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MOLECULAR PHYLOGENY AND CHARACTER EVOLUTION OF *DIDYMOCARPUS* (*GESNERIACEAE*) IN THAILAND

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Until recently the genus *Didymocarpus* Wall. (*Gesneriaceae*) was used in an unwarrantably wide sense and included more than 180 species. It has now been remodelled and restricted to around 70 species. Of these, 18 species and one variety are known to occur in Thailand. To clarify the relationships among Thai species of *Didymocarpus* we sequenced the internal transcribed spacers (ITS) of nuclear ribosomal DNA (nrDNA) from a sample of 23 taxa, including 15 from Thailand, four from China, three from Malaysia and one from Bhutan. Seventeen morphological characters were coded for all 23 taxa and optimized onto a retention index (RI) reweighted maximum parsimony (MP) tree. The phylogenetic analyses suggested that *Didymocarpus* taxa formed a strongly supported monophyletic clade, with several supported subclades. The combination of molecular phylogeny and optimization of morphological characters suggests the presence of three distinct groups: the first, corresponding to *Didymocarpus* sect. *Elati* Ridl., includes plants with tall stems, yellow or white flowers and one-celled conoid or two-celled headed pigment glands; the other two groups, which represent *Didymocarpus* sect. *Didymocarpus*, both contain plants with dwarfed stems and violet or purple flowers, but are distinguished by the presence of both four-celled conoid or one-celled globose glands in one, and the absence in the other. Optimization of geographical locality onto the phylogeny led us to propose the hypothesis that, based on this sample, the geographical origin of *Didymocarpus* is the Malay Peninsula, and the ancestral corolla colour is white/yellow. Subsequent dispersal northward through southern and northern Thailand to China and Bhutan was accompanied by the evolution of a purple/violet corolla colour.

Keywords. Character evolution, *Didymocarpus*, internal transcribed spacers, molecular phylogenetics, Thailand.

INTRODUCTION

The genus *Didymocarpus* Wall. is in subfamily *Didymocarpoideae* Endl. (Weber, 2004), tribe *Didymocarpeae* Endl. of the family *Gesneriaceae*. It was originally circumscribed as a large genus, eventually to include more than 180 species (Burtt & Wiehler, 1995). Weber & Burtt (1998) split *Didymocarpus* into three genera:

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Didymocarpus s.s., *Henckelia* Spreng., and *Hovanella* Weber & B.L.Burtt. Recently Weber *et al.* (2000) remodelled and restricted the genus *Didymocarpus* s.s. to include only 70 species. It was reduced by the exclusion of the Madagascan (now *Hovanella*), southern Indian, and most Malesian species. The geographical range of the redefined genus is now from northwest India, eastwards through Nepal, Bhutan, northeastern India, Burma, southern China (southern and southeastern Xizang, Yunnan, western and southwestern Sichuan, eastern and southern Guangxi, and western Guangdong), Vietnam, Thailand, and the Malay Peninsula, with one species (*D. cordatus* Wall. ex DC.) reaching northern Sumatra.

Weber & Burtt (1998) divided *Didymocarpus* into two sections: section *Elati* Ridl., including *D. corchorifolius* Wall. ex DC., *D. antirrhinoides* Weber, *D. sulphureus* Ridl. and *D. robustus* Ridl., characterized by tall stems with somewhat lignified axes, well-developed internodes, pigmented glands having a single-celled head and tuberculate seeds; and section *Didymocarpus*, typically perennial plants producing herbaceous annual stems that die after fruiting with next season's stems forming while the fruits are ripening. Almost all other species are included here. However, the sectional position of six species, *Didymocarpus aureoglandulosus* C.B.Clarke, *D. cordatus*, *D. elatior* Prain, *D. platycalyx* C.B.Clarke, *D. barbinervius* C.B.Clarke and *D. rufipes* C.B.Clarke, was uncertain. Moreover, the sectional position of *Didymocarpus citrinus* Ridl., *D. purpureus* Ridl., *D. ovatus* Barnett and *D. megaphyllus* Barnett, all placed in *Didymocarpus* sect. *Didymocarpus*, was also doubtful since they have tuberculate seeds and well-developed internodes.

Following the classification of Weber & Burtt (1998) the genus *Didymocarpus* s.s. may form a natural group. Its members share the characteristics of an essentially perennial deciduous herb with annual or monocarpic flowering stems with innovation shoots and resting shoots (Weber & Burtt, 1998) being produced in the rainy season and developing into flowering shoots in the following season. Their leaves are opposite, decussate and mostly unequal within pairs (anisophylly). The inflorescence is cymose, pedunculate, with few to many flowers, and with a pair of oval, often wine-coloured, bracts. The calyx is united for more than half of its length or is free to the base. The corolla is salverform, funnelform or personate, widening towards the mouth with a bilabiate limb, the lobes are rounded, and the colour is somewhat mauve, claret, violet or yellow/white. The flower has two stamens with slender filaments and cohering anthers. It also has two to three, minute, staminodes and a cup-shaped disc with an irregularly lobed rim. The ovary is cylindrical with an entirely capitate stigma while the capsules are straight, orthocarpic, bivalved, and dehisce loculicidally. Pigment glands are also found in some species, densely covering the lower side of leaf blades and sparsely so on other organs. Their morphology ranges from one-celled and globose or conoid, with a two-celled head, to four-celled and conoid. Many *Didymocarpus* species grow on limestone bedrocks and in seasonal rainfall areas.

In Thailand *Didymocarpus* is widespread from the south to the north. Barnett (1962) was the first taxonomist to enumerate the Thai species. She listed 22 species; five of these were later transferred to *Henckelia* by Weber & Burtt (1998) based on

fruit and karyotype characteristics. Most recently, Burtt (2001) has listed 16 Thai species and noted that the genus is in need of a thorough revision. A thorough revision of *Didymocarpus* in Thailand has now been completed and includes 18 species and one variety (Palee & Maxwell, 2006). The genus has not previously been the subject of a detailed molecular study.

Molecular phylogenetic studies of other genera of *Gesneriaceae* have been performed successfully using sequences of the internal transcribed spacer (ITS) regions of nuclear ribosomal DNA (nrDNA). For example, in *Saintpaulia* Wend. (African violets), ITS sequence data gave valuable information suggesting the evolution of this genus from *Streptocarpus* Ridl. (Möller & Cronk, 1997a, 2001a, 2001b). Although a follow-up study on *Saintpaulia* by the authors (Möller & Cronk, 1997b) revealed limitations in the use of ITS sequences to elucidate recent radiations of poorly differentiated species, the molecular data did provide valuable information for conservation issues within the genus. Other ITS studies in *Gesneriaceae* at the species level include *Aeschynanthus* Jack (Denduangboripant *et al.*, 2001; Mendum *et al.*, 2001), *Cyrtandra* J.R.Forst. & G.Forst. (Atkins *et al.*, 2001; Bramley *et al.*, 2004) and *Agalmyla* Blume (Chapman, 2003). Given the widespread and successful use of ITS in phylogenetic studies at the species level in *Gesneriaceae* it seemed the most appropriate region for this study.

In this study a molecular investigation was undertaken using sequences of both the ITS1 and ITS2 regions of nrDNA on 23 *Didymocarpus* taxa, with a particular focus on species from Thailand (15 taxa). We also scored 19 characters, including 17 morphological features, for each sample included and optimized their evolution on phylogenetic trees. The specific aims of this study were to reconstruct a phylogenetic hypothesis for *Didymocarpus* to (i) test the monophyly of the genus, (ii) test the integrity of the two taxonomic sections of Weber & Burtt (1998), (iii) investigate relationships between the taxa included, (iv) trace geographic patterns of distribution within the genus for the species from Thailand, and (v) elucidate the evolution of morphological characters. Results from this study will be extremely valuable for the taxonomy of *Didymocarpus* and for an understanding of evolutionary processes that shaped the genus.

Materials and Methods

Plant materials

Ingroup selection. Samples of 23 taxa of *Didymocarpus* were selected as ingroup taxa, spanning the north–south distribution range of the genus *sensu stricto*. Fifteen of these were collected in Thailand (Fig. 1), including two new taxa (Palee & Maxwell, 2006). Fresh leaves were taken during field trips and rapidly dried in silica gel for DNA analysis. Voucher herbarium specimens were prepared and deposited at Chiang Mai University (CMU) Herbarium, Thailand, and the Royal Botanic Garden Edinburgh, UK (E). Voucher information for the samples used in this study,

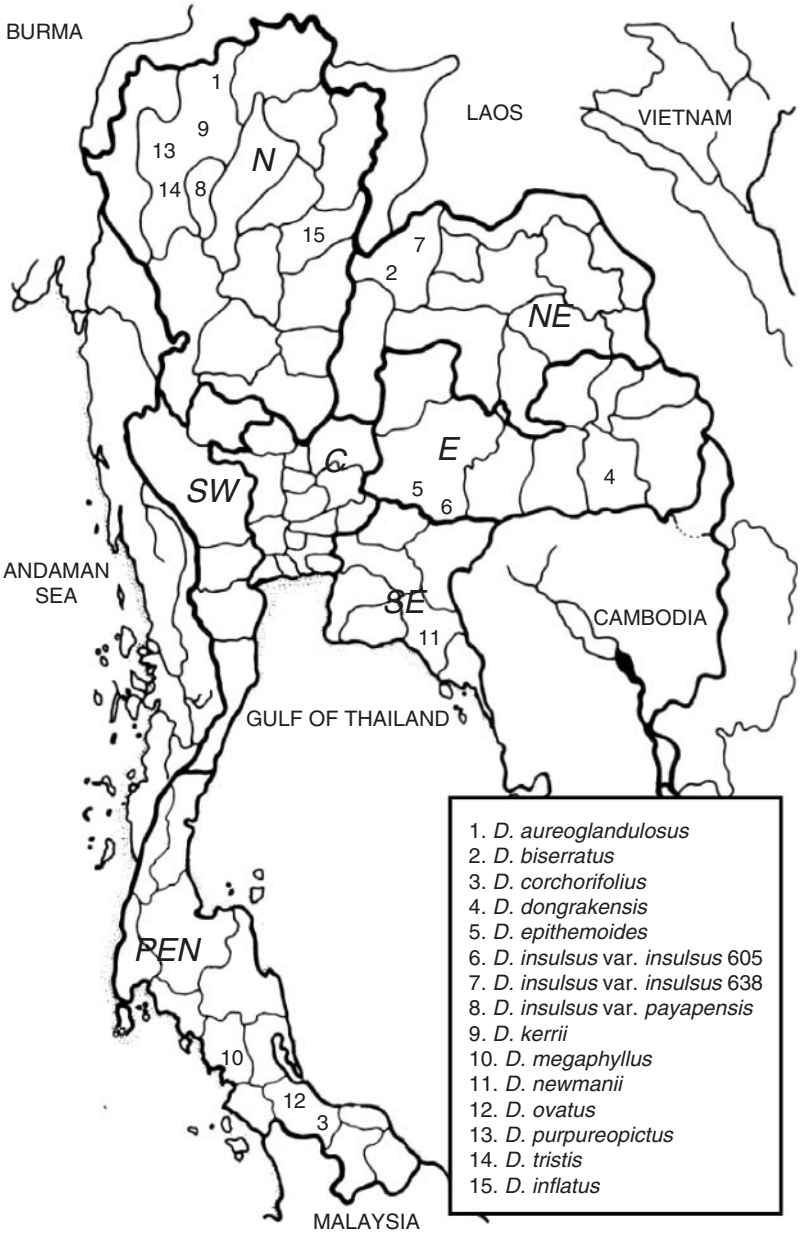

FIG. 1. The localities of Thai *Didymocarpus* taxa used in this study. The floristic-region map of Thailand is modified from *Thai Plant Names* (Smitinand, 2001).

including collection locality and GenBank accession numbers of their respective ITS sequences, is shown in Table 1. To investigate the phylogeographic relationships among the Thai species four additional species from China, three from the Malay Peninsula, and one from Bhutan were also included in the analyses.

TABLE 1. Taxon list, with specimen number and locality information for specimens used in this study, including GenBank accession for ITS sequences. (CMU = Chiang Mai University Herbarium, E = Royal Botanic Garden Edinburgh, K = Royal Botanic Gardens, Kew)

Taxon	Specimen no.	Locality	GenBank no.
Briggsia muscicola Craib	1995-2229 (K)	China: SE Xizang, NW Yunnan	DQ912665
Chirita asperifolia (Blume) B.L.Burtt	1995-1205 (E)	China: Guangxi (ex Smithsonian Institute 94-087)	DQ912668
Chirita caerulea R.Br.	C8252H (E)	Thailand: Chumphon	DQ912666
Chirita involucrata Craib	*Palee* 557 (CMU)	Thailand: Krabi	DQ912667
Oreocharis auricula (S.Moore) C.B.Clarke	*Möller* 03-376 (E)	China: Guizhou, Furongba, Xujia	DQ912664
Didymocarpus antirrhinoides Weber	1965-0167 (E)	Malay Peninsula: Perak	DQ912671
Didymocarpus aureoglandulosus C.B.Clarke	*Palee* 549 (CMU)	Thailand: Chiang Mai	DQ912685
Didymocarpus biserratus Barnett	*Palee* 639 (CMU)	Thailand: Loei	DQ912681
Didymocarpus citrinus Ridl.	1983-0510 (E)	Malay Peninsula: Perlis, Kedat Peak	DQ912669
Didymocarpus corchorifolius Wall. ex A.DC.	*Palee* 662 (CMU)	Thailand: Songkla	DQ912670
Didymocarpus cordatus Wall. ex A.DC.	*Weber* 860816-2/1 (WU)	Malay Peninsula	DQ912673
Didymocarpus dongrakensis B.L.Burtt	*Palee* 658 (CMU)	Thailand: Sri Saket	DQ912690
Didymocarpus epithemoides B.L.Burtt	*Palee* 603 (CMU)	Thailand: Nakorn Nayok	DQ912680
Didymocarpus inflatus Maxw. & Palee	*Palee* 642 (CMU)	Thailand: Pitsanulok	DQ912677
Didymocarpus insulsus Craib var. *insulsus*	*Palee* 605 (CMU)	Thailand: Nakorn Nayok	DQ912691
Didymocarpus insulsus Craib var. *insulsus*	*Palee* 638 (CMU)	Thailand: Loei	DQ912689
Didymocarpus insulsus var. *payapensis* Palee & Maxw.	*Palee* 641 (CMU)	Thailand: Lampoon	DQ912686
Didymocarpus kerrii Craib	*Palee* 550 (CMU)	Thailand: Chiang Mai	DQ912683
Didymocarpus megaphyllus Barnett	*Palee* 686 (CMU)	Thailand: Trang	DQ912672
Didymocarpus mengtze W.W.Sm.	*Zhang* G11 (E)	China: Yunnan	DQ912678
Didymocarpus cf. *mengtze* W.W.Sm.	*Möller* 03-238 (E)	China: Guizhou, Leishan	DQ912679
Didymocarpus newmanii B.L.Burtt	*Palee* 622 (CMU)	Thailand: Chantaburi	DQ912674
Didymocarpus ovatus Barnett	*Palee* 553 (CMU)	Thailand: Krabi	DQ912675
Didymocarpus podocarpus C.B.Clarke	NPSW-193 (E)	Bhutan: Deothang District	DQ912688
Didymocarpus purpureobracteatus W.W.Sm.	*Möller* 01-70 (E)	China: Yunnan, Pingbian	DQ912676
Didymocarpus purpureopictus Craib	*Palee* 567 (CMU)	Thailand: Chiang Mai	DQ912682
Didymocarpus stenanthos C.B.Clarke	*Möller* 01-156 (E)	China: Yunnan, Binshuan	DQ912687
Didymocarpus tristis Craib	*Palee* 551 (CMU)	Thailand: Chiang Mai	DQ912684

Outgroup selection. Five members of the tribe *Didymocarpeae* were selected as outgroup species to test the monophyly of the ingroup taxa. These included three species of *Chirita* Don, namely *C. asperifolia* (Blume) B.L.Burtt (section *Liebigia* (Endl.) C.B.Clarke), *C. caerulea* R.Br. and *C. involucrata* Craib (section *Microchirita* C.B.Clarke), *Briggsia muscicola* Craib, and *Oreocharis auricula* (S.Moore) C.B.Clarke. *Chirita* species share morphological features, such as diandry, with the ingroup taxa, but differ in other characteristics such as stigma morphology. Both *Oreocharis* Benth. and *Briggsia* Craib have four stamens. They were chosen on the basis of preliminary results of an extended ITS analysis, including representatives of all Asian tribes of *Gesneriaceae*. A larger analysis based on chloroplast DNA sequences supported the ITS results (M. Möller, unpublished data). The same analysis also showed that species previously included in the genus *Didymocarpus* and transferred to *Henckelia* and *Hovanella* are not closely related to species of *Didymocarpus* s.s. The genus *Hovanella* is closely related to *Streptocarpus* and has evolved from within this genus (Möller, 2003). As ITS sequences of *Henckelia* were not unambiguously alignable with those of *Didymocarpus* s.s. and those of *Hovanella* unobtainable (Möller, 2000, and unpublished data) they were excluded from the analysis here.

DNA extraction, PCR amplification and sequencing

Total genomic DNA from dried leaf material was extracted using the DNeasy®Plant Mini Kit (QIAgen Ltd, Dorking, Surrey, UK) according to the manufacturer's protocol. The DNA was collected in 1.5 ml Eppendorf tubes and stored at –20°C until required. The genomic DNA of each sample was quantified by agarose gel electrophoresis. The whole ITS region, including the 5.8S gene, was amplified using the modified ITS primers, ITS 5P (5′-GGA AGG AGA AGT CGT AAC AAG G-3′) and ITS 8P (5′-CAC GCT TCT CCA GAC TAC A-3′), following Möller & Cronk (1997a). PCR products were purified using the QIAquick PCR Purification Kit (QIAgen Ltd) according to the manufacturer's recommendations.

The PCR product for each taxon was sequenced in both forward and reverse reactions with two primers for complete sequence confirmation; forward sequencing was performed using primers ITS 5P and ITS 3P (5′-GCA TCG ATG AAG AAC GTA GC-3′), and reverse sequencing was done with primers ITS 4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) and ITS 2G (5′-GTG ACG CCC AGG CAG ACG T-3′) (Möller & Cronk, 1997a). The PCR products were sequenced on an ABI PRISM®3700 DNA Analyzer (Applied Biosystems) by Macrogen Inc. (Seoul, Korea).

Sequence alignment and phylogenetic analysis

Chromas version 1.45 (Conor McCarthy, School of Health Science, Griffith University, Gold Coast Campus, Southport, Queensland, Australia) was used to

clean up and align the forward and reverse sequence files. Determination of ITS spacer boundaries was carried out on published sequences for *Gesneriaceae* on GenBank. The newly determined sequences have been submitted to GenBank. Sequence alignments were manually constructed in PAUP*4.0b10 (Swofford, 2002).

Maximum parsimony (MP) analyses were performed in PAUP*. The matrix included no ambiguous alignment regions and thus all molecular characters of ITS1 and ITS2 were included and analysed unordered. Although a small amount of variation was found in the 5.8S gene it was not included in the analyses because of the great difference in sequence evolution between the gene and the spacers, a situation which could be problematic for the model-based maximum likelihood analysis. Indels (insertion/deletion of bases) were treated as missing data. However, where the indels were informative and their boundaries consistent across the matrix, they were coded according to the simple method of Simmons & Ochoterena (2000) and added to the end of the sequence matrix. The consistency index (CI) (Kluge & Farris, 1969), retention index (RI) and rescaled consistency index (RC) (Farris, 1989) were calculated using PAUP* while the transition/transversion ratio was obtained through MacClade version 3.07 (Maddison & Maddison, 1997). Molecular character state changes were either weighted equally or reweighted according to the RI using best fitting values recovered from the most parsimonious trees obtained in the unweighted MP analysis. Reweighting emphasizes those molecular characters that are congruent with the recovered tree topology. This method often results in fewer most parsimonious trees obtained and a higher resolution within the trees, which is important for a meaningful interpretation of morphological character optimization exercises.

Because of the moderate number of taxa a heuristic searching strategy was implemented using factory settings and 10,000 random addition sequence replicates with tree-bisection-reconnection branch swapping (TBR) and MulTrees options on, and Steepest Descent off, saving all most parsimonious trees. The trees were rooted on *Oreocharis* based on results from the preliminary analysis. Branch support was calculated as bootstrap values (Felsenstein, 1985) of 1000 replicates using full heuristic search with TBR and MulTrees on, and Steepest Descent off. Decay indices (Bremer, 1988) were also calculated using the default settings in AutoDecay version 4.0 (Eriksson, 1999).

A maximum likelihood (ML) analysis was performed to test the topology recovered from unweighted and reweighted MP analyses, using the model and parameter settings suggested by ModelTest version 3.06 (Posada & Crandall, 1998). From the 56 models tested SYM+G was selected (Zharkikh, 1994; with a rate matrix of 0.7156, 1.6166, 1.4720, 0.5513, 3.6188 and an α-parameter of the gamma distribution, G = 1.3294). The parameter settings were transferred to PAUP* and the analysis was executed with random sequence addition and TBR branch-swapping on. Branch support was calculated over 100 replicates of random addition, with TBR and MulTrees on in PAUP.

Morphological character coding and character optimization

To investigate the direction of character evolution in *Didymocarpus* the topology of the RI reweighted MP tree was used in MacClade to optimize the character state changes under DELTRAN (delayed character transformation, favouring parallelisms) and ACCTRAN (accelerated character transformation, favouring reversals).

In total 19 characters were scored for the 23 *Didymocarpus* taxa and five outgroup taxa. These characters were the sectional assignment of the taxon, detailed geography, and 17 morphological characters (Table 2). The morphological characters were examined mainly from herbarium specimens. The placement of species in sections (character 1) follows Weber & Burtt (1998). *Didymocarpus inflatus* Maxw. & Palee was classified as *Didymocarpus* sect. *Didymocarpus* as it has reticulate seeds. The geographical information was scored in detail dividing Thailand into seven floristic regions (character 2) (Fig. 1; Smitinand, 2001).

Seed surface structure (character 3) was treated as a multistate character (Beaufort-Murphy, 1983). Corolla colour (character 4) and corolla shape (character 5) were also coded as multistate characters with three states each. Colour coding is always difficult, and is particularly problematic in *Gesneriaceae*. Thus we chose to combine purple and violet colours into one state (state 0), and separate states for yellow (state 1) and white (state 2). The character states of the calyx lobes (character 6) were assigned as either 'free', when the lobes were free to the base (state 0), 'united up to middle', when the lobes were fused up to half of their lengths (state 1), or 'united more than half', when the lobes were almost entirely fused (state 2). In some species the upper (adaxial) three calyx lobes were smaller and almost entirely fused to form a 3-lobed unit; these species were coded according to the lower lobes. The asymmetry between the adaxial and abaxial lobes was treated as an additional character (character 7) with an asymmetric calyx (as in *Didymocarpus mengtze* W.W.Sm.) coded as state 1, and a symmetric or regular calyx coded as state 0, where all lobes were equal in size. Types of pigment glands (character 8) were coded as a multistate character with five states. In *Didymocarpus* pigment glands usually appear on the lower leaf surface, petiole, stem, peduncle and pedicel. When the glands were present, the gland types were constant among organs within a species. Only a few taxa possessed glands on the outside of the calyx. Their presence/absence was coded as a separate character (character 9). The indumentum on floral structures [anther (character 11), calyx (character 14) and pedicels (character 15)] and on leaves [lower blades (character 17) and lower nerves (character 19)] were scored as absent (state 0), glandular (state 1) or eglandular (state 2). The hairs on upper blades (character 16) and on upper nerves (character 18) were present in all taxa and these characters were scored as glandular (state 0) or eglandular (state 1). Hairs on the filaments (character 10) were scored as absent (state 0) or present (state 1) and hairs on the ovary (character 12) and the outside of the corolla (character 13) were scored as absent (state 0) or present (state 1).

TABLE 2. Character and character-state coding for *Didymocarpus* and outgroup taxa investigated in this study

Character	Character name	Character state
1	Section	0 = *Didymocarpus*, 1 = *Elati*, 2 = uncertain position, 3 = other genera
2	Geography	0 = Bhutan, 1 = China, 2 = Malaysia, 3 = N Thailand, 4 = Sumatra, 5 = NE Thailand, 6 = E Thailand, 7 = SE Thailand, 8 = Thai Peninsula
3	Seed surface; cell face	0 = striated, 1 = tuberculate, 2 = verrucate, 3 = smooth
4	Corolla colour	0 = violet/purple, 1 = yellow, 2 = white
5	Corolla shape	0 = salverform, 1 = funnelform, 2 = personate
6	Calyx lobes	0 = free, 1 = united up to middle, 2 = united to more than half
7	Calyx shape	0 = symmetric, 1 = asymmetric
8	Gland type	0 = absent, 1 = one-celled globose, 2 = one-celled conoid, 3 = two-celled head, 4 = four-celled conoid
9	Glands outside calyx	0 = absent, 1 = present
10	Hairs on filament	0 = absent, 1 = present
11	Hairs on anther	0 = absent, 1 = glandular, 2 = eglandular
12	Hairs on ovary	0 = absent, 1 = present
13	Hairs on corolla outer surface	0 = absent, 1 = present
14	Hairs on calyx outer surface	0 = absent, 1 = glandular, 2 = eglandular
15	Hairs on pedicel	0 = absent, 1 = glandular, 2 = eglandular
16	Hairs on upper blade	0 = glandular, 1 = eglandular
17	Hairs on lower blade	0 = absent, 1 = glandular, 2 = eglandular
18	Hairs on upper nerves	0 = glandular, 1 = eglandular
19	Hairs on lower nerves	0 = absent, 1 = glandular, 2 = eglandular

RESULTS

Sequence and matrix characteristics

The matrix characteristics of the ITS sequences are summarized in Table 3. The spacer lengths ranged from 204 to 236 base pairs (bp) for ITS1 and 211 to 241 bp for ITS2. The 5.8S region was 164 bp for all taxa. The GC content of the spacers was lower (47.8% for ITS1; 48% for ITS2) compared with the 5.8S gene (53.5%). The alignment of combined ITS1 and ITS2 sequences resulted in a matrix of 530 bp. In total, 16 informative gap characters were added to the matrix, 12 in ITS1 and four in ITS2. The aligned sequence matrix included 199 constant sites (37.5%), 108 uninformative sites (20.4%) and 223 potentially phylogenetically informative sites (42%). ITS1 and ITS2 were very similar in their proportions of variable and invariable sites (Table 3).

TABLE 3. Sequence characteristics of *Didymocarpus* and outgroup taxa analysed in this study

Parameters	ITS1	5.8S	ITS2	Combined ITS1+ITS2
Number of taxa	28	27*	28	28
Total length range (bp)	204–236	164	211–241	436–472
Mean	226.2	–	231.2	457.4
Sequence length range (outgroup) (bp)	226–236	164	216–238	448–469
Mean	230.0	–	230.3	460.3
Sequence length range (ingroup) (bp)	204–234	164	210–240	436–472
Mean	226.9	–	234.4	461.3
Total aligned matrix length (bp)	265	–	265	530
G+C content (%)	47.8	53.5	48.0	48.0
Number of informative indels	12	–	4	16
Number of uninformative indels	15	–	23	38
Number of constant sites (%)	95 (35.8)	138 (84.1)	104 (39.2)	199 (37.5)
Number of variable sites (%)	170 (64.2)	26 (15.9)	161 (60.8)	331 (62.5)
Number of uninformative sites (%)	57 (21.5)	14 (8.5)	51 (19.2)	108 (20.4)
Number of informative sites (%)	113 (42.6)	12 (7.3)	110 (41.5)	223 (42.0)
Sequence divergence (outgroup) (%)				1–99 0.2–23
Sequence divergence (in/outgroup) (%)				87–121 20–26.6
Sequence divergence (ingroup) (%)				9–90 1.9–19.7
Transitions : transversions (minimum)				349 : 286
Ts/Tv ratio				1.22
Most parsimonious trees (with gap matrix)				148
Length, steps (with gap matrix)				823
Average steps per character				1.55
CI				0.6209
RI				0.6397
RC				0.3972

*Excluding *Chirita caerulea*.

Sequence divergence across the combined spacer sequences within the outgroup taxa was between 0.2% (1 molecular character change) for the pair *Chirita caerulea* and *C. involucrata* and 23% (99 changes) for *C. asperifolia* and *Oreocharis auricula*. Divergence between in- and outgroup taxa was relatively high, with the lowest between *C. asperifolia* and *D. citrinus* (20%, 87 changes) and highest for *D. purpureobracteatus* W.W.Sm. and *O. auricula* (26.6%, 121 changes). Within the ingroup, sequences of *D. epithemoides* B.L.Burtt and *D. biserratus* Barnett were most similar (1.9%, 9 changes), while *D. cordatus* and *D. insulsus* var. *payapensis* Palee & Maxw. showed the highest divergence (19.7%, 90 changes). The transition/transversion ratio was 1.22.

Tree topologies

The MP analysis on the combined ITS sequence data, including the gap matrix, resulted in 148 most parsimonious trees of 823 steps length (CI = 0.6209, RI = 0.6397 and RC = 0.3972). The randomly selected phylogram shows the distribution of branch lengths between the samples and indicated that the in- and outgroup taxa were separated by 29 steps. In the strict consensus tree (Fig. 2) bootstrap branch support values ranged from 67% to 100%, with 13 out of 17 branches having a bootstrap support > 90%. The analysis on RI reweighted characters resulted in a single most parsimonious tree (Fig. 3) with fully resolved relationships among the taxa. The additional branches compared with the unweighted analysis received little or no branch support. Otherwise, the topology of the reweighted tree was congruent with the tree topology of the strict consensus tree of the unweighted analysis.

All 23 *Didymocarpus* taxa analysed by MP in this study formed a monophyletic group with high branch support (BS = 100%; DI (decay indices) = 12 & 13.8, Figs 2 and 3). One large clade (Clade I) including 17 taxa was recognized within the ingroup taxa which was strongly supported (BS = 96 & 99%; DI = 6 & 6.3). The other six taxa remained as sister on unsupported branches (*Didymocarpus cordatus*, *D. newmanii* B.L.Burtt, *D. ovatus*), or formed a strongly supported clade (Clade II) comprising *D. citrinus*, *D. corchorifolius* and *D. antirrhinoides* (BS = 100%; DI = 11 & 10.9). Two of these, *D. corchorifolius* and *D. antirrhinoides*, are members of *Didymocarpus* sect. *Elati* while the other is at present of uncertain section assignation. Note that *Didymocarpus newmannii* has been corrected to *D. newmanii*, named after Mark Newman, under Art. 60 of the International Code of Botanical Nomenclature (Greuter *et al.*, 2000).

Within Clade I, one supported subclade was present (Subclade Ia) (BS = 51 & 73%; DI = 2 & 1.7) containing species from north or northeast Thailand. In addition, a few strongly supported sister pairs were found in Clade I: a pair of the Chinese samples of *Didymocarpus mengtze* (BS = 100%; DI = 12 & 8.1), the Chinese/Thai pair *D. purpureobracteatus* and *D. inflatus* Maxw. & Palee (BS = 96 & 98%; DI = 7 & 6), the Thai pair *D. epithemoides* and *D. biserratus* (BS = 100%; DI = 11 & 9.5), and the Bhutanese/Chinese pair *D. podocarpus* C.B.Clarke and *D. stenanthos* C.B.Clarke (BS = 96 & 99%; DI = 6 & 4.8). A sister relationship between the latter two pairs was indicated (BS = 57 & 67%; DI = 1 & 2).

The topology of the maximum likelihood tree (data not shown) was greatly congruent with that of the reweighted MP tree, except for a polytomic position of *Didymocarpus cordatus* and *D. newmanii*, and the position of the sister pair *D. purpureobracteatus*/*D. inflatus*, while *D. tristis* C.B.Clarke and *D. aureoglandulosus* formed a sister pair. Furthermore, the position of *Didymocarpus insulsus* Craib var. *insulsus* 638 as a sister to *D. dongrakensis* B.L.Burtt changed to a sister position with *D. insulsus* var. *insulsus* 605. However, these changes exclusively involved branches with no or very low bootstrap support in the latter case (BS = < 52%).

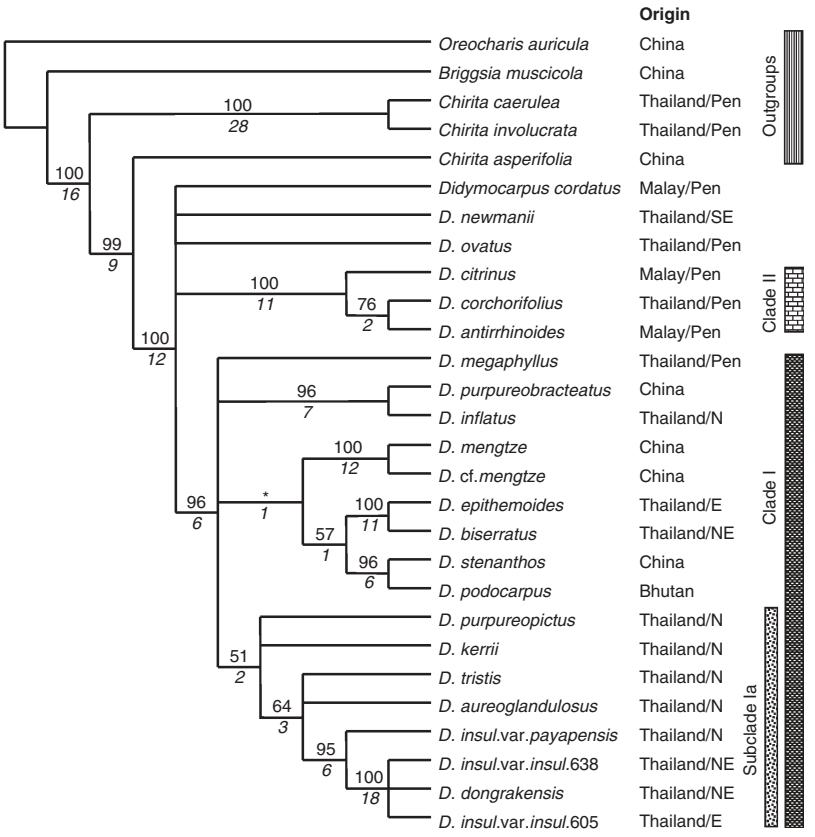

FIG. 2. Strict consensus tree of 148 most parsimonious trees based on combined ITS1 and ITS2 sequence data plus an alignment gap matrix. Numbers above branches indicate bootstrap support, numbers below branches (in italics) are decay indices (DI). Asterisks (*) indicate branches with less than 50% bootstrap support. Origin of taxa and clade assignment are indicated to the right.

Morphological character evolution

For the 19 characters used in this analysis, only five (0.9%) of the total of 532 cells were coded as missing (Table 4). Of the characters scored for the in- and outgroup taxa and optimized onto the reweighted tree, half were revealed to be highly homoplastic (Table 5) and generally had CIs lower than or equal to 0.33. These were mainly characters associated with corolla shape (character 5), calyx (characters 6 and 7) and the hairs on floral parts (characters 10 to 15). These characters did not strongly support any grouping of *Didymocarpus* taxa and appeared to have evolved in a random fashion. Appearance of the hairs on the leaf surface (characters 16 to 19) was more interesting as it supported the split of the Chinese taxa; both

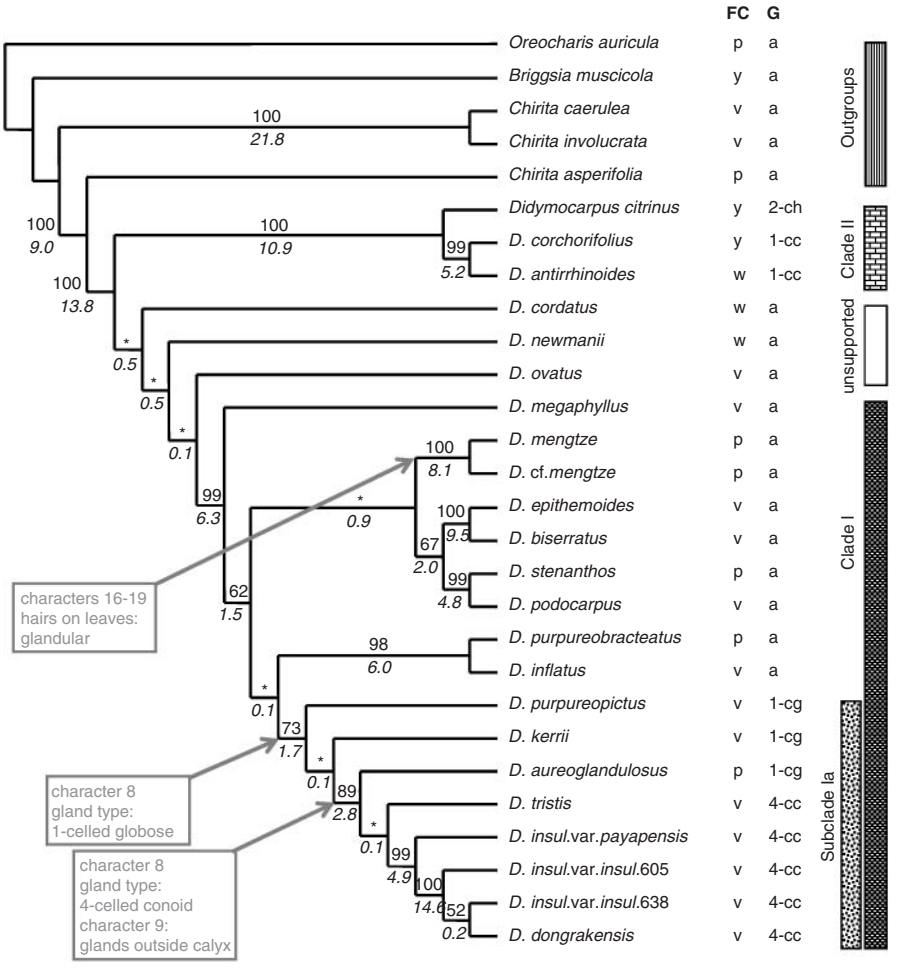

FIG. 3. Cladogram of the single most parsimonious tree after reweighting characters according to RI values, based on the combined ITS1 and ITS2 sequence data plus an alignment gap matrix. Numbers above branches indicate bootstrap support, numbers below branches (in italics) are decay indices (DI). Asterisks (*) indicate branches with less than 50% bootstrap support. Flower colour, gland type and clade assignment are indicated to the right. Significant character states supporting clades are indicated in grey boxes. FC = flower colours: p = purple, v = violet, w = white, y = yellow; G = gland types: 1-cc = one-celled conoid, 1-cg = one-celled globose, 2-ch = two-celled head, 4-cc = four-celled conoid, a = absent.

D. mengtze specimens had glandular hairs on both leaf surfaces, a synapomorphy for this pair.

Characters which are more complex, but potentially of more taxonomic diagnostic value, are described in more detail below.

Section assignation (character 1) supported *Didymocarpus* sect. *Elati* as containing a monophyletic pair of *Didymocarpus corchorifolius* and *D. antirrhinoides*. The

TABLE 4. Data matrix of the morphological characters coded for *Didymocarpus* and outgroup taxa investigated in this study

Taxon	Character number																		
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Briggsia muscicola	3	1	?	1	1	0	0	0	0	1	0	0	0	2	2	1	2	0	2
Chirita caerulea	3	8	?	0	1	0	1	0	0	0	1	0	1	1	1	1	0	0	2
C. involucrata	3	8	0	0	1	0	0	0	0	0	1	0	1	1	1	1	2	0	2
C. asperifolia	3	4	?	0	1	2	0	0	0	0	1	1	1	2	2	1	2	0	2
Oreocharis auricula	3	1	0	0	0	0	0	0	0	0	0	0	1	2	2	1	2	0	2
Didymocarpus antirrhinoides	1	2	1	2	2	2	0	2	0	1	2	1	0	0	0	1	2	0	2
D. aureoglandulosus	2	3	3	0	1	0	1	1	1	0	0	1	0	1	1	1	2	0	2
D. biserratus	0	5	0	0	1	2	0	0	0	0	1	0	0	0	0	1	2	0	2
D. citrinus	2	2	1	1	1	0	0	3	0	1	0	1	0	0	0	1	2	0	2
D. corchorifolius	1	8	2	1	2	0	0	2	0	1	2	1	0	0	0	1	2	0	2
D. cordatus	2	2	1	2	1	0	0	0	0	0	1	0	0	0	0	1	2	0	2
D. dongrakensis	0	6	0	0	1	0	0	4	0	0	0	0	0	0	1	1	0	0	0
D. epithemoides	0	6	0	0	0	2	0	0	0	0	1	0	0	0	1	1	2	0	2
D. inflatus	0	3	2	0	1	2	0	0	0	0	2	0	0	0	0	1	0	0	2
D. insulsus var. *insulsus* 605	0	6	0	0	0	0	0	4	1	0	2	0	0	0	1	1	0	0	0
D. insulsus var. *insulsus* 638	0	5	0	0	0	0	0	4	1	0	2	0	0	0	1	1	0	0	2
D. insulsus var. *payapensis*	0	3	3	0	0	1	0	4	1	0	2	0	0	1	1	1	0	0	2
D. kerrii	0	3	3	0	0	2	0	1	0	0	0	0	0	1	1	1	2	0	2
D. megaphyllus	0	8	1	0	1	2	0	0	0	1	2	1	1	1	0	1	2	0	2
D. mengtze	0	1	?	0	0	1	1	0	0	0	1	0	0	0	1	0	1	1	1
D. cf. *mengtze*	0	1	2	0	0	1	1	0	0	0	1	0	0	0	1	0	1	1	1
D. newmanii	0	7	3	2	1	0	0	0	0	1	1	0	0	0	0	1	2	0	2
D. ovatus	0	8	3	0	0	0	0	0	0	0	2	0	0	0	1	1	2	0	2
D. podocarpus	0	0	1	0	1	1	0	0	0	0	1	0	1	0	0	1	2	0	2
D. purpureobracteatus	0	1	?	0	1	2	1	0	0	0	0	0	0	0	0	1	2	0	2
D. purpureopictus	0	3	0	0	1	2	0	1	0	1	1	1	0	0	1	1	2	0	2
D. stenanthos	0	1	3	0	0	2	1	0	0	0	1	0	0	0	0	1	2	0	2
D. tristis	0	3	3	0	0	0	0	4	1	0	0	0	0	0	1	1	2	0	2

delimitation of *Didymocarpus* sect. *Didymocarpus* is unclear and is only monophyletic (albeit with no branch support) when *D. ovatus* and *D. cordatus*, of uncertain position, are included. However, these and *Didymocarpus newmanii* of *Didymocarpus* sect. *Didymocarpus* are in an unsupported basal position to Clade I. The position of *Didymocarpus aureoglandulosus*, previously not assigned to any section, suggests an inclusion in *Didymocarpus* sect. *Didymocarpus*.

Mapping fine scale geographic data (character 2) onto the tree resulted in a complex pattern, with an equivocal ancestral character state for the genus

TABLE 5. Characteristics of characters across the single most parsimonious tree based on reweighted characters using RI. Min = minimum steps; Max = maximum steps

Character		Type	% missing	Weight	States	Min Steps	Max	CI
1.	Section	Unordered	0	1	4	3 4	10	0.75
2.	Geography (detailed)	Unordered	0	1	9	8 14	22	0.57
3.	Seed surface; cell face	Unordered	17.9	1	4	3 10	15	**0.30**
4.	Corolla colour	Unordered	0	1	3	2 5	6	0.40
5.	Corolla shape	Unordered	0	1	3	2 9	13	**0.22**
6.	Calyx lobes	Unordered	0	1	3	2 7	14	**0.29**
7.	Calyx shape	Unordered	0	1	2	1 5	6	**0.20**
8.	Gland type	Unordered	0	1	5	4 4	11	1.00
9.	Glands outside calyx	Unordered	0	1	2	1 2	5	0.50
10.	Hairs on filament	Unordered	0	1	2	1 5	7	**0.20**
11.	Hairs on anther	Unordered	0	1	3	2 10	16	**0.20**
12.	Hairs on ovary	Unordered	0	1	2	1 5	7	**0.20**
13.	Hairs on outside corolla	Unordered	0	1	2	1 4	6	**0.25**
14.	Hairs on outside calyx	Unordered	0	1	3	2 6	9	**0.33**
15.	Hairs on pedicel	Unordered	0	1	3	2 6	14	**0.33**
16.	Hairs on upper blade	Unordered	0	1	2	1 1	2	1.00
17.	Hairs on lower blade	Unordered	0	1	3	2 4	8	0.50
18.	Hairs on upper nerves	Unordered	0	1	2	1 1	2	1.00
19.	Hairs on lower nerves	Unordered	0	1	3	2 3	4	0.67

Didymocarpus under DELTRAN optimization, but suggesting a Peninsular Malaysian origin of *Didymocarpus* under ACCTRAN optimization. The genus appeared to have originated in Peninsular Malaysia (ACCTRAN), and appears to have spread northwards into the southern part of Thailand, then further north into northern Thailand, Bhutan and China. The geographic data indicated two dispersal events into China (under both ACCTRAN and DELTRAN optimizations), with a reversal of species distribution into northeast and east Thailand. The presence of *Didymocarpus purpureobracteatus* in China appeared to be the result of a separate, isolated event. The majority of Subclade Ia is distributed in north Thailand and the terminal cluster consisting of *D. dongrakensis* and *D. insulsus* var. *insulsus* accessions appears to have dispersed into northeast and east Thailand.

The ancestral state for the seed surface (character 3) was tuberculate for *Didymocarpus*. This state was present in basal taxa of Clade II and *D. cordatus* and as reversals in two taxa, *D. megaphyllus* and *D. podocarpus*. The tuberculate seed surface changed to smooth as the ancestral state of Clade I, plus *Didymocarpus cordatus* and *D. newmanii*. Striate and verrucate surface appeared to be homoplastic, with three changes to the striated surface in Clade I and three changes to verrucate surfaces across the genus.

The ancestral state for corolla shape (character 5) was funnelform for the genus *Didymocarpus*. Within Subclade Ia, the corolla shape character was homoplastic, with three changes from a funnelform to a narrow salverform tube and four reversals to funnelform. Clade II also included one change from funnelform to personate flowers (from both DELTRAN and ACCTRAN).

Corolla colour (character 4) and gland characters were generally more informative and their character state distribution was more congruent with the reweighted tree topology than the seed surface and the corolla shape. All taxa in Clade I and *Didymocarpus ovatus* had corollas with purple or violet colours (Fig. 3). Purple and violet colours appear to be a derived state within the genus. White and yellow flower colours were present in Clade II (in *Didymocarpus* sect. *Elati*), while the white corolla colour was also present in *Didymocarpus cordatus* and *D. newmanii*.

Pigment glands on plant parts, except the calyx (character 8), were present only in taxa in Subclade Ia and Clade II, with no overlap of states. The three basal taxa in Subclade Ia possessed one-celled globular glands, while four-celled conoid glands were present in the remaining five taxa. In Clade II *Didymocarpus citrinus* had two-celled glands and *D. corchorifolius* and *D. antirrhinoides* had one-celled conoid glands. The presence of glands outside the calyx (character 9) can be correlated with the appearance of four-celled conoid glands in Subclade Ia, except for *Didymocarpus dongrakensis* where the glands are absent, and *D. aureoglandulosus* which had one-celled globular glands on other plant parts as well.

Discussion

Sequence evolution

The average lengths of the ITS regions in *Didymocarpus* (ITS1 = 226.9 bp and ITS2 = 234.4 bp) were similar to those of other *Gesneriaceae* genera, such as *Streptocarpus* (ITS1 240.3 bp and ITS2 229.9 bp) (Möller & Cronk, 1997a) and *Aeschynanthus* (ITS1 225.3 bp and ITS2 239.3 bp) (Denduangboripant *et al.*, 2001). The evolution of the ITS region in *Didymocarpus* species included base changes as well as insertion and deletion events. Unlike in *Streptocarpus* and *Aeschynanthus*, there were no larger deletions at the 5′-end of ITS2, except for a 31 bp deletion in *D. inflatus*. The shared deletion in three genera demonstrates the relative redundancy of this region of ITS2 for proper functioning of post-transcriptional processes.

Generally, the GC content is higher than 50% for nuclear genes (Page & Holmes, 1998). This value is known to be consistent for related groups of plants but can vary between lineages (Hershkovitz *et al.*, 1999). *Didymocarpus*, however, had relatively low values (48%) for its ITS sequences, significantly lower than those of other *Gesneriaceae*, for example *Aeschynanthus*, 55% (Denduangboripant *et al.*, 2001); *Streptocarpus*, 56.1% (Möller & Cronk, 1997a); *Cyrtandra*, 58% (Atkins *et al.*, 2001; Bramley *et al.*, 2004) or *Agalmyla*, 63.4% (Chapman, 2003). The significance of this finding is not known.

Phylogeny and geography of Didymocarpus *in SE Asia*

From the result of the phylogenetic analysis a number of observations can be made, irrespective of whether unweighted, reweighted MP or ML analyses were performed:

1 The taxa of *Didymocarpus* s.s. included in this study formed a strongly supported monophyletic clade.

2 Two strongly supported clades were always recovered, Clade I, including most taxa of *Didymocarpus* sect. *Didymocarpus*, and Clade II, corresponding to the 'broader' concept of the *Didymocarpus* sect. *Elati* of Weber & Burtt (1998) which suggested the inclusion of *D. citrinus*. *Didymocarpus aureoglandulosus*, unclassified by Weber & Burtt (1998), always fell within Clade I, suggesting that this taxon belongs to *Didymocarpus* sect. *Didymocarpus*. The branch support within Clade I was relatively variable and the relationships therein varied between the analyses performed.

3 Subclade Ia was recovered in both MP and ML analyses, and included mainly northeast, north and east Thai species. This clade was also supported by morphological characters (see below).

Different relationships among sister pairs within Clade I between MP and ML analyses were apparent, suggesting that some phylogenetic relationships observed may be unstable. This instability may be linked to the very short internal branches which suggest a burst of rapid radiation at this point in the evolution of *Didymocarpus*. This hypothesis is supported by the fact that the strongly supported sister pairs include taxa from adjacent geographic regions (e.g. China/Bhutan, China/N Thailand). Addition of further taxa may help to stabilize the tree topology. Irrespective of the conflicting topologies represented by the ML and MP analyses, inferences on the geographic history of *Didymocarpus* can be made. The genus has a possible origin on the Malay Peninsula from which it moved northwards through the Thai Peninsula, south Thailand into north and northeast Thailand. There the taxa from China perturbate the picture since our results suggest they were not the result of a single colonization event, but rather their occurrence in China was the result of several independent dispersal events. The south–north trend in the evolution of *Didymocarpus* species, however, is clearly indicated. Thus, the Thai species do not form a monophyletic group, irrespective of method of analysis.

Section assignment and evolution of morphological characters

This survey of morphological characters established that some characters gave more grouping information and support to phylogenetic relationships than others. Characters relating to the calyx and the indumentum on floral parts were uninformative as they were highly homoplastic, often appearing to have evolved several times independently across the reweighted MP phylogenetic tree used for morphological character optimization. Using the ML tree did not alter this aspect

(data not shown). This pattern of evolution indicates that parallelisms in *Didymocarpus* are frequent. Nevertheless, these homoplastic characters could be useful to distinguish closely related species and for regional keys. The distribution of glandular hairs on the leaves is more interesting as it divided the taxa from China. The two samples of *Didymocarpus mengtze* were unique with their glandular hair type, while *D. stenanthos* and *D. purpureobracteatus* had eglandular hairs as for the rest of the taxa included. This supported the phylogenetic results which suggested that the Chinese species have a different evolutionary history (see above). Inclusion of more specimens from this region is necessary to obtain a complete picture of the evolutionary history of the genus in China and to test whether the bifurcation in hair type in Chinese taxa is real or a sampling artefact as only four out of 31 described Chinese species were included (Wang *et al.*, 1998).

Essentially, two characters, the corolla colour and the gland type, were found to be most effective in correlating taxonomy and molecular phylogeny across the genus. These characters were also used by Weber & Burtt (1998) to define *Didymocarpus* sect. *Elati* which was well supported by the molecular analyses in this study. From our phylogenetic results we therefore suggest that the genus *Didymocarpus* in Thailand contains at least three groups. The first group is *Didymocarpus* sect. *Elati* which, with the addition of *D. citrinus*, comprised the three members of Clade II and can be defined by yellow or white flowers, either one-celled and conoid or two-celled head pigment glands, and tall stems with well-developed internodes. The other section, *Didymocarpus* sect. *Didymocarpus*, can be split into two groups and has not been suggested by Weber & Burtt (1998). One (group 2) includes eight members of Subclade Ia from east, north and northeast Thailand with the distinguishing characters violet and purple flowers, with either four-celled and conoid or one-celled and globose glands, and dwarfed stems which often die after fruiting. *Didymocarpus aureoglandulosus* also belongs in this section. The other (group 3) includes all remaining taxa comprising a complex mix of members from Malaysia, Thailand, China and Bhutan. This group possesses the habit of *Didymocarpus* sect. *Didymocarpus*, but has no pigment glands. Elucidation of whether this group represents a new section, however, will have to await the addition of further taxa from China, north and northeast India and Nepal to our analyses.

Summary and Conclusions

Taxa of the remodelled genus *Didymocarpus* s.s. (Weber & Burtt, 1998) included in the present study form a strongly supported monophyletic clade. The genus is split into three groups, distinguished by geography, absence/presence and morphology of glands, and corolla colour. The phylogeny suggested strong support for *Didymocarpus* sect. *Elati* sensu Weber & Burtt (1998), including *D. citrinus*. Taxa from Thailand do not form a monophyletic group, as taxa from Bhutan, China and the Malay Peninsula cluster amongst them. Our results suggest that, based on this

sample, the geographical origin of the genus was in the Malay Peninsula, and the ancestral corolla colour was yellow/white. The ancestral Malay taxa dispersed northwards to southern and eastern Thailand, then progressively northwards into China and Bhutan. This northward expansion coincided with the appearance of purple- and violet-coloured corollas. The northern Thai species additionally evolved novel morphological character states, four-celled and conoid or one-celled and globose pigment glands. It would be premature to propose an updated taxonomic classification at this point, however, as only about one third of the taxa in the genus are included here (23 out of c.70) and more samples are required to test our hypothesized groupings. Further analyses on additional characters, such as basic chromosome numbers, would be useful to investigate natural groups of species in the genus. The chromosome numbers of only 16 taxa have been determined, but even this small sample produced seven different basic numbers (Möller & Kiehn, 2004).

Nonetheless, the combination of molecular phylogeny and morphological character state change optimization provided a useful tool in unravelling historical events in the evolution of the genus. Even though only a single locus was used for our molecular analysis, several morphological characters supported the groupings found. Although this study focused predominantly on species from Thailand, it has resulted in findings that are relevant to the systematics of *Didymocarpus* s.s. The present study provides the basis from which future sampling can be structured to guarantee that other morphological characters that may be found to be important sectional or subsectional indicators can be included.

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