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RESEARCH ARTICLE

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Molecular phylogeny of *Dioscorea* (*Dioscoreaceae*) in East and Southeast Asia

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Key words

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matK
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rbcL
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Abstract The phylogenetic relationships of *Dioscorea*, including sections *Botryosicyos*, *Combilium*, *Enantiophyllum*, *Lasiophyton*, *Opsophyton*, *Shannicorea* and *Stenophora*, are reconstructed with chloroplast *trnL-F*, *matK*, *rbcL* and *atpB-rbcL* sequence data, covering a total of 72 accessions including 48 ingroup species and five outgroup species from East and Southeast Asia. The seven Asian sections do reflect the genetic relationships among the species that they include. In summary, *D.* sect. *Combilium* and sect. *Shannicorea* are recognized as closely related sections with moderate support. The results also support Burkills treatment of subsect. *Euopsophyton* (*D. bulbifera*) as sect. *Opsophyton* and subsect. *Macrourae* (*D. sansibarensis*) as sect. *Macroura*. In addition, there is a well-supported sister relationship between *D.* sect. *Lasiophyton* and sect. *Botryosicyos*. The level of morphological characters and molecular divergence within *D.* sect. *Enantiophyllum* is low, but the members of this group could still be distinguished from each other.

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INTRODUCTION

Dioscorea L. is the largest genus of *Dioscoreaceae* with about 600 species distributed in Southeast Asia, Africa, Central America, South America and other tropical and subtropical regions (Huber 1998). *Dioscorea* includes important vegetatively reproducing tuber crops, known as yams. Yams have played a significant role in the advent of agriculture in Southeast Asia including Taiwan (e.g., *D. alata* and *D. esculenta*) and equatorial Africa (e.g., *D. cayenensis*, *D. dumetorum* and *D. rotundata*) (Ayensu & Coursey 1972, Coursey 1981). *Dioscorea* species are used for food and pharmaceutical products. Species such as *D. nipponica* and *D. zingiberensis* contain diosgenin, which provides one of the important raw materials for the synthesis of steroid hormone drugs (Zhai et al. 2009, Zhang et al. 2010). It has been reported that *D. bulbifera* could be effective for curing thyroid diseases and cancer (Liu et al. 2009).

Since *Dioscorea* is a large genus, many researchers have proposed infrageneric classifications of *Dioscorea*. Knuth (1924) has proposed 58 sections in *Dioscorea*, most of which are still used today. Prain & Burkill (1936, 1938) presented several new sections for the Asian members of *Dioscorea*. In comparison to Kuth (1924) they placed greater emphasis on seed characters, underground organ and male inflorescence morphology. Burkill (1960) proceeded to the arrangement of the Old World sections of the genus *Dioscorea*, dividing 220 species into 23 sections. Recently, Huber (1998) has proposed 28 sections of *Dioscorea* including *Borderea*, *Epipetrum*, *Rajania*, *Tamus* and *Testudinaria*. However, more detailed studies of the infrageneric classification of *Dioscorea* have revealed that several sections seem to be artificial groupings, and many species are not fit to their section boundaries. For example, compound-leaved yams in the Old World (*D.* sect. *Lasiophyton*, sect. *Trieuphorostemon* and sect. *Botryosicyos*) have been treated as one to three sections by different systematists (Knuth 1924, Prain & Burkill 1936, Ding & Gilbert 2000).

Phylogenetic relationships of *Dioscorea* have presented a challenge to systematists for many years because of the difficulties in species identification, which is due to a continuous variability of morphological characters, especially of aerial parts, such as leaves (Pavan Kumar et al. 2007, Wilkin et al. 2005). Further, many morphological characters are shared by different species, which make the identification and classification of the genus a rather difficult task. For example, some classifications have considered *D. batatas*, *D. doryphora* and *D. potanini* as synonyms of *D. polystachya*, because those species have many morphological characters in common (Ding & Gilbert 2000).

A further question is whether these morphological groups correctly reflect their genetic relationships within *Dioscorea*. Recent studies have analysed molecular datasets to provide additional indications of the relationships within this genus. The phylogenetic relationships of six species (*D. gracillima*, *D. nipponica*, *D. quinqueloba*, *D. septemloba*, *D. tenuipes* and *D. tokoro*) in *D.* sect. *Stenophora* were investigated based on DNA sequences of the phosphoglucose isomerase (Kawabe et al. 1997). It was reported that *D. tenuipes* and *D. tokoro* were clustered into a clade, while the rest species formed a separate clade.

Furthermore, chloroplast sequence data has been used to examine the phylogenetic relationships within *Dioscorea*. Wilkin & Caddick (2000) found that the palaeotropical compound-leaved yams were classified into two monophyletic groups based on a combined analysis of chloroplast sequence data and morphological characters. Later, the phylogenetic relationships of 67 *Dioscorea* taxa were reconstructed based on chloroplast *rbcL* and *matK* sequence data (Wilkin et al. 2005). They found that the main Old World groups (such as the left-twining *D.* sect. *Stenophora* and the right-twining *D.* sect. *Enantiophyllum*) were monophyletic. However, these studies included a limited sampling of Asian species and the obtained phylogenetic resolution

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Table 1 List of sample sources and Genbank accession numbers of the sequences used in this study.

Taxon	Location	Voucher	GenBank accession numbers			
			trnL-F	*matK*	*rbcL*	*atpB-rbcL*
Dioscorea alata L. 1	Nantou, Taiwan	Chen 56 (TCB)	JQ733816	JQ733662	JQ733739	JQ733585
D. alata L. 2	Phitsanulok, Thailand	Y.S. Liang D. 29 (TCB)	JQ733843	JQ733689	JQ733766	JQ733612
D. alata L. 3	Bali, Indonesia	Hsu 242 (TCB)	JQ733870	JQ733716	JQ733793	JQ733639
D. batatas Decne.	Taipei, Taiwan	Hsu 201 (TCB)	JQ733824	JQ733670	JQ733747	JQ733593
D. benthamii Prain & Burkill 1	Hong Kong, China	Venus 101 (TCB)	JQ733820	JQ733666	JQ733743	JQ733589
D. benthamii Prain & Burkill 2	Hong Kong, China	Venus 102 (TCB)	JQ733849	JQ733695	JQ733772	JQ733618
D. benthamii Prain & Burkill 3	Hong Kong, China	Venus 103 (TCB)	JQ733850	JQ733696	JQ733773	JQ733619
D. bulbifera L. 1	Zhanghua, Taiwan	Hsu 99 (TCB)	JQ733821	JQ733667	JQ733744	JQ733590
D. bulbifera L. 2	Dhaka, Bangladesh	Lu 16195 (TCB)	JQ733830	JQ733676	JQ733753	JQ733599
D. bulbifera L. 3	Guizhou, China	Y.S. Liang 1034 (TCB)	JQ733834	JQ733680	JQ733757	JQ733603
D. bulbifera L. 4	Phitsanulok, Thailand	Y.S. Liang 2147 (TCB)	JQ733841	JQ733687	JQ733764	JQ733610
D. bulbifera L. 5	Guizhou, China	Ann 3836 (TNM)	JQ733867	JQ733713	JQ733790	JQ733636
D. bulbifera L. 6	Bali, Indonesia	Hsu 241 (TCB)	JQ733869	JQ733715	JQ733792	JQ733638
D. cirrhosa Lour.	Yunlin, Taiwan	Chen 51 (TCB)	JQ733817	JQ733663	JQ733740	JQ733586
D. cirrhosa var. *cylindrica* C.T.Ting & M.C.Chang 1	Guangdong, China	Y.S. Liang 2656 (TCB)	JQ733883	JQ733729	JQ733806	JQ733652
D. cirrhosa var. *cylindrica* C.T.Ting & M.C.Chang 2	Guangdong, China	Y.S. Liang 2691 (TCB)	JQ733884	JQ733730	JQ733807	JQ733653
D. collettii Hook.f. 1	Nantou, Taiwan	Hsu 101 (TCB)	JQ733818	JQ733664	JQ733741	JQ733587
D. collettii Hook.f. 2	Lanyu, Taiwan	Hsu 76 (TCB)	JQ733882	JQ733728	JQ733805	JQ733651
D. collettii var. *hypoglauca* (Palib.) C.Pei & C.T.Ting	Hunan, China	Zhang 90619057 (TAIF)	JQ733874	JQ733720	JQ733797	JQ733643
D. cumingii Prain & Burkill	Lanyu, Taiwan	Chen 18 (TCB)	JQ733822	JQ733668	JQ733745	JQ733591
D. doryphora Hance 1	Zhanghua, Taiwan	Hsu 56 (TCB)	JQ733823	JQ733669	JQ733746	JQ733592
D. doryphora Hance 2	Lanyu, Taiwan	Chen 22 (TCB)	JQ733847	JQ733693	JQ733770	JQ733616
D. esculenta (Lour.) Burkill	Luzon, Philippines	Lu 20826 (TCB)	JQ733878	JQ733724	JQ733801	JQ733647
D. esculenta var. *spinosa* (Roxb. ex Prain & Burkill) R.Knuth	Jiayi, Taiwan	Chen 20 (TCB)	JQ733833	JQ733679	JQ733756	JQ733602
D. exalata C.T.Ting & M.C.Chang 1	Guizhou, China	Y.S. Liang 1037 (TCB)	JQ733835	JQ733681	JQ733758	JQ733604
D. exalata C.T.Ting & M.C.Chang 2	Guangdong, China	Lu 21091 (TCB)	JQ733879	JQ733725	JQ733802	JQ733648
D. fordii Prain & Burkill	Yunnan, China	Shui 3526 (TNM)	JQ733860	JQ733706	JQ733783	JQ733629
D. formosana Knuth	Taoyuan, Taiwan	Chen 42 (TCB)	JQ733845	JQ733691	JQ733768	JQ733614
D. futschauensis Uline ex R.Knuth	Mazu, Taiwan	Hsu 32 (TCB)	JQ733825	JQ733671	JQ733748	JQ733594
D. gracillima Miq.	Jiangxi, China	Tan 95288 (TNM)	JQ733863	JQ733709	JQ733786	JQ733632
D. hamiltonii Hook.f.	Taipei, Taiwan	Hsu 202 (TCB)	JQ733832	JQ733678	JQ733755	JQ733601
D. hemsleyi Prain & Burkill 1	Yunnan, China	Yang 18467 (TNM)	JQ733855	JQ733701	JQ733778	JQ733624
D. hemsleyi Prain & Burkill 2	Yunnan, China	Zhu 7106 (TAIF)	JQ733875	JQ733721	JQ733798	JQ733644
D. hispida Dennst. 1	Nantou, Taiwan	Hsu 211 (TCB)	JQ733826	JQ733672	JQ733749	JQ733595
D. hispida Dennst. 2	Bali, Indonesia	Hsu 244 (TCB)	JQ733872	JQ733718	JQ733795	JQ733641
D. japonica Thunb. 1	Pingdong, Taiwan	Hsu 94 (TCB)	JQ733819	JQ733665	JQ733742	JQ733588
D. japonica Thunb. 2	Guizhou, China	Y.S. Liang 1046 (TCB)	JQ733837	JQ733683	JQ733760	JQ733606
D. japonica Thunb. 3	Kyoto, Japan	Hsu 231 (TCB)	JQ733844	JQ733690	JQ733767	JQ733613
D. kamoonensis Kunth 1	Guizhou, China	CHC 7539 (TCB)	JQ733838	JQ733684	JQ733761	JQ733607
D. kamoonensis Kunth 2	Hunan, China	Ma 1145 (TNM)	JQ733866	JQ733712	JQ733789	JQ733635
D. lepcharum Prain & Burkill	Dhaka, Bangladesh	Lu 16156 (TCB)	JQ733829	JQ733675	JQ733752	JQ733598
D. martini Prain & Burkill	Yunnan, China	Yang 14136 (TNM)	JQ733864	JQ733710	JQ733787	JQ733633
D. melanophyma Prain & Burkill 1	Guizhou, China	CHC 9203 (TCB)	JQ733842	JQ733688	JQ733765	JQ733611
D. melanophyma Prain & Burkill 2	Yunnan, China	Yang 14137 (TNM)	JQ733865	JQ733711	JQ733788	JQ733634
D. nipponica Makino	Hunan, China	Tan 71 (TAIF)	JQ733851	JQ733697	JQ733774	JQ733620
D. nitens Prain & Burkill	Guangdong, China	Y.S. Liang 2628 (TCB)	JQ733887	JQ733733	JQ733810	JQ733656
D. nummularia Roxb.	Luzon, Philippines	Lu 20549 (TCB)	JQ733877	JQ733723	JQ733800	JQ733646
D. pentaphylla L. 1	Dhaka, Bangladesh	Lu 16206 (TCB)	JQ733831	JQ733677	JQ733754	JQ733600
D. pentaphylla L. 2	Yunnan, China	Yang 20890 (TNM)	JQ733858	JQ733704	JQ733781	JQ733627
D. polystachya Turcz. 1	Mazu, Taiwan	Hsu 31 (TCB)	JQ733827	JQ733673	JQ733750	JQ733596
D. polystachya Turcz. 2	Tianjin, China	Ching 189 (TNM)	JQ733853	JQ733699	JQ733776	JQ733622
D. potanini Prain & Burkill	Hunan, China	Huang 644 (TNM)	JQ733856	JQ733702	JQ733779	JQ733625
D. quinqueloba Thunb	Kyushu, Japan	Yonekura 6186 (TNM)	JQ733859	JQ733705	JQ733782	JQ733628
D. sansibarensis Pax 1	Yunnan, China	Hsu 221 (TCB)	JQ733839	JQ733685	JQ733762	JQ733608
D. sansibarensis Pax 2	Iringa, Tanzania	G. Massawe 285 (TNM)	JQ733852	JQ733698	JQ733775	JQ733621
D. scortechinii var. *parviflora* Prain & Burkill	Hanoi, Vietnam	Lu 19238 (TCB)	JQ733868	JQ733714	JQ733791	JQ733637
D. sp. A	Phitsanulok, Thailand	Y.S. Liang D. 26 (TCB)	JQ733840	JQ733686	JQ733763	JQ733609
D. sp. B	Guizhou, China	Y.S. Liang 1050 (TCB)	JQ733846	JQ733692	JQ733769	JQ733615
D. sp. C	Jiangxi, China	Tan 97894 (TNM)	JQ733854	JQ733700	JQ733777	JQ733623
D. sp. D	Phitsanulok, Thailand	Y.S. Liang 3539 (TCB)	JQ733880	JQ733726	JQ733803	JQ733649
D. sp. E	Hong Kong, China	Y.S. Liang D. 178 (TCB)	JQ733881	JQ733727	JQ733804	JQ733650
D. sp. F	Guangdong, China	Y.S. Liang 2594 (TCB)	JQ733885	JQ733731	JQ733808	JQ733654
D. sp. G	Luzon, Philippines	Lu 20548 (TCB)	JQ733876	JQ733722	JQ733799	JQ733645
D. sp. H	Bali, Indonesia	Hsu 243 (TCB)	JQ733871	JQ733717	JQ733794	JQ733640
D. subcalva Prain & Burkill 1	Guizhou, China	Y.S. Liang 1045 (TCB)	JQ733836	JQ733682	JQ733759	JQ733605
D. subcalva Prain & Burkill 2	Guizhou, China	Wang 1-0588 (TAIF)	JQ733873	JQ733719	JQ733796	JQ733642
D. subcalva var. *submollis* C.T.Ting & P.P.Ling 1	Yunnan, China	Y.S. Liang D. 39 (TCB)	JQ733848	JQ733694	JQ733771	JQ733617
D. subcalva var. *submollis* C.T.Ting & P.P.Ling 2	Yunnan, China	Chen 6177 (TNM)	JQ733861	JQ733707	JQ733784	JQ733630
D. tokoro Makino	Honshu, Japan	C.H. Chen 5805 (TNM)	JQ733862	JQ733708	JQ733785	JQ733631
D. velutipes Prain & Burkill	Guangdong, China	Y.S. Liang 2609 (TCB)	JQ733886	JQ733732	JQ733809	JQ733655
D. wallichii Hook.f.	Dhaka, Bangladesh	Lu 16155 (TCB)	JQ733828	JQ733674	JQ733751	JQ733597
D. yunnanensis Prain & Burkill	Yunnan, China	Ma 7140 (TNM)	JQ733857	JQ733703	JQ733780	JQ733626
Stemona tuberosa Lour.	Nantou, Taiwan	Hsu 401 (TCB)	JQ733892	JQ733738	JQ733815	JQ733661
Stenomeris borneensis Oliv.	Luzon, Philippines	Lu 20601 (TCB)	JQ733888	JQ733734	JQ733811	JQ733657
Tacca chantrieri André	Cultivated	Hsu 302 (TCB)	JQ733890	JQ733736	JQ733813	JQ733659
Tacca leontopetaloides (L.) Kuntze	Pingdong, Taiwan	Hsu 301 (TCB)	JQ733889	JQ733735	JQ733812	JQ733658
Tacca plantaginea (Hance) Drenth	Cultivated	Hsu 303 (TCB)	JQ733891	JQ733737	JQ733814	JQ733660

was relatively low. Therefore the phylogenetic relationships among the species of Asian *Dioscorea* have not been well established (e.g., in *D.* sect. *Shannicorea*). Further studies to resolve both the limits of their species and the phylogenetic relationships between them are necessary.

A complete plastid genome of a *Dioscorea* species is available (Hansen et al. 2007) and this provides a rich source of phylogenetic tools to unravel the genetic relationships within *Dioscorea*. Based on chloroplast genes including *trnL-F*, *matK*, *rbcL* and *atpB-rbcL* sequence data, the objectives of this study are to further clarify infrageneric classification of Asian *Dioscorea* and provide information for the genetic conservation of wild and cultivated yams. We examine currently recognized species within seven sections (sect. *Botryosicyos*, *Combilium*, *Enantiophyllum*, *Lasiophyton*, *Opsophyton*, *Shannicorea* and *Stenophora*) from East and Southeast Asia and investigate the relationships amongst these sections. We compare our results to recent studies of *Dioscorea* and the molecular phylogeny of *Dioscorea* in East and Southeast Asian is discussed.

MATERIALS AND METHODS

Taxon sampling

Our analysis of chloroplast *trnL-F*, *matK*, *rbcL* and *atpB-rbcL* covered a total of 72 accessions of 48 ingroup species and five outgroup species (Table 1). These five outgroup taxa were part of *Tacca* and *Stenomeris* in *Dioscoreaceae* and *Stemona* in *Stemonaceae* (Caddick et al. 2002).

DNA extraction, amplification and sequencing

DNA was extracted from fresh leaves, dried leaves, or herbarium sheets using a Puregene DNA Purification kit (Gentra Systems, Minneapolis, MN, USA). Four gene products were amplified by primers *trnL*-5 (5’-CGAAATCGGTAGACGCTACG-3’) and IGS-3 (5’-ATTTGAACTGGTGACACGAG-3’) for *trnL-F*, *matK*-F (5’- ACCCCATCCCATCCATCTGGAAAT-3’) and *matK*-R (5’-TATCCAAATACCAAATGCGTCCTG-3’) for *matK*, *rbcL*-F (5’-GTTGGATTCAAAGCTGGTGTTAAAGAT-3’) and *rbcL*-R (5’-CGTCCCTCATTACGAGCTTG-3’) for *rbcL*, and *atpB*-2 (5’-AGCGTTGTAAATATTAGGCATCTT-3’) and *rbcL*-2 (5’-ATCTTTAACACCAGCTTTGAATCCAAC-3’) for *atpB-rbcL*, respectively. A total volume of 50 µl PCR reaction contained 1 µl of template DNA (50–100 ng extracted genomic DNA), 1 µl of 10 mM of each primer, 2.5 µl of PCR buffer, 1 µl of 10 mM dNTPs, 2.5 µl of 25 mM MgCl$_2$ and 1 U of *Taq* polymerase. PCR reactions were performed in a PCR thermocycler (GeneAmp 9700 PCR system; Applied Biosystems, Foster City, CA, USA) and carried out in the following conditions: an initial denaturation step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 52 °C for 1 min and 72 °C for 2 mins, with a final extension of 72 °C for 7 min. The PCR amplified products were checked on a 1 % agarose gel electrophoresis stained with ethidium bromide. Using Micro-Elute DNA Clean/Extraction Kit (GeneMark, Taiwan), the PCR products were purified and dissolved in 10 µl ddH$_2$O. The purified PCR products were sequenced with the PCR primer pairs in both directions by an ABI Model 3100 DNA sequencer (Applied Biosystems, USA) with BigDye terminator cycle sequencing reagent (Applied Biosystems, USA).

Sequence analyses

The sequences were aligned and edited using BioEdit 7.0.1 (Hall 1999). The alignments of the concatenated sequence datasets were obtained by using CLUSTAL-X version 1.83 (Thompson et al. 1997) with manual adjustments for accuracy. Statistical analyses of the alignments were performed using MEGA v. 4 (Tamura et al. 2007).

Phylogenetic analyses

After alignment, phylogenetic analyses were conducted with PAUP* 4.0b10 (Swofford 2002) using the methods of distance and maximum parsimony (MP). Bayesian inference (BI) analyses were conducted with MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). The optimal model of nucleotide substitution was evaluated by a likelihood ratio test with MODELTEST 3.7 (Posada & Crandall 1998). The K81uf+I+G model with proportion of invariable sites (I) = 0.3661 and gamma distribution shape parameter (G) = 0.9624 was selected as the best model for the concatenated DNA sequence of *trnL-F*, *matK*, *rbcL* and *atpB-rbcL* genes.

Based on this model, a distance tree was constructed with the neighbor-joining (NJ) algorithm. In the MP analysis, characters were equally weighted and a heuristic search option with tree bisection reconnection (TBR) branch-swapping and 10 random stepwise additions was used (gaps were treated as missing data). All bootstrap values were based on 1 000 replicates performed for NJ and MP. The BI analysis was run for 2× 106 generations, with a sample frequency of 100. The first 2 000 trees were discarded and 18 000 trees were applied in the final consensus tree. The posterior probabilities (calculated with MrBayes) were recorded to represent the support for nodes.

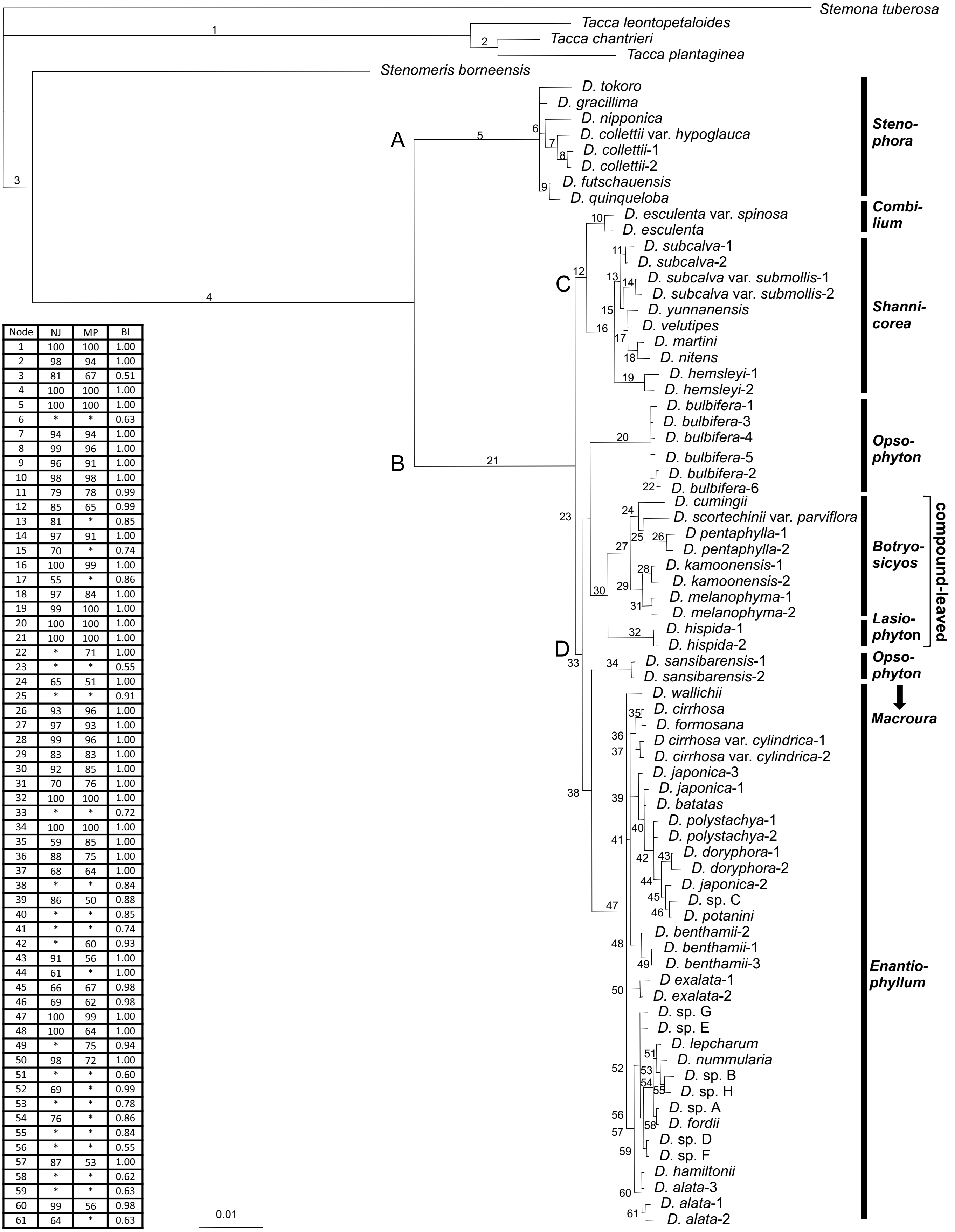
RESULTS

Sequence characteristics and variations

For all *Dioscorea* and the outgroup species, the sequenced *trnL-F* region was 640–745 bp, the *matK* region 895–901 bp, the *rbcL* region 1 159 bp and the *atpB-rbcL* region 690–838 bp. The lengths of the alignments are given in Table 2. The pairwise distances (*p*-distances) among the seven *Dioscorea* sections ranged from 0.007 to 0.042 for combined datasets including chloroplast genome *trnL-F*, *matK*, *rbcL* and *atpB-rbcL* DNA sequences. The average *p*-distance in all the sampled *Dioscorea* species was 0.017 for *trnL-F*, 0.020 for *matK*, 0.012 for *rbcL* and 0.015 for *atpB-rbcL*. The *p*-distance within each section ranged from 0.000 to 0.006 for *trnL-F*, 0.000 to 0.007 for *matK*, 0.000 to 0.006 for *rbcL* and 0.000 to 0.006 for *atpB-rbcL*. The most divergent section was *D.* sect. *Opsophyton* (including the *D. bulbifera* group and *D. sansibarensis* group), in which the *p*-distance was 0.006 for the combined dataset (data not shown).

Table 2 Tree statistics and sequence information of the *trnL-F*, *matK*, *rbcL*, *atpB-rbcL* and combined datasets.

	trnL-F	*matK*	*rbcL*	*atpB-rbcL*	Combined dataset
Aligned sequence length (bp)	896	907	1160	938	3901
G+C content (%)	33.9	31.8	44.7	29.8	34.1
No. parsimony informative sites	253	160	94	139	646
Tree length (MP)	651	390	323	374	1601
Consistency index (CI)	0.69	0.80	0.52	0.85	0.78
Retention index (RI)	0.83	0.92	0.80	0.92	0.90



Node	NJ	MP	BI
1	100	100	1.00
2	98	94	1.00
3	81	67	0.51
4	100	100	1.00
5	100	100	1.00
6	*	*	0.63
7	94	94	1.00
8	99	96	1.00
9	96	91	1.00
10	98	98	1.00
11	79	78	0.99
12	85	65	0.99
13	81	*	0.85
14	97	91	1.00
15	70	*	0.74
16	100	99	1.00
17	55	*	0.86
18	97	84	1.00
19	99	100	1.00
20	100	100	1.00
21	100	100	1.00
22	*	71	1.00
23	*	*	0.55
24	65	51	1.00
25	*	*	0.91
26	93	96	1.00
27	97	93	1.00
28	99	96	1.00
29	83	83	1.00
30	92	85	1.00
31	70	76	1.00
32	100	100	1.00
33	*	*	0.72
34	100	100	1.00
35	59	85	1.00
36	88	75	1.00
37	68	64	1.00
38	*	*	0.84
39	86	50	0.88
40	*	*	0.85
41	*	*	0.74
42	*	60	0.93
43	91	56	1.00
44	61	*	1.00
45	66	67	0.98
46	69	62	0.98
47	100	99	1.00
48	100	64	1.00
49	*	75	0.94
50	98	72	1.00
51	*	*	0.60
52	69	*	0.99
53	*	*	0.78
54	76	*	0.86
55	*	*	0.84
56	*	*	0.55
57	87	53	1.00
58	*	*	0.62
59	*	*	0.63
60	99	56	0.98
61	64	*	0.63

Fig. 1 Bayesian tree of *Dioscorea* specimens reconstructed with combined chloroplast genome *trnL-F*, *matK*, *rbcL* and *atpB-rbcL* DNA sequences. Statistical supports for each node (node numbers on the branches of the tree) in NJ, MP and BI analyses is shown in the table on the left. An asterisk (*) indicates a node value < 50 %.

Phylogenetic analyses

The MP analysis of the combined dataset resulted in a single tree of 1 601 steps with CI = 0.78 and RI = 0.90. The phylogenetic tree based on the cpDNA combined datasets as reconstructed by the Bayesian method with statistical supports for each node in NJ, MP and BI analyses is shown in Fig. 1. There were no supported contradictions between the topologies of NJ, MP and Bayesian consensus tree. The *Dioscorea* species formed a monophyletic group with maximum support at node 4. Within *Dioscorea*, there were two strongly supported clades, clade A (node 5, 100/100/1.00) and clade B (node 21, 100/100/1.00). Clade B was further divided into two clades (C and D). Clade C included two strongly supported sections, *D.* sect. *Combilium* (node 10, 98/98/1.00) and *D.* sect. *Shannicorea* (node 16, 100/99/1.00), which were moderately supported (node 12, 85/65/0.99) as sister to each other. Clade D includes five strongly supported sections. Within *D.* sect. *Opsophyton*, 6 individuals of *D. bulbifera* and two individuals of *D. sansibarensis* were not clustered together, but formed monophyletic clades with strong support (node 20 and 34, 100/100/1.00) individually. Next, the *D.* sect. *Botryosicyos* clade was strongly supported as monophyletic group (node 27, 97/93/1.00), and its sister *D.* sect. *Lasiophyton* was also strongly supported as monophyletic group (node 32, 100/100/1.00). Finally, *D.* sect. *Enantiophyllum* was also strongly supported as monophyletic (node 47, 100/99/1.00).

DISCUSSION

Systematic implications of the molecular phylogeny

Based on their twining stems, compound leaves, underground organ morphology, hairs, male flowers, capsule and seed characters, the species of Asian *Dioscorea* can be divided into nine sections (sect. *Botryosicyos*, *Combilium*, *Enantiophyllum*, *Lasiophyton*, *Opsophyton*, *Paramecocarpa*, *Shannicorea*, *Stenocorea* and *Stenophora*). A total of seven out of nine sections (except *D.* sect. *Stenocorea* and *Paramecocarpa*) were included in our analysis and the phylogenetic tree of *Dioscorea* was reconstructed by cpDNA combined datasets. Our results show general support for the infrageneric classification of *Dioscorea*.

The Stenophora clade

As shown in Fig. 1, all *Dioscorea* species formed a monophyletic group with two distinct, strongly supported clades (clade A and B). This confirms that *D.* sect. *Stenophora* (clade A) is sister to the rest of *Dioscorea* (clade B) in the systematics of the genus as reported in Wilkin et al. (2005). Many ancestral characteristics of the genus are also present in *D.* sect. *Stenophora* including rhizome, diploid chromosome number and single pollen aperture (Pei et al.1979, Chin et al.1985, Schols et al. 2003). Because its fossil record is the earliest of the genus *Dioscorea*, sect. *Stenophora* has been proposed as the oldest section in *Dioscorea* (Burkill 1960).

Furthermore, *D. collettii* was reported as a Sino-Himalayan species in Thapyai et al. (2005). Burkill (1960) had distinguished an additional species from *D. collettii*, which he called *D. hypoglauca*. However, in the most recent treatment of this species, Ding & Gilbert (2000) defined *D. hypoglauca* as a variety of *D. collettii*, *D. collettii* var. *hypoglauca*. These two taxa exhibit continuous morphological variations and show sympatric distribution in China. Gao et al. (2008) suggested that *D. collettii* var. *collettii* and *D. collettii* var. *hypoglauca* were sister to each other with only weak support. In this study, these two taxa were also sister to each other, but with strong support (Fig. 1 node 7). In addition, the specimens of *D. collettii* var. *collettii* sampled from Taiwan and Lanyu Island showed three stable transversions within cpDNA *trnL-F* and *matK* regions. Thus, denser sampling is required to evaluate the intraspecific classification of *D. collettii* var. *collettii* in the future.

The Combilium and Shannicorea clades

Dioscorea sect. *Combilium* and *D.* sect. *Shannicorea* show some morphological characters in common, such as producing one or several annually renewed storage tubercles, capsules which are longer than their wide and distally-winged seeds. In the arrangement of the Old World sections of the genus *Dioscorea*, Burkill's (1960) has divided 220 species into 23 sections. He has emphasized on the seed characters, underground organ morphology and development, and male inflorescence morphology as the defining characteristics in his report. Describing the relationships among these 23 sections, he indicated that *D.* sect. *Combilium* and *D.* sect. *Shannicorea* were closely related. This is also supported by our result in which a novel sister relationship of *D.* sect. *Combilium* to *D.* sect. *Shannicorea* was found with moderate support (Fig. 1 node 12). Furthermore, our study is the first analysis showing the internal topology of the *Shannicorea* with strong support (Fig. 1 node 16). Within this monophyletic clade, four taxa are endemic to southern China (*D. martini*, *D. nitens*, *D. subcalva* and *D. yunnanensis*), one is distributed in Northern Thailand, Myanmar and southern China (*D. velutipes*) and one is distributed from central China to Indochina (*D. hemsleyi*). These five species plus one variety (*D. subcalva* var. *submollis*) are grouped together and sister to *D. hemsleyi* (Fig. 1 node 16). Within this clade, a major branch is found in the NJ and BI tree, but not in the MP tree (Fig. 1 node 13). *Dioscorea martini* and *D. nitens* were grouped together and sister to *D. yunnanensis* and *D. velutipes*, these four species were closer to *D. subcalva* var. *submollis* than to *D. subcalva*. *Dioscorea* sect. *Shannicorea* comprises eight species, of which a total of six species and one variety were investigated for their phylogenetic relationships in this study. With regard to the species within *D.* sect. *Shannicorea* not included in this study *D. pseudonitens* Prain & Burkill was not sister to *D. tentaculigera* Prain & Burkill in the phylogenetic tree presented by Wilkin et al. (2005). Recently, Wilkin & Thapyai (2011) have reported that *D. pseudonitens* is conspecific with *D. nitens*. In summary, this study should be able to provide a framework for *Shannicorea* clade but it would need further study to evaluate the phylogenetic position of *D. tentaculigera* in the future.

The Opsophyton clade

Dioscorea bulbifera is the main species of *D.* sect. *Opsophyton* distributed in the wild state in both Asia and Africa. The formation of many axillary tubers (bulbils) is the distinct characteristic of *D. bulbifera*, but intraspecific classifications are still diverse. Prain & Burkill (1936) have treated the African form with angular bulbils as a single variety, *D. bulbifera* var. *anthropophagorum*, and the Asian form with globular bulbils has been divided into nine varieties according to highly variable characters such as the colour, shape, and dimension of bulbils and leaves. In this study, we found six different haplotypes of the Asian form of *D. bulbifera* (data not shown). Two accessions from Bangladesh and Indonesia (*D. bulbifera*-2 and *D. bulbifera*-6) were grouped together and sister to the rest of *D. bulbifera* in clade D (Fig. 1).

Interestingly, Knuth (1924) has treated *D.* sect. *Opsophyton* into three subsections: 1) *Euopsophyton*; 2) *Isocanthae*; 3) *Macrourae*. Two species of this section, *D. bulbifera* (*Euopsophyton*) and *D. sansibarensis* (*Macrourae*), were also surveyed in this study. It was found that *D. bulbifera* and *D. sansibarensis* were not closely related but individually formed well-supported monophyletic clades (node 20 and 34 in Fig. 1). Consequently, the subsectional classification proposed by Knuth (1924) for

D. bulbifera (*Euopsophyton*) and *D. sansibarensis* (*Macrourae*) was not agreed in our results, instead our data supported Burkill's treatment of subsect. *Euopsophyton* (*D. bulbifera*) as sect. *Opsophyton* and subsect. *Macrourae* (*D. sansibarensis*) as sect. *Macroura* (Burkill 1937).

The Botryosicyos and Lasiophyton clades

These two sections show many morphological characters in common including perennial crown with annual tubers, left-twining, usually pubescent and spiny, compound leaves and capsules that are longer than their wide. Prain & Burkill (1936) have combined these two sections and treated the members of *D.* sect. *Botryosicyos* within *D.* sect. *Lasiophyton*. However, these two sections show clear morphological differences to each other, such as the variations in leaflet venation, male bracts, and stamen number. Thus, the obtained phylogenetic relationships seem to be well-supported by morphological characters. As shown in Fig. 1, *D.* sect. *Botryosicyos* and *D.* sect. *Lasiophyton* were both identified in the tree as well-supported clades within the compound-leaved clade (Fig. 1 node 27 and 32). The members of *D.* sect. *Botryosicyos*, characterized by one main vein per leaflet, were sister to those of *D.* sect. *Lasiophyton*, which had several veins per leaflet.

The Enantiophyllum clade

In Wilkin et al. (2005), twelve species of *D.* sect. *Enantiophyllum* were sampled and found to form a monophyletic clade with strong support. Our study was based on a sampling of 24 taxa of sect. *Enantiophyllum* and obtained a similar result with the monophyly of the section also strongly supported (Fig. 1 node 47). *Dioscorea* sect. *Enantiophyllum* is consistently defined by right-twining stems and usually opposite leaves. This section is the largest in terms of the number of species, with about 120 species, distributed mainly in tropical Asia and Africa (Prain & Burkill 1938), but still many species are often not clearly distinguished. There are two main groups under *Enantiophyllum* section, an Asian-Oceanian group and an African group. Wilkin et al. (2005) reported that the African species *D. schimperiana* Hochst. ex Kunth and the Asian species were clearly separated. In addition, it was suggested by Tostain et al. (2006) that the haplotypes of African species were different from those of the Asian-Oceanian species based on data derived from SSR markers. In this study, Asian species of this section were investigated and several groups of which relationships were not clear in previous studies were clearly identified (Fig. 1). Malapa et al. (2005) proposed that *D. alata*, the most important cultivated yam in Asia, should be grouped with *D. nummularia* and *D. transversa* together representing a southeast Asian-Oceanian gene pool, rather than to *D. persimilis* (as a synonym of *D. hamiltonii*) as reported in Wilkin et al. (2007). However, our study has surveyed three typical species, *D. alata*, *D. nummularia* and *D. hamiltonii*, and the result showed that *D. alata* and *D. hamiltonii* were grouped together with strong support (Fig. 1 node 60) and sister to the rest of Asian-Oceanian species.

Many species identification and nomenclatural problems of the group, from *D. japonica to D. potanini*, have been mentioned in previous studies. For example, Ding & Gilbert (2000) considered that *D. batatas*, *D. doryphora* and *D. potanini* should be regarded as a synonym of *D. polystachya*. In this study, it was shown that they could be distinguished from each other (Fig. 1 nodes 40, 42, 44). However, further experiments with population-based sampling would be necessary to verify clearly the phylogenetic relationships among *D. batatas*, *D. doryphora* and *D. potanini*. In addition, it is interesting to notice that three different haplotypes of *D. japonica* are found and do not form a monophyletic group within the *Enantiophyllum* clade (Fig. 1). Additional synonyms and varieties of *D. japonica* were also reported in Prain & Burkill (1938). Therefore, a denser sampling is required to evaluate the intraspecific classification of *D. japonica* in the future. Finally, Fig. 1 shows that eight right-twining species (*D.* sp. A–H) fall within the *Enantiophyllum* clade. The results also are congruent with those of Wilkin et al. (2005), the right-twining habit has clearly only evolved once in Asia.

In summary, this study shows that the molecular phylogenetic results are generally congruent with past morphology-based infrageneric classifications of *Dioscorea*. The resolution of the available phylogeny within *Dioscorea* was improved by adding information from the cpDNA *trnL-F*, *matK*, *rbcL* and *atpB-rbcL* combined datasets in our results. The low levels of molecular divergence within some clades (as measured by the short branch lengths) indicated that radiation might be relatively recent or at a slower rate. This hypothesis warrants further evaluation with a more extensive sample and even a higher resolution.

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