whole *matK-trnK* region, the internal primers 390F and 1326R were also used for some problematic amplifications and all sequencing (Cuénoud et al. 2002). Most taxa were amplified successfully with a premelt of 2 mins 30 secs at 94°C, followed by 28 cycles of 1 min denaturation at 94°C, 45 secs annealing at 52°C, 2 mins 30 secs extension at 72°C, plus a final extension of 7 mins at 72°C. For problem taxa, a 4 min premelt was used, followed by 30 cycles of 1 min at 94°C, 1 min at 52°C, 2 mins 30 secs at 72°C, and a 7 minute final extension at 72°C. in these cases, each 50 µl reaction contained 6 µl 25 mmol/L MgCl$_2$, 5 µl 10x Mg-free DNA polymerase buffer (Promega, Madison, Wisconsin, USA), 2 µl 0.4 % bovine serum albumen (BSA), 1 µl 10 mmol/L dNTP, 0.7 µl each primer, 0.5 µl 5 units/µl Taq DNA-polymerase (Promega), 2 µl template DNA and 32.1 µl ddH$_2$0.

All PCR products were cleaned using 'Wizard' mini-columns, following manufacturer's instructions (Promega, Crawley, West Sussex, UK). Amplification products were sequenced directly with modified dideoxy cycle sequencing with dye terminators according to the manufacturers' protocol (Applied Biosystems, ABI, Warrington, Cheshire, UK). Sequencing reactions were run on an ABI 377 automated sequencer according to the manufacturer's protocols. Sequence files were edited and assembled using Sequence Navigator and Autoassembler (ABI) or Sequencer 4.1 (Gene Codes Corp., Ann Arbor, Michigan, USA). Low numbers of insertions and deletions in *matK* and their absence in *rbcL* made alignment by eye possible. The part of the *trnK* intron sequenced was not used in analyses. Coding of indels as presence/absence characters was not undertaken. The matrix is available on email request from PW and MWC. All sequences have been submitted to GenBank and the aligned data matrix to TreeBASE (study accession S1370, matrix accessions M2432).

Analyses. Parsimony analyses were carried out using PAUP* 4.0b10 software (Swofford 2001). For *rbcL*, missing data at the 3' and 5' ends were excluded such that only base pair positions 31–1398 were used. Similarly, base pair positions 1–32 were omitted from the *matK* analysis. All characters were treated as equally weighted. The species of *Burmannia* and *Thismia* (Table 1) were specified as the outgroup following the tree topology obtained in Caddick et al. (2002b). A heuristic search consisted of 1000 random taxon-additions and tree-bisection-reconnection (TBR) branch swapping but holding no more than 10 trees per replicate to avoid swapping on large numbers of suboptimal trees, with the Mul-Trees option in effect. Branches were collapsed if minimum length = 0 ("amb-"). The trees generated were then used in a second phase of analysis with no tree limit in effect. Support for clades was estimated using the bootstrap (Felsenstein 1985), which consisted of 1000 bootstrap replicates, simple taxon addition, and TBR branch swapping. Again, no more than 10 trees were saved per replicate. Groups were retained with bootstrap percentages (BP) >50%.

Morphological Character Optimization. In addition to the DNA datasets, the following macromorphological characters and states were optimized onto the tree presented here using Mac-Clade 4.05 (Maddison and Maddison 2002); the characters were not not used in the combined analysis because there are too few of them to have an effect.

1. Underground parts rhizomatous (0), perennial tuber (1), annually replaced tuber (2).
2. Plants with all flowers hermaphrodite (0), plants dioecious (1) (terminology follows Richards 1986).
3. Stem twining direction left (0), right (1).
4. Fruit a dry, thin-walled capsule (0), fruit a fleshy berry, at least until submature (1), fruit a leathery berry (2).
5. Seed winged all round margin (0), seed wing basal only (1), seed wing apical only (2), seed winged only at base and apex (3).

Character state data were obtained from the authors' personal observations. All of the characters were important parts of the infrageneric classifications of *Dioscorea* of Knuth (1924) and Burkill (1960). Underground part morphology was used to delimit sections extensively by Burkill, but only to a very limited extent by Knuth. Both used dioecy as a character to delimit *Dioscorea*. Stem twining direction exhibited a particularly clear division between two groups of Burkill's Old World sections. Fruit morphology was used to separate *Rajania* and *Tamus* from *Dioscorea* since the era of Linnaeus. Burkill (1960) also used the presence of fleshy capsule walls to define *D.* sect. *Pachycapsa* Burkill & H. Perr., and seed wing position used by all authors as far back as Kunth (1850) as a generic or subgeneric character. Thus the character optimization was seen as a valuable exercise at this point in our understanding of *Dioscorea* phylogenetics, particularly for assessing the congruence of characters used to define existing infrageneric taxa with the trees obtained in this study, or at least those parts with good support.

RESULTS

Separate analyses of the *rbcL* and *matK* datasets were undertaken first. Tree statistics for these analyses are shown in Table 2. Taxa with missing data from entire regions were excluded from the single gene analyses (*D.* c.f. *adenocarpa*, *D. amaranthoides*, *D. calcicola*, *D. coriacea*, *D. glomerulata*, *D. karatana*, *D. lagoa-santa*, *D. larecajensis*, *D. namorokensis*, *D. ovinala*, *D.* sp. A, *D.* sp. B, *D.* sp. C, *D. subhastata*, and *Burmannia biflora* for *rbcL*; *D. cirrhosa*, *D. pyrenaica*, *Thismia rodwayi*, and *Tacca artocarpfolia* for *matK*). No hard incongruence (i.e., incongruence with high BP) was observed in the bootstrap consensus trees obtained for the two genes, so the datasets were combined. This approach is the same as that followed in, for example, Whitten et al. (2000), Reeves et al. (2001) and Simpson et al. (2003). One tree from the combined plastid gene analysis is presented in Fig. 1. This is based on a final alignment of 3,321 sites, of which 1,041 are variable and 680 (20.5 %) po-

TABLE 2. Tree statistics for analyses of the *matK, rbcL,* and combined datasets.

Gene	No. of sites	No. potentially phylogenetically informative	% potentially phylogenetically informative	Tree length	No. of trees	CI	RI
rbcL	1368	187	13.7	608	More than 30000	0.59	0.76
matK	1953	493	25.2	1345	More than 30000	0.70	0.83
Combined dataset	3321	680	20.5	1977	15868	0.66	0.81

tentially phylogenetically informative. The total number of equally most parsimonious trees generated was 15,868, each with 1,977 steps, CI = 0.66 and RI = 0.81. Figure 1 also shows those branches that collapse in the strict consensus tree (dashed lines) and identifies nodes with weak or strong bootstrap support. *Dioscorea*, including all of the dioecious taxa (as in Caddick et al. 2002b) is resolved as monophyletic within Dioscoreaceae (BP = 95). Resolution among the genera of Dioscoreaceae is unclear. Within *Dioscorea*, the base of the tree and the early branching clades (A) are mostly strongly supported. It is clear that *D.* sect. *Stenophora* Uline is sister to the rest of *Dioscorea* (the clade labelled Stenophora); its monophyly has BP 100, and its sister clade BP 92. There are also two strongly supported New World clades (NW) that are not each other's closest relatives (BP 88). The spine of the tree has much shorter branch lengths above the base. Several, such as the African southern and montane clade (Afr.) and the European clade (EU) form a polytomy with the larger NW clade in the strict consensus tree from the combined analysis. In the African southern and montane clade, *D. sylvatica* and *D. elephantipes* are sister-species with strong bootstrap support. Both were once placed in the genus *Testudinaria* Burch, and they share large, perennial, woody tubers.

The remaining clade, from *D. tentaculigera* to *D. oryzetorum* (B), comprises entirely Old World taxa, with the sole exception of *Rajania* (*Dioscorea*) *cordata*. Its monophyly has weak support (BP 53). Overall, the degree of resolution of these taxa by *matK* and *rbcL* is mixed, with a significant polytomy at the node above *D. tentaculigera* and a number of unresolved taxa, but two well-supported subclades within the B clade can be identified (Malagasy and Enantiophyllum). *Dioscorea tentaculigera* is sister to all of the B clade taxa, on a rather long branch. The first of the two main subclades is one wholly comprising endemic species from Madagascar (Malagasy; BP 91). It can also be seen that it is further divided into two strongly supported sister clades. The first, with three taxa, is relatively divergent from the rest. The accession of *D.* c.f. *tanalarum*, which came from eastern Madagascar, but not littoral forest, may be *D. arcuatinervis* or a close relative, but unfortunately was sterile. The second clade has *D. fandra* sis-

ter to the rest of its many taxa and conspicuously short internal branches. A compound-leafed clade (CL) was recovered, but it lacks BP support. The two main compound-leafed groups each have an entire-leafed sister species: *D. antaly* in the case of *D. dumetorum*/*D. hispida* and *D. bulbifera* for *D. arachidna*/*D. pentaphylla*. The final major subclade of the B clade is *D.* sect. *Enantiophyllum* (Enantiophyllum; BP 98). The African species *D. schimperiana* is sister to the rest of the clade. Like the Malagasy clade, the internal branches are short. It should be noted that in the optimizations of the morphological characters (Figs. 2–5), state changes occur wholly or mainly at the better supported nodes. The clade comprising *D. birmanica* (Birmanica) and its possible allies is not robust. In some trees *D. petelotii*, *D. pseudonitens*, and *D. esculenta* also fall within this clade, but all of these taxa collapse to the base of the B clade in the strict consensus tree, as does *Rajania* (*Dioscorea*) *cordata*.

DISCUSSION

Dioscorea *and* D. *sect*. Stenophora. The sister relationship of *D.* sect. *Stenophora* to the rest of *Dioscorea* has appeared in every analysis of *Dioscorea* to date, highlighting the pivotal position of the section in the systematics of the genus. This study is the first in which there is BP support for this topology. *Dioscorea* sect. *Stenophora* was divided into two taxa by Knuth (1924), *D.* sect. *Eustenophora* R. Knuth and *D.* sect. *Macropoda* Uline. Burkill (1936, 1960) united them, and suggested their importance in the evolution of the genus. What he could not have known was their degree of sequence divergence from the rest of *Dioscorea*, indicated by the data from the two plastid genes studied here (Fig. 1). All 20–25 species of this taxon have underground parts consisting solely of a branching horizontal rhizome. In this respect they are similar to *Trichopus sempervirens* and several members of *Tacca*. Figure 2 shows underground part morphology optimized onto the tree in Fig. 1. The optimization of flower sex is shown in Fig. 3. Dioecy is clearly a synapomorphy for the genus *Dioscorea*. However, several species of *D.* sect. *Stenophora* display polymorphism for this character: a few immature capsules occurring on a male plant, or male flowers on a female inflorescence (e.g.,
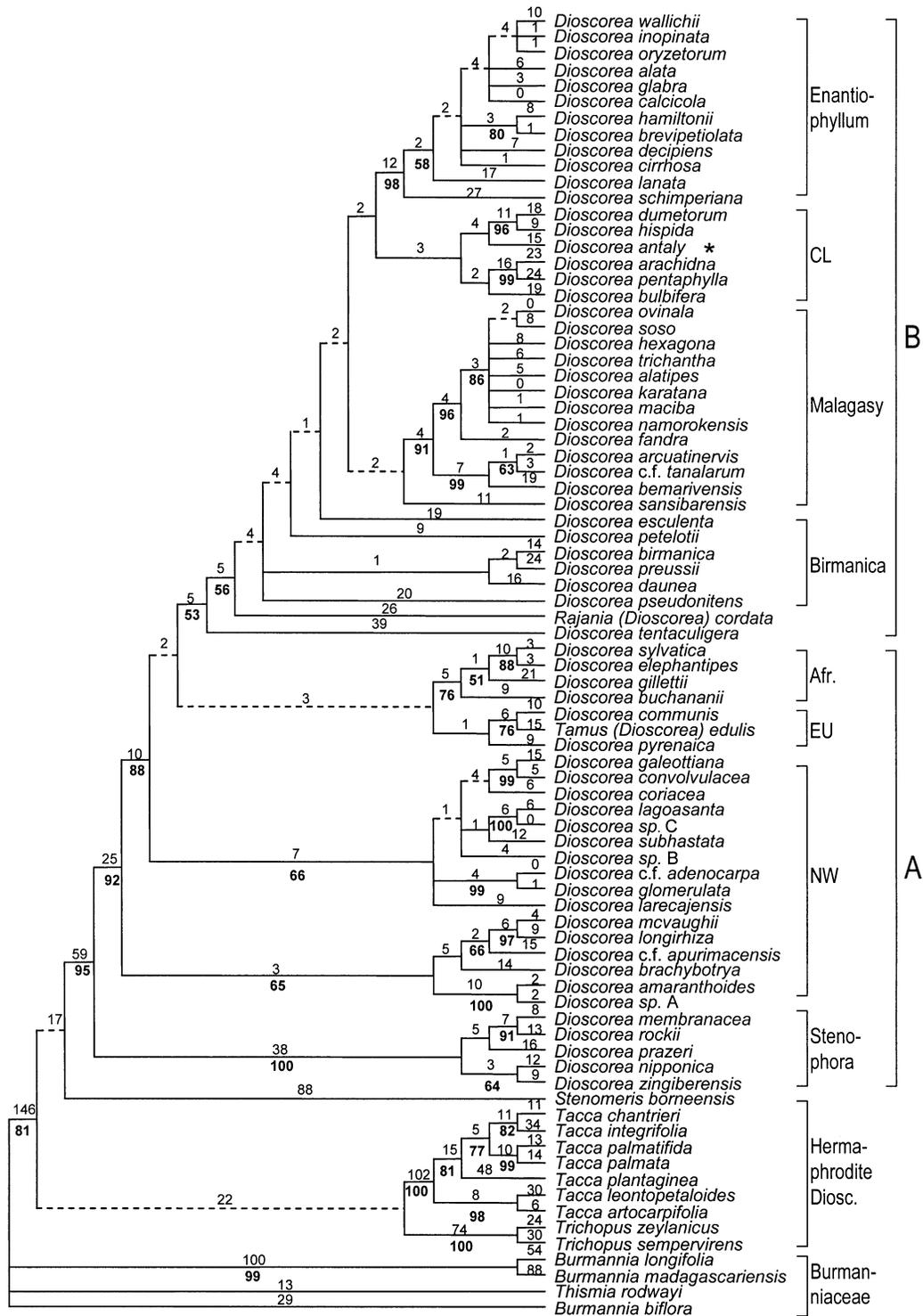
FIG. 1.   One of the 15868 trees from the combined *rbcL* and *matK* analysis of *Dioscorea*. Figures above lines are branch lengths (DELTRAN optimization) and below (bold) bootstrap percentages (BP). Dashed lines indicate the nodes that collapse in the strict consensus tree. NW = New World, EU = Europe, CL = compound-leafed, A = early branching clades, B = B clade. *D. antaly* (marked *) is the only endemic species from Madagascar that does not fall in the Malagasy clade.
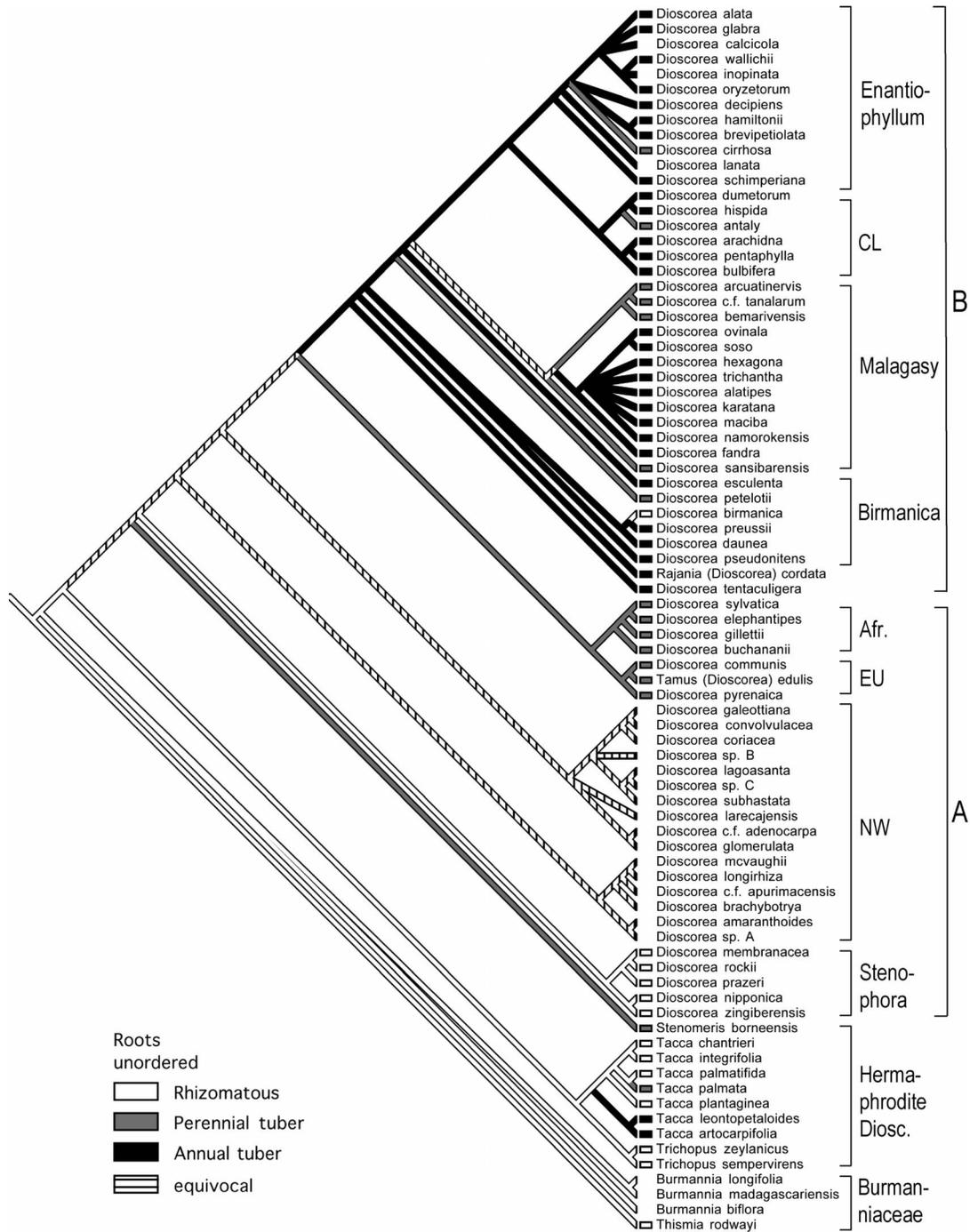
FIG. 2. Underground part morphology optimized onto one of the 15868 most parsimonious trees. Missing data are indicated by the lack of a shaded box at the branch tip.

D. rockii [pers. obs.] and *D. zingiberensis* [M.G. Gilbert pers. comm.]). Unstable dioecy has also been reported in a few Neotropical species such as *D. convolvulacea* (Sosa et al. 1987), which are also members of early branching clades. This indicates that the developmental mechanisms that control floral development are not as stable in these clades as in the rest of *Dioscorea*. As currently understood, the species of *D.* sect. *Stenophora* are restricted to temperate and subtropical areas of the northern hemisphere, through the Caucasus, Himala-
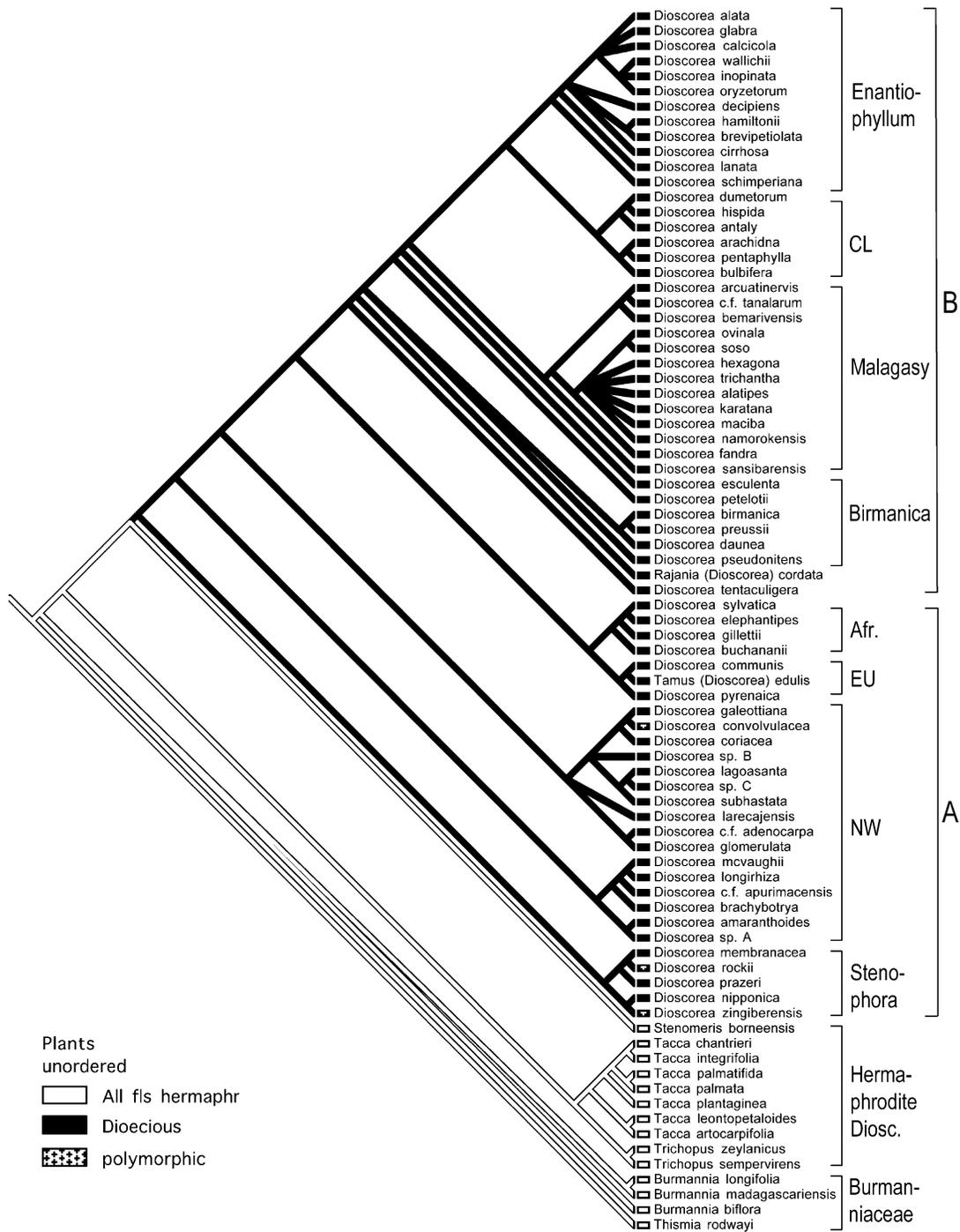
FIG. 3. Flowers hermaphrodite/plants dioecious optimized onto one of the 15868 most parsimonious trees. Missing data are indicated by the lack of a shaded box at the branch tip.

yas, China and Japan to North America. Their southernmost point of distribution is probably the Isthmus of Kra in Thailand. Two more species may belong to this group (*D. ridleyi* Prain & Burkill from Sarawak and *D. palawana* Prain & Burkill from the Philippines) but

unfortunately they are not included. They are rare, and their underground parts are not fully known (Wilkin et al. 2002). The diversity and pantropical distribution of *Dioscorea* as a whole (ca. 450 species) when compared with the restricted distribution and fewer spe-

cies of *D.* sect. *Stenophora* suggest that the origins of the diversity may lie in the combination of dioecy and tuber possession, but not dioecy alone. Burkill (1960) suggested that the evolution of the ability to inhabit niches with limited or more seasonal rainfall was the most important factor promoting diversity in *Dioscorea*, not dioecy. *Dioscorea* is light-demanding, but of insufficient stature to be a major element of wet tropical forests. Consequently selection favoured forms that could thrive in drier, more open habitats.

New World Taxa. The tree topology in Fig. 1 with two monophyletic stem clades of New World taxa is similar to that found by Bharathan et al. (2001) for *trnL-F* and *ndhF*. Clearly, much greater sampling of New World taxa is necessary to increase confidence in the results obtained. Nevertheless, it appears that only *Rajania* falls among the otherwise exclusively Old World B clade. Research by Lauren Raz (pers. comm.) indicates that a small number of Neotropical *Dioscorea* species allied to *Rajania* also nest there. Analysis of the combined data obtained by Raz and in this study is already planned. Figure 4 shows that the right-twining (dextrorse in the sense of Burkill 1960) habit has arisen at least twice in *Dioscorea*, with both clades and their positions having weak or strong bootstrap support (Fig. 1). Further sampling may show multiple New World origins of right-twining, but it is present in only one clade here. The right-twining habit has clearly only evolved once in the Old World (see Enantiophyllum below). Underground part morphology (Fig. 2) is an area in which the New World taxa are data-deficient; *D. convolvulacea* may be rhizomatous (Sosa et al. 1987), but field-based research on underground part morphology is desirable.

Southern and Montane Africa. The biogeographical link between the species in this clade (Afr.) species makes further study particularly important. They have not previously been thought to form a taxon.

European Taxa. This clade (EU) is not present in the combined strict consensus tree, perhaps because *D. pyrenaica* has only been sequenced for *rbcL* (we need to collect more extractable material). It is found in the strict consensus tree for *rbcL* alone, although only the *D. communis/Tamus* (*Dioscorea*) *edulis* clade has weak bootstrap support. It is mainly of interest because the genus *Tamus* was separated from *Dioscorea* on the basis of it having a fleshy berry, whereas *Dioscorea* has a dry capsule. Berry-like fruits are also encountered in at least two other lineages in Madagascar, although in both *D. ovinala* and *D. antaly* (Fig. 1) the thick-walled fruit dries out and dehisces in the final stages of development. The first author has also seen a herbarium specimen from Bolivia that appeared to have a berry, although it was not fully mature and the fruits had been dried rather than preserved in spirit. Thus, this shift of dispersal strategy in *Dioscorea* does not merit

generic segregation. *Tacca*, with many fewer species than *Dioscorea*, has much greater diversity in fruit morphology.

B Clade. This clade is the most economically important within *Dioscorea* because the major cultigens and many of the edible wild species are found in this clade. This is probably because many of the species in this part of the tree have annual tubers, in marked contrast to the clades at the basal nodes, which usually have perennial tubers (Fig. 2). Unfortunately, information on the underground parts of the New World taxa of *Dioscorea* is lacking. It is probable, based on the optimization in Fig. 2, that tuber evolution is an important source of diversity within the B clade of *Dioscorea*. For example, the compound-leafed species (CL clade) studied by Wilkin and Caddick (2000) have a diverse array of tuber forms. The low levels of molecular divergence among some of the B clade (*D. tentaculigera* to *D. oryzetorum*) shown by the short branch lengths suggests that the limit of resolution of the two genes studied has been reached for these taxa. Branch lengths for *D. tentaculigera* and *Rajania* (*Dioscorea*) *cordata* are noticeably higher than for other taxa in this part of the tree. The former in particular appears to be in a systematically isolated position. Prain and Burkill (1936) tentatively placed *D. tentaculigera* in *D.* sect. *Stenophora* on the basis of its capsule shape, though they had not seen its underground organs, but this study and its paired, vertically oriented tubers (Thapyai, pers. obs.) both contradict this.

Madagascar. One of the clades nested within the B clade comprises endemic taxa from Madascar (Malagasy). Only one endemic species from Madagascar does not fall in this clade, *D. antaly*. The clade of endemic Malagasy species is composed of two well-supported sister subclades. The first of these (*D. bemarivensis–D. c.f. tanalarum*) has seeds with a wing all round the margin (Fig. 5; the plesiomorphic condition). The underground parts of *D. arcuatinervis* and *D. bemarivensis* appear similar, with long, terete tubers that spread horizontally through the soil and have swollen apices. *Dioscorea bemarivensis* is from western, seasonally dry forests, whereas *D. arcuatinervis* is found in littoral forest of the eastern coast, which receives year-round rainfall. The second subclade comprises species with basally winged seeds, in which rotational flight appears to improve dispersal distance when compared with the gliding flight of seeds that are winged all round the margin (Burkill 1960). This adaptive change appears to have multiple evolutionary origins (Fig. 5). All species of the second subclade possess two vertically oriented tubers (one shrinking, one growing) of even thickness. As in the *D. bemarivensis–D. c.f. tanalarum* clade, *D. fandra*, which is sister to the rest of the second clade, is a species from the dry southwest. The low levels of molecular divergence within this clade
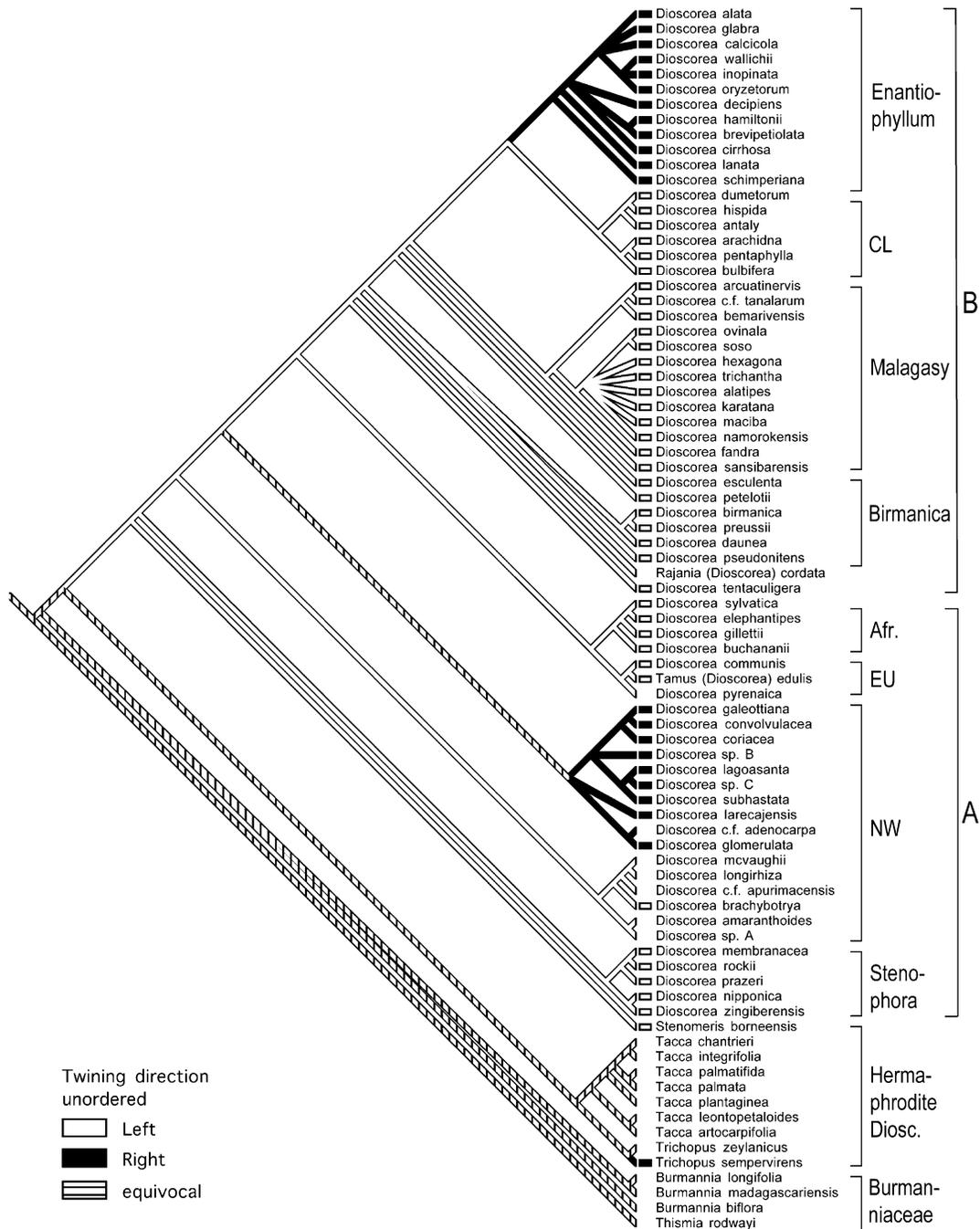
FIG. 4. Stem twining direction optimized onto one of the 15868 most parsimonious trees. Missing data are indicated by the lack of a shaded box at the branch tip.

(as measured by the short branch lengths) may also indicate that it may have been a relatively recent radiation or rate change, in which changes in life history associated with shifts into wetter zones and changes in flower and fruit morphology played key roles. This hypothesis needs to be evaluated with a better sampled and resolved tree.

The position of *D. sansibarensis* is unresolved. It is found in the lowland tropics of Madagascar and Africa, especially in riverine habitats. It has an unusual seed wing; the seed is winged all around the margin, but expanded towards the base and apex of the capsule locule, making it narrowly oblong (Wilkin 2001). Whether it is native to Madagascar is open to question;
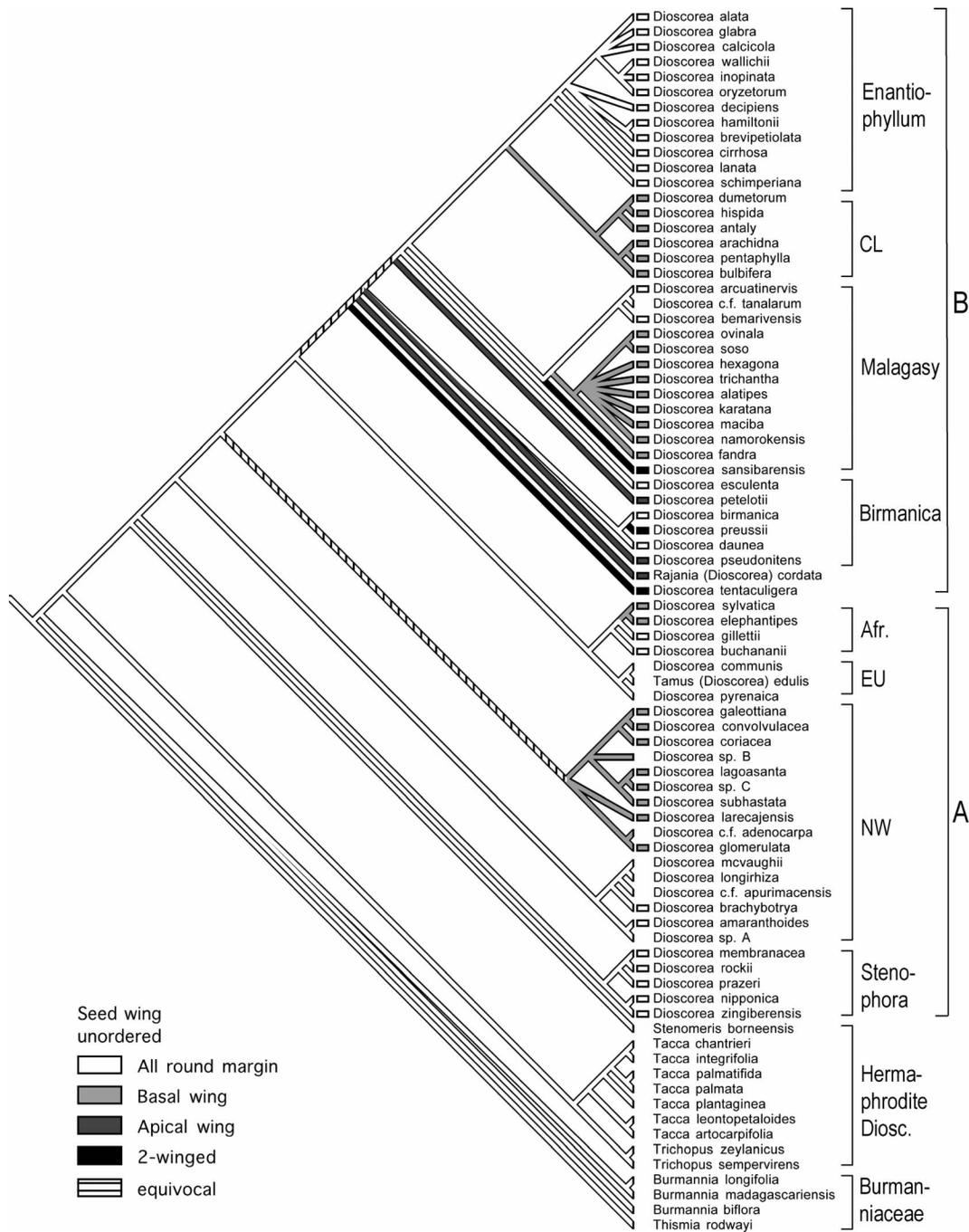
Fig. 5.   Seed wing morphology optimized onto one of the 15868 most parsimonious trees. Missing data are indicated by the lack of a shaded box at the branch tip.

it is a bulbil-producer and thus easily dispersed. In the past it has been cultivated as both a famine food and a source of poison (Burkill 1985).

There is no support in Fig. 1 for the sectional classification used by Burkill and Perrier de la Bâthie (1950) for the Malagasy species. *Dioscorea* sects. *Car-*

diocapsa Uline and *Madagascarienses* R. Knuth appear to represent a single taxon (one subclade). The other subclade includes the monotypic *Dioscorea* sect. *Pachycapsa* Burkill & H. Perr. (*D. ovinala*, characterised by fruit with inflated, fleshy wings that only become dry shortly before dehiscence), *D.* sect. *Campanuliflorae* Burkill &

H. Perr. (e.g., *D. maciba*, with a campanulate torus), *D.* sect. *Seriflorae* Burkill & H. Perr. (e.g., *D. karatana*, described as possessing claviform buds), and *D.* sect. *Brachyandra* Uline, in which the majority of species were placed. Species of *D.* sect. *Brachyandra* have a flat torus and a capsule in which the wings are thin and relatively dry throughout their development; *D. fandra*, *D. soso*, and *D. trichantha* were all placed there (Fig. 1). Although the limit of resolution of *rbcL* and *matK* has been reached in this clade, it is nevertheless probable that the three smaller sections listed above nest within the larger *D.* sect. *Brachyandra*. Further support for this hypothesis is found in the existence of a Malagasy species with shallow bowl-shaped torus (Weber and Wilkin in prep.) intermediate in form between the flat disc of *D.* sect. *Brachyandra* and the campanulate torus found in *D.* sect. *Seriflorae* or *Campanuliflorae*. The topology of Fig. 1 in general, and this clade in particular, indicates that substantial simplification of the whole Knuth/Burkill infrageneric classification will be possible when an adequate number of species have been sampled.

Compound-leafed Taxa and their Allies. Although compound leaves have arisen at least twice in the Old World tropics (CL clade and *D. bemarivensis* in Madagascar) as well as in the New World, the majority of compound-leafed species are found in the CL clade. A previous study (Wilkin and Caddick 2000) using *rbcL* and morphology indicated that *D. dumetorum* and its allies with several veins per leaflet (*D.* sect. *Lasiophyton*) were sister to *D. pentaphylla* and its allies with one main vein per leaflet (*D.* sect. *Botryosicyos*), although *rbcL* alone did not show this relationship. The two sections are recovered in the combined strict consensus tree (Fig. 1) as well-supported clades within the CL clade (BP 96 and 99, respectively). In the analysis of *matK* alone the two clades are sister with weak bootstrap support. It appears that *matK* is congruent with morphology for these taxa, but that there is insufficient variation in *rbcL* to show their sister relationship.

Figure 1 indicates that entire-leafed species may be related to *D.* sect. *Lasiophyton* and *D.* sect. *Botryosicyos*. In the case of *D.* sect. *Lasiophyton*, *D. antaly* is the sister taxon with weak bootstrap support (BP 50). *Dioscorea antaly* is the only endemic Malagasy species not in the main Malagasy clade. It has always been thought to be unusual; it has unique branching tubers and a fruit thickened along its axis. Burkill and Perrier de la Bâthie (1950) separated it as *D.* sect. *Xylinocapsa* Burkill & H. Perr. It therefore appears that Madagascar has two endemic lineages of *Dioscorea*, one of which has radiated (the Malagasy clade) and one of which has not (*D. antaly*). *D.* sect. *Botryosicyos* has *D. bulbifera* as their sister species. Although this clade is not robust, it is present in the strict consensus tree obtained from *rbcL* alone. *Dioscorea bulbifera* is unresolved at the base of a combined compound-leafed clade in the strict consensus tree obtained from *matK* alone. All of these six taxa have seeds winged at the base only (Fig. 5).

Enantiophyllum. This clade is congruent with past classifications; both Knuth (1924) and Burkill (1960) recognized the Old World right-twining species (Fig. 4) as *D.* sect. *Enantiophyllum* Uline. All of the species sampled (except the unusual Thai limestone endemic *D. inopinata*) also have opposite leaves, whereas the left-twiners are usually alternate. Prain and Burkill (1936) believed *D. inopinata* to be a hybrid between *D. bulbifera* and a right-twining species, but they may have been misled by its narrow, alternate leaves. The clade is clearly defined by right-twining stems and usually opposite leaves, but the limits of its ca. 80 species are often unclear. All of the species of this clade have putatively retained the plesiomorphic seed condition (winged all round the margin, Fig. 5), unlike many other B clade taxa of *Dioscorea*. African *D. schimperiana*, with its stellate hairs, appears to be sister to the remaining species in this clade. Similar stellate hairs are also found in four other African species and *D. orbiculata* Hook. f. from Asia, all of which are right-twiners. The African species with stellate hairs were placed in a separate section or sections by previous authors (e.g., *D.* sect *Asterotricha* Uline; Burkill 1960), but this is not supported by our results. Like the Malagasy clade, it appears from the low levels of molecular divergence that this group is a recent radiation or has a slower rate, but this needs further investigation.

The position of one species in this clade is of particular economic significance; *D. alata*, the winged yam, which is the most important cultivated yam in Asia. It has also been distributed westward and can be encountered in Africa and even the Americas. Prain and Burkill (1938) placed it in a group of species from Southeast Asia that share a flexuous male inflorescence. A recent AFLP study (Malapa et al. 2006) indicated that its affinities were with *D. nummularia* Lam., an edible species of the Malay Archipelago east to New Guinea. *D. nummularia* was thought to be a close relative of *D. glabra* by Prain and Burkill (1938). The sequence data used here produces a similar result; the species with a flexuous male inflorescence are represented by *D. hamiltonii* (with which *D. persimilis*, studied by Malapa et al. 2006, is synonymous) and *D. brevipetiolata*. These two species are resolved as sister species with BP 85. *Dioscorea alata*, however, does not fall within this clade when either the Fuse and Tamura (2000) *matK* sequence or one generated in this study are used.

In overall terms, the phylogenetic trees presented and discussed here appear to provide a framework to guide further research on *Dioscorea*. Monophyletic taxa such as *D.* sects. *Stenophora* and *Enantiophyllum* and the Malagasy clade need further work to resolve both the

limits of their species and the phylogenetic relationships between them. It is envisaged that this work will include both study of morphology and sequencing of at least one low-copy nuclear gene such as those used successfully in palms (e.g., Lewis and Doyle 2002). Research on the macromorphology of the Malagasy species is already well advanced, and a pilot study using ITS has been started for the genus. The taxon sampling will be substantially increased by adding New World taxa and gap-filling among the Old World groups, especially the main cultivated and edible taxa and their close relatives. The morphological character optimizations show that comparative studies need to be targeted towards underground parts and seeds to try to uncover homologous characters for systematic use.

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