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RELATIONSHIPS AMONG GENERA OF THE INFORMAL *DICHROSTACHYS* AND *LEUCAENA* GROUPS (MIMOSOIDEAE) INFERRED FROM NUCLEAR RIBOSOMAL ITS SEQUENCES

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Abstract

Previous attempts to elucidate sister group relationships among the genera of the informal *Dichrostachys* and *Leucaena* groups of the tribe Mimoseae have been hampered by incomplete taxon sampling, incomplete knowledge and poor circumscription of a number of the constituent genera, primary reliance on a limited set of morphological characters, and uncertainty about sister group relationships across the Mimoseae as a whole. Here we present a densely sampled informal *Dichrostachys* and *Leucaena* group analysis that includes all the constituent genera and 72 of the 91 species using a new DNA sequence data set from the nrDNA 5.8S and flanking internal transcribed spacer regions (ITS1 and ITS2). This analysis confirms the previously proposed realignment of the informal *Leucaena* group to include *Leucaena, Desmanthus, Schleinitzia* and *Kanaloa*, and the *Dichrostachys* group to include *Dichrostachys, Gagnebina, Alantsilodendron* and *Calliandropsis*, as well as the exclusion of *Neptunia* from these groups. The analysis also provides the first species-level molecular phylogeny for the genera of the *Dichrostachys* group, and species relationships within this group are discussed in relation to morphology and generic delimitation. The pattern of ITS variation within *Desmanthus* indicates incomplete sampling of ITS diversity limiting the usefulness of the current ITS gene tree to infer species relationships within the genus.

Introduction

Lewis and Elias (1981) recognised 12 informal groups within the tribe Mimoseae. They distinguished the *Leucaena* group, comprising the genera *Leucaena* and *Schleinitzia*, by presence of an involucel and peltate floral bracts, and the *Dichrostachys* group, comprising *Desmanthus*, *Dichrostachys*, *Gagnebina* and *Neptunia*, by the presence of

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staminodial flowers at the base of the inflorescence. This classification (Table 1) has provided the starting point for a series of more explicitly phylogenetic analyses of sister group relationships among genera of the informal *Leucaena* and *Dichrostachys* groups undertaken over the last 10 years (Luckow, 1993, 1995, 1997; Harris *et al.*, 1994; Hughes, 1998; Luckow *et al.*, 2000). These subsequent studies have highlighted the inconstancy of the morphological characters used by Lewis and Elias, and questioned the monophyly and composition of these two informal groups, prompting Luckow (1997) to suggest an alternative arrangement of genera (Table 1). However, all these recent analyses have been hampered by incomplete taxon sampling, incomplete knowledge and poor circumscription of a number of the constituent genera, primary reliance on a limited set of morphological characters, and uncertainty about sister group relationships across the Mimoseae as a whole. For example, initial analyses focused either on the genera of the *Dichrostachys* group (Luckow 1993, 1995) or the *Leucaena* group (Harris *et al.*, 1994) alone and were based solely on morphological data (Luckow, 1993, 1995; Hughes, 1998). The only molecular data sets, although encompassing genera from both informal groups, have included sparse sampling. For example, only 17 out of 89 taxa were included in the cpDNA restriction site analysis of Luckow (1997) and 20 out of 89 taxa in the *trn*L-*trn*F DNA sequence analysis of Luckow *et al.* (2000). These limitations suggest that further development of phylogenetic hypotheses is needed to establish the relationships within and among these genera.

Since the classification of Lewis and Elias (1981), knowledge of the constituent genera of the *Leucaena* and *Dichrostachys* groups has grown, with new field collections and monographic treatments of *Desmanthus* (Luckow, 1993) and *Leucaena* (Hughes, 1998), and the Old World genera of the *Dichrostachys* group (Luckow, unpubl. data). This has rectified significant species delimitation problems associated with these genera, and strengthened our previously fragmentary knowledge of some taxa, and particularly the previously very poorly known Malagasy taxa. In addition, three new genera with affinities to these groups have been described during the last decade. The Madagascan genus *Alantsilodendron* segregated by Villiers (1994) shows clear affinities to other Malagasy genera (*Dichrostachys* and *Gagnebina*) of the *Dichrostachys* group and these have been confirmed by analyses of morphological (Luckow, 1995; Hughes, 1998) and molecular data (Luckow *et al.*, 2000). However, the affinities of the other two new genera, *Calliandropsis* described by Hernández and Guinet (1990), and *Kanaloa*, described by Lorence and Wood (1994), were not so readily apparent when they were originally described. The lack of any generic diagnosis provided for either genus is symptomatic of the confusion surrounding the diagnostic characteristics of the genera of the two informal groups. Subsequent work has suggested that the monotypic Mexican endemic genus *Calliandropsis* belongs within the primarily Malagasy *Dichrostachys* group (Luckow, 1995, 1997; Hughes, 1998; Luckow *et al.*, 2000) while the monotypic Hawaiian endemic *Kanaloa* belongs with *Desmanthus* and *Schleinitzia* in the informal *Leucaena* group (Luckow *et al.*, 2000) *sensu* Luckow (1997).

Here we present the results of an analysis of a new DNA sequence data set from the 5.8S subunit and flanking internal transcribed spacer regions ITS1 and ITS2 of nuclear ribosomal DNA. Our aim has been to assemble a new DNA sequence data set to test the monophyly of the genera and the revised *Leucaena* and *Dichrostachys* groups *sensu* Luckow (1997), and also to sample much more densely across all the constituent genera in order to provide a hypothesis of relationships among species and genera within these groups.

Materials and methods

Seventy-two of the 91 species currently known to comprise the genera of the informal *Leucaena* and *Dichrostachys* groups are included in the ITS data set (sampling summarised in Table 1). Multiple accessions are included for a number of taxa. Accessions, taxon authorities, voucher details and Genbank numbers are listed in

TABLE 1. Genera of the informal *Dichrostachys* and *Leucaena* groups.

Genus	Species sampled / species in genus	Distribution	Informal group (Lewis & Elias, 1981)	Revised groups (Luckow, 1997)
Leucaena Benth.	17/22	New World, USA to Peru	*Leucaena* group	*Leucaena* group
Schleinitzia Warb. ex Nevling & Niezgoda	2/3(–4)	W Pacific basin	*Leucaena* group	*Leucaena* group
Desmanthus Willd.	21/24	New World, USA to Argentina	*Dichrostachys* group	*Leucaena* group
Kanaloa Lorence & K.R. Wood	1/1	Endemic to Hawaii	Post-Lewis & Elias (1981)	Not included
Alantsilodendron Villiers	6/8	Endemic to Madagascar	Post-Lewis & Elias (1981)	*Dichrostachys* group
Calliandropsis H.M. Hern. & P. Guinet	1/1	Endemic to central Mexico	Post-Lewis & Elias (1981)	*Dichrostachys* group
Dichrostachys Wight & Arn.	10/13	Madagascar, NE Africa, 1 pantropical, 1 Australia	*Dichrostachys* group	*Dichrostachys* group
Gagnebina Neck.	7/7	Madagascar, Mascarene and Comoros Islands	*Dichrostachys* group	*Dichrostachys* group
Neptunia Lour.	7/12	Pantropical, mainly tropical America and Australia	*Dichrostachys* group	*Incertae sedis*

TABLE 2. Plant material, voucher specimens and Genbank accession numbers.

Species	Voucher and herbarium	Location	Genbank Number
Alantsilodendron alluaudianum (R.Vig.) Villiers	*Luckow* 4114 BH	Madagascar	AF458796
Alantsilodendron brevipes (R.Vig.) Villiers	*Luckow* 4324 BH	Madagascar	AF458797
Alantsilodendron mahafalense (R.Vig.) Villiers	*Luckow* 4360 BH	Madagascar	AF458801
Alantsilodendron pilosum Villiers	*Luckow* 4162 BH	Madagascar	AF458800
Alantsilodendron ramosum Villiers	*Du Puy* 433 K	Tokara, Madagascar	AF458802
Alantsilodendron villosum (R. Vig.) Villiers	*Luckow* 4437 BH	Madagascar	AF458803
Calliandropsis nervosus (Britton & Rose) H.M. Hern. & P. Guinet	*Hughes* 1784 FHO	Puebla, Mexico	AF458819
Desmanthus acuminatus Benth.	*Luckow* 3527 TEX	Texas, USA	a-AF458832 b-AF458850
Desmanthus balsensis J.L. Contr.	*Hughes* 1825 FHO	Guerrero, Mexico	AF458824
Desmanthus bicornutus S. Watson-1	*Luckow* 2980 TEX	Sinaloa, Mexico	a-AF458829 b-AF458847
Desmanthus bicornutus S. Watson-2	*Luckow* 3502 TEX	Guerrero, Mexico	a-AF458828 b-AF458831
Desmanthus covillei (Britton & Rose) Wiggins ex B.L. Turner	*Luckow* 2806 TEX	Sonora, Mexico	AF458848
Desmanthus fruticosus Rose	*Hughes* 1532 FHO	Baja California Sur, Mexico	AF418018
Desmanthus glandulosus (B.L. Turner) Luckow	*Luckow* 2731 TEX	Texas, USA	a-AF458837
Desmanthus illinoiensis (Michx.) MacMill. ex Robinson & Fernald	*Luckow* s.n. TEX	Texas, USA	AF458836
Desmanthus interior (Britton & Rose) Bullock	*Luckow* 3511 TEX	Jalisco, Mexico	a-AF458846
Desmanthus leptolobus Torr. & A. Gray	*Grimes* 3025 TEX	Oklahoma, USA	AF458835
Desmanthus leptophyllus Humb., Bonpl. & Kunth-1	*Luckow* 3159 TEX	Veracruz, Mexico	AF458839
Desmanthus leptophyllus Humb., Bonpl. & Kunth-2	*Luckow* 3032a TEX	Veracruz, Mexico	AF458838
Desmanthus obtusus S. Watson	*Luckow* 2738 TEX	Texas, USA	AF458842
Desmanthus oligospermus Brandegee	*Luckow* 2824 TEX	Baja California Sur, Mexico	AF458844
Desmanthus paspalaceus (Lindm.) Burkart	*Ginzberg* 387 TEX	Corrientes, Argentina	AF458825
Desmanthus pernambucanus (L.) Thell.	*Luckow* s.n. BH	Seeds from University of Hawaii, G. G. Hynes	AF458840
Desmnathus pringlei (Britton & Rose) F.J. Herm.	*Luckow* 2640 TEX	Nuevo León, Mexico	a-AF458834
Desmanthus pubescens B.L. Turner	*Luckow* 3137 TEX	Veracruz, Mexico	a-AF458826 b-AF458830

Table 2. continued

Desmanthus pumilus (Schltdl.) J.F. Macbr.	*Hughes* 1809 FHO	Puebla, Mexico	AF458845
Desmanthus reticulatus Benth.	*Luckow* 3593 TEX	Texas, USA	AF458849
Desmanthus tatahuyensis Hoehne	*Ginzberg* 39 TEX	Corrientes, Argentina	AF458833
Desmanthus velutinus Scheele	*Luckow* 2707 TEX	Texas, USA	AF458827
Desmanthus virgatus (L.) Willd.-1	*Hughes* 1768 FHO	Oaxaca, Mexico	AF458843
Desmanthus virgatus (L.) Willd.-2	*Renvoize* 3551 K–mixed collection of *Neptunia* and *D. virgatus*	Argentina	AF458841
Dichrostachys akataensis Villiers	*Luckow* 4439 BH	Madagascar	AF458811
Dichrostachys arborescens (Bojer ex Benth.) Villiers	*Luckow* 4289A BH	Madagascar	AF458807
Dichrostachys cinerea (L.) Wight & Arn.	*Barnes* 4761 FHO	Bulawayo, Zimbabwe	AF458820
Dichrostachys paucifoliolata (S. Elliot) Drake	*Luckow* 4157 BH	Fort Dauphin, Madagascar	AF458812
Dichrostachys richardiana Baill.	*Luckow* 4239 BH	Madagascar	AF458798
Dichrostachys scottiana (Drake) Villiers	*Luckow* 4161 BH	Madagascar	AF458809
Dichrostachys spicata (F. Muell.) Domin.	Seeds from Austr. Nat. Herb. *Dunlap* 5853 BH	Northern Territory, Australia	AF458821
Dichrostachys tenuifolia Benth.	*Luckow* 4387 BH	Madagascar	AF458810
Dichrostachys unijuga Baker	*Luckow* 4279 BH	Madagascar	AF458808
Dichrostachys venosa Villiers	*Luckow* 4339 BH	Madagascar	AF458799
Gagnebina bakoliae Luckow & Du Puy	*Luckow* 4225 BH	Madagascar	AF458804
Gagnebina bernieriana (Baill.) Luckow	*Luckow* 4243 BH	Madagascar	AF458805
Gagnebina calcicola (R. Vig.) Renvoize-1	*Luckow* 4441 BH	Madagascar	AF458816
Gagnebina calcicola (R. Vig.) Renvoize-2	*Lewis* 2156 K	Antsiranana, Madagascar	AF458817
Gagnebina commersoniana (Baill.) R. Vig.-1	*D. Potter* 43809-01 BH	Aldabra	AF458815
Gagnebina commersoniana (Baill.) R. Vig.-2	*Du Puy* 252 K	Antsiranana, Madagascar	AF458814
Gagnebina myriophylla (Baker) G.P. Lewis & P. Guinet	*Du Puy* 233 K	Sambirano, Madagascar	AF458818
Gagnebina pervilleana (Baill.) G.P. Lewis & P. Guinet	*Luckow* 4412 BH	Madagascar	AF458806
Gagnebina pterocarpa (Lam.) Baill.	*Luckow* s.n. BH	Cultivated NTBGarden, Kauai, Hawaii, USA via Lorence	AF458813
Kanaloa kahoolawensis Lorence & K.R. Wood		Cultivated NTBGarden, Kauai, Hawaii, USA via Lorence	AF458822
Leucaena collinsii Britton & Rose ssp. *collinsii*	*Hughes* 527 FHO	Chiapas, Mexico	AF418020
Leucaena collinsii Britton & Rose ssp. *zacapana* C.E. Hughes-1	*Hughes* 1096 FHO	Chiquimula, Guatemala	AF418023

Table 2. continued

Leucaena collinsii Britton & Rose ssp. *zacapana* C.E. Hughes-2	*Hughes* 299 FHO	El Progreso, Guatemala	AF418021
Leucaena collinsii Britton & Rose ssp. *zacapana* C.E. Hughes-3	*Hughes* 1120 FHO	Zacapa, Guatemala	AF418022
Leucaena cuspidata Standl.	*Hughes* 1583 FHO	Hidalgo, Mexico	AF418024
Leucaena esculenta (Sessé & Moc. ex DC.) Benth.	*Hughes* 894 FHO	Guerrero, Mexico	AF418096
Leucaena greggii S. Watson	*Hughes* 1057 FHO	Nuevo León, Mexico	AF418066
Leucaena lanceolata S. Watson var. *lanceolata*-1	*Hughes* 631 FHO	Michoacán, Mexico	AF418031
Leucaena lanceolata S. Watson var. *lanceolata*-2	*Hughes* 613 FHO	Sinaloa, Mexico	AF418029
Leucaena lanceolata S. Watson var. *lanceolata*-3	*Hughes* 1577 FHO	Sonora, Mexico	AF418030
Leucaena lanceolata S. Watson var. *lanceolata*-4	*Hughes* 913 FHO	Veracruz, Mexico	a-AF418050
Leucaena lanceolata S. Watson var. *sousae* (Zárate) C.E. Hughes	*Hughes* 872 FHO	Oaxaca, Mexico	AF418051
Leucaena lempirana C.E. Hughes	*Hughes* 1447 FHO	Yoro, Honduras	AF418032
Leucaena macrophylla Benth. ssp. *macrophylla*	*Hughes* 1179 FHO	Guerrero, Mexico	AF418034
Leucaena macrophylla Benth. ssp. *istmensis* C.E. Hughes	*Hughes* 580 FHO	Oaxaca, Mexico	AF418033
Leucaena magnifica (C.E. Hughes) C.E. Hughes-1	*Hughes* 412 FHO	Chiquimula, Guatemala	AF418035
Leucaena magnifica (C.E. Hughes) C.E. Hughes-2	*Hughes* 720 FHO	Chiquimula, Guatemala	AF418036
Leucaena matudae (Zárate) C.E. Hughes	*Hughes* 879 FHO	Guerrero, Mexico	AF418100
Leucaena multicapitula Schery	*Hughes* 1025 FHO	Los Santos, Panama	AF418037
Leucaena pueblana Britton & Rose	*Hughes* 1648 FHO	Oaxaca, Mexico	AF418099
Leucaena pulverulenta (Schltdl.) Benth.-1	*Hughes* 1611 FHO	Hidalgo, Mexico	a-AF418076
Leucaena pulverulenta (Schltdl.) Benth.-2	*Bendeck* 22/86	Nuevo León, Mexico	a-AF418075 b-AF418085
Leucaena pulverulenta (Schltdl.) Benth.-3	*Hughes* 1593 FHO	San Luis Potosí, Mexico	a-AF418062
Leucaena pulverulenta (Schltdl.) Benth.-4	*Hughes* 1866 FHO	Veracruz, Mexico	a-AF418080
Leucaena pulverulenta (Schltdl.) Benth.-5	*Hughes* 1058 FHO	Texas, USA	AF418063
Leucaena retusa Benth.	*Bendeck* 23/86	Coahuila, Mexico	AF418065
Leucaena salvadorensis Standl. ex Britton & Rose	*Hughes* 1407 FHO	Estelí, Nicaragua	AF418038
Leucaena shannonii Donn. Sm.-1	*Hughes* 507 FHO	Campeche, Mexico	AF418042
Leucaena shannonii Donn. Sm.-2	*Hughes* 1676 FHO	Chiapas, Mexico	AF418039
Leucaena shannonii Donn. Sm.-3	*Hughes* 1417 FHO	Jutiapa, Guatemala	AF418041
Leucaena shannonii Donn. Sm.-4	*Hughes* 239 FHO	Comayagua, Honduras	a-AF418049
Leucaena shannonii Donn. Sm.-5	*Hughes* 1714 FHO	Yoro, Honduras	AF418040
Leucaena trichandra (Zucc.) Urb.-1	*Hughes* 1682 FHO	Chiapas, Mexico	AF418045
Leucaena trichandra (Zucc.) Urb.-2	*Hughes* 1654 FHO	Oaxaca, Mexico	AF418044
Leucaena trichandra (Zucc.) Urb.-3	*Hughes* 1106 FHO	Guatemala, Guatemala	AF418043
Leucaena trichodes (Jacq.) Benth.	*Hughes* 997 FHO	Manabi, Ecuador	AF418046

Table 2. continued

Microlobius foetidus (Jacq.) M. Sousa & G. Andrade	*Macqueen* 432 FHO	Guerrero, Mexico	AF458783
Mimosa guatemalensis (Hook. & Arn.) Benth.	*Macqueen* 190 FHO	Colima, Mexico	AF458784
Neptunia dimorphantha Domin	*Krosnick* 00-51 BH	Seeds from Waterhouse & Puttock 11028, Australia	AF458790
Neptunia gracilis Benth.	*Krosnick* 00-55 BH	Austr. Tropical Forages CQ2881 93-028 Australia	AF458787
Neptunia lutea Benth.	*S.M. Tracy* 8511 BH	Cameron, LA, USA	AF458794
Neptunia monosperma F. Muell.-1	*Sands* 4871 K	Western Australia, Australia	AF458788
Neptunia monosperma F. Muell.-2	*Krosnick* 00-50 BH	Seed from B. Jackes, Wambiana Station, Australia	AF458789
Neptunia oleracea Lour.	*Krosnick* 00-57 BH	Seed from herbarium sheet, D.B. Pickel, Aug. 1931	AF458791
Neptunia plena (L.) Benth.-1	*Graham* s.n. K	Singapore	AF458792
Neptunia plena (L.) Benth.-2	*Luckow* 3332 TEX	Puerto Rico	AF458793
Neptunia pubescens Benth.	*Luckow* 3401 TEX	Texas, USA	AF458795
Prosopis articulata S. Watson	*Hughes* 1559 FHO	Baja California Sur, Mexico	AF458786
Prosopis palmeri S. Watson	*Hughes* 1553 FHO	Baja California Sur, Mexico	AF458785
Schleinitzia insularum (Guill.) Burkart	*Rinehart* 17441 K	Guam	AF458823
Schleinitzia novoguineensis (Warb.) Verdc.	*Chaplin* 57/84	Munda, Solomon Islands	AF418019

Table 2. Accessions within taxa are numbered 1, 2, 3…, and sequences from different clones within accessions are labelled with letters, a, b. Thus the first clone from the first accession of a particular taxon is labelled '*Genus species*-1a'.

Gaps in sampling due to lack of DNA samples are as follows: three species of *Desmanthus* – *D. cooleyi* (Eaton) Trelease from the southwestern USA, *D. painteri* (Britton & Rose) Standl., and *D. hexapetalus* (M. Micheli) Macbride. However, the latter species differs only in unusual stem morphology from *D. paspalaceus* and may be no more than an unusual teratology (Luckow, 1993). Two species of *Schleinitzia* were not included, one of which (*S. fosbergii* Nevling & Niezgoda) is very similar to *S. insularum* (Nevling and Niezgoda, 1978), while the other (*S. megaladenia* (Merr.) P. Guinet & Nielsen from the Philippines) is notably distinctive (Guinet and Nielsen, 1980) but could not be included due to difficulties of obtaining DNA from older herbarium material and lack of recently collected material. Restriction of sampling within *Leucaena* in this study to the 17 diploid species and exclusion of sequences of the five known tetraploid species is justified due to the complex patterns of within accession ITS polymorphism found for these species which are attributable to reticulate origins of the taxa. The full ITS gene tree for *Leucaena* and potential origins of the polyploid taxa are discussed in detail elsewhere (Hughes *et al.*, 2002). Silica-dried leaf material of several species of *Dichrostachys* has not yet been collected in the field and DNA isolation from dried herbarium material was unsuccessful. These include *D. dehiscens* Balf. and *D. kirkii* Benth. from Socotra and Somalia respectively, and *D. dumetaria* Villiers & Du Puy from southern Madagascar. The Malagasy endemic *D. perrieriana* R. Vig. has not been collected in recent years, despite intensive searches. Most of the habitat in which this species had previously been collected is now destroyed and the species is probably extinct (Luckow, unpubl. data). Two putative species of *Alantsilodendron* were also unavailable for study. Attempts to isolate DNA from silica-dried samples of *A. decaryanum* (R. Vig.) Villiers were unsuccessful. *Alantsilodendron glomeratum* Villiers is known only from the type specimen, and may represent an anomalous collection of *A. humbertii*. Although it would be desirable to include representatives of these five taxa, there is little doubt that they belong within the *Dichrostachys* group and their exclusion, while possibly influencing relationships within the group, are not likely to affect overall generic relationships. There are also still several gaps in our sampling of *Neptunia*. *Neptunia amplexicaulis* Domin. and *N. major* (Benth.) Windler are clearly related to the other Australian species, and *N. microcarpa* Rose was once considered a variety of *N. pubescens*, so absence of these taxa is not likely to influence our conclusions. No material suitable for DNA extraction was available for the two Asian species, *N. acinaciformis* (Span.) Miq. and *N. triquetra* (Vahl) Benth. Since these are the only exclusively Asian representatives in the genus, it would be most desirable to include these taxa in future analyses.

Lack of a well-supported hypothesis of generic relationships both within the tribe Mimoseae and indeed across the subfamily Mimosoideae as a whole (Luckow *et al.*, 2000), has hampered the search for sister groups that might be used as outgroups in analyses of the *Dichrostachys* and *Leucaena* groups. Previous analyses of these groups have used *Parkia* (Luckow, 1995, 1997; Hughes, 1998) and *Xylia* (Hughes, 1998). The recent *trn*L-*trn*F analysis by Luckow *et al.* (2000), and the combined *trn*L-*trn*F/*mat*K analysis by Luckow *et al.* (2003), although indicating that *Xylia* at least is distantly related, do little to ease this uncertainty due to lack of resolution among the genera and groups of genera close to the *Dichrostachys* and *Leucaena* groups. In this analysis four outgroup sequences from amongst these largely unresolved sister groups (*Microlobius foetidus*, *Mimosa guatemalensis*, *Prosopis articulata* and *P. palmeri*) were used. Given the lack of previous evidence that the *Dichrostachys* and *Leucaena* groups together form a monophyletic group, it is quite possible that inclusion of additional genera (e.g. *Prosopidastrum* — see Luckow *et al.*, 2003) could alter our results.

DNAs were extracted from fresh leaves of plants grown from seed, herbarium specimens, or silica gel dried samples of field collected leaf material (Table 2). DNA isolation followed the CTAB technique of Doyle and Doyle (1987) or a DNeasy kit

(QIAGEN Inc., Santa Clarita, CA). Most of the *Leucaena* DNA samples were further purified using caesium chloride gradients (Maniatis *et al.*, 1982) and DNAs were resuspended in TE or water and stored at -20°C.

Polymerase chain reactions (PCR) were run using Qiagen (QIAGEN Inc., Santa Clarita, CA) *Taq* polymerase (final concentrations: c. 1.5 units *Taq*, 100 µM of each dNTP, 1X PCR buffer, 1X Q solution, and 0.5 µM of each primer). Amplifications were performed on a Progene thermocycler (Techne Limited, Cambridge UK). Several combinations of ITS4 / ITS5 (White *et al.*, 1990) and 17SE / 26SE (Sun *et al.*, 1994) primers were used to obtain amplifications from all the taxa of interest. All amplifications began with a three minute 94°C denaturation step, followed by 35 rounds of 1) one minute 94°C denaturation; 2) one minute annealing at 48°C (primer combinations ITS4+ITS5 and 17SE+ITS4), or 53°C (primer combination ITS5+26SE); and 3) a one minute 72°C extension. This protocol was modified for species of *Neptunia*, to include combinations of ITS2 / ITS3 primers (White *et al.*, 1990) using 45 rounds with an annealing temperature of 55°C. PCR products were cleaned using the Concert Purification System (Life Technologies, Paisley UK) or Qiagen Gel Extraction Kits for direct sequencing or cloning. Both strands were sequenced for the majority of sequences using the PCR primers and 'Big Dye' termination chemistry (Applied Biosystems Inc, Warrington UK). Overlapping traces for several *Desmanthus* templates were cloned (pGEM; Promega Corporation, Madison WI) using one half the reaction volume described by the manufacturer. Clones were screened for the presence of an ITS insert using the PCR amplification primers, and subsequently sequenced.

Sequence traces from PCR products or clones were edited and joined into consensus sequences using Sequencher (Gene Codes Corp.). Complete sequences were provisionally aligned using ClustalX ver. 1.8 (Thompson *et al.*, 1997) and then adjusted by eye in WinClada (Nixon, 1999). ClustalX default parameters for multiple alignments were changed to a gap opening cost of 8 and gap extension cost of 6 to generate reasonable starting alignments. Contiguous gaps were scored as characters as advocated by Simmons *et al.* (2001) using the 'simple gap coding' method formalised by Simmons and Ochoterena (2000). Individual gap positions were scored as missing data. Sequences are available in GenBank (Table 2), the sequence alignment is available in the EMBL nucleotide alignment database (accession Align_000328 at **ftp://ftp.ebi.ac.uk/pub/databases/embl/align/**) and the complete data matrix with aligned sequences and gap characters can be obtained from the first author.

Parsimony analysis was conducted using NONA (Goloboff, 2000) spawned from WinClada (Nixon, 1999) using 1000 random addition sequences, tree bisection and reconnection (TBR), holding 100 trees per replicate and attempting to swap to completion (hold/100; mult*1000; max*). All characters were scored as unordered and equally weighted. The strict consensus bootstrap approach was used to assess branch support (Davis *et al.*, 1998). The bootstrap analysis used 1000 replications each with 10 random additions holding 10 in each replicate, with a maximum of 100 trees saved per replication (1000 replications; mult*10; hold/10). Strict consensus bootstrap values rounded to the nearest percentage were mapped to the strict consensus tree in WinClada.

The presence of a number of unusually long branches and several instances of polymorphism within accessions in the *Desmanthus* clade, along with previous detection of numerous pseudogene sequences in *Leucaena* (Hughes *et al.*, 2002) prompted us to analyse patterns of ITS sequence divergence in order to detect putative non-functional pseudogene sequences. Identification of potential pseudogene sequences can shed light on alignment, branch attraction and sampling problems. To do this we used a tree-based approach (C.D. Bailey *et al.*, unpubl., University of Oxford) to record the number of putative substitutions found in the 5.8S subunit relative to the total ITS (ITS 1, ITS 2 and 5.8S) variation (i.e., the observed percentage 5.8S contribution) for each branch longer than 10 steps on one of the equally most parsimonious trees. The expected change for a freely evolving nrDNA pseudogene branch was calculated based

on the percentage of 5.8S bases optimised to each branch (5.8S bp / ITS region bp corrected for indel regions). If the observed 5.8S percentage change along the branch was comparable to that expected of a relatively unconstrained region, *i.e.* a pseudogene, the branch, and terminal(s) derived from it, were marked as potential pseudogenes. These calculations were carried out using the complete aligned matrix within *Desmanthus* and *Leucaena*. Although statistical testing of these comparisons would be desirable, no suitable tests to do this are available as yet. However, putative non-functional pseudogene sequences are quite distinct from functional copies (see Hughes *et al.* (2002) for detailed analysis of *Leucaena* pseudogenes).

Results

A total of 108 ITS sequences from 102 accessions of 78 taxa were generated for the ITS analysis. Five sequences (*Desmanthus bicornutus* 1a and 2b, *Dichrostachys spicata*, *Dichrostachys venosa*, and *Neptunia lutea*) were incomplete with up to a maximum of 120 bp missing data. Alignment was complicated by length variation among sequences, which range from 588 to 710 bp. The final matrix included 718 aligned bases representing 371 potentially informative substitution characters and 70 potentially informative gap characters. A single region of ITS 1 from positions 73–167 of the aligned matrix was problematic to align and was excluded from the analysis. In addition, one cloned sequence of *Desmanthus pringlei* was unalignable outside the 5.8S subunit and was discarded from the data set prior to analysis. Standard parsimony analysis swapped to completion recovering 324 equally parsimonious trees (L=1290, CI=0.46, RI=0.87). The strict consensus tree is presented in Fig. 1, with strict consensus bootstrap values above nodes.

The most striking feature of the ITS analysis is that the revised *Leucaena* and *Dichrostachys* groups *sensu* Luckow (1997) (Table 1) are resolved as monophyletic sister groups with high bootstrap support (Fig. 1). The *Dichrostachys* group comprising *Alantsilodendron*, *Calliandropsis*, *Dichrostachys* and *Gagnebina* has strong (100%) bootstrap support. Within this group, three moderately or strongly supported clades are resolved, one comprising all species of *Alantsilodendron* plus *Dichrostachys richardiana* and *D. venosa* with 64% bootstrap support, a second group comprising the Malagasy *Dichrostachys* species with 98% bootstrap support, and a group comprising *Dichrostachys cinerea* and *D. spicata* (100% bootstrap support). The placements of *Calliandropsis* and *Gagnebina pterocarpa* are weakly supported.

The *Leucaena* group *sensu* Luckow (1997) comprising *Leucaena*, *Desmanthus*, *Kanaloa*, and *Schleinitzia* is resolved as monophyletic with 98% bootstrap support in the ITS analysis. Within the *Leucaena* group two large subclades are resolved, a monophyletic *Leucaena* with strong 99% bootstrap support, and a moderately supported (74% bootstrap value) group comprising the genera *Schleinitzia*, *Desmanthus* and *Kanaloa*, a result mirrored exactly in the analysis of *trn*L-*trn*F and *mat*K sequence data (Luckow *et al.*, 2000, 2003).

Four cases of ITS polymorphism within accessions of *Desmanthus acuminatus*, *D. pubescens*, *D. bicornutus* and *Leucaena pulverulenta* were detected. The three *Desmanthus* species are polyphyletic on the ITS gene tree. In the case of the three *Desmanthus* species, these sequences represent divergent copy types derived from cloned PCR products where direct sequencing had produced overlapping sequence traces. We have also detected ITS polymorphism within individuals of four of the five tetraploid and one diploid species of *Leucaena* (Hughes *et al.*, 2002). In the case of the one diploid species *Leucaena pulverulenta*, the different ITS sequence types found within accessions form a monophyletic group with sequences of other accessions of *L. pulverulenta* (Fig. 1; Hughes *et al.*, 2002).

Five *Desmanthus* sequences (*D. acuminatus* a and b, *D. pringlei*, *D. tatahuyensis*, and *D. velutinus*) and four *Leucaena pulverulenta* sequences (1, 2a and 2b, and 4) whose

observed percent divergences from the 5.8S subunit closely match the values expected for a relatively unconstrained region were identified as potentially non-functional pseudogene sequences.

Discussion

This analysis, which includes 80% of the known species of the *Leucaena* and *Dichrostachys* groups, is by far the most comprehensively sampled study of sister group relationships among these genera undertaken to date. The ITS analysis supports the re-circumscription of the informal *Leucaena* group to include *Desmanthus*, *Kanaloa*, *Leucaena* and *Schleinitzia*, and the *Dichrostachys* group comprising *Alantsilodendron*, *Calliandropsis, Dichrostachys* and *Gagnebina*, as well as the exclusion of *Neptunia* from the *Dichrostachys* group as proposed by Luckow (1997). Both the informal groups are

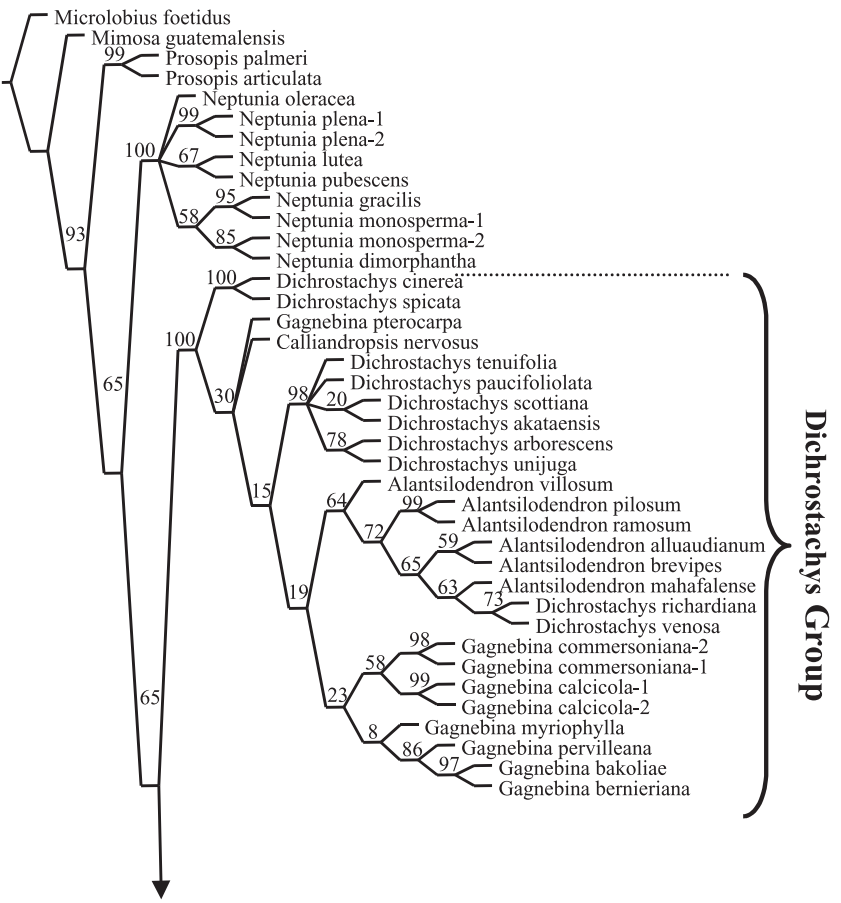

Fig. 1 cont'
Leucaena Group

Fig. 1 cont' - Dichrostachys Group

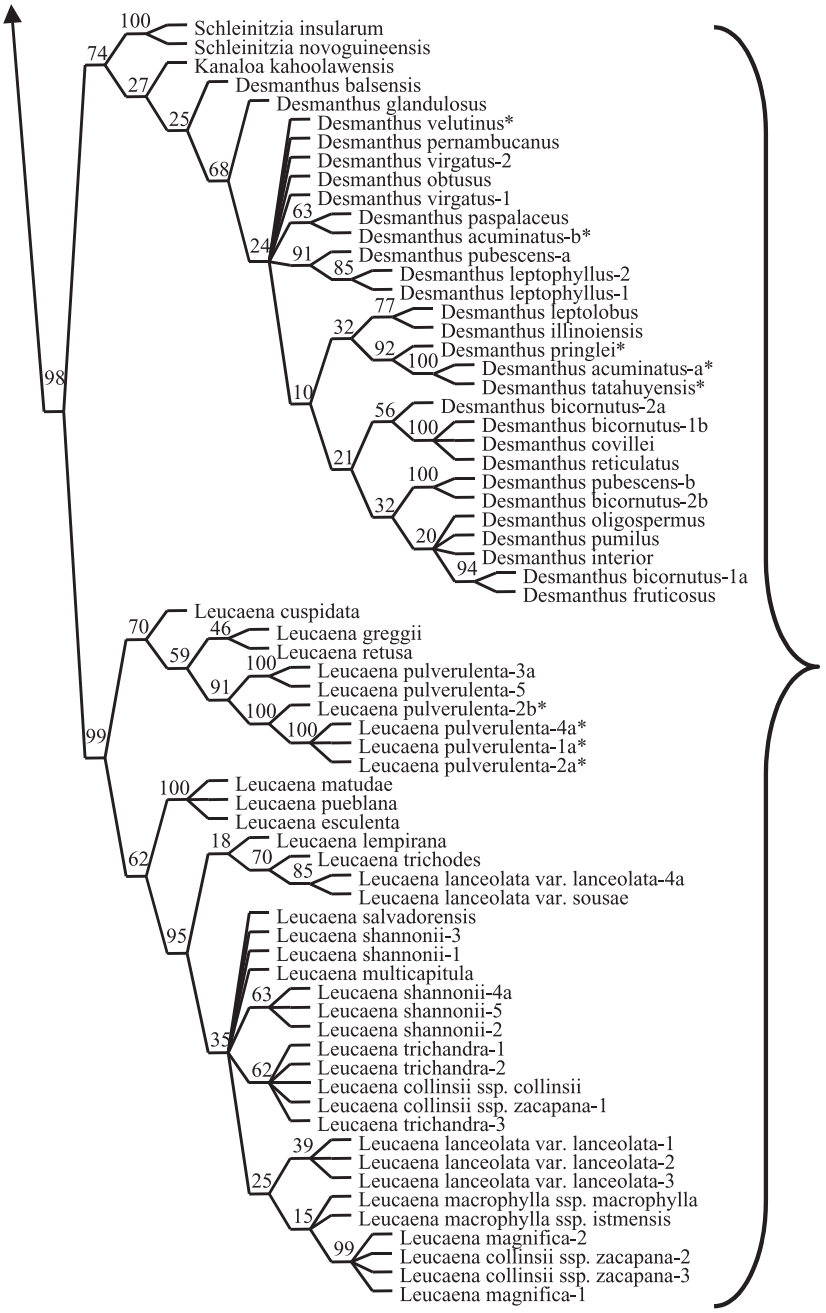

FIG. 1. Strict consensus of 324 equally parsimonious trees (length=1290 steps; CI=0.46; RI=0.87) with strict consensus bootstrap values rounded to the nearest % above nodes. Potential pseudogene sequences are marked with asterisks.

monophyletic in the ITS analysis and were also supported in the analysis of *trn*L-*trn*F cpDNA sequence data by Luckow *et al.* (2000) and combined *trn*L-*trn*F/*mat*K cpDNA sequence data sets by Luckow *et al.* (2003). There is only moderate bootstrap support for these groups in the *trn*L-*trn*F analysis, but support is stronger (98% bootstrap for the *Leucaena* group and 100% bootstrap for the *Dichrostachys* group) in the ITS analysis. Both the wider *trn*L-*trn*F analysis of the Mimoseae, and the combined *trn*L-*trn*F/*mat*K analysis of the mimosoids as a whole included a much sparser (20%) sample from the *Dichrostachys* and *Leucaena* group taxa precluding simultaneous analysis of the two data sets combined. However, the fact that the same groups are inferred independently from both cpDNA *trn*L-*trn*F, *mat*K, and nrDNA ITS sequence data lends confidence to these results.

This analysis with its limited sampling of genera outside the *Leucaena* and *Dichrostachys* groups does not address the higher-level relationships of these groups which were assessed by Luckow *et al.* (2000, 2003). While the inclusion of other taxa from the informal *Prosopis* and *Piptadenia* groups would be feasible and desirable, it is likely that the utility of ITS for higher level studies will be limited within the Mimosoid legumes due to alignment difficulties posed by length variation. ITS sequences for *Entadopsis polystachya* and *Xylia torreana* were impossible to align with the matrix analysed here.

***Dichrostachys* group**

Results of the current analysis agree with previous studies in showing the *Dichrostachys* group as monophyletic and strongly supported (100% bootstrap value). Many of the relationships in the ITS tree are congruent with the morphological evidence, some of it as yet unpublished (Luckow, unpubl. data). *Dichrostachys* has proved polyphyletic in all cladistic analyses to date and this one is no exception. However, the monophyly of the clade containing the bulk of *Dichrostachys* which is exclusively Malagasy is strongly supported in the ITS analysis (98% bootstrap support) and is also supported by characters such as mauve staminodia and coriaceous fruits that curl post-dehiscence. *Dichrostachys cinerea* and *D. spicata* (African and Australian, respectively) share distinctive characters such as spines, indehiscent woody fruits, acalymmate polyads, and long-stipitate anther glands. *Alantsilodendron* is monophyletic with the inclusion of *D. richardiana* and *D. venosa*, a relationship supported by characters such as connate petals and adaxial distribution of stomata. *Dichrostachys richardiana* and *D. venosa* share a distinctive leaflet anatomy, having an enlarged, sclerified bundle sheath (Luckow, 2002), which is consistent with a sister relationship between them. Morphology also supports sister relationships between *Gagnebina commersoniana* and *G. calcicola* (indehiscent winged fruits, linear anthers), and *G. bakoliae* and *G. bernieriana* (see Luckow and Du Puy, 2000).

In contrast, some of the relationships portrayed in the ITS tree within the *Dichrostachys* group are at odds with previous work. For example, whereas morphological and cpDNA analyses (Luckow, 1995; Luckow *et al.*, 2000) strongly support the monophyly of *Gagnebina*, direct interpretation of the ITS gene tree as a species tree would require the segregation of *G. pterocarpa* from the remaining species of *Gagnebina*, although this relationship is only weakly supported. Such a relationship is unlikely, given the many synapomorphies this species shares with other species of *Gagnebina* (e.g. indehiscent, winged fruits [with *G. calcicola* and *G. commersoniana*], subulate stipules, resting buds instead of brachyblasts). The nesting of *Calliandropsis* among the Old World species in the group is also somewhat problematic. *Calliandropsis* was sister to all other taxa in the *Dichrostachys* group in tribal-level cpDNA studies (Luckow *et al.*, 2000, 2003). *Calliandropsis* shares a number of morphological features with *Alantsilodendron* (no staminodial flowers, elastically dehiscent valves, anther appendages, capitate inflorescences), and is sister to this genus in a previous morphological study (Luckow, 1995). However, in addition to geographic considerations, there are a number of morphological features that mitigate against its

inclusion within the group of Old World taxa. Most notably, *Calliandropsis* possesses typical tricolporate monad pollen units whilst all other species in the *Dichrostachys* group possess pollen in polyads (the acalymmate monads of *D. cinerea* are quite different and clearly derived from polyads, see Luckow, 1995). Nonetheless, the possibility remains that this monotypic endemic Mexican genus is sister to an exclusively Malagasy clade. The relationships of *Gagnebina* and *Calliandropsis* are only weakly supported by ITS characters and more data are needed to definitively resolve these relationships. Such a global morphological/molecular study is in progress and will be published as part of the forthcoming revision of this group (Luckow, unpubl. data).

***Leucaena* group**

Leucaena is strongly supported (99% bootstrap value) as monophyletic with three main clades resolved within the genus, a result that is congruent with previous analysis of multiple data sources (Harris *et al.*, 1994; Hughes *et al.*, 2002). Relationships within *Leucaena* and the origins of the five tetraploid species are analysed in greater detail elsewhere (Hughes *et al.*, 2002) using the full ITS data set, including the variable ITS 1 region excluded in the analysis presented here.

The placement of *Desmanthus*, *Kanaloa*, and *Schleinitzia* in a clade that is sister group to *Leucaena* is in line with a number of other studies. Luckow (1993) pointed out the close similarity in pollen and anther gland morphology of *Desmanthus balsensis* to *Schleinitzia,* first suggesting a need to re-evaluate the relationships between the *Dichrostachys* and *Leucaena* groups. The placement of *Schleinitzia* as sister group to *Desmanthus*, rather than to *Leucaena* as proposed by Lewis and Elias (1981), was also suggested by Harris *et al.* (1994) and Luckow (1997) based on separate analyses of cpDNA restriction sites and by Hughes (1998) based on a morphological analysis. Furthermore, *Desmanthus* and *Schleinitzia* are placed in a clade together with *Kanaloa* in the analysis of *trn*L-*trn*F sequence data (Luckow *et al.*, 2000). Thus, there is now overwhelming evidence from multiple data sources to support the two clades within the *Leucaena* group as shown in Fig. 1.

The Hawaiian endemic *Kanaloa kahoolawensis* was described from two known individuals growing on the 'Ale'ale sea stack off the coast of the small island of Kaho'olawe by Lorence and Wood (1994). At that time they refrained from placing *Kanaloa* firmly in either the *Leucaena* or *Dichrostachys* groups, because of its apparent affinities to both *Desmanthus* (then placed in the *Dichrostachys* group) and *Leucaena.* The placement of *Kanaloa* in the *Leucaena* group, in a clade with *Schleinitzia* and *Desmanthus*, in both the ITS analysis presented here and the earlier *trn*L-*trn*F analysis of Luckow *et al.* (2000), and the recent *trn*L-*trn*F/*mat*K analysis of Luckow *et al.* (2003) confirms the affinities to these genera suggested by Lorence and Wood (1994). A number of morphological features, including flowers in heads and flowers subtended by persistent peltate bracts support the placement of *Kanaloa* in the *Leucaena* group. In addition the tricolporate rugulate pollen of *Kanaloa* matches pollen of some *Desmanthus* species, even though pollen is extremely variable across the *Leucaena* group as a whole with both monads and polyads occurring within both *Desmanthus* (Luckow, 1993) and *Leucaena* (Hughes, 1997). The placement of *Kanaloa* as sister to *Desmanthus* is weakly supported in the ITS analysis and the precise relationships of *Kanaloa* to *Desmanthus* and *Schleinitzia* remain uncertain. The branches supporting *Schleinitzia*, *Kanaloa* and the basal *Desmanthus balsensis* are all long. *Kanaloa* remains in some respects poorly known in that hermaphrodite flowers have not yet been found. Finally, omission of the highly distinctive *Schleinitzia megaladenia* from the Philippines from this analysis due to lack of material is potentially significant. All these considerations suggest a need for further work to establish the precise relationships among these genera with greater certainty. However, whatever the precise arrangement of these genera, the close relationship of the Hawaiian *Kanaloa* to both *Schleinitzia* from the W. Pacific basin and *Desmanthus* from the Americas presents an intriguing biogeographic relationship.

The only previous phylogenetic analysis of species relationships within *Desmanthus* (Luckow, 1993) relied on a cladistic analysis of 22 morphological characters. More recently Pengelly and Liu (2001) investigated patterns of diversity in a subset of *Desmanthus* species using RAPDs. Twenty-one of the 24 species of *Desmanthus* species were included in the ITS analysis providing the first species-level molecular phylogeny of that genus. However, the utility of the ITS gene tree to infer species relationships appears to be limited by what we conclude is almost certainly incomplete sampling of ITS diversity within accessions of some species. Our analysis of ITS sequence divergence patterns across the ITS gene tree suggest that five sequences (*D. acuminatus* a and b, *D. pringlei*, *D. tatahuyensis*, and *D. velutinus*) are potential pseudogenes. While inclusion of pseudogene sequences in analysis is desirable and should not in itself be a cause for concern, in this case, all four of these species are currently represented in the ITS gene tree only by potentially non-functional copy types; no functional copy types have yet been detected and sequenced for these taxa. This strongly suggests that the ITS gene tree is under-sampled, particularly given that several accessions (*D. acuminatus*, *D. bicornutus* 1 and 2, and *D. pubescens*) show divergent ITS copy types. The detrimental influence of incomplete sampling of gene trees in cases where paralogous copies are present, on species tree inference are well documented and widely appreciated (Sanderson and Doyle, 1992).

This undersampling may explain at least in part the general lack of congruence between the ITS gene tree and the morphological analysis of Luckow (1993). Beyond the congruent placement of the unusual *Desmanthus balsensis* at the base of the genus in both analyses, and a number of congruent pairs of species as sister species in both analyses, the ITS gene tree does not currently reflect relationships inferred from morphology.

The discovery of, as yet incompletely sampled, ITS polymorphism and lack of congruence between the ITS gene tree and morphological evidence suggests that further work to investigate species relationships within *Desmanthus* would be worthwhile. The majority of documented cases of ITS polymorphism have been associated with hybridization and polyploidy and /or multiple nucleolar organiser regions (Campbell *et al.*, 1997; Hershkovitz *et al.*, 1999). For *Desmanthus*, there are chromosome counts for only five of the 24 species (all 2n=28; Turner and Beaman, 1953; Smith, 1963) and additional chromosome counts are needed to assess whether any species in the genus are polyploids. Additional ITS sampling is needed to detect functional ITS copies for accessions and species where only potentially non-functional copies have so far been sampled. These additional data are needed to understand gene tree relationships prior to inferring species relationships. In addition, reassessment and analysis of the morphological data of Luckow (1993) to exclude *Neptunia* and include more appropriate outgroups would be desirable. Compared to *Leucaena*, where we know from multiple sources of evidence that hybridization has been an important process in the evolution of the genus, where chromosome counts are available for all species with five polyploid species documented, and where we have sampled ITS diversity much more extensively allowing us to draw specific conclusions about the underlying evolution of nrDNA polymorphism (Hughes *et al.*, 2002), we are at a much earlier stage in our understanding of patterns of nrDNA polymorphism and what this means for species relationships within *Desmanthus*.

Neptunia

Neptunia has generally been considered to be closely related to *Desmanthus* (Windler, 1966; Isley, 1970). However, the placement of *Neptunia* within *Desmanthus* in a series of morphological cladistic analyses (Luckow, 1993, 1995; Hughes, 1998) has been viewed as problematic as it necessitates extensive character reversals (see Luckow, 1993). Furthermore, analyses of cpDNA restriction site data (Luckow, 1997) and *trn*L-*trn*F sequence data including a wider sample of genera suggested that *Neptunia* does

not belong within *Desmanthus*, or indeed within the *Dichrostachys* group as suggested by Lewis and Elias (1981). Recent cladistic analyses of the subfamily Mimosoideae using cpDNA sequence data (*trn*L-*trn*F and *mat*K-*trn*K) indicate that *Neptunia* is more closely related to *Prosopidastrum*, a small genus in the informal *Prosopis* group, than it is to either the *Dichrostachys* or *Leucaena* groups (Luckow *et al.*, 2003). The ITS results show strong (100% bootstrap) support for a monophyletic *Neptunia* outside these groups providing further evidence to support the exclusion of *Neptunia* from the *Dichrostachys* group. This is supported by a number of morphological features. Firstly, the presence of sterile flowers at the base of the inflorescence was used by Lewis and Elias (1981) to distinguish the genera of the *Dichrostachys* group, but the staminodia in *Neptunia* are petaloid and yellow, and quite different from the filamentous white or pink staminodia of *Desmanthus, Dichrostachys* and *Gagnebina*. Secondly, data on floral ontogeny show that *Neptunia* is unique amongst the genera of the *Leucaena* and *Dichrostachys* groups studied so far in having simultaneous rather than helical order of sepal initiation (Ramirez-Domenech and Tucker, 1990).

The current sampling of species of *Neptunia* is incomplete and the relationships within *Neptunia* are largely unresolved. However, the two subclades that are resolved in the ITS tree show good correspondence with morphology and geography. The largest subclade groups the Australian taxa (*N. dimorphantha, N. monosperma,* and *N. gracilis*), all of which have five rather than ten stamens per flower. Furthermore, *N. lutea* and *N. pubescens* have traditionally been considered to be closely related (Windler, 1966; Krosnick, unpubl. data) as both species have bracts in the upper half of the peduncle and lack petiolar nectaries, a result reflected in the ITS tree.

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