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Molecular phylogenetics and the evolution of fruit and leaf morphology of *Dichaea* (Orchidaceae: Zygopetalinae)

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• *Background and Aims* The orchid genus *Dichaea*, with over 100 species found throughout the neotropics, is easily recognized by distichous leaves on long stems without pseudobulbs and flowers with infrastigmatic ligules. The genus has previously been divided into four sections based primarily on presence of ovary bristles and a foliar abscission layer. The aim of this work is to use DNA sequence data to estimate phylogenetic relationships within *Dichaea* and map the distribution of major morphological characters that have been used to delimit subgenera/sections.

• *Methods* Sequence data for the nuclear ribosomal internal transcribed spacers and plastid *matK*, *trnL* intron, *trnL-F* spacer and *ycf1* for 67 ingroup and seven outgroup operational taxonomic units were used to estimate phylogenetic relationships within *Dichaea*. Taxa from each of the four sections were sampled, with the greatest representation from section *Dichaea*, the most diverse and taxonomically puzzling group.

• *Key Results* Molecular data and morphology support monophyly of *Dichaea*. Results indicate that section *Dichaeopsis* is polyphyletic and based on symplesiomorphies, including deciduous leaves and smooth ovaries that are widespread in Zygopetalinae. There are at least three well-supported clades within section *Dichaeopsis*. Section *Pseudodichaea* is monophyletic and defined by setose ovaries and leaves with an abscission layer. Sections *Dichaea* and *Dichaeastrum* are monophyletic and defined by pendent habit and persistent leaves. Section *Dichaeastrum*, distinguished from section *Dichaea* primarily by a glabrous ovary, is potentially polyphyletic.

• *Conclusions* The leaf abscission layer was lost once, occurring only in the derived sections *Dichaea* and *Dichaeastrum*. The setose fruit is a more homoplasious character with several losses and gains within the genus. We propose an informal division of the genus based upon five well-supported clades.

Key words: *Dichaea*, *matK*, nrITS, Orchidaceae, *trnL* intron, *trnL-F* spacer, *ycf1*, Zygopetalinae.

INTRODUCTION

Dichaea Lindl. is a rarely cultivated orchid genus, closely related to some commonly cultivated, showy genera including *Zygopetalum* Hook., *Huntleya* Bateman ex Lindl. and *Pescatoria* Rchb.f. With approx. 100 species, *Dichaea* is found throughout the neotropics, reaching peak diversity in the equatorial Andes. *Dichaea* is the largest genus in subtribe Zygopetalinae with about 400 species (Chase *et al.*, 2003). Zygopetalinae form a strongly supported clade (Whitten *et al.*, 2000) within tribe Cymbidieae (Chase *et al.*, 2003). All members of this subtribe are part of an exclusively neotropical clade within the widespread tribe Cymbidieae that also includes Catasetinae, Coeliopsidinae, Cymbidiinae, Cyrtopodiinae, Eriopsidinae, Eulophiinae, Maxillariinae, Oncidiinae, Stanhopeinae and Vargasiellinae.

The unusual habit and floral morphology (Fig. 1) of *Dichaea* have made its systematic position controversial. Szlachetko (1995) placed *Dichaea* in the monogeneric subtribe Dichaeinae, part of a larger tribe Dichaeeae including *Vargasiella* C.Schweinf., *Fernandezia* Lindl. and *Pachyphyllum* Kunth, all with a similar vegetative habit. No sequence data have been published for *Vargasiella*, but it is now treated as the only member of subtribe Vargasiellinae (Romero and Carnevali, 1993; Pridgeon *et al.*, 2009). Molecular data clearly place *Fernandezia* and *Pachyphyllum* in Oncidiinae (Williams *et al.*, 2001). Although placement of *Dichaea* within Zygopetalinae was novel in the molecular analysis of Whitten *et al.* (2000), it was supported by single-flowered inflorescences, pollinarium structure and pseudobulbless stems (Dressler, 1993*b*).

Since Lindley (1833) described *Dichaea*, generic and subgeneric classifications have been problematic. Knowles and Westcott (1839) first addressed subgeneric categories within the genus. They erected a second genus, *Epithecia* Knowles & Westc., for species with articulate (deciduous) leaves. Pfitzer (1889) described another segregate genus, *Dichaeopsis*, to encompass species with articulate leaves, apparently ignoring the work of Knowles and Westcott. Kuntze (1904) reduced *Dichaeopsis* to a section within *Dichaea*. Cogniaux (1906) retained *Dichaea* as a single genus of four sections: *Dichaea* (as *Eudichaea*), *Dichaeastrum* Cogn., *Dichaeopsis* (Pfitzer) Kuntze and *Pseudodichaea* Cogn. Section *Dichaea* was distinguished by setose ovaries and non-articulate leaves, section *Dichaeastrum* by glabrous ovaries and non-articulate leaves, section *Dichaeopsis* by glabrous ovaries and articulate leaves

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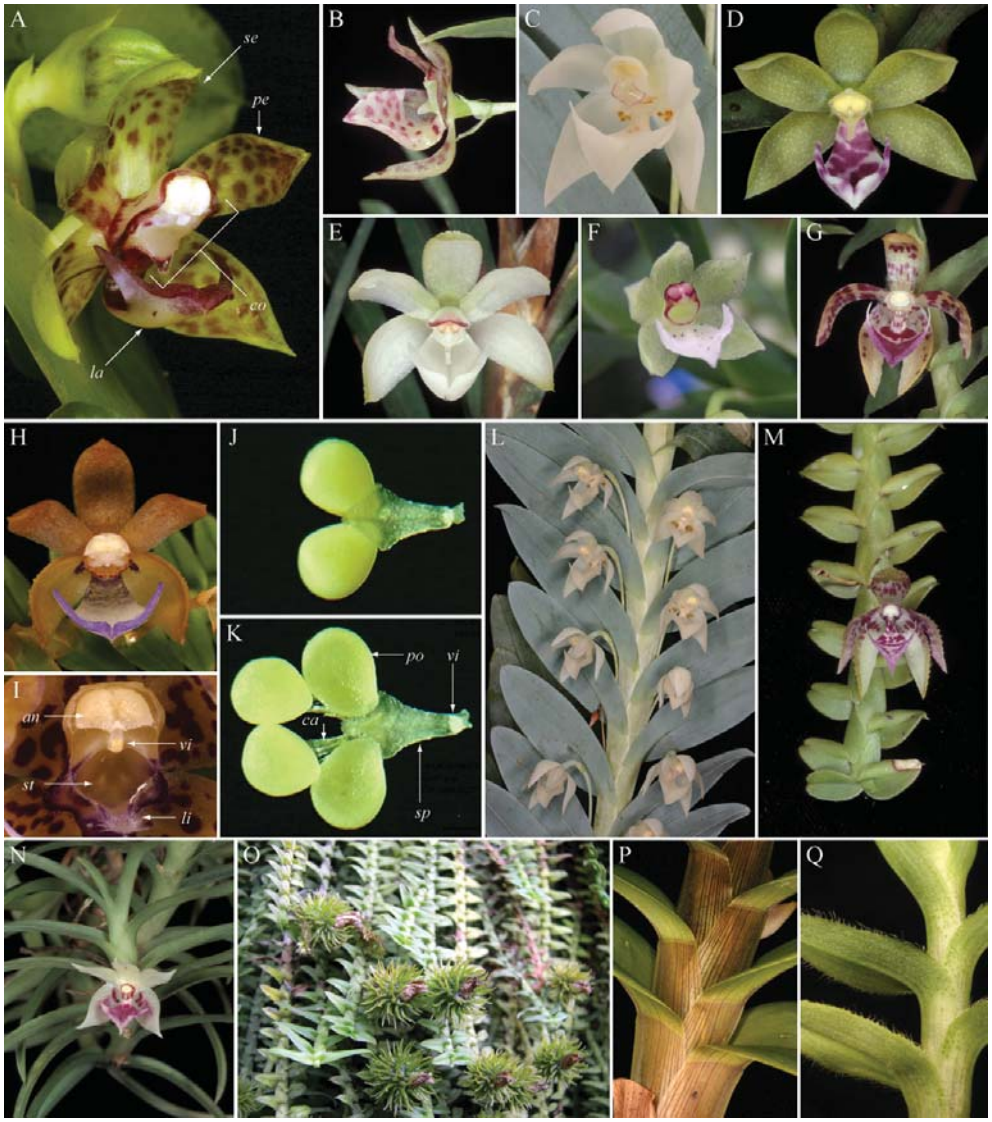

FIG. 1. Morphological features of *Dichaea*: (A) *Dichaea globosa* (section *Pseudodichaea*) (*co* = column, *pe* = petal, *la* = labellum, *se* = sepal); (B) *D. panamensis* (section *Dichaeopsis*); (C) *D. glauca* (section *Dichaeopsis*); (D) *D. trulla* (section *Dichaeopsis*); (E) *D. caveroi* (section *Dichaeopsis*); (F) *D. ancoraelabia* (section *Dichaeopsis*); (G) *D. poicillantha* (section *Dichaea*); (H) *D. squarrosa* (section *Dichaea*); (I) typical column (note the round stigma and pubescent instrastigmatic ligule; *an* = anther, *li* = infrastigmatic ligule, *st* = stigma, *vi* = viscidium); (J) pollinarium of *Dichaea* in natural configuration; (K) pollinarium pressed to show the elastic caudicles as the upper pollinia extend over the lower ones (*ca* = caudicle, *po* = pollinium, *sp* = stipe, *vi* = viscidium); (L) *D. glauca* (note erect stem and thickly glaucous leaves); (M) *D. cryptarrhena*; note strongly pendulous habit; (N) *D. ecuadorensis* (note semi-erect habit); (O) spiny fruits of section *Dichaea*; (P) leaves with an abscission layer between the sheath and blade (section *Pseudodichaea*); (Q) leaves lacking an abscission layer (section *Dichaea*).

and section *Pseudodichaea* by setose ovaries and articulate leaves. Although Cogniaux's treatment was limited to Brazilian *Dichaea*, his sections did encompass all combinations of the two characters (i.e. leaf abscission and setose ovaries). Schlechter (1914) accepted the four groups of Cogniaux but preferred the generic delimitation of Knowles and Westcott (1839). Therefore he placed sections *Dichaea* and *Dichaeastrum* in *Dichaea* and sections *Dichaeopsis* and *Pseudodichaea* in *Epithecia*. Kränzlin (1923) treated the group as a single genus with three sections: *Dichaea*, *Dichaeopsis* and *Maxillariopsis* Kränzl. However, the four species placed in section *Maxillariopsis* are currently treated as members of *Maxillariella* M.A.Blanco & Carnevali in subtribe Maxillariinae (Folsom, 1987, 1996). Senghas (1996), who followed Kränzlin's work closely, erected two subgenera in *Dichaea*. His subgenus *Dichaea* included section *Dichaea* and subgenus *Epithecia* included sections *Dichaeopsis* and *Maxillariopsis*.

There is no monograph, revision or even synopsis of the entire genus. Folsom (1987) monographed section *Dichaea*, the most taxonomically and morphologically diverse group in the genus. In addition to his monograph, he diagrammed his ideas of the relationships within section *Dichaea* in a non-cladistic manner. In his revision of Costa Rican *Dichaea*, Pupulin (2007) presented a morphological cladistic analysis (limited to Costa Rican taxa). Historically, infrageneric classifications of *Dichaea* were based on one to few characters

without a phylogenetic framework and with conflicting results. No sectional scheme of *Dichaea* has ever used clearly defined apomorphic characters to circumscribe subgeneric taxa. The objective of this study was to use DNA sequence data [nuclear ribosomal internal transcribed spacers (nrITS), the plastid *matK*, *trnL* intron, *trnL-F* intergenic spacer and *ycf1*] to reconstruct phylogenetic relationships in *Dichaea* and map the distribution of major morphological characters that have previously been used to delimit subgenera/sections. For purposes of clarity, the classification of Cogniaux (1906), as above, will be followed.

MATERIALS AND METHODS

Taxon sampling

Specimens were obtained from wild-collected and cultivated plants (Appendix). Sampling of *Dichaea* included 35 species, representing all four described sections of *Dichaea*. Outgroups included six other genera of subtribe Zygopetalinae and *Heterotaxis violaceopunctata* (Rchb.f.) F. Barros (subtribe Maxillariinae). Outgroups were chosen based on phylogenetic placement in previous work (Cameron, 2001, 2004; Cameron *et al.*, 1999; Chase *et al.*, 2003; Whitten *et al.*, 2000).

Extractions, amplification and sequencing

All freshly collected material was preserved in silica gel (Chase and Hills, 1991). Genomic DNA was extracted using a modified 2 × CTAB (cetyl trimethylammonium bromide) technique (Doyle and Doyle, 1987), scaled to a 1-mL volume reaction. Approximately 10 mg of dried tissue were ground in 1 mL of 2 × CTAB buffer and either 8 μL of β-mercaptoethanol or 10 μL of proteinase-K. Some total DNAs were then cleaned with Qiagen QIAquick PCR purification columns to remove any inhibitory secondary compounds (e.g. species of section *Pseudodichaea*). Amplifications were performed using a Biometra Tgradient or an Eppendorf Mastercycler EP Gradient S thermocycler and Sigma brand reagents in 25-μL volumes with the following reaction components for ITS: 0·5–1·0 μL template DNA (approx. 10–100 ng), 11 μL water, 6·5 μL 5M betaine, 2·5 μL 10 × buffer, 3 μL $MgCl_2$, 0·5 μL of 10 μM dNTPs, 0·5 μL each of 10 μM primers and 0·5 units *Taq*. For the plastid regions the following reaction components were used: 0·5–1·0 μL template DNA (approx. 10–100 ng), 16–17·5 μL water, 2·5 μL 10 × buffer, 2–4 μL $MgCl_2$, 0·5 μL of 10 μM dNTPs, 0·5 μL each of 10 μM primers and 0·5 units *Taq*.

nrITS (ITS 1 + 5·8S rDNA + ITS 2). This region was amplified with the parameters 99 °C, 10 min; 94 °C hold for *Taq* addition; 33 × (94 °C, 45 s; 65 °C, 1 min; 72 °C, 1 min); 72 °C, 3 min, with the primers 17SE and 26SE from Sun *et al.* (1994).

matK–trnK. This region includes the entire *matK* gene and the flanking 3′ *trnK* spacer. This region was amplified with the parameters 94 °C, 3 min; 33 × (94 °C, 45 s; 60 °C, 45 s; 72 °C, 2 min); 72 °C, 3 min, with primers –19F (Molvray *et al.*, 2000) and trnK2R (Johnson and Soltis, 1994). Internal sequencing primers were 308F and 1520R (Whitten *et al.*, 2007). Some outgroups were amplified using the primers 56F and 1520R (Whitten *et al.*, 2000) that yielded a shorter, but nearly complete sequence of *matK* (missing the 3′ spacer).

trnL–trnF. This region includes both the *trnL* intron and the spacer between *trnL* and *trnF* (hereafter collectively referred to as *trnL-F*). This region was amplified with the parameters 94 °C, 3 min; 33 × (94 °C, 1 min; 58 °C, 1 min; 72 °C, 1 min, 20 s); 72 °C, 6 min, with the primers c and f from Taberlet *et al.* (1991). Additional primers d and e were rarely required for sequencing.

ycf1. In *Phalaenopsis* Blume (GenBank: AY916449), this open reading frame is nearly 6 kb in length and may be the most variable coding region within the plastid genome (M. Moore, Oberlin College, OH, USA, pers. comm.). An approx. 1500-base-pair (bp) portion from the 3' end was sequenced. This region was amplified using a 'touchdown' protocol with the parameters 94 °C, 3 min; 8 × (94 °C, 30 s; 60–51 °C, 1 min; 72 °C, 3 min); 30 × (94 °C, 30 s; 50 °C, 1 min; 72 °C, 3 min); 72 °C, 3 min, with primers 3720F (TAC GTA TGT AAT GAA CGA ATG G) and 5500R (GCT GTT ATT GGC ATC AAA CCA ATA GCG). Additional internal primers intF (GAT CTG GAC CAA TGC ACA TAT T) and intR (TTT GAT TGG GAT GAT CCA AGG) were also required for sequencing.

Products were cleaned with Microclean™ (The Gel Company, San Francisco, CA, USA) following the manufacturer's protocols, eluted with 50 μL of 10 mM Tris–HCl (pH 8·5) and stored at 4 °C. Purified PCR products were then cycle-sequenced using the parameters 96 °C, 10 s; 25 × (96 °C, 10 s; 50 °C, 5 s; 60 °C, 4 min), with mix of 3 μL water, 1 μL fluorescent Big Dye dideoxy terminator, 2 μL Better Buffer™ (The Gel Company), 1 μL template and 0·5 μL primer. Cycle sequencing products were cleaned using ExoSAP™ (USB Corporation, OH, USA) following the maufacturer's protocols. Purified cycle sequencing products were directly sequenced using BigDye terminator reagents on an ABI 377, 3100 or 3130 automated sequencer according to the manufacturer's protocols (Applied Biosystems, Foster City, CA, USA). Electropherograms were edited and assembled using Sequencher 4·6™ (GeneCodes, Ann Arbor, MI, USA). All sequences were deposited in GenBank (Appendix).

Data analysis

Sequence data were manually aligned using Se-Al v2.0a11 (Rambaut, 1996). No sequence data were excluded from analyses. Indels (insertions/deletions) were coded as missing. Analyses were performed using PAUP* 4.0b10 (Swofford, 1999) with Fitch parsimony (unordered characters with equal weights; Fitch, 1971). A heuristic search strategy consisted of branch swapping by tree bisection reconnection (TBR), stepwise addition with 5000 random-addition replicates holding five trees at each step, and saving multiple trees (MulTrees). Levels of support were assessed using the bootstrap (Felsenstein, 1985). Bootstrap percentages were estimated with 1000 bootstrap replicates, using TBR swapping for five random-addition replicates per bootstrap replicate.

Maximum likelihood (ML) analyses were performed using the program Garli 0·95 (Zwickl, 2006), assuming a GTR + I + Γ model. Modeltest (Posada and Crandall, 1998) was used to determine the appropriate model for analysis using all combined data under the Akaike Information Criterion. However, because the results from maximum likelihood analyses are so similar to those found with parsimony searches, they are not presented here.

All analyses were performed for datasets including ITS only, plastid regions only and all data combined. Data congruence was tested using the partition homogeneity test in PAUP*4.0b10 (Swofford, 1999) as described by Johnson and Soltis (1998). Heuristic searches for the partition homogeneity tests were performed using 100 replicates and TBR branch-swapping. Probability values lower than 0·05 were used to identify data sets that were significantly different from one another.

RESULTS

The aligned length of the ITS data set was 758 bp. Of these, 270 were potentially parsimony informative (35·6 %). Fitch parsimony analysis of the ITS region found 53 equally parsimonious trees of 896 steps [consistency index (CI) = 0·58, retention index (RI) = 0·86]. The aligned length of the combined plastid dataset (*matK*, *trnL-F* and *ycf1*) was 4719 bp. Of these, 413 were potentially parsimony informative (8·8 %). Fitch analysis of the combined plastid regions found 3407 equally parsimonious trees of 1054 steps (CI = 0·79, RI = 0·92). Individually (trees not shown), *trnL-F* had a total of 1278 characters, with 106 potentially parsimony informative, giving trees of 276 steps (CI = 0·80, RI = 0·92); *matK* had a total of 1813 characters, with 126 potentially parsimony informative, giving trees of 316 steps (CI = 0·83, RI = 0·93); and *ycf1* had a total of 1628 characters, with 181 potentially parsimony informative, giving trees of 457 steps (CI = 0·77, RI = 0·91). Fitch parsimony analysis of all four regions found 725 equally parsimonious trees of 1982 steps (CI = 0·68, RI = 0·88). Maximum likelihood searches gave log likelihood scores (−lnL) of 6221·06, 13936·48 and 20857·29 for ITS only, plastid regions only and all five regions, respectively.

The partition homogeneity test comparing ITS and the combined plastid data showed significant incongruence compared with random partitions of the same size ($P = 0{\cdot}01$, $\alpha = 0{\cdot}05$). Various combinations of each of the four of individual datasets, however, did not indicate significant incongruence (ITS/*trnL-F* $P = 0{\cdot}07$; ITS/*matK* $P = 0{\cdot}16$; *matK*/*trnL-F* $P = 0{\cdot}30$; *ycf1*/*matK* $P = 0{\cdot}76$; *ycf1*/*trnL-F* $P = 0{\cdot}78$) except between ITS and *ycf1* ($P = 0{\cdot}01$). The combined plastid data did conflict with ITS, apparently due to the addition of *ycf1*. A visual comparison of bootstrap percentages between the ITS and plastid data sets shows that there are only three strongly supported cases of incongruence (e.g. the positions of *D. tuerckheimii* Schltr., *D. glauca* Lindl. and *D. elliptica* Dressler & Folsom; Fig. 2). Furthermore, the exclusion of *ycf1* from the total combined analysis yields little support for relationships among the sections of *Dichaea*. Because the partition homogeneity test has been demonstrated to be overly sensitive (Graham *et al.*, 1998; Reeves *et al.*, 2001) and because a total evidence approach yields a highly resolved and relatively strongly supported topology, all data were combined.

Monophyly of *Dichaea* and its placement within Zygopetalinae are both strongly supported by all analyses (Figs 2 and 3). The monophyly of combined sections *Dichaea* + *Dichaeastrum* is strongly supported. Also, section *Pseudodichaea* is strongly supported in all analyses. However, no individual or combined dataset supports the monophyly of section *Dichaeopsis.*

DISCUSSION

Although the placement of *Dichaea* within subtribe Zygopetalinae is strongly supported according to molecular data (Whitten *et al.*, 2005), the genus does not share many vegetative or floral features with other members of the subtribe. *Dichaea* species lack the distinctive multi-ridged labellar callus and the short sympodial growth typical of other members of Zygopetalinae. However, *Dichaea* does share a characteristic pollinarium structure of four pollinia attached by four caudicles to a broad stipe terminating in a small, hyaline viscidium (Fig. 1J, K). The monophyly of *Dichaea* is strongly supported by both molecular and morphological data. Synapomorphies of *Dichaea* include elongate, monopodial stems, reduced flower size (relative to most other Zygopetalinae except *Cryptarrhena* R.Br.) and infrastigmatic ligules (Fig. 1I).

Plants of *Dichaea* appear to be monopodial, in contrast to sympodial growth found in rest of Zygopetalinae. In monopodial plants, the axillary shoot has the potential for indefinite apical growth, as in most stems of *Dichaea*, which continue to elongate and root at nodes (Dressler, 1993*b*). Roots of *Dichaea* are usually small, delicate and sparse. Leaves consist of a sheath and blade, although in many taxa, the abscission layer has been lost. However, no developmental studies of this unusual feature have been made, so another explanation is the loss of the blade and development of a blade-like sheath. Inflorescences of *Dichaea* are single-flowered, a derived condition within Zygopetalinae, but also found in some derived genera of the closely related subtribe Maxillariinae *s.l.* (Whitten *et al.*, 2007). Flowers have distinctive anchor-shaped labella, which have a largely homogeneous papillose micromorphology (Davies and Stpiczynska, 2008). Fruits of *Dichaea* are capsules that split along two longitudinal lines of dehiscence (on only one side of the fruit), rather than having three to six lines of dehiscence observed in most orchids (Pupulin, 2007). The surface of the ovary and fruit can be glabrous, setose or spiny. Only sections *Dichaea* and *Pseudodichaea* have ornamentations on the fruit, which can become robust and prickle-like (Fig. 3).

In a recent systematic revision of the *Dichaea* species in Costa Rica, Pupulin (2007) performed parsimony analyses of 62 morphological and anatomical characters. The phylogenetic structure obtained with morphological characters is largely similar to that produced with molecular data. Both morphology and molecular data support the monophyly of section *Pseudodichaea*. Monophyly of sections *Dichaea* and *Dichaeastrum* (combined) is also supported. In some morphological trees, section *Dichaeastrum* is sister to section *Dichaea*, not polyphyletic as indicated by molecular data.

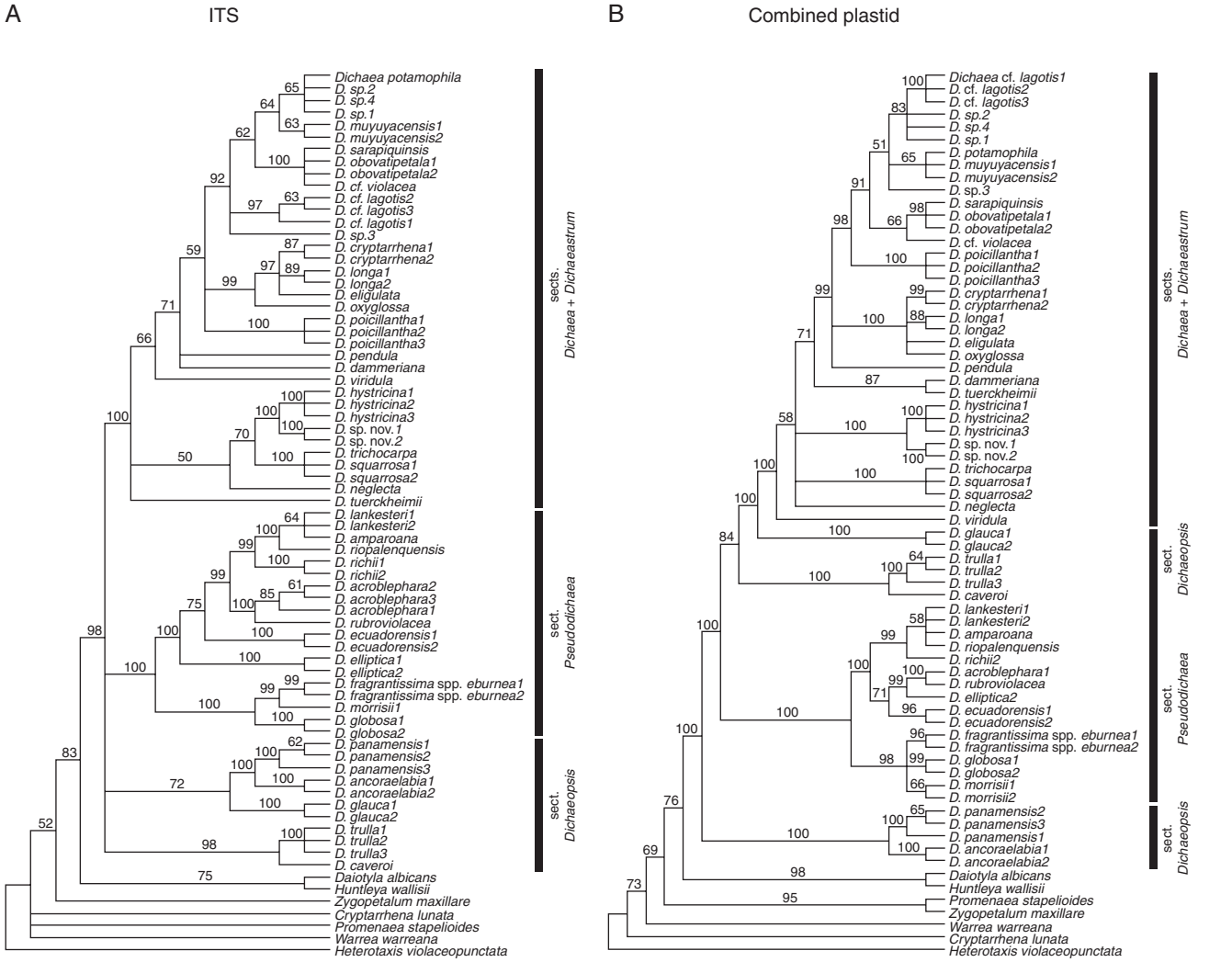

FIG. 2. Comparison of parsimony bootstrap consensus trees for (A) ITS dataset only and (B) plastid combined dataset. Bootstrap percentages are indicated above branches.

Most topological discrepancies between morphological and molecular data sets can be found among species groupings within section *Dichaea*. Morphological data also consistently support the paraphyletic topology of section *Dichaeopsis*, as discussed below.

Section Dichaeopsis

This section, as circumscribed in all classifications proposed to date, is not monophyletic. Cogniaux's more concise circumscription, including only those taxa with glabrous ovaries and an abscission layer in the leaves, consists of at least three clades that are not each other's closest relatives.

Dichaea panamensis Lindl. and *D. ancoraelabia* C. Schweinf. form a clade ('Panamensis group' in Fig. 3) sister to the rest of the genus with strong support. This group of at least eight species has relatively short and narrow leaves (i.e. similar to smaller species of section *Pseudodichaea*; see below), delicate stems, relatively thick roots, dark maroon anther caps, spotted perianth and reduced and bluntly triangular infrastigmatic ligules (Fig. 1B, F). These characters are shared by most species within this *Dichaeopsis* clade and represent putative synapomorphies. There are, however, many more described species not sampled in this study that are closely related to these two species, based on morphological similarity. These species include *D. campanulata* C.Schweinf., *D. dressleri* Folsom, *D. hutchisonii* D.E.Benn. & Christenson, *D. longipedunculata* D.E.Benn. & Christenson, *D. peruviensis* D.E.Benn. & Christenson and *D. picta* Rchb.f.

Another *Dichaeopsis* clade consists of two species sampled in this study, *D. trulla* Rchb.f. and *D. caveroi* D.E.Benn. & Christenson ('Trulla group' in Fig. 3). There is moderate support for this clade sister to the 'Glauca group' + sect. *Dichaea s.l.* This clade of species is well supported and easily diagnosable. The plants typically have erect or semi-erect stems with leaves that are relatively long (up to 10 cm) and narrow (many other species in the genus have leaves that are proportionally broader and 2–4 cm long). Also, the flowers typically have well-developed infrastigmatic ligules (Fig. 1D, E). Other species likely to be included in the 'Trulla group'

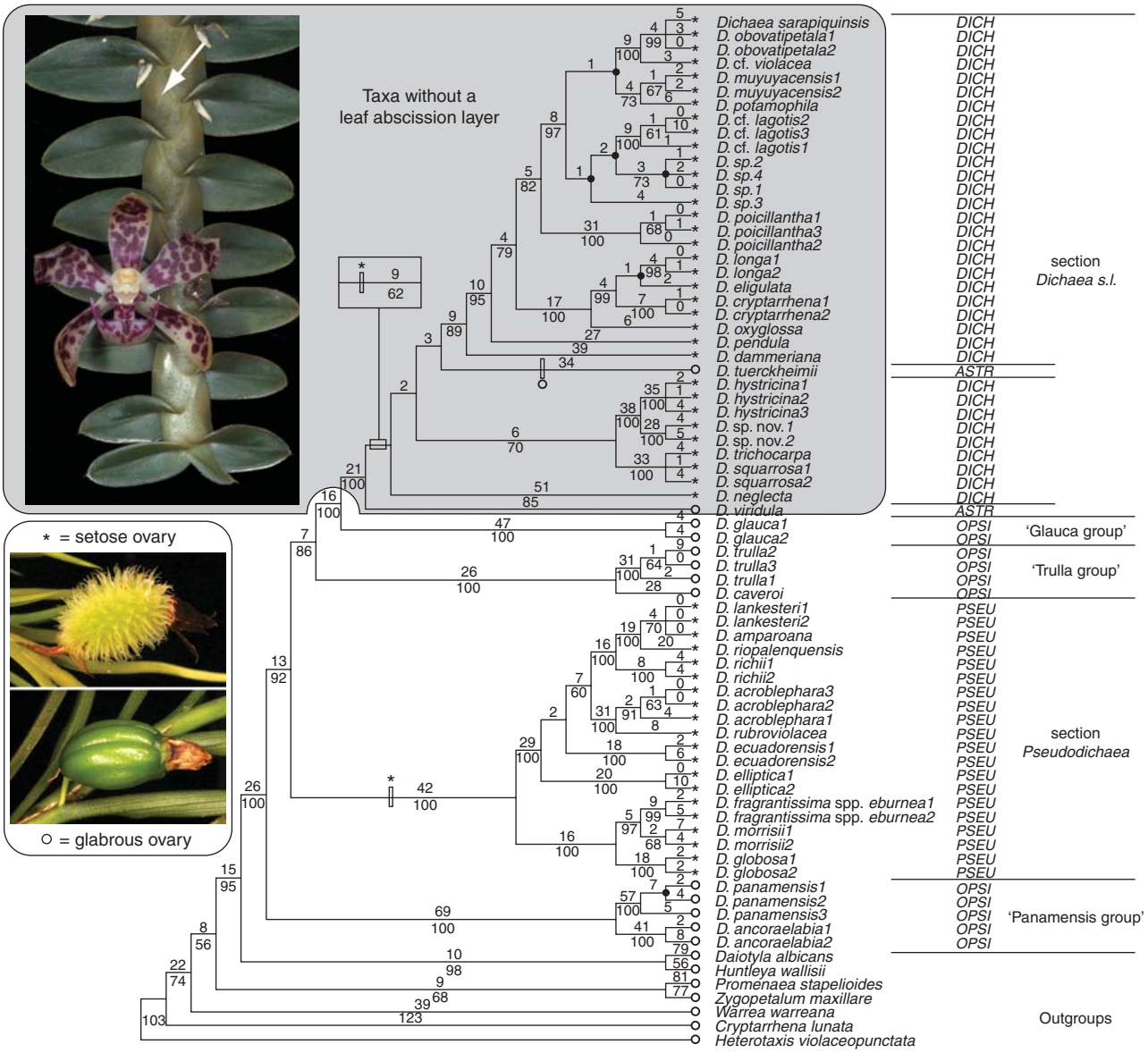

FIG. 3. One of 725 most-parsimonious trees (MPTs) of all molecular data combined (DELTRAN optimization). Branch lengths are above the branches, and bootstrap percentages are below the branches. Branches that collapse in the strict consensus tree are indicated by closed circles. Taxa missing a leaf abscission layer are enclosed within the box. Fruit ornamentation is also shown with character states [glabrous (o) vs. setose (*)] on branch terminals, and hash-marks in the tree represent apomorphies. Traditional sectional placement for all species is listed by abbreviation (sections *Dichaea*, DICH; *Dichaeastrum*, ASTR; *Dichaeopsis*, OPSI; *Pseudodichaea*, PSEU); present circumscription is listed on the far right.

are *D. calyculata* Poepp. & Endl., *D. powellii* Schltr. (likely a synonym of *D. trulla*) and *D. benzingii* Dodson.

Dichaea glauca (Sw.) Lindl. ('Glauca group' in Fig. 3) occurs in Central America and the Caribbean and is the tallest species in the genus. It has glaucous leaves and the thickest roots in the genus. This species also displays multiple inflorescences per stem simultaneously (Fig. 1L), an unusual feature for the genus. The ITS results place this species sister to *D. panamensis* and *D. ancoraelabia*, but with just moderate support. *Dichaea panamensis* also has glaucous leaves, an unusual feature in *Dichaea*. However, plastid results strongly support placement of *D. glauca* sister to sections *Dichaea* and *Dichaeastrum*, even though there is no apparent morphological basis for this relationship.

Section Pseudodichaea

Two characters define section *Pseudodichaea*: leaves with an abscission layer (plesiomorphic) and setose ovaries (apomorphic). Setose ovaries are also found in section *Dichaea*, making the character homoplasious within the genus (Fig. 3). Although circumscription of section *Pseudodichaea* is based on plesiomorphic and homoplasious characters, it is still monophyletic. Section *Pseudodichaea* is not as closely related to section *Dichaea* as might be supposed based on possession of setose ovaries. These two sections are separated by section *Dichaeastrum* (in part) and a paraphyletic grade of section *Dichaeopsis*.

Molecular results support two main, strongly supported sister groups in section *Pseudodichaea*. One group consists

of at least three species: *D. morrisii* Fawc. & Rendl., *D. fragrantissima* Folsom and *D. globosa* Dressler & Pupulin. These have stout, more or less horizontal stems and relatively large, broad leaves with strongly ancipitous sheaths (Fig. 1P). The other group is much more species rich, with relatively small, narrow leaves (Fig. 1N). The close relationship between *D. amparoana* Schltr. and *D. lankesteri* Ames shown by molecular data was previously suggested based on morphology (Dressler, 1993*a*; Pupulin, 2007). *Dichaea elliptica* Dressler & Folsom and *D. acroblephara* Schltr. are similar morphologically but not closely related according to these results. Section *Pseudodichaea* is particularly diverse in South America, and many of these species were not sampled in this study (e.g. *D. alcantarae* D.E.Benn. & Christenson, *D. angustisegmenta* Dodson, *D. chasei* Dodson, *D. cleistogama* Dodson, *D. delcastilloi* D.E.Benn. & Christenson, *D. galeata* Dodson, *D. luerorum* Dodson, *D. moronensis* Dodson, *D. sodiroi* Schltr., *D. suarezii* Dodson, *D. tamboensis* Dodson and *D. venezuelensis* Carnevali & I. Ramírez). This section shows high levels of sequence divergence and phylogenetic resolution. Because of the high degree of endemism, biogeographic patterns could probably be determined with increased taxon sampling of this section.

Section Dichaea sensu lato *(including section* Dichaeastrum*)*

The most coherent group within *Dichaea* based on morphology consists of sections *Dichaea* and *Dichaeastrum*. This group has leaves lacking an abscission layer, a generally pendulous or creeping habit (Fig. 1M), stems with indeterminate growth and setose (or more rarely, glabrous) ovaries (Fig. 1O). These two sections also form a strongly supported clade with all sampled DNA regions. Pfitzer (1889) and Kuntze (1904) recognized this entire group as section *Dichaea*. Cogniaux (1906) and Schlechter (1914) split the group into sections *Dichaea* and *Dichaeastrum*. This study does not support monophyly of either section *Dichaeastrum* or *Dichaea*. The two species from this study representing section *Dichaeastrum* (*D. tuerckheimii* and *D. viridula* Pupulin) have glabrous ovaries and are relatively diminutive plants. As these two species are not sister groups and are in a poorly supported portion of the tree, section *Dichaeastrum* should be treated as a part of section *Dichaea*. There are more species belonging to this glabrous-ovary group known as section *Dichaeastrum* (e.g. *D. escobariana* Dodson, *D. pumila* Barb.Rodr., *D. retroflexa* Kränzl. and *D. tenuifolia* Schltr.). Species in section *Dichaeastrum* usually have small, thin-textured, non-articulate leaves and glabrous ovaries. Therefore, with greater taxon sampling, a larger monophyletic group of what would be recognized as section *Dichaeastrum* may appear. The type of section *Dichaeastrum* was not sampled; morphological affinity of the type species to either *D. tuerckheimii* or *D. viridula* cannot be assessed at this time.

Section *Dichaea sensu stricto* was monographed by Folsom (1987, 1996), who attempted to diagram the evolutionary patterns within this group, with emphasis on clusters of species complexes. However, his non-cladistic approach to phylogenetic reconstruction leaves much to interpretation as it was based on subjective ideas of relationships among species. *Dichaea poicillantha* Schltr. is a distinct species on a long branch in all datasets. Folsom placed it near *D. schlechteri* Folsom, *D. cryptarrhena* Rchb.f. ex Kränzl. and *D. muricatoides* Hamar & Garay.

Dichaea oxyglossa Schltr., *D. eligulata* Folsom, *D. longa* Schltr. and *D. cryptarrhena* form a strongly supported complex of species in the present analyses. This group does not entirely agree with Folsom's diagram. He placed *D. oxyglossa* and *D. eligulata* near *D. obovatipetala* Folsom, *D. sarapiquinsis* Folsom and *D. retroflexiligula* Folsom. *Dichaea retroflexiligula* was not sampled in this study. *Dichaea obovatipetala* and *D. sarapiquinsis* are not closely related to this group, as postulated by Folsom (1987, 1996).

Dichaea obovatipetala and *D. sarapiquinsis* are strongly supported and part of a strongly supported clade of primarily South American taxa (including *D.* cf. *lagotis* Rchb.f., *D. muyuyacensis* Dodson, *D. potamophila* Folsom and *D.* cf. *violacea* Folsom). Folsom (1987, 1996) suspected that *D. obovatipetala* and *D. sarapiquinsis*, species endemic to Central America, were closely related to South American taxa based on morphology. *Dichaea obovatipetala* and *D. sarapiquinsis* lack clear cross-venation. Although some accessions sampled from this clade are unidentified, they do represent the South American group, most of which have clear cross-venation.

Folsom (1987, 1996) ignored some taxa that should properly be placed within section *Dichaea*. He referred to one such group as the '*Dichaea hystricina* complex,' including *D. hystricina* Rchb.f., *D. ciliolata* Rolfe and other unspecified species. This complex is monophyletic and distinguished by ciliate leaf margins. The exclusion of this complex from section *Dichaea* would make the latter paraphyletic. Pupulin (2005) studied the *D. hystricina* complex and showed that variation in vegetative morphology in Costa Rican specimens corresponded to a single species. The type of *D. ciliolata* is from Costa Rica, and Pupulin suggested that it is a synonym of *D. hystricina*, which is further corroborated by these data. Molecular and morphological data support the existence in Ecuador of a distinct, undescribed species that is sister to *D. hystricina*. However, more data need to be collected before it should be formally described.

Another distinct clade included *D. trichocarpa* (Sw.) Lindl. and *D. squarrosa* Lindl. *Dichaea intermedia* Ames & Correll is sometimes considered to be a hybrid between *D. trichocarpa* and *D. squarrosa* (Ames and Correll, 1985; Folsom, 1987). Taxon sampling was insufficient to determine the distinctiveness of these three species individually, but they form a clade. Among these taxa in section *Dichaea*, *D. neglecta* Schltr. was poorly supported as sister to the rest of section *Dichaea* with the exclusion of *D. viridula* (Fig. 3). Folsom also cited a few aberrant species that were not closely related to any other species. Species such as *D. pendula* (Aubl.) Cogn. and *D. dammeriana* Kraenzl. were sampled in this study and shown to be part of a paraphyletic grade relative to the core group of section *Dichaea*.

Evolution of leaves

According to the present results, the leaf abscission layer was lost once within the genus *Dichaea*. The absence of the

leaf abscission layer is a synapomorphy for section *Dichaea sensu lato*, with no apparent reversions. The lack of an abscission layer has no obvious adaptive value. This condition (marcescent leaves) is uncommon in tropical Orchidaceae, but is found in many Arecaceae and some temperate trees, such as *Fagus* L. Some studies of marcescent leaves in alpine tropical plants have shown that marcescent leaves protect the stem against tissue damage by freezing and desiccation (Smith, 1979). Species of *Dichaea* never occur at such high elevations (Pupulin, 2007), so this feature is unlikely to be an adaptation to freezing. The occurrence of marcescent leaves in sections *Dichaea* and *Dichaeastrum* is associated with a creeping or pendent habit. In these groups, the leaf bases are often twisted 90° so that their adaxial surfaces all face the same direction, making the stem complanate. This condition presumably maximizes the photosynthetic area exposed to light. However, the association among these leaf characters needs further study.

Evolution of fruits

Compared with other orchids, *Dichaea* is unusual in having setose or muricate fruits. Some members of the orchid subtribes Pleurothallidinae (e.g. *Pleurothallis* R.Br. subgenera *Kraenzlinella* Kuntze and *Aenigma* Luer; Luer, 1994), Laeliinae (e.g. *Pygmaeorchis* Brade; Pridgeon *et al.*, 2005) and Oncidiinae (e.g. *Saundersia* Rchb.f.) also have muricate ovaries, although these taxa are not closely related to *Dichaea*. Like almost all other orchid fruits, those of *Dichaea* are dehiscent capsules with wind-dispersed seeds.

It is possible that the spines on the fruits are used in dispersal by adhering to animals. Hooked spines that aid in dispersal are fairly common in many families of flowering plants. However, the setae of *Dichaea* fruits are not strongly uncinate, so it is unlikely that the fruits are exozoochorous. Also, *Dichaea* species have 'dust seeds' that are readily dispersed by wind, so exozoochory of fruits seems superfluous.

Many plants have setose or spiny fruits, which presumably protect seeds against herbivory. Spininess has been shown to inhibit herbivory by small mammals (Cooper and Ginnett, 1998) and by larger animals (Young and Augustine, 2007). However, the fruit setae of many species of sections *Dichaea* and *Pseudodichaea* are soft and could hardly inhibit predation by large or medium-sized herbivores. No assessment of the adaptive value, if any, of these setae has ever been performed. More puzzling than their apparent lack of adaptive value is the polyphyletic nature of their occurrence. Setose fruits appear to have evolved at least twice (Fig. 3), and the groups in *Dichaea* with setose fruits are also the most diverse in terms of species richness.

CONCLUSIONS

DNA sequence data were used to investigate the circumscription of sectional classification and species relationships of *Dichaea*. Characters used to separate the sections within the genus have been misleading. Section *Dichaeopsis* was found to be polyphyletic whereas section *Pseudodichaea* is monophyletic. We suggested a broader circumscription of section *Dichaea*, including section *Dichaeastrum*, because *Dichaeastrum* is polyphyletic and *Dichaea* and *Dichaeastrum* are similar vegetatively and together are easily distinguished from other sections. The marcescent leaves of section *Dichaea sensu lato* have been attained only once, whereas setae on fruits have been attained at least twice within the genus. However, there is no obvious adaptive value to the plants of these unique morphological features. Further investigation into the anatomical structure of the leaves in section *Dichaea* could address the developmental and evolutionary significance of the lack of an abscission layer between sheath and blade. Careful *in situ* observations of orchid taxa with fruit setae may yield more meaningful phylogenetic interpretations of this character. The question of how such a wide range of morphological diversity was shaped and the ecological role behind these features requires further probing in this group of plants.

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APPENDIX

Voucher information and GenBank accession numbers for all taxa used in this study

Species	Country	Voucher information (herbarium)	ITS	*matK* + *trnK*	*trnL-F*	*ycf1*
Cryptarrhena lunata R. Br.	Costa Rica	Whitten 98000 (FLAS)	AY870081	AY869982	AY869894	EU123733
Daiotyla albicans (Rolfe) Dressler	Panama	Whitten 1932 (FLAS)	AY870016	AY869917	AY869831	EU123734
Dichaea acroblephara Schltr. 1	Costa Rica	Pupulin 4795 (USJ-L)	EU123545	EU123611	EU123677	EU123735
D. acroblephara Schltr. 2	Panama	Whitten 2669 (FLAS)	EU123546	NA	NA	NA
D. acroblephara Schltr. 3	Panama	Blanco 2994 (FLAS)	EU123547	NA	NA	NA
D. amparoana Schltr.	Costa Rica	Bogarín 679 (USJ-L)	EU123548	EU123612	EU123678	NA
D. ancoraelabia C.Schweinf. 1	Ecuador	Neubig 1–2004 (FLAS)	EU123549	EU123613	NA	EU123736
D. ancoraelabia C.Schweinf. 2	Ecuador	Whitten 2542 (FLAS)	EU123550	EU123614	EU123679	EU123737
D. caveroi D.E. Benn. & Christenson	Ecuador	Whitten 2417 (FLAS)	EU123551	EU123615	EU123680	EU123738
D. cryptarrhena Rchb.f. ex Kraenzl. 1	Costa Rica	Pupulin 4436 (USJ-L)	EU123556	EU123620	EU123685	EU123743
D. cryptarrhena Rchb.f. ex Kraenzl. 2	Panama	Whitten 2610 (FLAS)	EU123557	EU123621	EU123686	EU123744
D. dammeriana Kraenzl.	Costa Rica	Bogarín & León-Páez 197 (USJ-L)	EU123558	EU123622	EU123687	EU123745
D. ecuadorensis Schltr. 1	Ecuador	Whitten 1799 (FLAS)	EU123559	EU123623	NA	EU123746
D. ecuadorensis Schltr. 2	Ecuador	Whitten 2416 (FLAS)	EU123560	EU123624	EU123688	NA
D. eligulata Folsom	Costa Rica	Pupulin 1094 (USJ-L)	EU123561	EU123625	EU123689	EU123747
D. elliptica Dressler & Folsom 1	Costa Rica	Pupulin 5133 (USJ-L)	EU123562	NA	NA	NA
D. elliptica Dressler & Folsom 2	Costa Rica	Pupulin 4945 (USJ-L)	EU123563	EU123626	EU123690	EU123748
D. fragrantissima Folsom ssp. *eburnea* Dressler & Pupulin 1	Costa Rica	Blanco 513 (USJ-L)	EU123564	EU123627	NA	EU123749
D. fragrantissima Folsom ssp. *eburnea* Dressler & Pupulin 2	Costa Rica	Pupulin 4601 (USJ-L)	EU123565	EU123628	EU123691	EU123750
D. glauca (Sw.) Lindl. 1	Costa Rica	Pupulin 4734 (USJ-L)	EU123566	EU123629	EU123692	EU123751
D. glauca (Sw.) Lindl. 2	Mexico	Neubig 9–2006 (FLAS)	EU123567	EU123630	EU123693	EU123752
D. globosa Dressler & Pupulin 1	Costa Rica	Pupulin 4517 (USJ-L)	EU123568	EU123631	EU123694	EU123753
D. globosa Dressler & Pupulin 2	Panama	Neubig 2–2005 (FLAS)	EU123569	EU123632	EU123695	EU123754
D. hystricina Rchb.f. 1	Costa Rica	Pupulin 3925 (USJ-L)	EU123570	EU123633	EU123696	EU123755
D. hystricina Rchb.f. 2	Costa Rica	Pupulin 2925 (USJ-L)	EU123571	EU123634	EU123697	EU123756
D. hystricina Rchb.f. 3	Costa Rica	Pupulin 4320 (USJ-L)	EU123572	EU123635	EU123698	EU123757
D. cf. *lagotis* Rchb.f. 1	Ecuador	Whitten 1801 (FLAS)	EU123552	EU123616	EU123681	EU123739
D. cf. *lagotis* Rchb.f. 2	Ecuador	Whitten 2477 (QCA)	EU123553	EU123617	EU123682	EU123740
D. cf. *lagotis* Rchb.f. 3	Ecuador	Whitten 2523 (QCA)	EU123554	EU123618	EU123683	EU123741
D. lankesteri Ames 1	Panama	Blanco 2993 (FLAS)	EU123573	EU123636	NA	EU123758
D. lankesteri Ames 2	Costa Rica	Pupulin 3030 (USJ-L)	EU123574	EU123637	EU123699	EU123759
D. longa Schltr. 1	Ecuador	Whitten 2684 (FLAS)	EU123575	EU123638	NA	EU123760
D. longa Schltr. 2	Ecuador	Whitten 2685 (FLAS)	EU123576	EU123639	EU123700	EU123761
D. morrisii Fawc. & Rendl. 1	Panama	Neubig 3–2004 (FLAS)	EU123577	EU123640	EU123701	EU123762
D. morrisii Fawc. & Rendl. 2	Ecuador	Neubig 8–2006 (FLAS)	NA	EU123641	EU123702	EU123763
D. muyuyacensis Dodson 1	Panama	Neubig 5–2004 (FLAS)	EU123578	EU123642	EU123703	EU123764
D. muyuyacensis Dodson 2	Ecuador	Whitten 1512 (FLAS)	EU123579	EU123643	NA	EU123765
D. neglecta Schltr.	Mexico	Higgins 1005 (FLAS)	EU123580	EU123644	EU123704	EU123766
D. obovatipetala Folsom 1	Costa Rica	Pupulin 5023 (USJ-L)	EU123581	EU123645	EU123705	EU123767
D. obovatipetala Folsom 2	Costa Rica	Pupulin 4202 (USJ-L)	EU123582	EU123646	EU123706	EU123768
D. oxyglossa Schltr.	Costa Rica	Bogarín & León-Páez 186 (USJ-L)	EU123583	EU123647	EU123707	EU123769
D. panamensis Lindl. 1	Costa Rica	Pupulin 3667 (USJ-L)	EU123584	EU123648	EU123708	EU123770
D. panamensis Lindl. 2	Ecuador	Whitten 2348 (FLAS)	EU123585	EU123649	EU123709	EU123771
D. panamensis Lindl. 3	Panama	Whitten 2556 (FLAS)	EU123586	EU123650	NA	EU123772
D. pendula (Aubl.) Cogn.	Costa Rica	Pupulin 3024 (USJ-L)	EU123587	EU123651	EU123710	EU123773
D. poicillantha Schltr. 1	Panama	Blanco 2981 (FLAS)	EU123588	EU123652	EU123711	EU123774
D. poicillantha Schltr. 2	Costa Rica	Pupulin 4662 (USJ-L)	EU123589	EU123653	EU123712	EU123775
D. poicillantha Schltr. 3	Panama	Whitten 2557 (FLAS)	EU123590	EU123654	EU123713	EU123776
D. potamophila Folsom	Peru	Neubig 4–2004 (FLAS)	EU123591	EU123655	EU123714	EU123777

D. richii Dodson 1	Ecuador	Whitten 1526 (FLAS)	EU123592	NA	NA	NA
D. richii Dodson 2	Ecuador	Whitten 2429 (QCA)	EU123593	EU123656	EU123715	NA
D. riopalenquensis Dodson	Ecuador	Whitten 2731 (FLAS)	EU123594	EU123657	EU123716	EU123778
D. rubroviolacea Dodson	Ecuador	Whitten 2945 (FLAS)	EU123595	EU123658	EU123717	EU123779
D. sarapiquinsis Folsom	Costa Rica	Pupulin 4856 (USJ-L)	EU123596	EU123659	EU123718	EU123780
D. squarrosa Lindl. 1	Costa Rica	Pupulin 5127 (USJ-L)	EU123604	EU123667	EU123726	EU123788
D. squarrosa Lindl. 2	Mexico	Neubig 4–2006 (FLAS)	EU123603	EU123666	EU123725	EU123787
D. trichocarpa (Sw.) Lindl.	Costa Rica	Bogarín 173 (USJ-L)	EU123605	EU123668	EU123727	EU123789
D. trulla Rchb.f. 1	Costa Rica	Whitten 2096 (USJ-L)	EU123607	EU123670	EU123729	EU123791
D. trulla Rchb.f. 2	Ecuador	Whitten 2474 (QCA)	EU123608	EU123671	EU123730	EU123792
D. trulla Rchb.f. 3	Ecuador	Whitten 2475 (FLAS)	EU123606	EU123669	EU123728	EU123790
D. tuerckheimii Schltr.	Costa Rica	Whitten 2097 (USJ-L)	EU123609	EU123672	EU123731	EU123793
D. cf. *violacea* Folsom	Panama	Neubig 6–2004 (FLAS)	EU123555	EU123619	EU123684	EU123742
D. viridula Pupulin	Costa Rica	Pupulin 4752 (USJ-L)	EU123610	EU123673	EU123732	EU123794
D. sp. 1	Ecuador	Whitten 2709 (FLAS)	EU123597	EU123660	EU123719	EU123781
D. sp. 2	Ecuador	Whitten 2434 (QCA)	EU123598	EU123661	EU123720	EU123782
D. sp. 3	Ecuador	Whitten 2435 (QCA)	EU123599	EU123662	EU123721	EU123783
D. sp. 4	Ecuador	Whitten 2476 (QCA)	EU123600	EU123663	EU123722	EU123784
D. sp. nov. 1	Ecuador	Neubig 6–2006 (FLAS)	EU123601	EU123664	EU123723	EU123785
D. sp. nov. 2	Ecuador	Whitten 2329 (FLAS)	EU123602	EU123665	EU123724	EU123786
Heterotaxis violaceopunctata (Rchb.f.) F. Barros	Brazil	Whitten 2294 (FLAS)	DQ210308	DQ210807	NA	EU123795
H. violaceopunctata (Rchb.f.) F. Barros	Suriname	Whitten 1980 (FLAS)	NA	NA	AY869911	NA
Huntleya wallisii (Rchb.f) Rolfe	Ecuador	Whitten 88026 (FLAS)	AY870074	EU123674	AY869887	EU123796
Promenaea stapelioides (Link & Otto) Lindl.	Brazil	Whitten 94102 (FLAS)	AY870101	AY870002	AY869905	EU123797
Warrea warreana (Lodd. ex Lindl.) C. Schweinf.	Costa Rica	Whitten 1752 (FLAS)	AF239321	EU123675	AF239513	EU123798
Zygopetalum maxillare Lodd.	Brazil	Whitten 94103 (FLAS)	AY870095	EU123676	AY869899	EU123799

Vouchers are deposited at FLAS (Florida Museum of Natural History Herbarium), USJ-L (University of Costa Rica, San José, Lankester Botanical Garden) and QCA (Pontifica Universidad Católica del Ecuador, Quito).