

Gender-bending aubergines: molecular phylogenetics of cryptically dioecious *Solanum* in Australia

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Abstract. The causes, consequences and correlates of dioecy have been the subject of much discussion since the days of Darwin. Several recent authors have stressed the importance of informing this body of theory, with studies focusing on lineages in which both dioecy and hermaphroditism are present. The genus *Solanum* is an ideal group for analysis, because dioecy, hermaphroditism and potential unisexual transitions (e.g. andromonoecy) among them all occur. Phylogenetic hypotheses are presented for the Australian species in *Solanum* subgenus *Leptostemonum* (the ‘spiny solanums’) section *Melongena*, which contains 10 of the 14 currently described dioecious species in the genus. Phylogenetic analysis of the ITS and *trnK–matK* gene regions supports a single origin of dioecy from andromonoecy in Australian *Solanum*. The causes, mechanisms, and maintenance of dioecy in Australian *Solanum* are explored, including the role of past climate change in the establishment of centres of endemism.

Introduction

Dioecy is present in only 6% of the ~250 000 species of angiosperms (Renner and Ricklefs 1995), although it is somewhat more common in some floras and taxonomic groups (Bawa 1980). Long considered a derived breeding system (as per Neal and Anderson 2005) within angiosperms (Lewis 1942), the causes for dioecy, and the potential pathways to it, have been the subject of scientific discourse since Darwin (1877). An impressive body of literature has been generated on the causes (Bawa and Opler 1975; Thomson and Barrett 1981; Givnish 1982; Charlesworth 1984; Thomson and Brunet 1990; Freeman *et al.* 1997), consequences (Baker 1984; Heilbut *et al.* 2001; Vamosi and Vamosi 2005; others), correlations (Darwin 1877; Yampolsky and Yampolsky 1922; Baker 1959; Bawa 1980; Givnish 1980; Fox 1985; Muenchow 1987; Steiner 1988; Anderson and Symon 1989; Thomson and Brunet 1990; Renner and Ricklefs 1995; Vamosi *et al.* 2003; Vamosi and Vamosi 2004) and distributions (Yampolsky and Yampolsky 1922; Charlesworth 1985; Renner and Ricklefs 1995) of dioecy. Several authors have recommended the application of phylogenetic analysis to investigate the origin of dioecy (Baker 1984; Charlesworth 1985; Donoghue 1989; Thomson and Brunet 1990; Weller and Sakai 1999; Vamosi *et al.* 2003); however, relatively few studies have used this approach as a means to trace the evolution of dioecy and associated natural history in specific taxonomic groups (e.g. Weller and Sakai 1990; Weiblen *et al.* 2000).

In the economically important plant family Solanaceae, dioecy is present in less than 1% of the species (~25 of 3000–4000) (Hunziker 2001; Anderson *et al.* 2006b; Brennan *et al.* 2006) scattered through the following five genera: *Solanum*

(e.g. Levine and Anderson 1986; Anderson and Symon 1989; Knapp *et al.* 1998), *Deprea* (e.g. Sawyer and Anderson 2000), *Dunalia* (e.g. Hunziker 1960, 2001), *Lycium* (e.g. Spies *et al.* 1993; Miller and Venable 2002) and *Withania* (e.g. Anderson *et al.* 2006b). Of the ~25 occurrences in the family, 14 are in *Solanum* (Anderson and Symon 1989; Knapp *et al.* 1998; Hunziker 2001; Martine 2006; Martine *et al.* 2006), the largest genus in Solanaceae. In total, 12 of the 14 described dioecious species of *Solanum* are in subgenus *Leptostemonum* (the so-called ‘spiny solanums’) and there are probably more remaining to be described, given that the taxonomy of the group still remains poorly known. Two of the dioecious spiny solanums are Antillean in distribution (Whalen 1984; Knapp *et al.* 1998), with the remaining 10 represented by short-lived woody shrubs inhabiting the semiarid tropics of northern Australia. These 10 species are concentrated in two centres of Australian endemism defined by Crisp *et al.* (2001), namely the ‘North Kimberley’ area (of the Kimberley Plateau, Western Australia) and ‘Kakadu–Alligator River’ area (of the upper coastal region of the Northern Territory).

The dioecious Australian solanums are androdioecious morphologically (Symon 1979a), and the studies by Anderson and Symon (1989) clarified that they are functionally dioecious. In this system, individual plants bear either staminate flowers or morphologically hermaphrodite flowers. Despite producing anthers and pollen, hermaphrodite flowers are functionally pistillate because the pollen grains are inaperturate and ingerminal (Zavada and Anderson 1997; Zavada *et al.* 2000). This unusual, cryptic form of dioecy is the only manner in which dioecy is known in *Solanum* (Anderson 1979; Anderson and Levine 1982; Levine and Anderson 1986; Anderson and

Symon 1989). All four occurrences in the New World apparently are autapomorphic, with each representing an independent origin among quite distantly related taxa (Knapp *et al.* 1998). In the Old World, dioecy in *Solanum* occurs only in the ~10 Australian eggplant relatives, placed by Symon (1981) in subgenus *Leptostemonum* section *Melongena*. In this group, dioecy could have evolved as many times as there are dioecious species (Anderson and Symon 1989) or as infrequently as once or twice (Whalen 1984; Anderson and Symon 1989; Martine *et al.* 2006; Martine and Anderson 2007).

Determining the precise number of times that dioecy evolved in this group is of particular interest evolutionarily because there is a tendency for dioecious clades to show higher likelihoods of extinction and/or lower rates of speciation (Baker 1959; Heilbut 2000; Vamosi and Otto 2002; Vamosi and Vamosi 2005). If dioecy evolved only once in Australian *Solanum*, that single evolutionary event would have preceded a radiation of at least 10 more species.

The evolution of dioecy from hermaphroditism within a lineage is postulated to involve a transition in which either male or female function is lost in some hermaphrodite flowers (Ross 1970; Bawa 1980; Charlesworth 1984). Previous workers in *Solanum* subgenus *Leptostemonum* have hypothesised a pathway from hermaphroditism to dioecy via the transitional breeding system of andromonoecy, which is considered to be

ancestral in many *Leptostemonum* groups (Whalen 1984; Whalen and Costich 1986; Anderson and Symon 1989; Connolly and Anderson 2003; Levin *et al.* 2006). Until recently, this hypothesis had not been tested in a phylogenetic context. The analyses of Martine *et al.* (2006) provided some phylogenetic support for this evolutionary pathway in the Australian spiny solanums; however, they were based on just a single gene region (ITS).

The monophyly of subgenus *Leptostemonum* (as well as an Old World Clade consisting of all the *Leptostemonum* species from Africa, Asia and Australia) has been well established (Levin *et al.* 2006); however, relationships of the Australian dioecious species remain problematic. In his monograph of Australian *Solanum*, Symon (1981) placed all dioecious species with several andromonoecious species from both Australia and Africa, as well as with *S. melongena*, the cultivated eggplant (Table 1). Whalen (1984) later placed all but one of the Australian dioecious species along with their proposed closest Australian andromonoecious ancestors in his 'dioicum group' (Table 1). Martine *et al.* (2006) found no support either for the monophyly of section *Melongena sensu* Symon (1981) or for Whalen's *dioicum* group. Instead, they resolved a series of five clades for the 20 species considered by Symon and Whalen (Table 1). The relationships among these five clades were unresolved, however, and this made further work with additional datasets necessary.

Table 1. Taxonomic organisation of members of *Solanum* subgenus *Leptostemonum*, considered here on the basis of the treatments of Symon (1981), Whalen (1984) and Martine *et al.* (2006)

Taxon	Range	Symon (1981)	Whalen (1984)	Martine <i>et al.</i> (2006)	Breeding system
<i>S. dioicum</i> W. Fitzg.	Australia	Section <i>Melongena</i>	Dioicum group	Dioicum complex (Clade 1)	Dioecious
<i>S. cunninghamii</i> Benth.	Australia	Section <i>Melongena</i>	Dioicum group	Dioicum complex (Clade 1)	Dioecious
<i>S. petraeum</i> Symon	Australia	Section <i>Melongena</i>	Dioicum group	Dioicum complex (Clade 1)	Dioecious
<i>S. 'Longini'</i> (undescribed)	Australia	n.a.	n.a.	Dioicum complex (Clade 1)	Dioecious
<i>S. carduiforme</i> Mueller	Australia	Section <i>Melongena</i>	Dioicum group	Dioicum complex (Clade 1)	Dioecious
<i>S. vansittartense</i> C.A.Gardner	Australia	Section <i>Melongena</i>	Dioicum group	Dioicum complex (Clade 1)	Dioecious
<i>S. tudununggae</i> Symon	Australia	Section <i>Melongena</i>	Dioicum group	Dioicum complex (Clade 1)	Dioecious
<i>S. leopoldensis</i> Symon	Australia	Section <i>Melongena</i>	Hystrix group	Dioicum complex (Clade 1)	Dioecious
<i>S. cataphractum</i> A.Cunn. ex Benth.	Australia	Section <i>Melongena</i>	Dioicum group	Dioicum complex (Clade 1)	Dioecious
<i>S. asymmetriphyllum</i> Specht	Australia	Section <i>Melongena</i>	Dioicum group	Kakadu clade (Clade 5)	Dioecious
<i>S. sejunctum</i> K.Brennan, Martine & Symon	Australia	n.a.	n.a.	Kakadu clade (Clade 5)	Dioecious
<i>S. beagleholei</i> Symon	Australia	Section <i>Melongena</i>	Dioicum group	Bush tomatoes (Clade 3)	Andromonoecious
<i>S. phlomoides</i> A. Cunn. ex Benth.	Australia	Section <i>Melongena</i>	Dioicum group	Bush tomatoes (Clade 3)	Andromonoecious
<i>S. chippendalei</i> Symon	Australia	Section <i>Melongena</i>	Dioicum group	Bush tomatoes (Clade 3)	Andromonoecious
<i>S. diversiflorum</i> Mueller	Australia	Section <i>Melongena</i>	Dioicum group	Bush tomatoes (Clade 3)	Andromonoecious
<i>S. eburneum</i> Symon	Australia	Section <i>Melongena</i>	Dioicum group	Bush tomatoes (Clade 3)	Andromonoecious
<i>S. melanospermum</i> Mueller	Australia	Section <i>Melongena</i>	Dioicum group	Melanospermum + clarkiae (Clade 4)	Andromonoecious
<i>S. clarkiae</i> Symon	Australia	Section <i>Melongena</i>	Dioicum group	Melanospermum + clarkiae (Clade 4)	Andromonoecious
<i>S. oedipus</i> Symon	Australia	Section <i>Melongena</i>	Hystrix group	Oedipus + heteropodium (Clade 2)	Andromonoecious
<i>S. heteropodium</i> Symon	Australia	Section <i>Melongena</i>	Dioicum group	Oedipus + heteropodium (Clade 2)	Andromonoecious
<i>S. aculeastrum</i> Dunal	Africa	n.a.	Incanum group	n.a.	Andromonoecious
<i>S. linnaeanum</i> Hepper & P.-M.L.Jaeger	Africa	Section <i>Melongena</i>	Incanum group	n.a.	Andromonoecious
<i>S. hoplopetalum</i> Bitter & Summerh.	Australia	Section <i>Oliganthes</i>	Hystrix group	n.a.	Hermaphroditic
<i>S. cinereum</i> R.Br.	Australia	Section <i>Melongena</i>	Unplaced	n.a.	Andromonoecious
<i>S. stupefactum</i> Symon	Australia	n.a.	n.a.	n.a.	Andromonoecious
<i>S. elaeagnifolium</i> Cav.	Americas	Section <i>Oliganthes</i>	Ellipticum group	n.a.	Andromonoecious

Here, we present the results of phylogenetic analyses of the Australian species placed by Symon (1981) in the section *Melongena*, conducted by using DNA sequence data from the ITS and *matK* regions, plus the 5' and 3' *trnK* introns. The major goals of the present study were to clarify relationships among the five clades identified by Martine *et al.* (2006) and test further the monophyly of Symon's section *Melongena*. These inferences will then inform hypotheses regarding breeding-system transitions within Australian *Solanum*, and generate ideas about the speciation history of the group in light of its breeding-system evolution.

Materials and methods

Taxon sampling

Taxon sampling followed Martine *et al.* (2006) and focussed primarily on the 19 Australian species included in the section *Melongena sensu* Symon (1981), and on one new and one undescribed species. Although extractions were attempted for all 21 taxa, we were able to generate clean *trnK*–*matK* sequence data for just 14, including *Solanum asymmetriphyllum*, *S. carduiforme*, *S. chippendalei*, *S. cinereum*, *S. clarkiae*, *S. dioicum*, *S. diversiflorum*, *S. leopoldensis*, *S.* 'Longini', *S. melanospermum*, *S. oedipus*, *S. petraeum*, *S. sejunctum* and *S. tudununggae*. The sampling also included two related species from Africa (*S. aculeastrum* and *S. linnaeanum*), one from the Americas (*S. elaeagnifolium*) and two 'non-*Melongena*' Australian species (*S. hoplopetalum* and *S. stupefactum*). Authorities and higher-level taxonomy are given in Table 1. More than 250 field collections were made in May and June of 2004 during expeditions in the area of Kakadu National Park, Northern Territory (C. Martine, D. Symon, H. Toelken and K. Brennan) and much of the Kimberley region, from the area of Keep River National Park, Northern Territory, to the coastal edge of the Great Sandy Desert (C. Martine and W. R. Barker). The new collections mostly represented Australian taxa included by Symon (1981) in section *Melongena*. Field-collected specimens were supplemented with herbarium material on loan from the Plant Biodiversity Centre, Adelaide Botanic Gardens (AD). Vouchers are listed in Appendix 1.

Leaf samples from freshly collected field specimens were placed immediately in cetyltrimethylammonium bromide (CTAB) gel preservative (Rogstad 1992) in 30-mL high-density polyethylene (HDPE) bottles (Nalgene, Rochester, NY). Associated voucher specimens were annotated by D. Symon. Herbarium voucher specimens are deposited at CONN and AD, with regionally appropriate vouchers also submitted to PERTH and DNA (Darwin).

DNA amplification and sequencing

Total genomic DNA was extracted following the protocols described in Martine *et al.* (2006). Amplification and sequencing of the internal spacer (ITS) region of nuclear rDNA, including ITS1, the 5.8 S gene and ITS2 (Baldwin 1992), was previously reported by Martine *et al.* (2006). A subset of those sequences was used in the combined dataset.

PCR was used to amplify the complete maturase K gene (*matK*) and the 5' and 3' *trnK* introns, by using primer combinations listed in Appendix 2. At least five separate reactions were used for each accession, with each amplification

targeting 500–600-bp segments of the ~2500-bp region. Cycle sequencing was performed with the same sets of forward and reverse primers. PCR amplification reactions were split into two mixes. The first, which was made up of 15 µL, contained 5.0 µL of dH₂O, 2.0 µL of 50 mM MgCl₂, 2.5 µL 10×NH₄ buffer, 2.5 µL dimethyl sulfoxide (DMSO), 1.0 µL of each primer and 1.0 µL of DNA template. Tubes containing this mix were given a 'hot start' at 94°C for 2 min, after which each tube received the second mix of 10 µL consisting of 8.5 µL of dH₂O, 1.25 µL of deoxyribonucleotide triphosphates (dNTPs) and 0.25 µL of polymerase. The PCR amplification program for the *trnK*–*matK* region was then run as follows: a single denaturing step at 94°C for 2 min, followed by 30 cycles consisting of 94°C for 20 s, an annealing step at 52°C for 40 s and a 3-min extension at 68°C. Successful PCR amplifications were cleaned with QIAquick PCR purification kit columns (Qiagen, Valencia, CA) following the manufacturer's protocol. Forward- and reverse-sequencing reactions of the purified PCR templates were made up to 10 µL with 4 µL dH₂O, 2 µL Big Dye terminator mix (Applied Biosystems, Foster City, CA), 0.5 µL DMSO, 0.5 µL of the primer and 3 µL of the template. Sequence-cycling settings were 28 cycles of 94°C for 20 s, 50°C for 15 s and 60°C for 4 min.

Forward and reverse sequences were analysed with an ABI 3100 DNA automated sequencer (Applied Biosystems). Cleaned *trnK*–*matK* sequences were acquired from a total of 25 accessions, representing 19 currently recognised species. See Appendix 1 for GenBank accession numbers.

Sequence alignment

Sequences were edited and contigs assembled for each species with the Sequencher 2.1 computer program (Gene Codes, Ann Arbor, MI). Sequences were aligned manually with MacClade 4.0 (Maddison and Maddison 2000). All base changes or ambiguous sites were checked individually by comparing the alignments with the raw chromatograms used to assemble the contigs from Sequencher.

Phylogenetic analyses

To determine whether the ITS and *trnK*–*matK* datasets should be combined, partition-homogeneity tests were performed, with a threshold of 0.05. Unknown states, inapplicable states or gaps represented ~10% of the data matrix cells. Data were partitioned to permit analysis of each dataset separately, or in combination, including analyses where only the *matK* coding region was weighted. An equally weighted maximum parsimony analysis was performed with PAUP* version 4b10 (Swofford 2002) following the methods used in Martine *et al.* (2006). Starting trees from 500 random addition sequence replicates were swapped using TBR. For each random addition sequence replicate, 1000 trees were saved and swapped to completion using the 'nchuck' and 'chuckscore' commands in PAUP*. A maximum limit of 500 000 trees was set using the 'maxtrees' option. Additional heuristic searches also were conducted in which the coding region of *matK* was weighted in the following manner: (A) first positions: transitions (Ts)=1 step, transversions (Tv)=3 steps; (B) second positions: Ts=2, Tv=4; and (C) third positions: Ts=1, Tv=2.

The use of the andromonoecious *S. elaeagnifolium* of the New World as the outgroup taxon followed the recommendation of L. Bohs (pers. comm.), and is based on ongoing and published research by Bohs and colleagues (Levin *et al.* 2006; Weese and Bohs 2007) on the overall phylogeny of *Solanum* subgenus *Leptostemonum*.

The strict consensus topology obtained from the two datasets combined was used to compare different degrees of support for the branch representing a single origin of dioecy by forcing a topology that assigned a sister-group relationship to the two previously published clades of dioecious species (Martine *et al.* 2006). Branch support was assessed by bootstrap values obtained by branch and bound search (1000 replicates).

To provide a second estimate of support with a model-based approach, the datasets were also analysed separately and in combination with MrBayes ver. 3.0b (<http://mrbayes.csit.fsu.edu/>). MrModeltest (Nylander 2002) was used for model selection. A single run of 2 000 000 generations was performed under this modelling scenario, with a burn-in of 200 000 generations (2000 trees) discarded from the analysis. Each run consisted of three heated chains (with temperatures set to the default settings in MrBayes) and one cold chain. Samples were drawn from the cold chain every 100 generations. A majority-rule consensus was constructed with the 'sumt' command in MrBayes.

Results

The final dataset consisted of 3.1 kb of DNA sequence data. The resulting *trnK-matK* alignment was 2685 bp in length. Of these, 217 bp (8%) were excluded as trailing, leading or otherwise difficult to align regions. Of the remaining 2468 characters, 39 were parsimony informative. ITS sequences and alignments (669 bp) generated previously (Martine *et al.* 2006) were matched to the accession list used for the *matK* region and then combined with the *trnK-matK* data into an alignment of 3137 bp in length, of which a total of 111 characters were parsimony informative.

Parsimony analyses were run for each of the three molecular datasets, including ITS, *trnK-matK* and both combined. For the ITS dataset, parsimony generated 1997 equally short trees of 240 steps, with a consistency index (CI) of 0.6500, CI_{exc} (excluding uninformative characters) of 0.5130 and a retention index (RI) of 0.6693. These were combined in PAUP to yield a strict consensus topology. The unweighted *trnK-matK* dataset, when analysed alone, generated 2362 equally parsimonious trees of 167 steps in length ($CI=0.8430$, $CI_{exc}=0.6389$, $RI=0.7374$). In the analysis of *trnK-matK* and ITS combined, three equally shortest trees (414 steps) were recovered ($CI=0.7174$, $CI_{exc}=0.5410$, $RI=0.6686$) and used to estimate the strict consensus topology (Fig. 1). Weighted parsimony analyses, in which substitutions in the first, second and third codon positions in *matK* were weighted differently, also were run for *trnK-matK* and *trnK-matK*+ITS combined. Bootstrap support values generally were slightly higher with the weighting scheme.

Bayesian analyses were run on individual and combined datasets, and resulted in similar topologies. MrModeltest (Nylander 2002) selected the Jukes-Cantor model as the best fit for the data. Bayesian results for *trnK-matK*+ITS are shown in

Figs 2 and 3. Support for the branch representing the single origin of dioecy reached a maximum bootstrap of 50% in the parsimony analyses (for the combined, weighted DNA dataset). In the Bayesian analysis, the posterior probability for this same branch reached a maximum of 0.99 (DNA data combined). Bayesian posteriors are known occasionally to be inflated for short branches (Lewis *et al.* 2005); however, the support for this particular branch is credible, given the strength of the characters in the dataset. Although only 111 of 3137 characters were found to be parsimony informative, those characters are robust enough to generate congruent topologies, regardless of the data combinations or the phylogenetic criterion used (see Les *et al.* 1999).

Combined dataset

Partition-homogeneity tests for *trnK-matK* v. ITS (0.08) showed the datasets to be congruent, so the datasets were combined. The combined dataset (ITS plus *trnK-matK*) was analysed with weighted and unweighted parsimony. Each analysis generated the same two shortest MP trees of 690 steps. Overall, bootstrap support was higher for the weighted scheme than for the unweighted scheme.

The analyses combining the two datasets (Figs 1–3) recovered trees of better resolution than those published previously by Martine *et al.* (2006). Specifically, the present study recovered the same five main clades of dioecious and andromonoecious species as identified in the previous study. However, the addition of new sequence data now provides a reasonable degree of confidence in the relationships among those clades and, in particular, a sister group relationship for the two previously identified dioecious clades (Clades 1 and 5 in Martine *et al.* 2006).

These results support a common dioecious ancestry of the Kimberley species and the Kakadu-Alligator River species. Support for the branch leading to this larger clade increased when data were combined.

Clades 2, 3 and 4 of Martine *et al.* (2006), representing the remaining *Solanum* species considered by Symon (1981) to be the Australian members of subgenus *Leptostemonum* section *Melongena*, formed a larger monophyletic group of andromonoecious species of uncertain relationship to the dioecious clade.

The topologies generated by the parsimony and Bayesian analyses are congruent. The few differences between the two sets of topologies are reflected in overall stronger branch support in the Bayesian analyses.

Discussion

Relationships among the 20 Australian species of subgenus *Leptostemonum* section *Melongena* (Symon 1981) have been an issue since the group was first recognised formally (Whalen 1984; D. E. Symon, pers. comm.). The addition of a new molecular dataset in the present study has improved the resolution of previously published topologies that used ITS data only (Martine *et al.* 2006). In so doing, the present analyses have facilitated a systematic understanding of this problematic group, and shed further light on the patterns associated with the distribution and evolution of dioecy in *Solanum*.

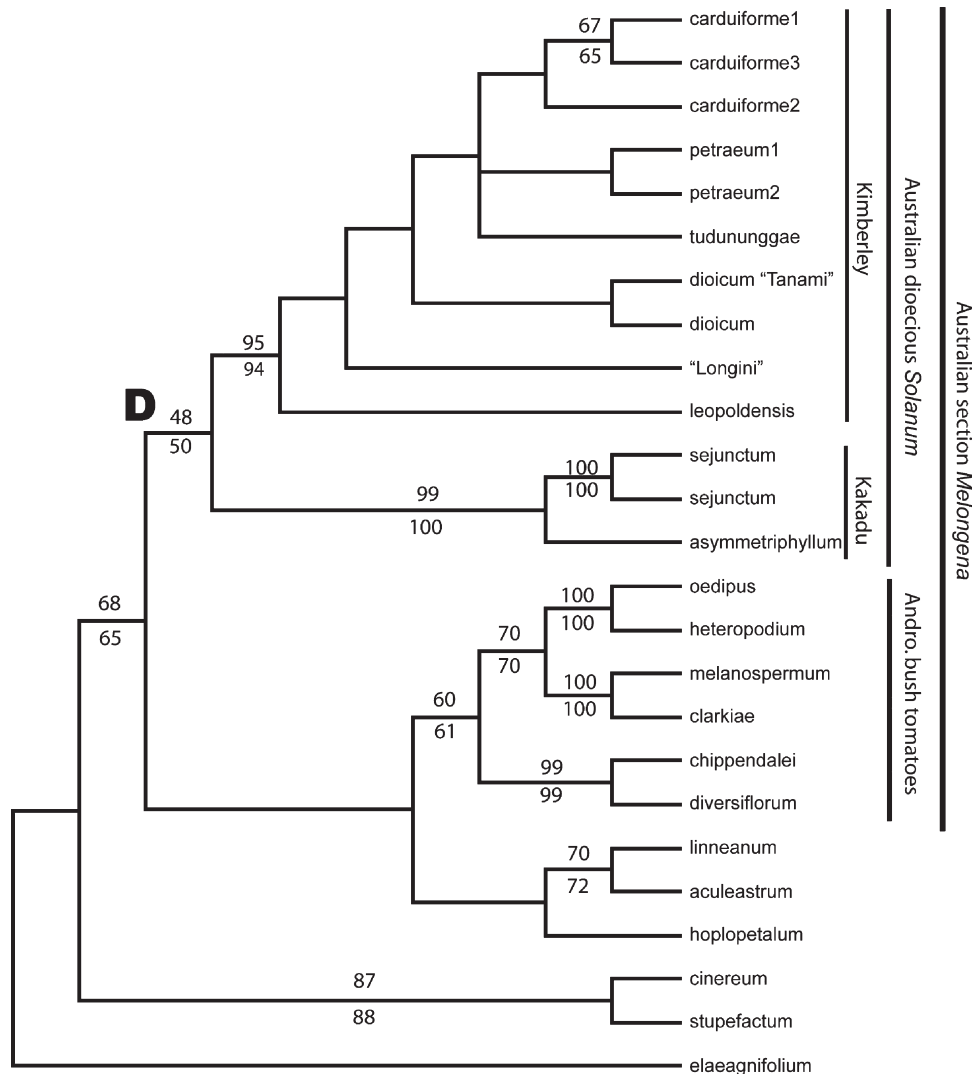


Fig. 1. ITS+trnK-matK strict consensus of the three most parsimonious (414 steps) maximum-parsimony trees. Numbers above branches are bootstrap support values (1000 replicates) when matK coding regions were unweighted. Numbers below branches are bootstrap values (1000 replicates) with coding regions weighted. Bootstraps are not shown where values are less than 50%. 'D' marks the branch where a single origin of dioecy is hypothesised to have arisen.

Monophyly of section *Melongena* sensu *Symon*

The monophyly of Australian species of the subgenus *Leptostemonum* section *Melongena* (Symon 1981) remains unclear and is likely to require a large-scale phylogeny, including most Old World spiny *Solanum*, for clarification. Similarly, additional taxon sampling is needed to compare Symon's classification adequately to the arrangement proposed by Whalen (1984; Table 1), who placed most of the same Australian species in his *dioicum* group. The broad sampling of the subgenus used in Levin *et al.* (2006), although sufficient to resolve several higher-level relationships in the New World taxa, resulted in a polytomy for Old World species. This unresolved set of relationships is consistent with the findings of the present study as well as with previous inferences on the relationships among African and Australian spiny solanums (Symon 1981; Whalen

1984; Jaeger 1985; Anderson *et al.* 2006a; Levin *et al.* 2006; Martine *et al.* 2006) and is likely a reflection of recent diversification within the Old World Clade. The present study shows no support for a monophyletic Australian section *Melongena sensu* Symon, mostly owing to the inclusion of African species (Whalen's (1984) *incanum* group) and the Australian *S. hoplopetalum* (Whalen's *hystrix* group) (see Table 1).

Support for and relationships among the five clades

In a phylogenetic analysis of ITS data, Martine *et al.* (2006) concluded that the 20 species included by Symon in the Australian section *Melongena* comprise five distinct clades, including two (Clades 1 and 5) consisting of dioecious species and three (Clades 2, 3 and 4) of andromonoecious species. However, the ITS data did not resolve the relationships among those five clades.

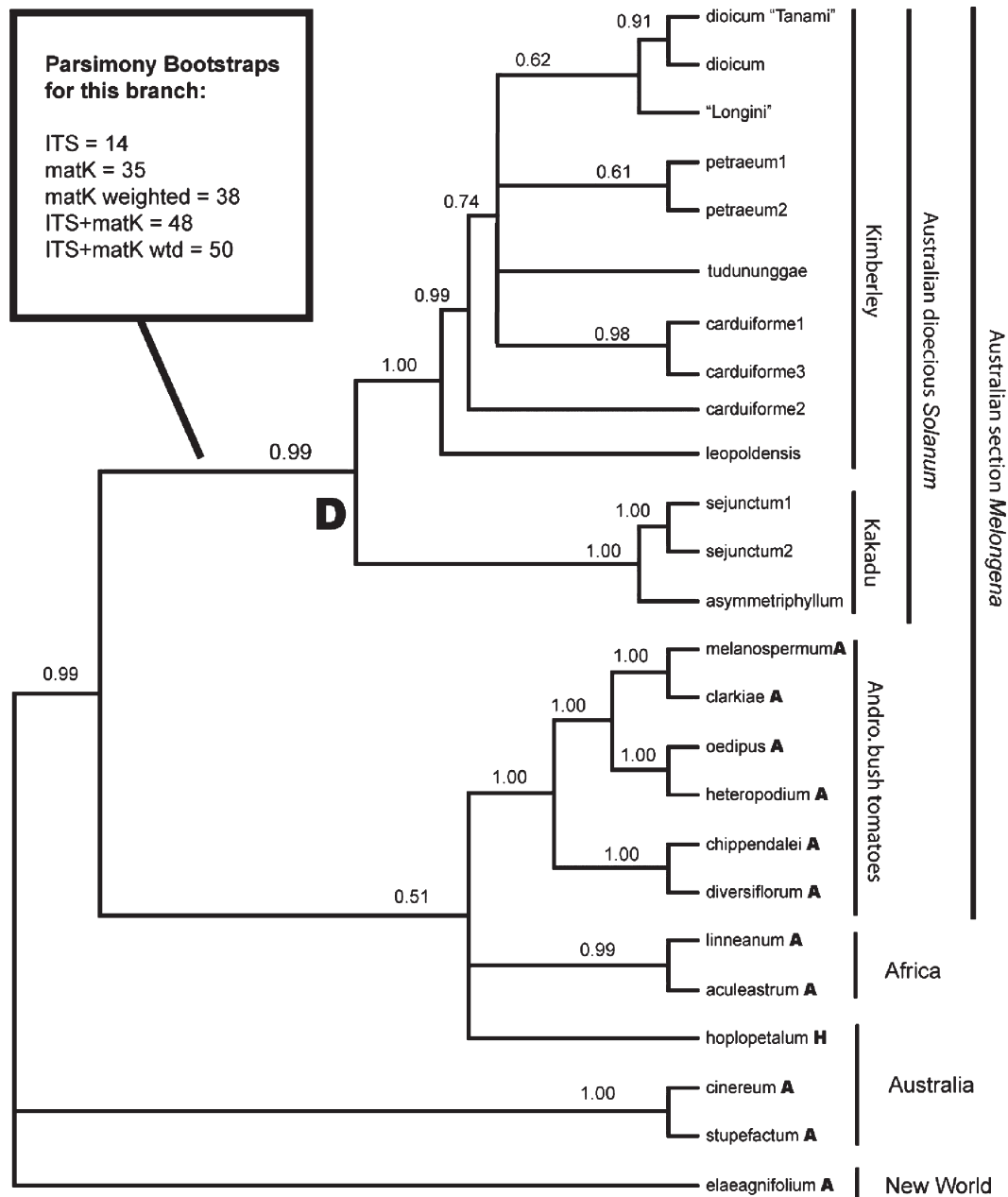


Fig. 2. Tree resulting from the Bayesian analysis of ITS+trnK-matK data, from a run of 2 000 000 generations with a burn-in of 2000 trees. Support values above branches are Bayesian posterior probabilities. Parsimony analyses for ITS+trnK-matK recovered the same topology. Parsimony bootstrap supports (1000 replicates) for the monophyly of dioecious Australian species are shown. 'D' marks the branch where a single origin of dioecy is hypothesised to have arisen. (A)=andromonoecious, (H)=hermaphrodite.

We found the same poor resolution of relationships among the five clades when the *trnK-matK* and ITS datasets were analysed individually, and improved resolution when they were analysed together. The increased resolution of these relationships brings with it several interesting inferences, the most notable of these being a well-resolved clade consisting of the andromonoecious species of section *Melongena sensu* Symon (Clades 2, 3 and 4 of Martine *et al.* 2006) and a sister relationship between the two dioecious clades (Clades 1 and 5 of Martine *et al.* 2006).

Andromonoecious bush tomatoes (expanded) – ((Clade 3, 'Bush Tomatoes': *S. chippendalei*, *S. diversiflorum*, *S. beagleholei*, *S. phlomoides*, *S. eburneum*) + (Clade 2: *S. oedipus* and *S. heteropodium* + Clade 4: *S. melanospermum* and *S. clarkiae*))

On the basis of ITS data alone, Martine *et al.* (2006) recognised three clades among the above Australian andromonoecious species. Here, those three clades make up a

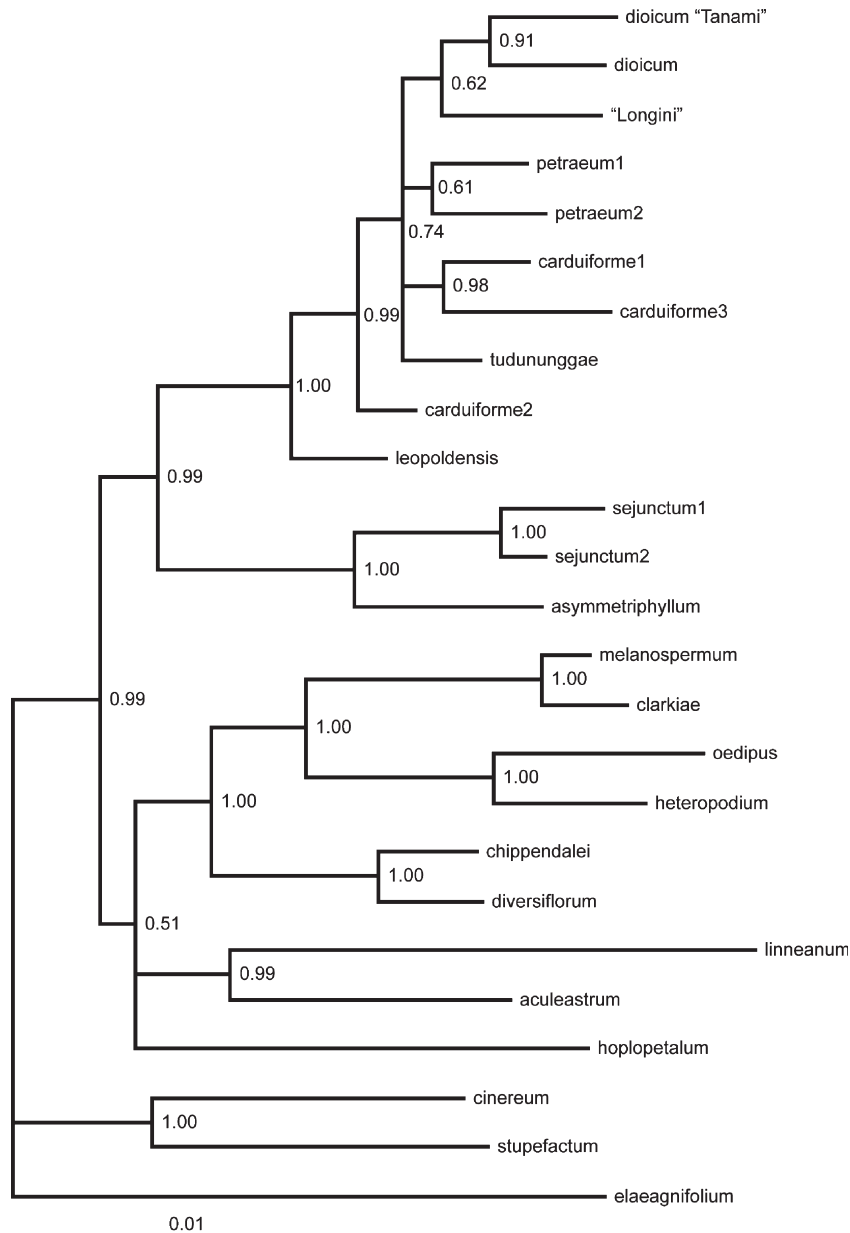


Fig. 3. Phylogram resulting from the Bayesian analysis of ITS+trnK-matK data, from a run of 2 000 000 generations with a burn-in of 2000 trees. Support values at nodes are Bayesian posterior probabilities. Parsimony analyses for ITS+trnK-matK recovered the same topology. See Fig. 2 for full details of the Bayesian results.

single monophyletic group. This result provides further evidence that Symon (1981) correctly concluded that these andromonoecious species belonged to the same taxonomic group. However, the close alliance with the cultivated eggplant and African relatives that Symon (1981) and D'Arcy (1979) proposed will remain unclear until a comprehensive phylogeny of Old World spiny *Solanum* is generated. The results of the present study show a close relationship among *S. clarkiae* (Kakadu region), *S. oedipus* (northern coastal Kimberley), *S. melanospermum* (north-western Queensland) and *S. heteropodium* (northern coastal Kimberley), a clade of

four species sharing long styles (13–20 mm) and corollas of 3–5 cm. This clade is sister to the typical bush tomatoes (represented in the combined analyses by *S. chippendalei* and *S. diversiflorum*), a clade of five species with shorter styles (10–12 mm), corollas of 3–5 cm and generally larger and fleshier fruits. Some topologies show weak support for a sister relationship between an expanded version of this larger andromonoecious clade and the Australian dioecious solanums. This taxonomic arrangement would inspire confidence in a single Australian transition from andromonoecy to dioecy. However, both parsimony and Bayesian trees place two andromonoecious

African species (*S. linnaeanum* and *S. aculeastrum*) plus a hermaphroditic species from a separate taxonomic section (section *Oliganthes sensu* Symon (1981); 'hystrix group' *sensu* Whalen (1984)) within a clade that also includes the Australian members of the section *Melongena*, as circumscribed by Symon. These results correspond with the previously published ITS topologies and offer further evidence that the Australian section *Melongena* as circumscribed by Symon (1981), is not monophyletic.

Australian dioecious Solanum – ((Clade 1, 'Kimberley Clade': *S. dioicum*, *S. cunninghamii*, *S. tudununggae*, *S. 'longini'*, *S. petraeum*, *S. cataphractum*, *S. carduiforme*, *S. leopoldensis*) + (Clade 5, 'Kakadu–Alligator River Clade': *S. asymmetriphyllum*, *S. sejunctum*))

Support for this clade, and for a single origin of dioecy in Australian *Solanum*, increased when the data were combined. It is reasonable to assume that subsequent additions of data would continue to improve branch support, as long as the datasets are congruent (as per Les *et al.* 1999). The improved branch support with additional data is likely because the two datasets used here (3137 characters) supply only 111 parsimony-informative characters. The contribution of new molecular datasets, particularly the WAXY gene, is currently being explored (L. Bohs).

The combined datasets also improved branch support for each of the previously identified dioecious clades and the relationships within them. The increase in resolution is especially relevant to the 'Kimberley Clade', which was previously supported only weakly (Martine *et al.* 2006) whereas here it was resolved with significantly higher support (e.g. maximum parsimony bootstrap (MP BS)=94, Bayesian posterior probability (pp.)=1.00; Figs 1, 2). Improved internal resolution also occurred within the 'dioicum complex' (including the placement of *S. leopoldensis* as sister to the rest of the Kimberley species) where an unresolved polytomy dominated the clade in previous work. *S. leopoldensis* is somewhat divergent from the rest of the complex, a point emphasised by Whalen's (1984) placement of this species in an entirely different group.

Evolution and distribution of dioecy

The most significant aspect of the present results is that they may provide support for the hypothesis that radiation of dioecious Australian *Solanum* followed a single origin. The evolutionary history and distribution of breeding systems in the group might be best considered as a three-step process, including the following: (1) the origin of dioecy in the common ancestor of the clade from within an andromonoecious lineage in Australia; (2) the geographic split within the dioecious lineage, leading to the establishment of one clade in the area of Kakadu–Alligator River and one clade concentrated in the Kimberley region; and (3) a radiation of new species in the Kimberley group. Each stage is addressed below within the context of a plausible evolutionary history for the group.

(1) Single origin of dioecy

Each of the consensus topologies generated with combined datasets recovered a sister relationship between the two dioecious

subclades. Furthermore, branch support for a combined dioecious clade improved as data were added, an indication that this is likely to be a true relationship. An andromonoecious progenitor to the dioecious clade is likely, given the phylogenetic topologies recovered here, and inferences provided by previous studies identifying andromonoecy as the plesiomorphic breeding system in many groups within the subgenus *Leptostemonum* (Symon 1981; Whalen 1984; Hunziker 2001; Levin *et al.* 2006).

The transition from andromonoecy to dioecy presumably has evolved to avoid inbreeding in this group (Whalen and Anderson 1981; Anderson and Stebbins 1984; Anderson and Symon 1989) and a further enforcement of outcrossing may have been the impetus for the origin of separate sexes in Australian *Solanum*. If this transition occurred from andromonoecy, as proposed here, one could conclude that the andromonoecious ancestor was affected negatively by inbreeding (see Holsinger 1993, 2000), and that those deleterious effects were severe enough for dioecy to succeed, at least in the short term. For a more detailed discussion of the transition from andromonoecy to dioecy in Australian *Solanum*, see Martine and Anderson (2007).

(2) Establishment of two lineages

The phylogenies based on the combined data show the split between the two main dioecious lineages. This deep bifurcation is strengthened by the significant number of synapomorphies, both molecular and morphological, that occur on the branch leading to the Kakadu–Alligator River Clade. The morphological differences between the two lineages are obvious and led Symon (1981) to note that *S. asymmetriphyllum* (at the time, the only dioecious species described from the Kakadu region) did not appear to be closely related to any of the other dioecious species. Placing the morphological characters on the combined data tree further supports this conclusion by showing that several reproductive and vegetative characters not present in the Kimberley Clade are shared by the two species, *S. asymmetriphyllum* and *S. sejunctum* (the recently described species of Brennan *et al.* (2006)), endemic to the Kakadu region. Among these characters are a sigmoid style, a scantily armed female calyx and lack of prickles on leaves and male calyces. Growth form also is quite different in the Kakadu species, with each rather woody ramet growing stiffly upright and often not branching for at least the first metre of growth. The conspicuous differences between the two lineages may be evidence of an early split, and they probably illustrate the geographic isolation of the Kakadu Clade in a small region of the upper Northern Territory. At least one of the species, *S. sejunctum*, occurs in relict rainforest habitats (Brennan *et al.* 2006), which may point to a formerly wide distribution for the clade before the shift to a subarid monsoonal climate that occurred in much of northern Australia about 3 million years ago (White 1990; Crisp *et al.* 2004). The more extensive former distribution of the rain forests (and its *Solanum* flora) may have been wide enough to bridge the large geographic gap that now exists between the Kakadu species and the dioecious solanums in the Kimberley lineage. Subsequently, the two dioecious species of the Kakadu region may have evolved in isolation within the refugia of the formerly widespread rainforest. This conjecture is supported by the number of uniquely shared characters of the Kakadu Clade. Climatic and geologic dynamics

also may have played a role in the establishment of genetic and morphological differentiation in the Kimberley lineage (see below).

(3) Radiation in the Kimberley lineage

Solanums of subgenus *Leptostemonum* are likely to have arrived in northern Australia by a combination of island-hopping and cross-continental movement on the tail-end of a long-term expansion of the lineage which began in the Neotropics, extended into sub-Saharan Africa, and then moved around the Indian Ocean Basin and south into Australia (Whalen 1984; Olmstead and Palmer 1997). The movement of taxa from a centre of origin (outside of Australia), followed by subsequent dispersal into Australia, is considered important in the Australian flora, with colonisation, adaptation and divergence following the relatively recent arrivals of several groups to the arid zone (Barker and Greenslade 1982). In fact, the recent re-evaluation of the divergence times by using molecular data provides strong support for long-distance dispersal (Givnish and Renner 2004; de Queiroz 2005; McGlone 2005; Cowie and Holland 2006) as being more important than post-Gondwanan vicariance in influencing distributions of Old World groups in the Indian Ocean Basin, including north-south migrations of plant groups into Australia (Crisp *et al.* 2004; Bartish *et al.* 2005; Cook and Crisp 2005; Yuan *et al.* 2005).

The trend towards postdispersal adaptation and divergence is pronounced in the northern Kimberley, where several endemic and uncommon species of *Solanum* are known from small populations with limited ranges. Examples include *S. cataphractum*, a dioecious species known only from a few collections along the coastline near Vansittart Bay (Symon 1981), and *S. tudumunggae*, a dioecious species found in a handful of small populations over quartz on the floodplain of the King James Edward River around Kalumburu (Symon 1981; C. T. Martine, pers. obs.). Apparently, the continual divergence of lineages has occurred historically in this part of Australia, given that the north Kimberley bioregion has been identified as an Australian Biodiversity 'hotspot', partly on the basis of its species richness and a significant presence of threatened, endangered and endemic species (Department of the Environment and Heritage 2004).

The current distributions of dioecious *Solanum* in the northern part of the Kimberley Plateau and elsewhere in northern Australia can be explained by the biogeographic framework proposed by several recent studies focusing on patterns of endemism in the continental flora. In total, 2 of the 11 recognised centres of endemism in Australia (Crisp *et al.* 2001; Laffan and Crisp 2003) encompass areas in which many of the dioecious species occur. These two centres, like all of the major centres of endemism recognised by Crisp *et al.* (2001), are near-coastal.

Coastal proximity is likely to be a consequence of climatic shifts that occurred during the Pliocene and Pleistocene periods during the course of the last 5 million years. During the early Pliocene, much of northern Australia had a stable, mild wet climate (Dodson and MacPhail 2004), a situation believed to have supported not only the evolution of floral diversity in general, but a higher rate of endemism as well (Crisp *et al.* 2001). The current system of extreme wet-dry glacial cycles was established during the mid-Pliocene (2.9 million years ago;

Crisp *et al.* 2001). This seasonally wet condition was largely uninterrupted until ~18 000 years ago, when the last polar glacial maximum expanded the Australian central desert well beyond its current boundaries in all directions (White 1990; Crisp *et al.* 2001). As the desert edges crept outward, widespread species probably retreated to edges and perhaps refugia deep in the mountain ranges, whereas narrow endemics likely became extinct (Crisp *et al.* 2001).

Today, the present centres of endemism on the continent lie outside of the expanded arid zone present during the last glacial maximum. In the Kimberley and the northern edge of the Northern Territory, the narrow coastal zone where nearly all of the dioecious and narrowly endemic *Solanum* species occur corresponds with the regional edge of this expanded desert. Thus, the distribution of these endemics illustrates the divergence of species that survived in refugia of a northern tropical zone that remained stable even as the southern latitudes dried out (Nix and Kalma 1972; White 1990).

The dioecious species in Australia that do not appear to be near-coastal relicts are *S. leopoldensis* and *S. carduiforme* (both inland endemics), and a triplet of similar species made up of *S. dioicum*, *S. cunninghamii*, and *S. petraeum*. Both *S. leopoldensis* and *S. carduiforme* likely were more widespread before the widening of the arid zone and each currently is extant in habitats that probably were refugia 18 000 years ago. *Solanum leopoldensis* is now known from two regions, the King Leopold Ranges and the Buccaneer Archipelago, where it is limited to gullies and rocky slopes. Crisp *et al.* (2001) suggested that endemism in Australia is likely to be linked with topographic relief due to habitat variability and availability of specialised sites like gullies, slopes, and peaks. The present disjunct range of *S. leopoldensis* offers evidence for a previously continuous distribution that may have been disrupted by the onset of desertification, forcing the retreat of the species to the upland refugia it occupies today. A similar hypothesis might also apply to *S. carduiforme*. This species currently is known from just four widely disjunct locations (Symon 1981; Martine *et al.* 2006): Purnululu National Park in Western Australia (which includes the Bungle Bungle Range), Keep River National Park in western Northern Territory, and two areas of northern interior Queensland. At least in Western Australia (Bungle Bungles) and the western Northern Territory (Keep River), *S. carduiforme* occurs in habitats considered to be remnants of ancient tropical forests, a conclusion supported by the co-occurrence of palm trees in each of these sites. These refugial oases, nestled amongst beehive-shaped Devonian sandstone deposits, may have served the same function for *S. carduiforme* as did the upland gullies for *S. leopoldensis*.

The group of species made up of *S. dioicum*, *S. petraeum*, and *S. cunninghamii* are set apart from the rest of Australian dioecious *Solanum* by their high morphological similarity to one another and more widespread distributions (D. Symon [pers. comm.] considers them intergrading). All three species have fairly extensive ranges (relative to other dioecious species), particularly *S. dioicum*, which occurs throughout the Kimberley region, and *S. cunninghamii*, which is found from the south-west Kimberley and west along the coast into the north-eastern Pilbarra region. These species likely were not narrow endemics before

desert expansion and may have retreated from the expanding arid zone and then moved back into the southern Kimberley as the wet–dry sub-arid cycle was re-established. Like many spiny solanums, these species are able to colonise recently disturbed sites (Symon 1981; Whalen 1984). *Solanum dioicum* and *S. cunninghamii* also appear to be rather well adapted to survival during the dry season and may have become further adapted to these conditions during the post-glacial period.

Wheeler and Byrne (2006) suggested that significant climate changes in south-western Australia between the late Pliocene (~3–5 myr bp) and mid-late Pleistocene (~1 myr bp) led to fragmentation and divergence of *Eucalyptus* lineages. The resultant divergences are still detectable today even in cases where secondary contact has been made (Wheeler and Byrne 2006). The *S. dioicum*-*S. cunninghamii*-*S. petraeum* group may have a similar history of divergence during the glacial period, with subsequent contact leading to the apparently intergrading populations seen in the present day.

(4) Isolation and speciation

Although a general pattern of colonisation, adaptation and divergence in Australian dioecious *Solanum* might not be surprising, given the recent climatic history of northern Australia and the nature of spiny solanums, it does contradict theory regarding the likelihood for dioecious lineages to persist and/or speciate. A recent survey by Vamosi and Vamosi (2005) supports previous conclusions that dioecious lineages are not likely to exhibit higher speciation rates. This generalisation is based on observations that dioecious species generally have a lower species richness than do non-dioecious clades (Baker 1959; Heilbuth 2000). Furthermore, dioecious plants are obligate outcrossers and such breeding systems generally exhibit higher gene flow and, thus, reduced levels of interpopulation diversity (Hamrick and Godt 1996; Loveless *et al.* 1998). A lower likelihood of speciation, when coupled with a potentially higher risk of extinction owing to reduced mate assurance (Pannell and Barrett 1998), the ‘seed shadow handicap’ (Heilbuth *et al.* 2001), and the reliance on large and unreliable pollinator pools (Vamosi and Otto 2002), may lead to clades with a reduced species richness relative to related co-sexual clades (Vamosi and Vamosi 2005).

The solanums of northern Australia may not fit the typical profile of dioecious species for several reasons, the most important being the intimate link between the spread of the lineage and landscape history. The Kimberley lineage, in taking advantage of both the stable early Pliocene climate and a landscape rife with microhabitats produced by erosion, may have simply spread and diverged so quickly that the resultant lineages avoided the pitfalls common to many other dioecious clades. A key species could have been *S. dioicum*, the most widespread of the dioecious Kimberley species. That species occurs across much of the Kimberley region and is best described as a habitat generalist, given that it exploits everything from sand plains and alluvial flats at the bases of rock masses to hummock grasslands and open woodlands (Symon 1981).

Theoretically, for a dioecious species to disperse and colonise it must have an advantage in its ability to persist and spread (Heilbuth *et al.* 2001). *S. dioicum*, like many nightshades, often inhabits disturbed habitats, exploiting areas of reduced

competition through clonal spread and perennial persistence. Its wide adaptability may have enabled peripheral isolates to have arisen and later speciated (and become endemic) through local adaptation and divergence. The isolation of populations along the range of this widely distributed species (or its ancestral forms) would explain the number of endemic sibling species documented (e.g. *S. tudununggae* and *S. cataphractum*) as well as the polytomous pattern of relationships seen for *S. dioicum* and close relatives in the topologies presented herein. A similar role in the advent of peripheral isolation has been proposed for *S. caripense* Dunal, a variable and widespread species in South America from which several new species have diverged in the montane valleys of the Peruvian Andes (Anderson *et al.* 2006c).

Solanum dioicum, like *S. caripense*, is quite variable, and Symon (1981) found that the species intergrades in parts of its range with *S. cunninghamii*, *S. petraeum* and a handful of distinctive forms such as *S. dioicum* ‘Tanami’ of the sandy plains on the eastern edge of the Kimberley. Apparently, divergence and speciation is ongoing in the Kimberley solanums, and could blur the ‘typical’ pattern expected for dioecious lineages (as in Vamosi and Vamosi 2005). Likewise, Whalen (1984) cited the distributions of several spiny *Solanum* species in montane habitats throughout South America as evidence that *Solanum* subgenus *Leptostemonum* is especially prone to local speciation in parapatry and even in sympatry.

Conservation implications

It is possible that the Australian dioecious solanums have not yet produced the expected pattern of extinction because their radiation was recent (and in some cases, continuing). This conclusion is feasible given the polytomous branching pattern seen in the *S. dioicum* complex. Similarly, an unresolved pattern of relationships among clades of Australian species of *Pultenaea* (Fabaceae) was considered as evidence of a rapid and recent radiation (Orthia *et al.* 2005). It also is possible that *Solanum* species that arose when climate conditions were more stable and competition was less severe are now gradually losing ground to the perils associated with dioecy. There is some qualitative evidence in support of this possibility. A visit by some of us (C. Martine and W. R. Barker) to one of the few known populations of *S. tudununggae* in June 2004 found there to be no seedling recruitment and just one fertile female individual, which bore a single malformed fruit.

Indeed, the biological limitations of dioecy might account for the decline of some species, although there also are potential human-induced changes to be considered. There is mounting evidence for the continuing loss of species and disruption of community assemblages throughout the Kimberley, owing to changing fire regimes, feral animals, exotic weeds and grazing pressures (Kimberley Natural Resource Management Plan 2004). It is not hard to imagine that these anthropogenic effects might be particularly detrimental to dioecious species (e.g. Percy and Cronk 1997), which probably are more susceptible to fluctuations in pollinator communities (Farwig *et al.* 2004) as well as to competition from co-sexual species where every plant can bear fruit and produce new recruits (Wilson and Harder 2003). Only one dioecious *Solanum* (*S. leopoldensis*) is considered a species

belonging to priority-listed flora in the most recent draft of the Kimberley Natural Resource Management Plan (2004). Because of their limited distributions and the factors described above, it also would be prudent to list *S. carduiforme*, *S. cataphractum*, *S. tudununggae* and *S. vansittartensis*. *Solanum* 'Longini,' a taxon yet to be described because of limited collections, is another candidate for listing.

Additional natural-history surveys need to be conducted in the Kimberley and Kakadu regions to establish better the distributions and estimated population of rare and/or endemic solanums, as well as to expand on the few studies that have attempted to determine their pollinators (e.g. Symon 1979a; Anderson and Symon 1988) and seed-dispersal mechanisms (Symon 1979b). Little is known about the specific insect species in the northern Australian *Solanum* pollinator fauna, although Anderson and Symon (1988) did establish that the flowers are mainly visited by four genera of pollen-foraging bees (*Nomia*, *Trigona*, *Amegilla* and *Xylocopa*). Even less is known about seed dispersal, with the whole of our knowledge having been reviewed in a paper by Symon (1979b) who, with a few intriguing exceptions, established biotic dispersal as the main mechanism for seed movement in Kimberley *Solanum*. Unfortunately, animal dispersal has never been confirmed in this group, although rock wallabies have been proposed as a possibility (Martine and Anderson 2007; K. Brennan, pers. comm.) and *Solanum* seeds have been found in rock wallaby scat.

A general dearth of natural-history information exists for Australia's dioecious solanums. Although extensive collections have been made for a few species (e.g. *S. dioicum*), several species are known from only a few collections (e.g. *S. cataphractum* and the newly described *S. sejunctum*). It is highly likely that additional new species (or at least local variants) are yet to be found. Many collections, and thus much distributional data, are made from populations at easily accessible sites near access roads, trails and natural landmarks. For better or worse, access to many difficult areas in the Kimberley is improving and should lead to exciting new discoveries related to both the flora and fauna.

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Appendix 1. List of herbarium vouchers for specimens used for DNA extraction and amplification of the *trnK*–*matK* region, plus GenBank accession codes [in brackets]

DNA codes beginning with ‘C’ are deposited at CONN. DNA codes beginning with ‘AU’ are from sheets held at AD and loaned to CTM at CONN. See Martine *et al.* (2006) for voucher information for ITS sequences

Taxon (CTM DNA#)	Voucher information
<i>S. aculeastrum</i> (C110)	US: UConn EEB greenhouses, <i>Martine</i> 637 4.ii.2004, CONN [EU983573]
<i>S. asymmetriphyllum</i> (C098)	NT: Kakadu NP, E. Alligator R., <i>Martine</i> 723 18.v.2004, CONN [EU983570]
<i>S. carduiforme</i> -1 (C062)	NT: Keep River, Jarrmarm Escarp., <i>Martine</i> 773 25.v.2004, CONN [EU983556]
<i>S. carduiforme</i> -2 (AU10)	QL: Lawn Hill Gorge, <i>Symon</i> 15790 AD [EU983558]
<i>S. carduiforme</i> -3 (AU41)	WA: Bungle Bungles, <i>Kenneally s.n.</i> AD [EU983557]
<i>S. chippendalei</i> (AU13)	QL: Thornton Station, <i>Symon</i> 15796 AD [EU983562]
<i>S. cinereum</i> (AU14)	SA: Broadview Gorge, <i>Bates</i> 49377 AD [EU983575]
<i>S. clarkiae</i> (C135)	NT: Kakadu, Koongara Saddle, <i>Martine</i> 734 19.v.2004, CONN [EU983564]
<i>S. dioicum</i> (C014)	WA: Great Northern Hwy, <i>Martine</i> 879A 14.vi.2004, CONN [EU983553]
<i>S. dioicum</i> ‘Tanami’ (C044)	NT: Keep River NP, <i>Martine</i> 772 25.v.2004, CONN [EU983554]
<i>S. diversiflorum</i> (AU7)	WA: Pilbarra, <i>Mollemans</i> 2448 AD [EU983563]
<i>S. elaeagnifolium</i> (C140)	US: LA, Bossier Parish, <i>Thomas and Dorris</i> 96112, CONN [EU983576]
<i>S. heteropodium</i> (AU76)	WA: Wilson’s Head, <i>Mitchell</i> 3506 AD [EU983567]
<i>S. hoplopetalum</i> (AU125)	WA: Northam, <i>Dodd</i> 196 AD [EU983571]
<i>S. leopoldensis</i> (C022)	WA: Lennard River Gorge, <i>Martine</i> 860 10.vi.2004, CONN [EU983560]
<i>S. linnaeanum</i> (HER)	US: UConn EEB greenhouses, <i>Martine</i> 628 1.x.2003, CONN [EU983574]
<i>S. melanospermum</i> (AU80)	Northern Territory, <i>Muir</i> 5007 AD [EU983565]
<i>S. oedipus</i> (C142)	WA: Longini Landing, <i>Martine</i> 814B 2.vi.2004, CONN [EU983566]
<i>S. petraeum</i> (AU4)	WA: Bachsten Creek, <i>Handasyde</i> TH01/176b AD [EU983559]
<i>S. petraeum</i> (C007)	WA: Beverley Springs, <i>Martine</i> 857 10.vi.2004, CONN [EU983555]
<i>S. sejunctum</i> -1 (C046)	NT: Baroalba Creek, <i>Martine</i> 735 19.v.2004, CONN [EU983568]
<i>S. sejunctum</i> -2 (C102)	NT: Koongara Saddle, <i>Martine</i> 730 19.v.2004, CONN [EU983569]
<i>S. stupefactum</i> (AU21)	QL: Mt Binga, <i>Bean</i> 16060 AD [EU983572]
<i>S. tudunggae</i> (C034)	WA: Kalumburu, <i>Martine</i> 826 3.vi.2004, CONN [EU983552]
<i>S.</i> ‘Longini’ (C077)	WA: Longini Landing, <i>Martine</i> 805 1.vi.2004, CONN [EU983561]

Appendix 2. Primers used to amplify the *trnK*–*matK* region

All primer dilutions made from stock held in the Les Laboratory, Department of Ecology and Evolutionary Biology, University of Connecticut. Primers designed by Don Les (DL codes) or Nic Tippery (NT codes), unless noted otherwise

Les code	Location	Sequence
DL15 ^A	0822F	GGATCCTTTCATGCATT
DL23	0068F	TCTTTCAGGAGTATATTTATG
DL16	1175R	AATGGGATGCCCTAATAC
DL18	<i>trnK-F</i> (original 5')	GCTAACTCAATGGTAGAGTACTCGG
DL19	<i>trnK-F</i> (modified 5')	AGTACTCGGCTTTTAAGTGC
NT001	0504F	AAGAAACAGTATATTGATAA
NT002	1097F	GGATTTTCAGTCRTTATGGA
NT003	1749F	GTATGTGAATACGAATCCATC
NT005	1966R	CCGCTATGATAATGAGAAAAGA
NT006	1556R	CCTTGATACCTAACATAATGC
NT007 ^B	1F	ACTGTATCGCACTATGTATCA

^AJohnson and Soltis (1994), designed by Kelly Steele.

^BBremer *et al.* (2002).