

# Phylogeny and classification of *Eucalyptus* subgenus *Eudesmia* (Myrtaceae) based on nuclear ribosomal DNA, chloroplast DNA and morphology

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**Abstract.** Phylogenetic analysis of *Eucalyptus* subgenus *Eudesmia* is presented on the basis of the following three datasets: sequences of the internal transcribed spacer (ITS) and the external transcribed spacer (ETS) regions from nuclear rDNA, sequences of the *psbA-trnH* intergenic spacer region from chloroplast DNA, and morphological characters, including stamen bundling, operculum development, seeds and trichomes. Studies of floral development were essential for understanding the morphology of mature flowers and interpretation of synapomorphy and homoplasy. A summary phylogeny was constructed from a maximum parsimony analysis of those nodes coded as characters that had support in the molecular trees together with morphological characters. A revised infra-subgeneric classification is presented on the basis of the summary phylogeny, and compared with classifications of Hill and Johnson (1998) and Brooker (2000). Differences relate to relationships between clades and taxonomic rank (sections, series and subseries) and valid names of Brooker (2000) are conserved where possible. One main clade of 14 species (section *Limbatae*), many of mallee growth form, was found in all analyses; this clade is distributed in the South-West of Western Australia and adjacent Interzone and desert areas. A second main clade (section *Complanatae*) occurs in the northern and eastern tropical and subtropical regions of Australia, including Kimberley, Arnhem, Queensland and New South Wales. This section includes *E. tetradonta*, previously treated as an isolated taxon in a monotypic section; however, this species is related to *E. baileyana*, *E. similis*, *E. lirata* and series *Miniatae*. The hypothesised phylogeny provides a framework for further analyses of biogeography and ecology, including functional traits.

## Introduction

Classification of the eucalypts (Myrtaceae) has had a long history from the early writings of R. Brown, G. Bentham and F. von Mueller in the 19th century to the subsequent works of J. H. Maiden, W. T. Blakely, L. D. Pryor, L. A. S. Johnson and M. I. H. Brooker (see summary in Brooker 2000). In recent years, molecular phylogenetic studies have identified relationships and monophyletic groups, in particular confirming the monophyly of *Eucalyptus sensu stricto* (e.g. Ladiges *et al.* 1995; Udovicic *et al.* 1995; Sale *et al.* 1996; Steane *et al.* 1999; Udovicic and Ladiges 2000). Despite differences of opinion of generic limits, both Hill and Johnson (1998) and Brooker (2000) recognised similar groups, including *Eucalyptus* subgenus *Eudesmia* (R.Br.) L.A.S.Johnson & K.D.Hill.

*Eudesmia* currently includes 26 species and subspecies (Hill and Johnson 1998; Table 1) distributed across tropical and temperate regions of Western Australia, Northern Territory, Queensland and central arid deserts of Western Australia and South Australia (Fig. 1). *Eudesmia* is a heterogeneous group of trees and mallees, with a range of morphological variation, including bark, flowers, fruit, seed, seedlings and adult foliage (some are neotenous). Seedlings have distinctive trichomes, hairs that radiate from raised oil glands (termed rE trichomes), a character considered a synapomorphy for the subgenus

(Ladiges 1984), although *E. gamophylla* and *E. odontocarpa* lack trichomes, which has been interpreted as a secondary loss (Hill and Johnson 1998).

A combination of other morphological characters also supports the monophyly of subgenus *Eudesmia*. All eudesmids have grey-black seeds, with a ventral hilum and double seed coat (Gaub and Pryor 1959, 1961; Boland *et al.* 1980). All species have a corolline operculum (Drinnan and Ladiges 1989a, 1989b), with either free sepals on the rim of the hypanthium (Fig. 2a) or have small sepals carried to the top of the corolline operculum (Fig. 2b). In all species, stamens develop on the basal adaxial components (buttresses) of young corolline parts, the basal components becoming the staminophore of the mature flower. In some species, e.g. *E. pleurocarpa* (= *E. tetragona* in Drinnan and Ladiges 1989a) and *E. erythrocorys*, the corolline parts remain more or less free and the stamens develop as four distinct bundles on an undulating staminophore (bundles incorrectly termed fascicles by some authors, because the filaments are not fused). In other species, early corolline continuity leads to a more or less continuous ring of stamens, e.g. in *E. jucunda*, *E. lirata* and *E. gamophylla*. These differences seen in the mature flower, despite having an underlying similarity of development, have been emphasised in previous classifications of the subgenus.

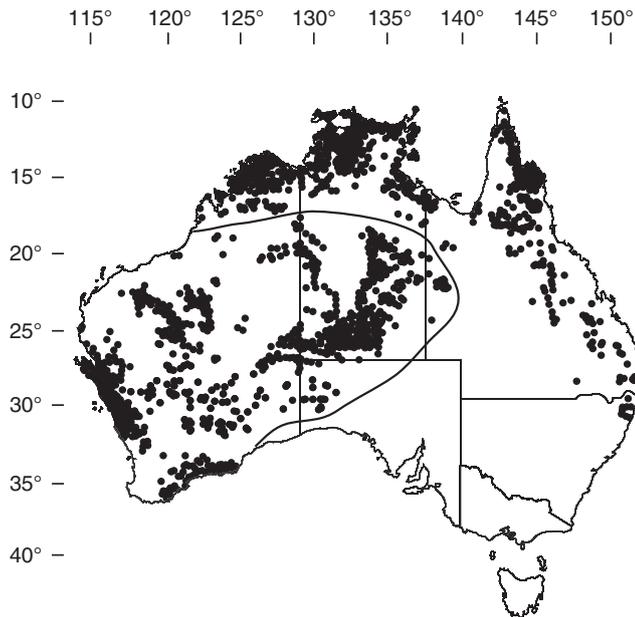
**Table 1. Comparison of classifications of subgenus *Eudesmia* by Hill and Johnson (1998) and Brooker (2000)**

Hill and Johnson (1998)	Brooker (2000)
<b>Subgenus <i>Eudesmia</i></b>	<b>Subgenus <i>Eudesmia</i></b>
<b>Section <i>Xeraria</i></b>	<b>Section <i>Limbatæ</i></b>
<i>E. gongylocarpa</i> Blakely	<b>Series <i>Heteropterae</i></b>
<b>Section <i>Odontaria</i></b>	Subseries <i>Patelliformes</i>
<i>E. odontocarpa</i> F.Muell.	<i>E. odontocarpa</i>
<i>E. gamophylla</i> F.Muell.	<i>E. gamophylla</i>
<b>Section <i>Quadraria</i></b>	Subseries <i>Tetraedrae</i>
<b>Series <i>Tetragonae</i></b>	<i>E. eudesmioides</i>
Subseries <i>Eudesmioidesae</i>	<i>E. gittinsii</i>
<i>E. eudesmioides</i> F.Muell.	<i>E. conveniens</i>
<i>E. pallida</i> L.A.S.Johnson & K.D.Hill	<i>E. pleurocarpa</i>
<i>E. selachiana</i> L.A.S.Johnson & K.D.Hill	<i>E. erythrocorys</i>
Subseries <i>Tetragonosae</i>	<i>E. roycei</i>
<i>E. gittinsii</i> Brooker & Blaxell subsp. <i>gittinsii</i>	<b>Series <i>Edentatae</i></b>
<i>E. gittinsii</i> subsp. <i>illucida</i> D.Nicolle	Subseries <i>Jucundae</i>
<i>E. conveniens</i> L.A.S.Johnson & K.D.Hill	<i>E. jucunda</i>
<i>E. pleurocarpa</i> Schauer	Subseries <i>Acetabuliformes</i>
<i>E. extrica</i> D.Nicolle	<i>E. gongylocarpa</i>
Subseries <i>Erythrocorythosae</i>	<b>Section <i>Complanatae</i></b>
<i>E. erythrocorys</i> F.Muell.	<i>E. tetrodonta</i>
<b>Series <i>Jucundae</i></b>	<b>Section <i>Ebbanoenses</i></b>
<i>E. jucunda</i> C.A.Gardner	<i>E. ebbanoensis</i>
<b>Series <i>Royceanae</i></b>	<b>Section <i>Reticulatae</i></b>
<i>E. roycei</i> S.G.M.Carr, D.J.Carr & A.S.George	<b>Series <i>Scutelliformes</i></b>
<b>Series <i>Ebbanoenses</i></b>	<i>E. baileyana</i>
<i>E. ebbanoensis</i> Maiden subsp. <i>ebbanoensis</i>	<b>Series <i>Miniatae</i></b>
subsp. <i>photina</i> Brooker & Hopper	Subseries <i>Variabilis</i>
subsp. <i>glauciramula</i> L.A.S.Johnson & K.D.Hill	<i>E. lirata</i>
<b>Section <i>Fibraria</i></b>	<i>E. similis</i>
<i>E. tetrodonta</i> F.Muell.	Subseries <i>Inclinatae</i>
<b>Section <i>Apicaria</i></b>	<i>E. miniata</i>
<b>Series <i>Baileyanae</i></b>	<i>E. gigantangion</i>
<i>E. baileyana</i> F.Muell.	<i>E. phoenicea</i>
<b>Series <i>Similes</i></b>	<i>E. ceracea</i>
<i>E. similis</i> Maiden	
<i>E. lirata</i> W.Fitzg. ex Maiden	
<b>Series <i>Miniatae</i></b>	
Subseries <i>Miniatosae</i>	
<i>E. miniata</i> A.Cunn. ex Schauer	
<i>E. gigantangion</i> L.A.S.Johnson & K.D.Hill	
<i>E. chartaboma</i> D.Nicolle	
Subseries <i>Phoeniceosae</i>	
<i>E. phoenicea</i> F.Muell.	
<i>E. ceracea</i> Brooker & Done	

*Taxonomic history of eudesmid eucalypts*

Robert Brown (1814) first described *E. tetragona*, placing it in the genus *Eudesmia* because he believed it was significantly different from other species of *Eucalyptus*. Bentham (1867) classified five species in *Eucalyptus* subseries *Eudesmiae*, and *E. miniata* in subseries *Robustae* (with *E. tetraptera* Turcz.).

Mueller (1879–1884) included *E. eudesmioides*, *E. tetragona*, *E. erythrocorys*, *E. miniata* and *E. phoenicea* in section *Parallelantherae*, and *E. baileyana* in section *Renantherae*, having mixed material with the stringybark *E. tindaliae* Blakely (see discussion in Hill and Johnson 1998). Maiden (1903–1931) placed nine species in subseries *Eudesmiae* within series *Non-corymbosae*, and *E. miniata* and



**Fig. 1.** Distribution of *Eucalyptus* subgenus *Eudesmia*, based on data from the Australian Virtual Herbarium. The line circumscribes the distribution of the western group of species (see Fig. 5).

*E. phoenicea* in an unnamed subseries within series *Corymbosae*. Blakely (1934) elevated the two subseries of Maiden (1903–1931) to two series, namely *Eudesmieae* and *Miniatae*. Carr and Carr (1968) divided the series *Eudesmieae* into the following two groups: Group A, with free persistent sepals on fruit, and Group B, with sepals carried on the corolline operculum (Fig. 2). Pryor and Johnson's informal taxonomic treatment (Pryor and Johnson (1971) classified the eudesmid eucalypts at the rank of subgenus *Eudesmia* with two sections, *Quadraria* and *Apicaria*. Chippendale (1988) followed Pryor and Johnson's classification (Pryor and Johnson (1971), and placed the known species into seven series, although with no additional ranks or indication of relationships.

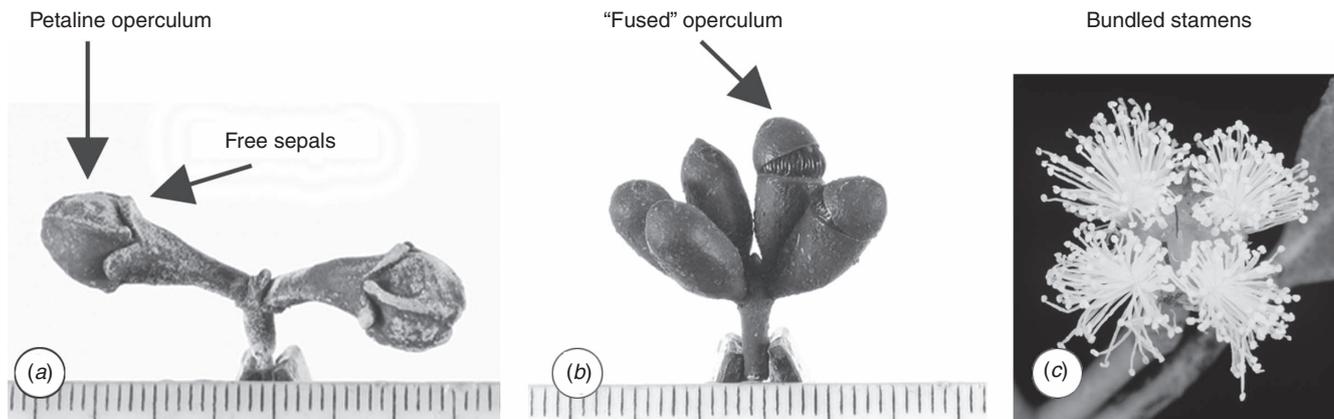
Hill and Johnson (1998) and Nicolle (2000) added nine new eudesmid taxa, most of which were subspecies of previously recognised species. Hill and Johnson's extracodical classification (see Table 1) recognised several monophyletic groups, congruent with those of Pryor and Johnson (1971), and the names of Hill and Johnson (1998) are used throughout the present paper for convenience. Nicolle (2000) used the name series *Heteroptera* Maiden (1903–1931) for Hill and Johnson's series *Tetragonae* and lumped some species; he regarded *E. pallida* as indistinct from *E. eudesmioides*, and *E. tetragona* as an intergrade between *E. pleurocarpa* and *E. extrica*.

The formal classification of subgenus *Eudesmia* by Brooker (2000) is the most recent and includes four sections, two of which are monotypic, four series and six subspecies (Table 1), characterised in part by seed characters. The hierarchy of these 14 higher taxa suggests relationships although the classification is not based on any published phylogeny.

#### Phylogenetic analyses

Molecular phylogenetic analyses have focussed on the broad eucalypt group and have included different combinations of a few eudesmid species (Sale *et al.* 1993, 1996; Ladiges *et al.* 1995; Udovicic *et al.* 1995; Steane *et al.* 1999; Udovicic and Ladiges 2000). Although based on a small sample of eudesmid species, analyses of Sale *et al.* (1993, chloroplast DNA) and Udovicic and Ladiges (2000, chloroplast and nuclear DNA) provided further support for the monophyly of the subgenus, in addition to morphology discussed above. In a large sample of ITS nuclear rDNA sequences for species of *Eucalyptus*, including six eudesmids, Steane *et al.* (2002) also found subgenus *Eudesmia* to be monophyletic, and related to subgenus *Eucalyptus* (the 'monocalypt' clade) and *E. tenuipes* (Maiden & Blakely) Blakely & C.T.White, although nodes lacked bootstrap support.

The only analysis to focus on relationships within *Eudesmia* is that of Hill and Johnson (1998). These authors, however, analysed only a small morphological dataset of 14 characters, with their eudesmid sections and series as terminal taxa together with outgroups. They found a high level of homoplasy and their



**Fig. 2.** The two groups of Carr and Carr (1968). (a) Free sepals on the rim of the hypanthium and petaline operculum (Group A; *Eucalyptus tetradonta*). (b) Sepals carried on the petaline operculum, sometimes visible on the top of the operculum (Group B; *E. jucunda*). (c) Bundled stamens are typical of several eudesmid taxa (*E. ebbanoensis*).

resulting cladogram was virtually unresolved except for two nodes.

The aim of the present paper is to determine the phylogenetic relationships of all taxa within subgenus *Eudesmia*, by using sequence data from both nuclear and chloroplast DNA, and morphology. A revised classification is presented on the basis of the phylogeny.

## Materials and methods

### Sampling

Location and accession details for the taxa investigated are listed in Appendix 1. Herbarium vouchers are housed at the seed supplier/institution or University of Melbourne Herbarium (MELU). Outgroup taxa included were *E. curtisii* Blakely & C.T.White (monotypic subgenus *Acerosae*), *E. tenuipes* (subgenus *Cuboidea*) and *E. cloeziana* F.Muell. (monotypic subgenus *Idiogenes*) based on previous eucalypt studies (Ladiges *et al.* 1995; Sale *et al.* 1996; Steane *et al.* 2002). As indicated by their treatment as subgenera by Brooker (2000), there is evidence from morphology and molecular data that these species are outside the main clades of *Eucalyptus* (subgenera *Eudesmia*, *Eucalyptus* and *Symphomyrtus*) and therefore they are useful as outgroups. Previous studies indicate that *E. curtisii* is sister taxon to all other taxa within *Eucalyptus sensu stricto* (Ladiges *et al.* 1995; Steane *et al.* 2002); *E. tenuipes* may be related to either subgenus *Eudesmia* or subgenus *Eucalyptus* (Steane *et al.* 2002) and *E. cloeziana* to subgenus *Eucalyptus*.

### DNA isolation and amplification

Leaf material was collected at Currency Creek Arboretum (South Australia), Kings Park (Western Australia) and Australian Botanic Gardens, or from glasshouse-grown seedlings (some seed from Top End seeds). Leaf tissue was manually disrupted in a mortar and pestle with liquid nitrogen. DNA was isolated from fresh leaf tissue with the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's handbook and stored at  $-20^{\circ}\text{C}$ .

### Choice of DNA regions

The ITS and ETS regions of nuclear rDNA were chosen on the basis of previous studies of eucalypts (Steane *et al.* 1999, 2002; Udovicic and Ladiges 2000; Whittock *et al.* 2003; Parra-O. *et al.* 2006; Ochieng *et al.* 2007). The *psbA-trnH* intergenic spacer region of chloroplast DNA was selected on the basis of Udovicic and Ladiges (2000), where *psbA-trnH* was found to be more informative than the *trnL* intron and *trnL-trnF* spacer regions, and more conserved than ITS.

PCR with nested primers was initially used for the entire ITS region by using the protocol of Udovicic and Murphy (2002); ITS26 and ITS18 primers were used for the initial PCR, followed by a second PCR with S3 and S4 primers and the product from the initial PCR. Because of poor reproducibility and with only three taxa successfully sequenced, the internal spacers were amplified and sequenced separately. Primers S3 and S5 were used for the ITS1 region, and S6 and ITS26 were used for the ITS2 region (Käss and Wink 1997). The ETS primers

used were ETS-18S (Wright *et al.* 2001) and ETSmyrtF (Lucas *et al.* 2007). The *psbA-trnH* intergenic primers were *psbAF* and *trnHR* (Sang *et al.* 1997).

The PCR reactions for ITS1+2 and *psbA-trnH* regions consisted of  $\times 10$  buffer (containing 15 mM  $\text{MgCl}_2$ ) (QIAGEN), 1.5  $\mu\text{L}$  of 25 mM  $\text{MgCl}_2$  (QIAGEN), 2  $\mu\text{L}$  of 2.5 mM dNTPs (Fisher Biotec, Perth, Australia), 0.5  $\mu\text{M}$  of each primer, 2.5U HotStarTaq DNA polymerase (QIAGEN), 20–50 ng of *Eucalyptus* DNA, and ultra-pure water to make a total volume of 25  $\mu\text{L}$ . A negative control for each batch of PCR reactions was always included to test whether the reagents were free of DNA contamination. PCR amplification reactions were conducted in a Mastercycler gradient thermal cycler (Eppendorf, Foster City, CA), beginning with incubation at  $95^{\circ}\text{C}$  for 15 min to activate the HotStarTaq DNA polymerase. PCR conditions for *psbA-trnH* and ITS1+2 were as follows: 30 cycles of  $94^{\circ}\text{C}$  for 30 s,  $55^{\circ}\text{C}$  for 30 s,  $72^{\circ}\text{C}$  for 10 s (*psbA-trnH*) or 20 s (ITS1+2), incubation at  $72^{\circ}\text{C}$  for 5 min, then held at  $4^{\circ}\text{C}$  (Käss and Wink 1997; Udovicic and Ladiges 2000). The PCR reagent concentrations and conditions for the ETS region are described in Parra-O. *et al.* (2006).

The QIAquick PCR Purification Kit (QIAGEN) was used to purify the PCR products, according to the manufacturer's handbook. The concentration of the purified DNA was determined by electrophoresis. Direct sequencing of the purified PCR products was conducted with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kits (Applied Biosystems, Foster City, CA), version 3.1. Sequencing reactions were analysed at the Australian Genome Research Facility in Brisbane. Contiguous sequences were edited with Sequencher version 3.0 (Gene Codes Corporation, Ann Arbor, MI), and deposited in GenBank (Accession Numbers FJ654340–FJ654428). Owing to sections of highly conserved sequence across all taxa, edited sequences were aligned manually with BioEdit version 7.0.1 (Hall 1999).

### Analysis of datasets

Both maximum parsimony and Bayesian inference methods of phylogenetic reconstruction were used. Molecular datasets were analysed separately and combined. Parsimony analyses were conducted with PAUP\* version 4.0b10 (Swofford 2002), with individual bases coded as unordered multi-state characters and gaps treated as missing data. All heuristic searches were conducted with 1000 random addition sequences by using tree bisection and reconnection (TBR) branch swapping, and trees were rooted by using outgroups. Multiple most parsimonious trees were summarised as a strict consensus tree. Branch lengths were calculated for one of the equally most parsimonious trees by using DELTRAN character-state optimisation. Node support was tested with bootstrap analyses using 1000 heuristic replicates. Nodes were considered supported with bootstrap values (bs) of  $\geq 50\%$ .

For Bayesian inference, Modeltest 3.7 (Posada and Crandall 1998) was used to select the model of nucleotide substitution that best fitted the data, from the 56 models available. The two model-selection tests used by Modeltest are the hierarchical likelihood ratio test (hLRT), and the Akaike information criterion (AIC). The two tests suggested different models for

each dataset. The hLRT was rejected because this test has been suggested to be dependent on the starting model and significance level, and may not select the best model (Posada and Buckley 2004).

The AIC test selected the TrN+I model as the best fit for the ITS2 region data. The estimated base frequencies (A=0.2078, C=0.3233, G=0.2803, T=0.1886), the substitution-rate matrix (A–C=1.0000, A–G=2.2544, A–T=1.0000, C–G=1.0000, C–T=4.0677, G–T=1.0000), the gamma-distribution shape (equal), and the proportion of invariable sites (0.6575) were defined. The AIC test selected the K81uf+I+ $\Gamma$  model as the best fit for the ETS region data. The estimated base frequencies (A=0.2722, C=0.2647, G=0.2763, T=0.1867), the substitution-rate matrix (A–C=1.0000, A–G=5.7694, A–T=0.3646, C–G=0.3646, C–T=5.7694, G–T=1.0000), the gamma-distribution shape (0.9177), and the proportion of invariable sites (0.6639) were defined. The AIC test selected the K81uf+ $\Gamma$  model as the best fit for the *psbA* region data. The estimated base frequencies (A=0.3111, C=0.1233, G=0.1575, T=0.4081), the substitution-rate matrix (A–C=1.0000, A–G=1.1036, A–T=0.6585, C–G=0.6585, C–T=1.1036, G–T=1.0000) and the gamma-distribution shape (0.2720) were defined.

Bayesian inference analyses were conducted with MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). Indels from the datasets were partitioned from the nucleotides to allow the Jukes Cantor plus gamma (JC+ $\Gamma$ ) model to be applied (Ronquist *et al.* 2005). The model and parameters estimated in Modeltest were used. To increase the mixing of chains and congruence, two replicate runs of 10 chains each were run for 2 000 000 generations with tree sampling every 100th generation (Martins and Hellwig 2005; Brown *et al.* 2006). To ensure that an analysis had run to completion, two runs needed to converge to a stationary distribution and the standard derivative of split frequencies approached zero (Ronquist *et al.* 2005). Trees sampled at the beginning of the run were discarded when log-likelihood values were increasing rapidly during the burn-in period, and remaining trees summarised as a 50% majority rule tree. Posterior probabilities (pp) were used as the measure of support (Larget and Simon 1999; Huelsenbeck *et al.* 2002); nodes were considered supported with pp  $\geq$  95% (Wilcox *et al.* 2002). Each Bayesian analysis was repeated five times to

ensure convergence to the same tree topology (Huelsenbeck *et al.* 2002).

### Morphological characters

The morphology of taxa was reviewed on the basis of herbarium specimens (on average, minimum of five samples per taxon) from AD, BRI, CANB, DNA, MEL, NSW, PERTH (see Appendix 1), field collections and literature. Light and scanning electron microscopy were used to investigate trichomes (including from glasshouse-grown seedlings) and seeds. Characters relating to size and shape of fruits, flower buds and leaves or bark are useful for identifying subspecies and species or small clades of sister taxa; however, they were difficult to score as non-overlapping discrete states for analysis across the whole subgenus. For cladistic analysis, 12 characters were scored across all taxa that were potentially informative of relationships within subgenus *Eudesmia* and that have been emphasised in previous infrageneric classifications. Nodes that had support in each of the nrDNA and cpDNA trees were coded as characters and added to the matrix of morphological characters. The matrix was analysed in PAUP using maximum parsimony, with multistate characters treated as unordered and an all-zero outgroup included.

## Results

### ITS1 region

The ITS1 region included the ITS1 intergenic spacer and 34 bp of the 5.8S rRNA gene. The region was more difficult to amplify, sequence and align than the ITS2 region. In part, this was due to five sequences identified as probable pseudogenes (*E. gamophylla*, *E. odontocarpa*, *E. pleurocarpa*, *E. extrica* and *E. lirata*) on the basis of an inferred increase in methylation-induced substitutions. The methylation-induced substitutions occur mainly at CpG and CpNpG sites, where N can be any nucleotide, followed by deamination (Gardiner-Garden *et al.* 1992). Deamination corresponds with a rise in A+T content, with the presumed pseudogenes in eudesmid eucalypts increasing up to 43.46–48.23%, compared with 33.93–39.92% in the ‘typical’ orthologous copies (Table 2). This corresponds with G+C content in the pseudogenes decreasing down to

**Table 2.** Sequence characteristics of the DNA regions ITS1, ITS2, ETS and *psbA-trnH*

Sequence characteristic	ITS1 region	ITS2 region	ETS region	<i>psbA-trnH</i> region
Aligned length (bp)	294	356	474	565
Unaligned length range (bp)	255–285	349–352	470–472	423–522
Base composition				
G+C%	60.07–66.08 51.25–56.54 <sup>A</sup>	59.43–62.75	50.85–54.68	25.93–29.25
A+T%	33.92–39.93 43.46–48.75 <sup>A</sup>	37.25–40.57	45.32–48.95	70.28–74.07
Sequence divergence % between ingroup and outgroup	9.25–18.57 <sup>A</sup>	7.65	5.12	5.25
Between ingroup taxa	10.97–22.68 <sup>A</sup>	8.32	4.82	5.46
No. of parsimony informative sites (% in parentheses)	35 (11.9%) 78 (26.5%) <sup>A</sup>	27 (7.56%)	28 (5.91%)	30 (6%)
No. of synapomorphic indels	1 <sup>A</sup>	1	0	2

<sup>A</sup>Characteristics for the pseudogenes for the ITS1 region.

51.77–56.54%, compared with 60.07–66.08% in the ‘typical’ orthologous copies. The details of the pseudogene sequences for *E. gamophylla*, *E. odontocarpa* and *E. lirata* have been reported by Bayly *et al.* (2008); none of the sequences in Steane *et al.* (2002) appears to be pseudogenes (M. Bayly, pers. comm.).

Pseudogenes are a potential problem because of comparison of non-orthologous copies in datasets (Bayly and Ladiges 2007); thus, they were excluded from the alignment. After their removal, ITS1 provided little resolution of phylogenetic relationships. For this reason, and because of the potential for other undetected paralogous ITS1 spacer copies within this dataset, the ITS1 region was not used in further analyses of the nrDNA dataset.

#### ITS2 region

The ITS2 region refers to the remaining 126 bp of the 5.8S rRNA gene, the ITS2 spacer and 26S rRNA gene. In all, 27 accessions representing 23 species of subgenus *Eudesmia* and three outgroup species were successfully aligned for the ITS2 region, including three sequences obtained from the GenBank (*E. ceracea*, *E. eudesmioides* and *E. curtisii*; Appendix 1). However, DNA for *E. gittinsii* subsp. *gittinsii* and *E. roycei* was unable to be sequenced successfully, despite repeated attempts, and could not be included in the analyses. The total alignment length of the ITS2 region was 356 bp (Table 2). In total, 28 characters, including one indel of 2 bp (Table 3), were found to be parsimony informative (7.65%).

#### ETS sequences

The ETS region from 20 accessions, representing 17 species of subgenus *Eudesmia* and three outgroup species, was aligned successfully. The ETS region for *E. roycei* was unable to be sequenced. M. Bayly (University of Melbourne) provided the ETS sequences for *E. similis* and *E. tenuipes*. ETS sequences for *E. pleurocarpa*, *E. curtisii* and *E. cloeziana* were from GenBank. The total alignment length of the ETS region was 474 bp and no pseudogenes were identified (Table 2). In total, 28 characters were found to be informative (5.91%) and no indels were coded for this dataset. The ETS region sequenced was longer than the ITS2 region, although it had the same number of informative characters.

#### Combined nrDNA analyses

The ITS2 and ETS regions were combined into a single matrix since analyses (not shown) of them separately were not in conflict. An heuristic search in PAUP\* identified 72 most parsimonious trees, each with a tree length of 161, CI=0.77,

RI=0.76. The parsimony strict consensus tree had 16 nodes, 11 resolved with  $bs \geq 50\%$ . The Bayesian tree had 18 nodes, 12 with  $pp \geq 95\%$ . This tree is illustrated in Fig. 3, with the sections and series of Hill and Johnson (1998, referred to as H&J in the text that follows) shown and nodes identified by parsimony analysis marked with an asterisk. The parsimony and Bayesian analyses produced largely congruent trees, except that Nodes 12 and 16 collapsed in the parsimony strict consensus tree.

*Eucalyptus phoenicea* and *E. ceracea* (series *Miniatae* subseries *Phoeniceosae* H&J) grouped with one of the outgroup species, *E. curtisii* (although Node 2 lacks support), rather than with members of series *Miniatae* (H&J). The phylogram of one of the most parsimonious trees (Fig. 4) illustrates relatively long branches leading to these two eudesmid taxa. Other relatively long branches lead to *E. gongylocarpa* (monotypic section *Xeraria* H&J), and the well supported clade of *E. tetradonta* (section *Fibraria* H&J) and *E. miniata*, *E. chartaboma* and *E. gigantangion* (series *Miniatae*). Series *Miniatae* is strongly supported at node 6 (bs 99% and pp 100%), including one indel.

Other well supported relationships are as follows: sister taxa *E. lirata* and *E. similis* (series *Similes* H&J) at Node 8 (bs 93% and pp 100%); the three subspecies of *E. ebbanoensis* at Node 10, (bs 84%, pp 100%); and the clade at Node 13 (bs 82%, pp 100%) that includes six species of series *Tetragonae* H&J (with subgroups well supported at Nodes 14 and 15), together with *E. odontocarpa* and *E. gamophylla* of series *Odontaria* H&J, which are shown as sister species at node 18 (bs 92%, pp 100%). Although Node 9 lacks bootstrap support (pp 95%), it suggests that all taxa from Western Australia and adjacent desert regions form a clade (here termed the Western clade). *E. erythrocorys* (part of the polytomy at Node 9) is outside the clade that includes the other members of series *Tetragonae* and its relationship is unresolved by this dataset.

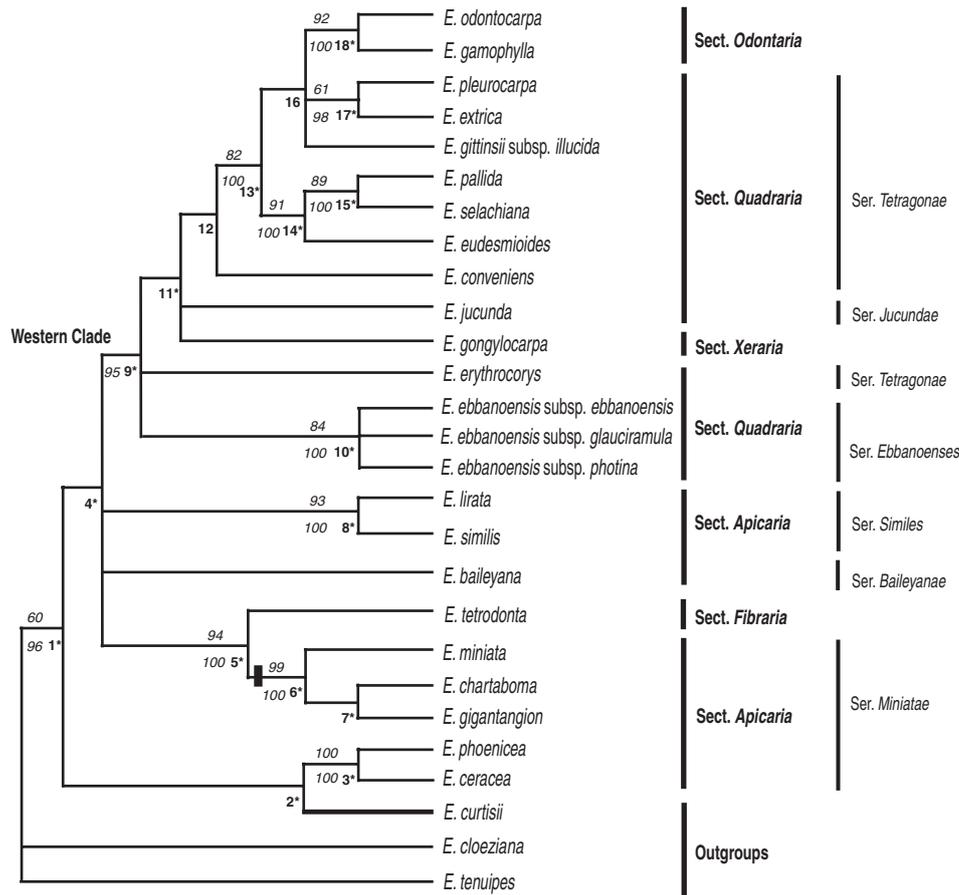
#### psbA–trnH intergenic spacer region

Sequences from the *psbA–trnH* intergenic spacer region from 26 accessions, representing the 23 species of subgenus *Eudesmia*, were aligned successfully. These included the sequence for *E. erythrocorys* obtained from the GenBank, and the three outgroup species (Appendix 1). The total alignment length of this spacer region was 565 bp (Table 2). Thirty characters, including two indels (Table 3), were found to be informative (6%), excluding regions of ambiguity. Regions of ambiguity (11.3%) included three different autapomorphic,

**Table 3. Indel characters for the ITS and *psbA–trnH* spacer regions**

Type is given relative to the outgroup taxa

Region	Indel	Base number	Size (bp)	Type	Species
ITS1	1	191–193	3	Insertion	<i>E. pleurocarpa</i> , <i>E. extrica</i>
ITS2	1	277–278	2	Insertion	<i>E. miniata</i> , <i>E. chartaboma</i> , <i>E. gigantangion</i>
<i>psbA–trnH</i> spacer	1	393–472	80	Deletion	<i>E. miniata</i> , <i>E. chartaboma</i> , <i>E. gigantangion</i> , <i>E. phoenicea</i> , <i>E. ceracea</i>
	2	500–507	8	Insertion	<i>E. selachiana</i> , <i>E. pallida</i> , <i>E. eudesmioides</i> , <i>E. conveniens</i> , <i>E. gittinsii</i> subsp. <i>gittinsii</i> , <i>E. gittinsii</i> subsp. <i>illucida</i> , <i>E. pleurocarpa</i> , <i>E. extrica</i> , <i>E. erythrocorys</i>



**Fig. 3.** Tree from the Bayesian analysis of the combined nrDNA region (ITS2 + ETS). Nodes are numbered in bold, and those that are also resolved by parsimony analysis are marked with an asterisk. Nodes 12 and 16 collapsed in the parsimony strict consensus tree. Numbers above the branches indicate bootstrap values  $\geq 50\%$  from the parsimony analysis, those below the branches indicate posterior probabilities  $\geq 95\%$  from the Bayesian analysis. Section and series names are according to Hill and Johnson (1998).

direct repeat sequence regions, one poly A repeat and one poly T repeat.

An heuristic search in PAUP\* identified 396 most parsimonious trees, with a minimum length of 112, CI=0.86, RI=0.88. The parsimony strict consensus tree had 13 nodes, 11 with bs  $\geq 50\%$ . The Bayesian tree had 14 nodes, 8 with pp  $\geq 95\%$  (Fig. 5). The parsimony and Bayesian analyses produced largely congruent trees, except that Nodes 2 and 4 (Fig. 5) in the Bayesian tree collapsed in the parsimony strict consensus tree, and Node 6 (bs 57%) in the parsimony tree was not identified by Bayesian inference. The phylogram of one of the most parsimonious trees showed overall short branches of five or fewer changes resolving clades and up to 11 changes for branches leading to terminal taxa.

On the basis of the *psbA-trnH* intergenic spacer dataset, the relationship of *E. baileyana* (series *Baileyanae* H&J) is unresolved at the base of the cladogram with one of the outgroups, *E. curtisii*, at a higher node. Basal Nodes 1 and 2 lack support, and monophyly of *Eudesia* is not rejected.

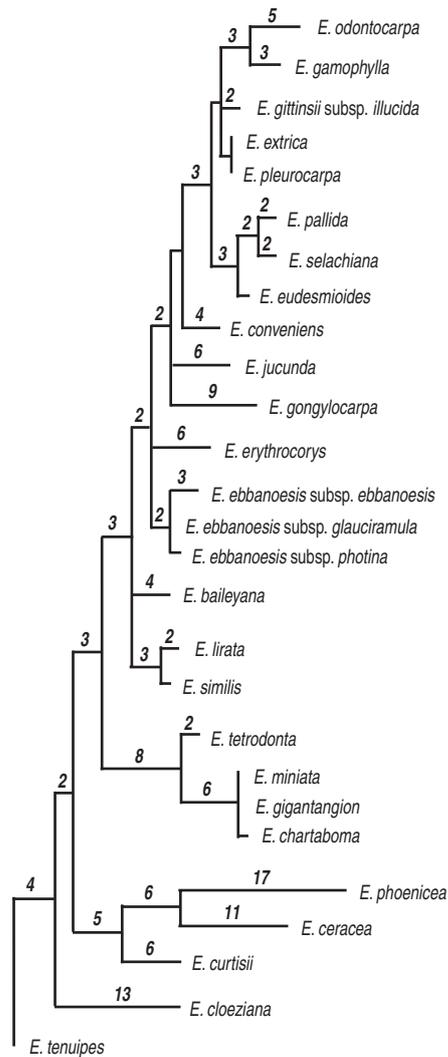
Of clades with support, series *Miniatae* (node 3, bs 92%, pp 100%) is characterised by a large indel (Table 3). Node 8 (bs 76%, pp 100%) confirms the Western clade. *E. roycei* (series

*Royceanae* H&J) and *E. gongylocarpa* (section *Xeraria*) at Node 8 are related to a supported clade of 15 other taxa. Again *E. odontocarpa* and *E. gamophylla* (section *Odontaria*) are sister taxa (Node 14, bs 75%, pp 100%) and, in contrast with the nuclear DNA data, series *Tetragonae* is monophyletic, supported by an indel character (Node 11, bs 85%, pp 100%).

One surprising result is the relationship of *E. tetradonta* with *E. similis* (Node 7). Examination of individual trees from the parsimony analysis shows for some a branch length of zero leading to Node 7, and sometimes these two species group with *E. lirata*, which has the longest terminal branch (11 steps) of all accessions.

#### Combined nrDNA and cpDNA analyses

The ITS2 + ETS and the *psbA-trnH* datasets were combined. An heuristic search in PAUP\* identified 1080 most parsimonious trees, with a minimum length of 289, CI=0.76, RI=0.77. The parsimony strict consensus tree had 14 nodes, 13 of which had bs  $\geq 50\%$ . The Bayesian tree had more resolved nodes, totalling 24, 16 with pp  $\geq 95\%$  (Fig. 6).



**Fig. 4.** One of the most parsimonious trees from the analysis (Fig. 3) of the combined nrDNA (ITS2+ETS) dataset shows branch lengths. Unlabelled branches are one step long.

Of the clades with support, series *Miniatae* (Node 4) is characterised by the large indel found in the *psbA-trnH* region (Table 3), and within that clade both subseries *Miniatosae* H&J (*E. miniata*, *E. gigantangion* and *E. chartaboma*) and *Phoeniceosae* (*E. phoenicea* and *E. ceracea*) are highly supported as monophyletic groups (Nodes 5 and 6, bs 99–100%, pp 100%). *E. tetradonta* (monotypic section *Fibraria*) is the sister taxon to series *Miniatae*, although it nests within that group in the parsimony strict consensus tree.

The clade at Node 9 includes three lineages, including *E. baileyana* (series *Baileyanae*), sister species *E. similis* and *E. lirata* (series *Similes*) and the Western clade including sections *Xeraria*, *Odontaria* and *Quadraria* (series *Royceanae*, *Ebbanoenses*, *Jucundae* and *Tetragonae*). Section *Odontaria* (*E. odontocarpa* and *E. gamophylla*) appears to be nested within *Tetragonae*, although Nodes 15–22 have low or no bootstrap support; the indel identified in the chloroplast data

supports *Tetragonae* as monophyletic and it may not be necessary to invoke a secondary loss of this character in *Odontaria*, which may be better placed at a lower node as in Fig. 4.

Nodes 2, 3, 4, 12, 15, 16, 19, 20, 21 and 22 (Fig. 6) in the Bayesian tree were collapsed in the parsimony strict consensus tree, and one node (8) in the parsimony tree was not identified by Bayesian inference. The collapse of more nodes in the parsimony tree suggests that the parsimony method more correctly reflected character conflict, and that some nodes resolved in the Bayesian analysis, although with low pp values, are artefactual. The incongruence length difference (ILD) test (Farris *et al.* 1994, the partition homogeneity test in PAUP) was applied to test for incongruence between the datasets. Heterogeneity was detected, suggesting conservatively that combining the dataset in this way into a single matrix may not be appropriate.

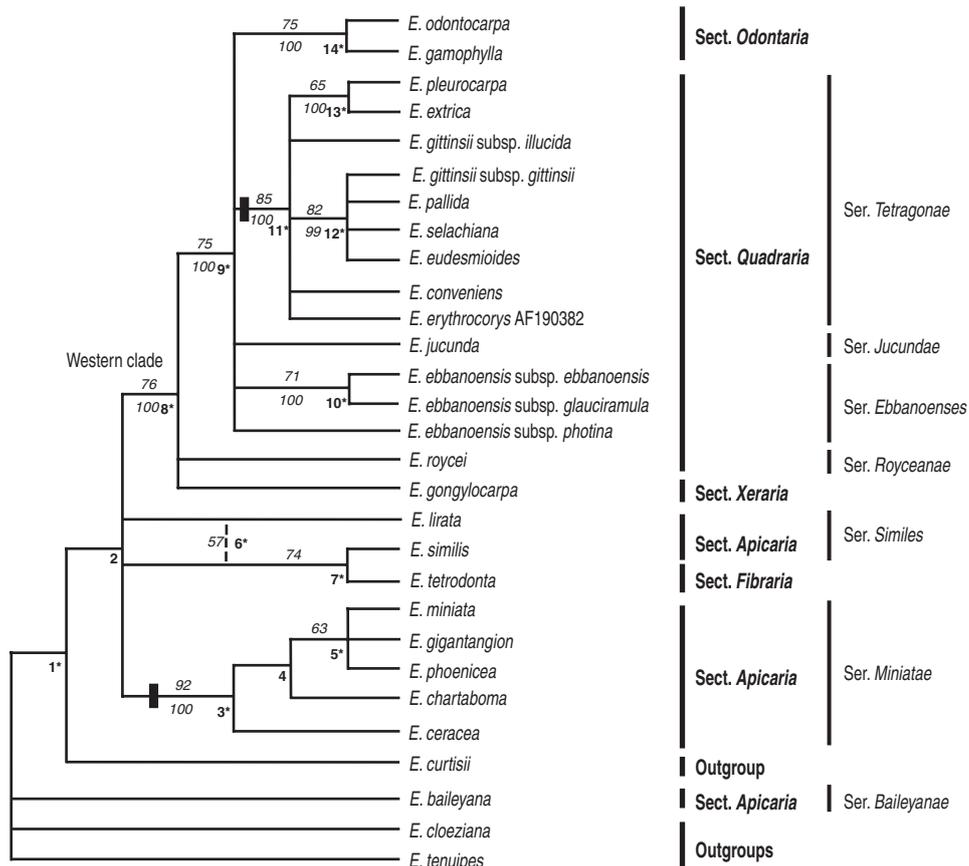
#### Combined morphology and molecules

An alternative way of analysing data in combination was to code as characters the nodes of each of the molecular trees (Figs 3, 5) that had bootstrap support  $\geq 50\%$  and posterior probability support  $\geq 95\%$ . In all, 19 characters were scored, and to these were added 12 morphological characters (Tables 4, 5). Character 8, grey-back seeds with ventral hilum, supported the monophyly of the subgenus (Node 1).

Parsimony analysis resulted in two trees, each of length 45, CI=0.82, RI=0.94 (Fig. 7). The two trees differed only with respect to the relationship of *E. tetradonta*, which was either unresolved at the base of the tree (Node 1) or resolved at Node 2 as sister to the other northern species on the basis of the presence of multicellular, blunt-ended hairs radiating from raised oil glands (Character 12). Radiating hairs of eudesmids (rE) have previously been described as one type (Ladiges 1984), but further investigation here identified the following three character states (Fig. 8): hairs may be unicellular blunt-ended (unique to *E. lirata*), multicellular blunt-ended (*E. tetradonta*, *E. baileyana*, *E. similis* and series *Miniatae*) or unicellular elongated-acute (Western clade except series *Odontaria*, see below).

The clade at Node 3 relates *E. baileyana*, sister species *E. similis* and *E. lirata* (bark termed ‘yellow jacket’, Character 2) and the five species in series *Miniatae*. Series *Miniatae* (at Node 4) is supported by the synapomorphy of orange stamens (Character 5), and the two subclades (Nodes 5 and 6) each have distinctive urceolate, ribbed fruits (illustrated in Fig. 9s–w). Series *Miniatae* is characterised also by woollybutt bark, large clavate buds, long peduncles, large seeds and large, fleshy cotyledons (not scored in matrix), in addition to the indel found in the *psbA-trnH* spacer region.

All of the taxa at Node 3 have the sepals carried up on the corolline operculum (Character 3), as do *E. jucunda* and *E. roycei* (Node 9) and *E. ebbanoensis* (Node 11). Carr and Carr (1968) emphasised this character to group these taxa as *Eudesmieae* B; however, the character is homoplasious (Fig. 7). If petals develop quickly after sepal initiation, petal growth can carry the sepals upwards, whereas if sepals grow to sufficient size before petal initiation, they remain on the rim of the hypanthium (Drinnan and Ladiges 1989c). Thus, homoplasy (either parallelism or reversal) may be simply the result of different growth rates. Those taxa



**Fig. 5.** Tree from the Bayesian analysis of the cpDNA *psbA-trnH* intergenic spacer region. Nodes are numbered in bold, and those that are also resolved by parsimony analysis are marked with an asterisk. Numbers above the branches indicate bootstrap values  $\geq 50\%$ , and those below the branches indicate posterior probabilities  $\geq 95\%$ . In the parsimony strict consensus tree, Nodes 2 and 4 collapsed, and *Eucalyptus lirata* is the sister taxon to *E. similis* + *E. tetrodonta* (Node 6, bs 57%, dashed line). The coloured blocks on the branches indicate indels. Section and series names are according to Hill and Johnson (1998).

with free and persistent sepals on the fruit are members of the clades at Nodes 13 and 18, plus *E. gongylocarpa* (Node 8) and *E. tetrodonta* (Node 2; Fig. 7), which Carr and Carr (1968) grouped in *Eudesmieae* A. *E. tetrodonta* may have a unique (apomorphic) pattern of development because the persistent sepals are located below the rim of the mature fruit, which extends upwards forming a narrow neck (Fig. 9o).

The Western clade (Node 8, Fig. 7), which has strong support from both the nrDNA and cpDNA analyses, is characterised by leaf waxes that are formed as tubes (Character 11; Hallam and Chambers 1970), winged seeds (Character 9, Fig. 10) and, as mentioned above, unicellular, long acute hairs (Character 12, State 3). Two of these characters are homoplasious. Seeds of *E. ebbanoensis* (Fig. 10g, h) lack a wing and are interpreted as autapomorphic (also described as uniquely 'obese' by Brooker 2000). Lack of hairs in *E. odontocarpa* and *E. gamophylla* is interpreted as a loss. These two species are sister taxa on the basis of several characters, including two rows of ovules (Character 7; Bohte and Drinnan 2005).

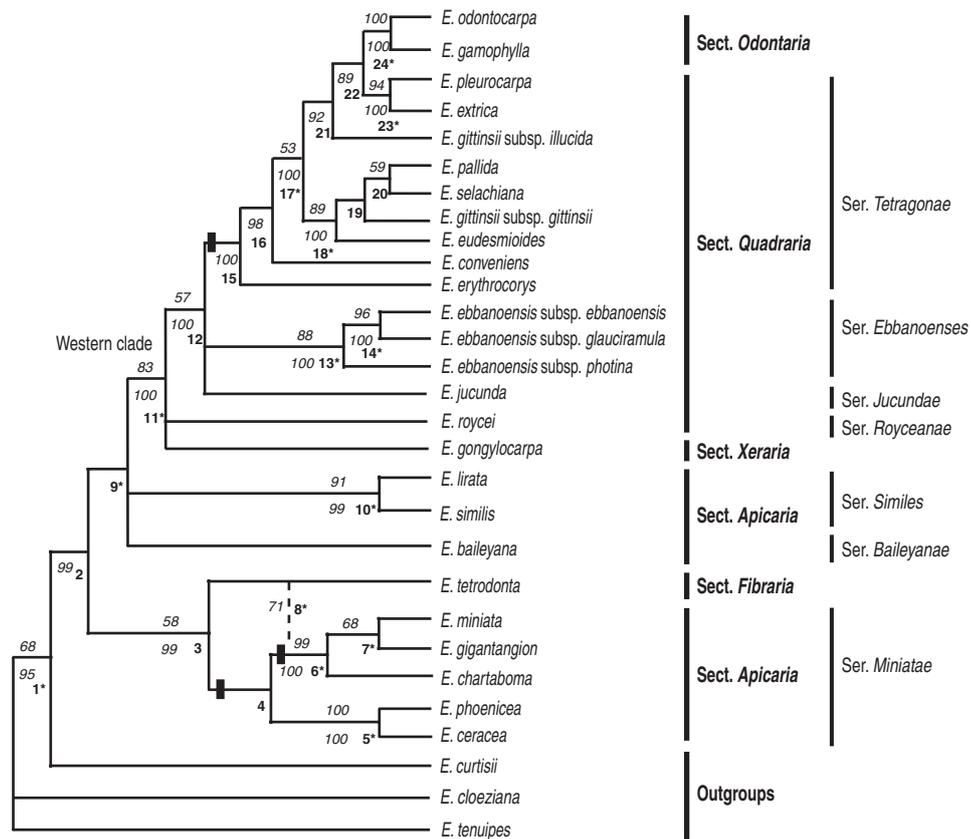
Within the Western clade, taxa at Node 9 are all mallees (Character 1), and those at Node 10 all have flowers in clusters

of three (Character 6, paralleled in *E. tetrodonta*). The clade (Node 11), consisting of taxa in series *Ebbanoensis* + *Tetragonae*, have stamens at anthesis in four distinct bundles on an undulating or discontinuous staminophore (Character 4). Bundling of stamens arises from four separate basal buttresses of the corolline whorl. All eudesmid species examined (Drinnan and Ladiges 1989a) develop these buttresses; a degree of stamen bundling is evident in flowers of *E. baileyana* and *E. tetrodonta* (Maiden 1922: vol. 5, p. 136) as well as *E. gongylocarpa*; however, at anthesis this is indistinct, the stamens forming a continuous ring owing to early meristematic fusion of the basal epipetalous buttresses (Drinnan and Ladiges 1989c). Various conditions in this character may be nothing more than the result of minor differences in timing of meristematic fusion.

## Discussion

### *Phylogenetic analyses and utility of the datasets*

The parsimony and Bayesian methods were mostly congruent, with all strongly supported nodes identified in the parsimony



**Fig. 6.** Tree from the Bayesian analysis of the combined nrDNA and cpDNA datasets. Nodes are numbered in bold, and those that are also resolved by parsimony analysis are marked with an asterisk. Nodes 2, 3, 4, 12, 15, 16, 21 and 22 collapsed in the parsimony strict consensus tree. Numbers above the branches indicate bootstrap values  $\geq 50\%$ , and those below the branches indicate posterior probabilities  $\geq 95\%$ . The coloured blocks on the branches indicate indels. Sectional and series names are according to Hill and Johnson (1998).

**Table 4. Morphological characters**

No.	Description of character state
1	Habit tree (0), usually mallee (1)
2	Bark not yellow jacket (0) yellow jacket (1)
3	Sepals free on rim of hypanthium (0), sepals carried with growth of corolline operculum, may be seen as small teeth at tip of operculum (1) (see text)
4	Stamens at anthesis in continuous ring (0), distinctly four bundled on undulating staminophore (1) (see text)
5	Stamen colour white/cream (0), yellow (1), orange (2)
6	Inflorescence 7-flowered (0), 3-flowered (1), >11-flowered (2)
7	Ovules and ovulodes in four rows (0), in two rows (1)
8	Seeds yellow-brown with terminal hilum (from anatropous ovules) (0), seeds grey-black with ventral hilum (from hemitropous ovules) (1)
9	Seed without wing (0), winged (1)
10	Seeds small (0), medium-large, thick, oval to cuboid (1), elongated ribbed (2), flattened, saucer shaped with dorsal keel (3)
11	Leaf waxes: plates (0), tubes, simple, branching occasionally at acute angles (1) (Hallam and Chambers 1970)
12	Trichomes (hairs on raised oil glands): absent (0), multicellular, blunt-ended (1), unicellular, short and blunt-ended (2), unicellular long, acute-ended (3)

analysis also found by Bayesian inference, although the latter consistently resolved more nodes.

All datasets have their advantages and limitations. The DNA regions sequenced here provided informative characters, including three indels. For the eudesmids, the ITS2 region had

greater sequence conservation than the ITS1 region, as reported for other taxa (Hershkovitz and Lewis 1996). The ETS region was easily aligned with no regions of ambiguity, and had a similar number of informative characters as did the ITS2 region. The combined dataset of the ITS and ETS regions

**Table 5. Morphological characters (see Table 4)**  
? = unknown

Species	Character number											
	1	2	3	4	5	6	7	8	9	10	11	12
<i>E. odontocarpa</i>	1	0	0	0	0	1	1	1	1	3	1	0
<i>E. gamophylla</i>	1	0	0	0	0	1	1	1	1	3	1	0
<i>E. eudesmioides</i>	1	0	0	1	0	1	0	1	1	2	1	3
<i>E. pallida</i>	1	0	0	1	0	1	0	1	1	2	?	3
<i>E. selachiana</i>	1	0	0	1	0	1	0	1	1	2	?	3
<i>E. gittinsii</i>	1	0	0	1	0	1	0	1	1	2	?	3
<i>E. conveniens</i>	1	0	0	1	0	1	0	1	1	2	?	3
<i>E. pleurocarpa</i>	1	0	0	1	0	1	0	1	1	2	1	3
<i>E. extrica</i>	1	0	0	1	0	1	0	1	1	2	?	3
<i>E. erythrocorys</i>	1	0	0	1	1	1	0	1	1	2	1	3
<i>E. ebbanoensis</i>	1	0	1	1	0	1	0	1	0	4	1	3
<i>E. roycei</i>	1	0	1	0	0	0	0	1	1	1	1	3
<i>E. jucunda</i>	1	0	1	0	0	0	0	1	1	1	1	3
<i>E. gongylocarpa</i>	0	0	0	0	0	0	0	1	1	1	1	3
<i>E. tetrodonta</i>	0	0	0	0	0	1	0	1	0	1	0	1
<i>E. baileyana</i>	0	0	1	0	0	0	0	1	0	1	0	1
<i>E. similis</i>	0	1	1	0	0	0	0	1	0	1	0	1
<i>E. lirata</i>	0	1	1	0	0	0	0	1	0	1	0	2
<i>E. miniata</i>	0	0	1	0	2	0	0	1	0	1	0	1
<i>E. gigantangion</i>	0	0	1	0	2	0	0	1	0	1	?	1
<i>E. chartaboma</i>	0	0	1	0	2	0	0	1	0	1	?	1
<i>E. phoenicea</i>	0	0	1	0	2	2	0	1	0	1	0	1
<i>E. ceracea</i>	0	0	1	0	2	2	0	1	0	1	?	1

produced a more resolved phylogeny than each dataset separately, a finding similar to what has been reported in other studies (e.g. Baldwin and Markos 1998; Parra-O. *et al.* 2006).

The *psbA-trnH* intergenic spacer region provided a number of informative characters similar to each of the nrDNA regions and yielded two informative indels, near the 5' end of the *trnH* gene, and several poly A/T repeats (Shaw *et al.* 2005). Direct repeats of sequence blocks of 6–12 nucleotides in the alignment were autapomorphic indels and thus not informative.

Limitations include the problems of pseudogenes (ITS), possible hybridisation with chloroplast capture confounding phylogenetic analyses and homoplasy in morphology that requires understanding of developmental processes. The identification of pseudogenes in the ITS-1 region meant that concerted evolution of this region cannot be assumed; thus, care needs to be taken when analysing ITS sequences. On the basis of analysis of chloroplast DNA, McKinnon *et al.* (1999) detected hybridisation between closely related eucalypt species sampled from the same geographic area, and concluded that cpDNA may provide information about geographic patterns rather than taxon relationships. This does not appear to be a general problem in *Eudesmia* although there may be exceptions. For example, the relationship of *E. tetrodonta* with *E. similis* and *E. lirata* according to the maternally inherited cpDNA was in conflict with the biparentally inherited nrDNA, where *E. tetrodonta* grouped closer to series *Miniatae*. *E. tetrodonta* overlaps the distribution range of these taxa in northern Queensland, and DNA may have been derived from plants with a history of hybridisation or introgression events. This widespread species needs further study across its broad distribution range to test for population variation and the possibility of hybridisation;

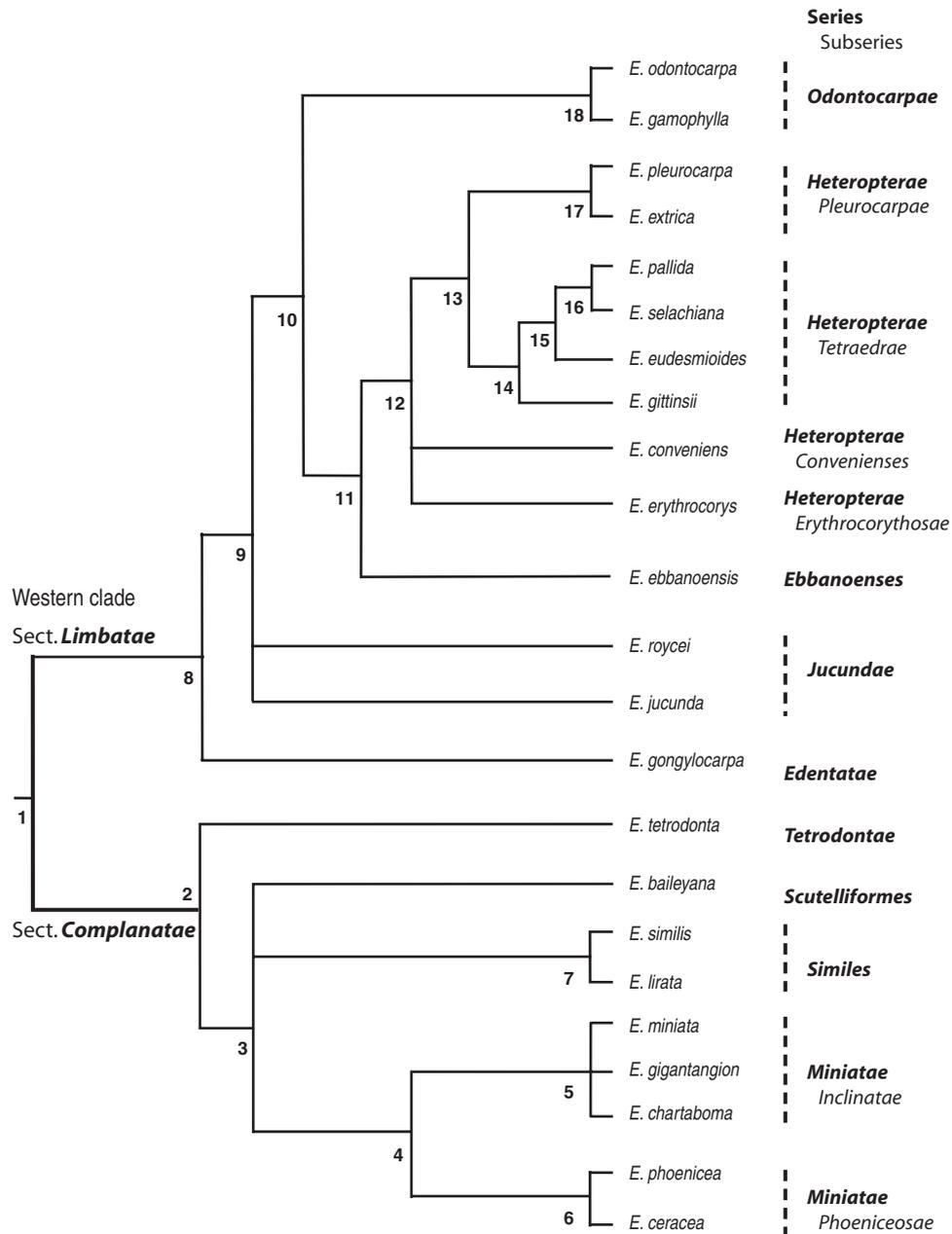
Bean (2006) recently recognised a new species *E. megasepala*, reportedly related to *E. tetrodonta*, which we have not sequenced.

Addition of morphological characters to supported nodes scored as characters from the nuclear trees, improved resolution of relationships. Morphology provided additional synapomorphies, particularly for the main clades (basal nodes). At the same time, evidence of relationships based on DNA sequencing helped identify homoplasy among morphological characters, and thus contributed to an understanding of characters that have in the past appeared problematic.

#### *Phylogeny of Eucalyptus subgenus Eudesmia and revised classification*

The summary phylogenetic tree (Fig. 7) is the basis of the revised classification in Table 6. Taxa are monophyletic and arranged in phyletic sequence, such that the phylogenetic tree can be constructed from the hierarchy of names. In phyletic sequencing (Wiley 1981), the first taxon in the list is the sister group to those below it of the same rank. For polytomous nodes, each lineage is given an equal rank. In our revised treatment, the valid names in the classification of Brooker (2000) are conserved where appropriate because the classification of Hill and Johnson (1998) was extracodical.

At the base of Tree 1 (Fig. 7), there are three possible relationships for *E. tetrodonta*, including sister taxon to all eudesmids, sister taxon to the Western clade or sister taxon to the other northern taxa. The evidence presented as Tree 2 (Fig. 7) points to its relationship with the northern group and, consequently, we have classified them together. This relationship is supported by multicellular hairs and nrDNA,



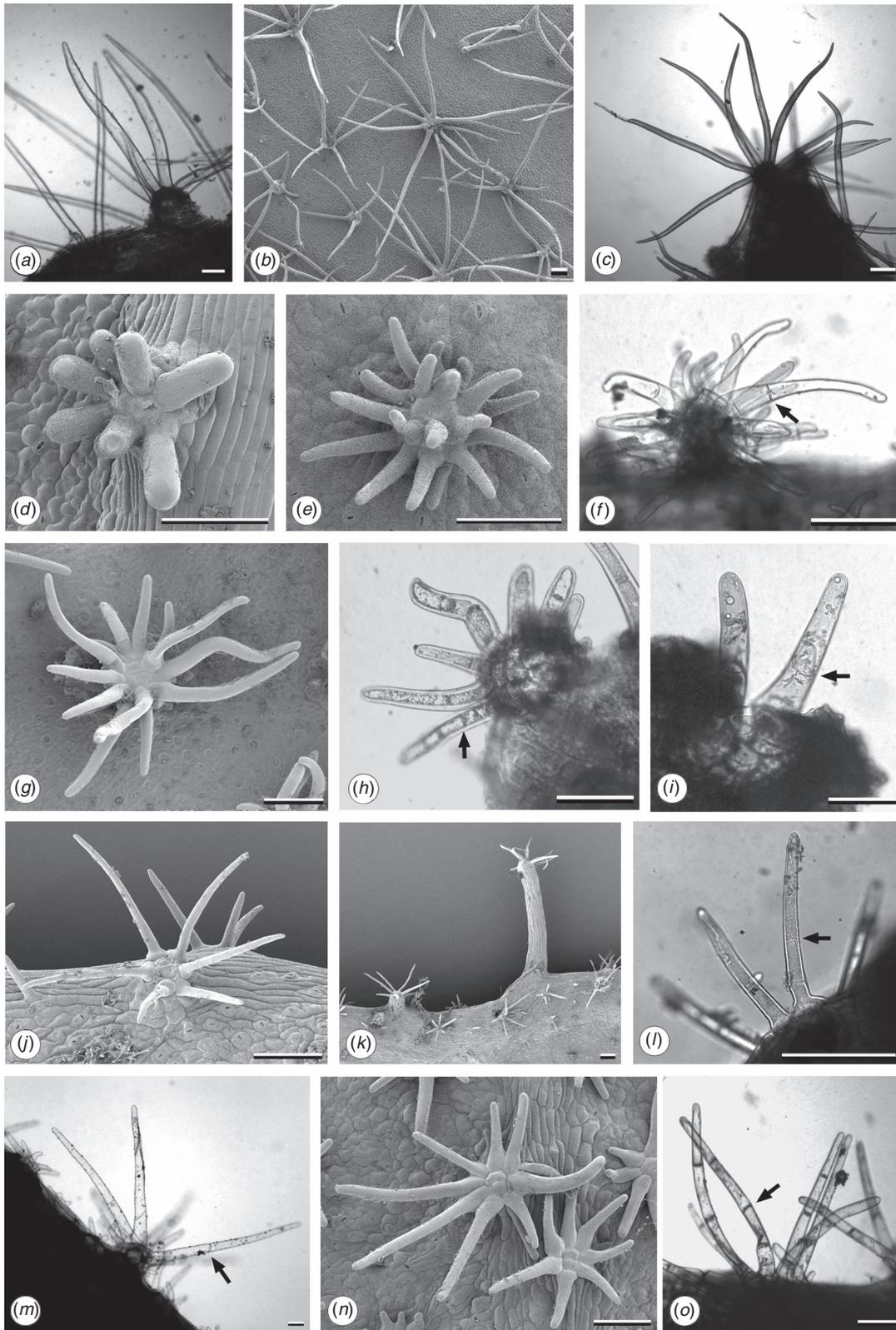
**Fig. 7.** Parsimony analysis of combined morphological characters and nodes coded as characters from nrDNA and cp DNA trees. The following two trees were found: Tree 1, with an unresolved basal polytomy; and Tree 2, shown here with *Eucalyptus tetradonta*, relates to the other northern species on the basis of multicellular, blunt-ended hairs. Our revised classification of sections, series and subseries is shown; compare Table 1 for the classifications of Hill and Johnson (1998) and Brooker (2000).

and these species also have rough persistent bark. The two main clades in Tree 2 (Fig. 7) are classified as two sections, *Complanatae* and *Limbatae*, conserving two sectional names of Brooker (Table 6). These two sections equate to informal sections *Quadraria* and *Apicaria* of Pryor and Johnson (1971), except for their placement of *E. tetradonta* in the former.

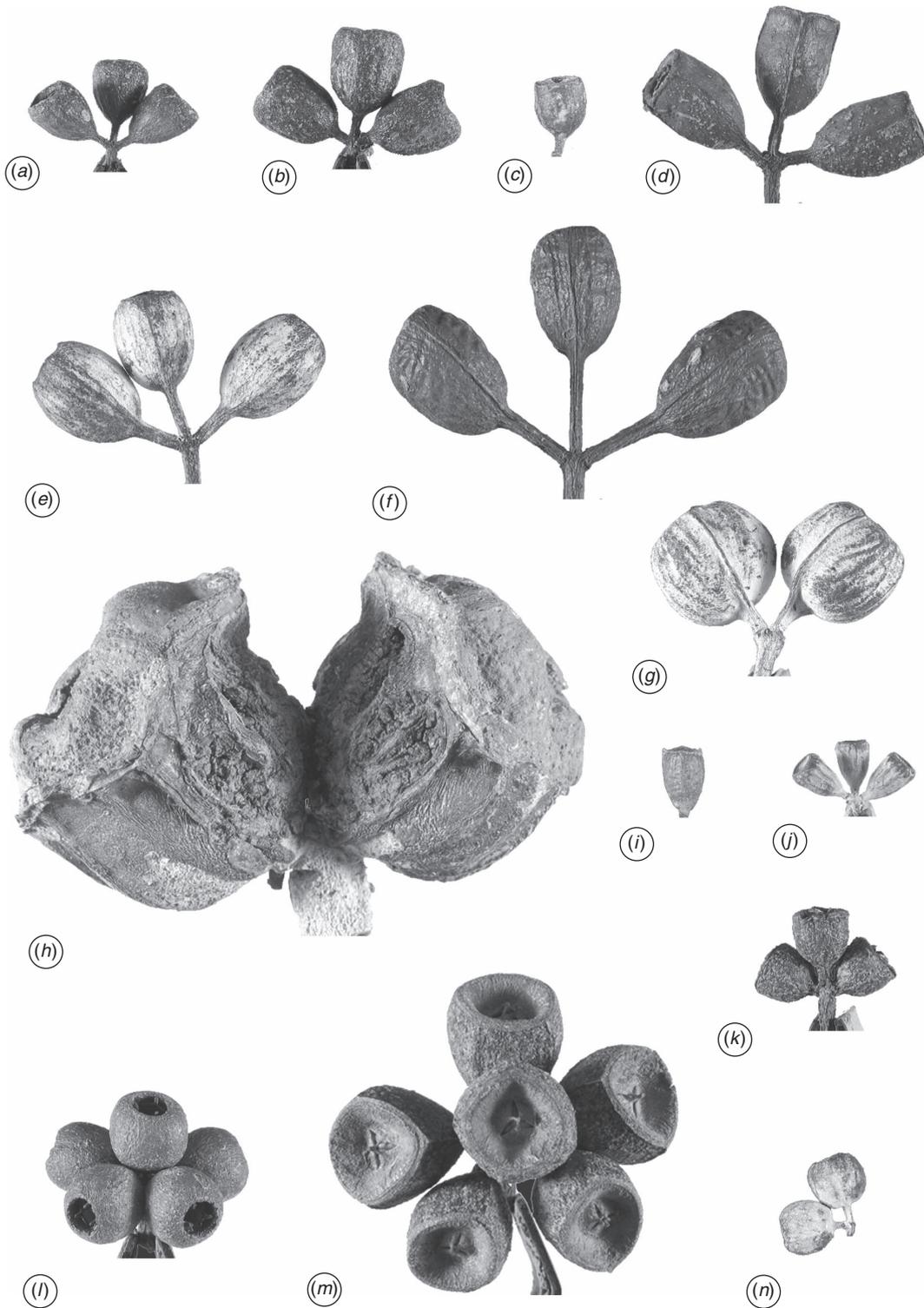
Section *Complanatae* is the clade of nine species from northern and eastern Australia, grouped in four series. In sequence, these are series *Tetradontae* (*E. tetradonta*), series

*Scutelliformes* (*E. baileyana*), series *Similes* (*E. similis* and *E. lirata*) and series *Miniatae*. Within series *Miniatae*, the clades *E. phoenicea* + *E. ceracea* and *E. miniata* + *E. gigantangion* + *E. chartaboma*, which are sister groups, are treated as two subseries *Phoeniceosae* and *Inclinatae*, respectively, similar to Hill and Johnson (1998).

Section *Limbatae* is the clade of 14 species from Western Australia. Within this section, we recognise six series and four subseries whereas Brooker's treatment included only



**Fig. 8.** Examples of trichomes of *Eucalyptus* subg. *Eudesmia*. Hairs radiate from raised oil glands. Unicellular, acute-ended hairs of (a) *E. extrica* and (b, c) *E. ebbanoensis*. (d) Unicellular, short, blunt-ended hairs of *E. lirata*. Multicellular, blunt-ended hairs of (e, f) *E. baileyana*, (g, h) *E. erythrocorys*, (i) *E. roycei*, (j–l) *E. tetradonta*, (m) *E. chartaboma* and (n, o) *E. miniata*. Cross-walls are indicated by arrows.



**Fig. 9.** Examples of fruits of *Eucalyptus* subgenus *Eudesmia* at life size. (a) *E. eudesmioides*, (b) *E. selachiana*, (c) *E. pallida*, (d) *E. gittinsii*, (e) *E. conveniens*, (f) *E. extrica*, (g) *E. pleurocarpa*, (h) *E. erythrocorys*, (i) *E. odontocarpa*, (j) *E. gamophylla*, (k) *E. ebbanoensis*, (l) *E. jucunda*, (m) *E. roycei*, (n) *E. gongylocarpa*, (o) *E. tetradonta*, (p) *E. similis*, (q) *E. lirata*, (r) *E. baileyana*, (s) *E. miniata*, (t) *E. chartaboma*, (u) *E. gigantangion*, (v) *E. phoenicea* and (w) *E. ceracea*.

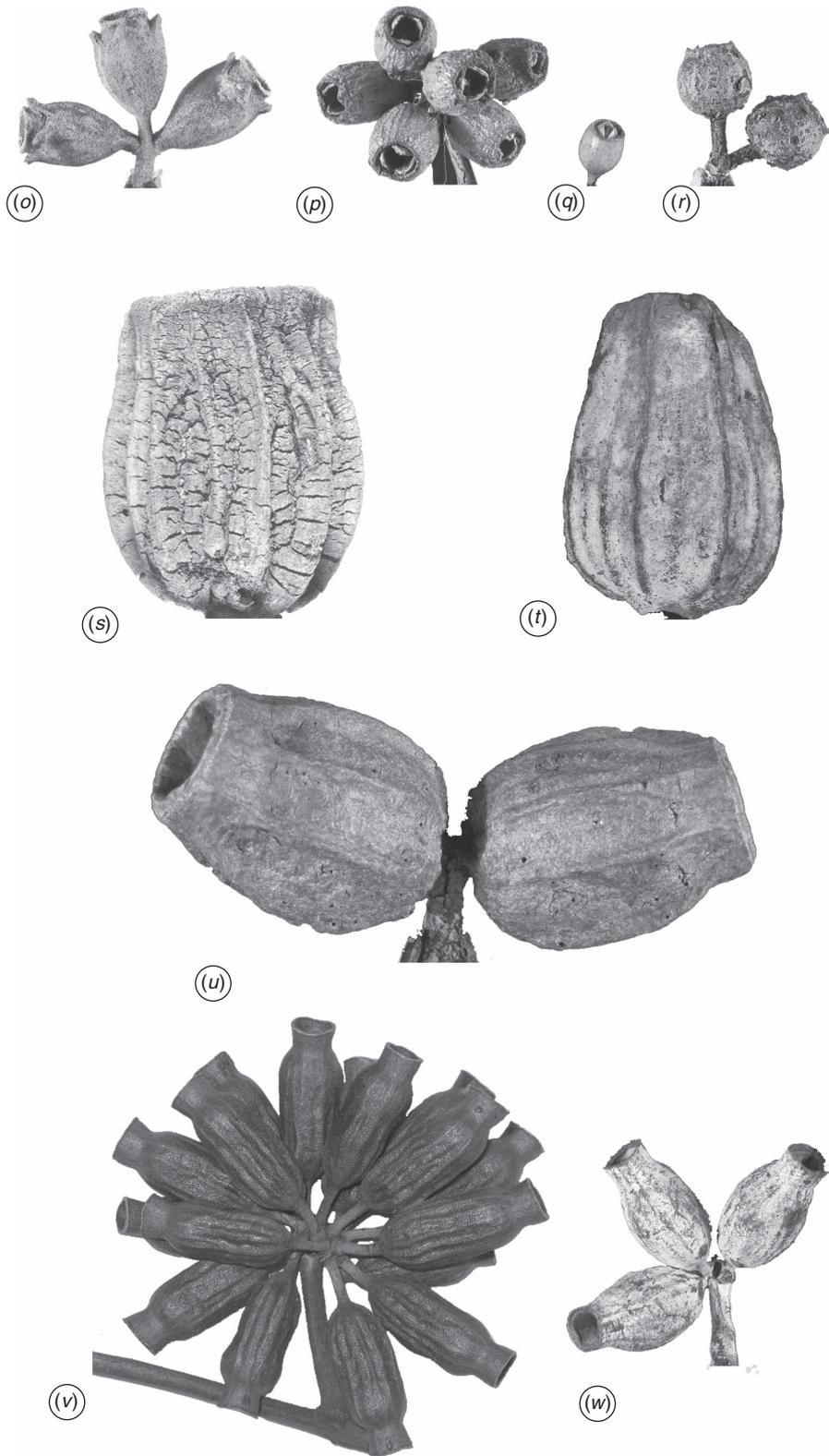
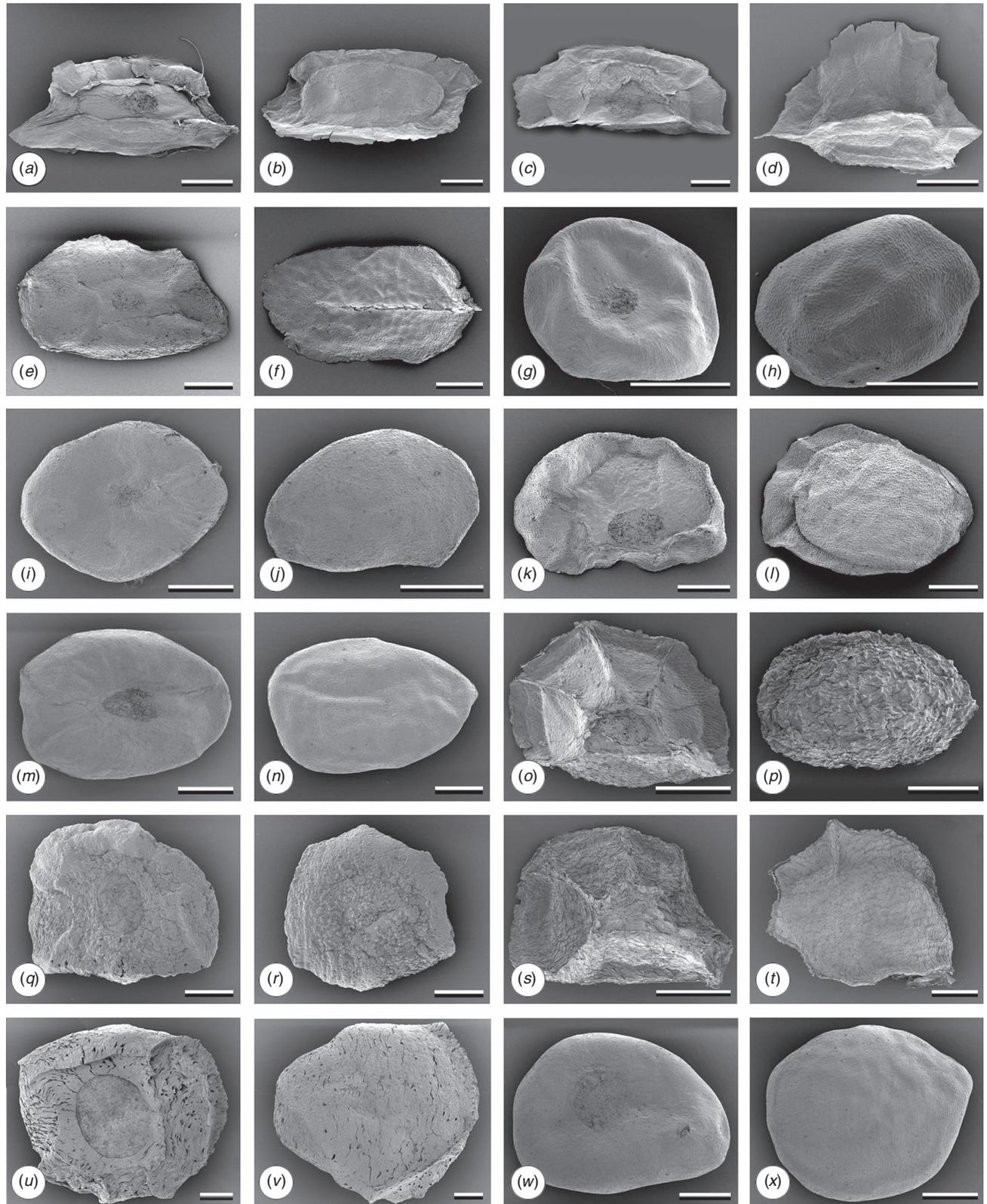


Fig. 9. (continued)



**Fig. 10.** Examples of seeds of *Eucalyptus* subg. *Eudesmia*. (a, b) *E. eudesmioides*, (c, d) *E. gittinsii*, (e, f) *E. odontocarpa*, (g, h) *E. ebbanoensis*, (i, j) *E. similis*, (k, l) *E. gongylocarpa*, (m, n) *E. baileyana*, (o, p) *E. jucunda*, (q, r) *E. tetradonta*, (s, t) *E. roycei*, (u, v) *E. chartaboma* and (w, x) *E. phoenicea*. The left photo in the pair is the ventral view and the right photo is the dorsal view. Scale bar = 100  $\mu$ m.

**Table 6. Revised classification of subgenus *Eudesmia* on the basis of Fig. 7*****Eucalyptus* sect. *Complanatae* Brooker**Typus: *E. tetradonta* F.Muell.[= sect. *Apicaria* + sect. *Apicaria* Hill & Johnson; subsumes sect. *Reticulatae* Brooker]***Eucalyptus* ser. *Tetradontae* Chippendale***E. tetradonta****Eucalyptus* ser. *Scutelliformes* Brooker**[= sect. *Baileyanae* Hill & Johnson]*E. baileyana****Eucalyptus* ser. *Similes* A.K.Gibbs & Ladiges, ser. nov.**

Arbores; cortex persistens flavo-brunneus; inflorescentiae 7-florae; sepala incremento operculi-corollini vecta; semina elliptica griseo-nigra exalata

Typus: *E. similis* Maiden

Trees with persistent, yellow-brown bark; unit inflorescences 7-flowered; sepals carried with growth of corolline operculum; seeds elliptical, greyish black, not winged

[= ser. *Similes* Hill & Johnson; ser. *Miniatae* subser. *Variables* Brooker]*E. similis**E. lirata****Eucalyptus* ser. *Miniatae* Blakely**Typus: *E. miniata* A.Cunn. ex Schauer***Eucalyptus* subser. *Inclinatae* Brooker pro parte***E. miniata**E. chartaboma**E. gigantangion****Eucalyptus* subser. *Phoeniceosae* A.K.Gibbs & Ladiges, subser. nov.**

Arbores; cortex persistens fibrosus; inflorescentiae &gt;11-florae; stamina aurantiaca in annulo disposita; sepala incremento operculi-corollini vecta; semina griseo-nigra exalata

Typus: *E. phoenicea* F.Muell.

Trees with persistent fibrous bark; unit inflorescences more than 11-flowered; stamens orange in continuous ring; sepals carried with growth of corolline operculum; seeds greyish-black, not winged

[= subser. *Phoeniceosae* Hill & Johnson; subser. *Inclinatae* Brooker pro parte]*E. phoenicea**E. ceracea****Eucalyptus* sect. *Limbatae* Brooker**Typus: *Eudesmia tetragona* R.Br. (= *Eucalyptus pleuropcarpa* Schauer)***Eucalyptus* ser. *Edentatae* Brooker**Typus: *E. gongylocarpa* Blakely[= sect. *Xeraria* Hill & Johnson; ser. *Edentatae* subser. *Acetabuliformes*]*E. gongylocarpa****Eucalyptus* ser. *Jucundae* Chippendale**Typus: *E. jucunda* C.A. Gardner[= ser. *Jucundae* Hill & Johnson; ser. *Edentatae* subser. *Jucundae* Brooker]*E. jucunda**E. roycei****Eucalyptus* ser. *Odontocarpae* Chippendale**Typus: *E. odontocarpa* F.Muell.[= sect. *Odontaria* Hill & Johnson; ser. *Heteropterae* subser. *Patelliformes* Brooker]*E. odontocarpa**E. gamophylla****Eucalyptus* ser. *Ebbanoenses* Chippendale**Typus: *E. ebbanoensis* Maiden[= sect. *Quadraria* ser. *Ebbanoenses* Hill & Johnson; sect. *Ebbanoenses* Brooker]*E. ebbanoensis* subsp. *ebanoensis*subsp. *photina*subsp. *glauciramula*

Table 6. (continued)

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***Eucalyptus* ser. *Heteropterae*** Maiden  
*Typus: Eudesmia tetragona* R.Br. (= *Eucalyptus pleurocarpa* Schauer)

***Eucalyptus* subser. *Erythrocorythosae*** A.K.Gibbs & Ladiges, subser. nov.  
 Arborea vel frutices ‘mallees’; cortex laevis; inflorescentiae 3-florae; operculum sepalinum rubrum 4-cristatum distincte; stamina flavo-virida  
*Typus: E. erythrocorys* F.Muell.  
 Trees or mallees, bark smooth; unit inflorescence 3-flowered, sepaline operculum red with four distinct ridges; stamens yellow-green  
 [= subser. *Erythrocorythosae* Hill & Johnson; subser. *Tetraedrae* Brooker pro parte]  
*E. erythrocorys*

***Eucalyptus* subser. *Convenienses*** A.K.Gibbs & Ladiges, subser. nov.  
 Frutices ‘mallees’; cortex laevis; surculi pruinosi; sepala libera; stamina in 4 fasciculis disposita  
*Typus: E. conveniens* L.A.S. Johnson & K.D. Hill  
 Mallees, bark smooth; shoots pruinose compared with *E. gittinsii*; sepals free, stamens in four bundles  
 [= subser. *Tetragonosae* Hill & Johnson pro parte; subser. *Tetraedrae* Brooker pro parte]  
*E. conveniens*

***Eucalyptus* subser. *Tetraedrae*** Brooker (pro parte)  
*Typus: E. eudesmioides* F.Muell.  
 [= subser. *Eudesmiodeosae* Hill & Johnson and subser. *Tetraedrae* pro parte]  
*E. gittinsii* subsp. *gittinsii*  
*E. gittinsii* subsp. *illucida*  
*E. eudesmioides*  
*E. pallida*  
*E. selachiana*

***Eucalyptus* subser. *Pleurocarpae*** A.K.Gibbs & Ladiges, subser. nov.  
 Frutices ‘mallee’; cortex laevis; surculi quadrangulares, pruinosi; folia adulta ovata vel elliptica; sepala libera; semina alata angulata, brunneo-nigra; fructus globosi  
*Typus: E. pleurocarpa* Schauer  
 Mallees, bark smooth, shoots quadrangular, pruinose, ovate to elliptic adult leaves; free sepals; stamens in four bundles; seeds angular, brownish-black with wing; globose fruits  
 [= subser. *Tetragonosae* Hill & Johnson; subser. *Tetraedrae* Brooker pro parte]  
*E. pleurocarpa*  
*E. extrica*

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two series, each with two subseries (Table 1). In comparison Hill and Johnson (1998) placed these species in three sections, one of which included four series and three subseries (Table 1). Some of these differences are a matter of rank, whereas others imply monophyletic groups not supported by our analysis. One significant change in our classification is the rank of the group of sister species *E. odontocarpa* and *E. gamophylla*. We treat them as series *Odontocarpae*, whereas Hill and Johnson (1998) elevated them to sectional level, implying a more basal phylogenetic position. Brooker (2000) treated them as a subseries related to subseries *Tetraedrae*, a relationship supported by our findings. Brooker’s treatment of *E. ebbanoensis* as a monotypic section isolated from its relatives identified here as the Western clade, is not supported.

Nicolle (2000) suggested that *E. pallida* was not significantly distinct from *E. eudesmioides*; however, on the basis of molecular sequences and morphology it is concluded to be a separate species from *E. eudesmioides* and most closely related to *E. selachiana*. Our results support the view of Nicolle (2000) that *E. extrica* is the closest relative of *E. pleurocarpa*, which we have classified as subseries *Pleurocarpae*.

## Conclusions

Phylogenetic analysis revealed several clades congruent across molecular datasets of ITS2, ETS and *psbA-trnH* spacer regions; however, no molecular dataset was sufficiently informative to resolve all sister group relationships. Limitations of datasets include pseudogenes in ITS, and the possibility that the cpDNA dataset is influenced by historic interspecific hybridisation and chloroplast-capture events. Morphological characters that have been emphasised in various classification schemes of eudesmids require interpretation through developmental studies, and provided further resolution of relationships. Our results provide a basis for a revised phylogenetic classification, recognising a level of congruence with previous formal and informal treatments (including those of Blakely, Chippendale, Hill and Johnson, and Brooker).

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**Appendix 1. DNA sources or published sequences used in analyses (shown here in bold), wet collections of flower buds and examples of voucher specimens examined for morphology**

AB, A. Bohte; AD, Adelaide Herbarium; AG, A. Gibbs; AND, A. N. Drinnan; CANB, Canberra Herbarium; CCA, Currency Creek Arboretum, South Australia; CL, Cookson Laboratory, The University of Melbourne; CSIRO, Commonwealth Scientific and Industrial Research Organisation, Australian Tree Seed Centre; FRI, CSIRO Division of Forest Research, Yarralumla; KPBG, Kings Park and Botanic Gardens, Perth; MELU, The University of Melbourne Herbarium; MIHB, M. I. H. Brooker; NSW, New South Wales; NT, Northern Territory; PYL, P. Y. Ladiges; Qld, Queensland; RBG, Syd., Royal Botanic Gardens, Sydney; SA, South Australia; Vic., Victoria; WA, Western Australia. Subgenera are according to Brooker (2000). For the Currency Creek material, vouchers numbers (DN#) are those of D. Nicolle and are for the parent tree from which first generation offspring were grown (see Nicolle 2003)

Taxon	Source
<b>Subgenus <i>Eudesmia</i></b>	
<i>E. baileyana</i> F.Muell.	<b>CCA DN665 ex Baryulgil, NSW; FJ654408 (ITS1), FJ654384 (ITS2), FJ654425 (ETS) &amp; FJ654358 (<i>psbA-trnH</i>)</b> ; seedlings grown from RBG Syd. Seedbank, seed parent 851642, ex Blackdown Tableland, Qld; Drinnan and Ladiges (1989c), MIHB 4805; CANB441046, 441048–49, 441058–59, 441060, 441070; MEL10607671, 2097666; NSW315585
<i>E. ceracea</i> Brooker & Done	<b>Steane <i>et al.</i> (1999) AF058459 (ITS2); MELU103818 KPBG, Perth, WA, Shade s.n. FJ654367 (<i>psbA-trnH</i>)</b> ; seedlings grown from RBG Syd. Seedbank, seed parent 841841, ex 103.2 km west of King George River Crossing, WA; CANB410319, 410742, 44759, 459004, 551306, 635034; MEL1560203
<i>E. chartaboma</i> D.Nicolle	<b>Seedling grown from RBG Syd. Seedbank, seed parent 981107, ex Undara Lodge car park, Qld; FJ654412 (ITS1), FJ654389 (ITS2) &amp; FJ654364 (<i>psbA-trnH</i>)</b> ; CANB414892, 414917, 414924; CBG43080, 7806166; MEL241393, 717505; NSW315617
<i>E. conveniens</i> L.A.S.Johnson & K.D.Hill	<b>CCA DN1162, ex Mt Adams, WA; FJ654402 (ITS1), FJ654378 (ITS2), FJ654419 (ETS) &amp; FJ654350 (<i>psbA-trnH</i>)</b> ; CBG7901894; NSW200793, 341194, 341196–7; PERTH1441264, 1455591, 4439899, 5031702
<i>E. ebbanoensis</i> Maiden subsp. <i>ebbanoensis</i>	<b>CCA DN293, ex Wongan Hills, WA; FJ654407 (ITS1), FJ654383 (ITS2), FJ654421 (ETS) &amp; FJ654352 (<i>psbA-trnH</i>)</b> ; CANB280331; MEL1609776, 1609778, 1609784, 1647091, 2011076; NSW341209; PERTH6476260
<i>E. ebbanoensis</i> subsp. <i>glauciramula</i> L.A.S.Johnson & K.D.Hill	<b>CCA DN2726, ex Balpe Lakes, WA; FJ654382 (ITS2) &amp; FJ654351 (<i>psbA-trnH</i>)</b> ; seedlings grown from RBG Syd. Seedbank, seed parent 866043, ex 10.6 km south of Diemals–Menzies road on Bullfinch Rd, WA; MEL1609782–3, 1611656; NSW201136, 341315; PERTH1341391, 5327725, 5483506
<i>E. ebbanoensis</i> subsp. <i>photina</i> Brooker & Hopper	<b>CCA DN270 ex Mt Michael, WA; FJ654406 (ITS1), FJ654381 (ITS2) &amp; FJ654353 (<i>psbA-trnH</i>)</b> ; seedlings grown from RBG Syd. Seedbank, seed parent 865997, ex Mt Michael, WA; CANB412305; MEL681970, 1600755, 1609775, 1609780; PERTH1138510, 1340883, 1370979
<i>E. erythrocorys</i> F.Muell.	<b>Grown from seed parent CCA DN265, ex Eneabba, WA; FJ654403 (ITS1), FJ654380 (ITS2) &amp; FJ654422 (ETS)</b> ; Udovicic and Ladiges (2000) <b>AF190382 (<i>psbA-trnH</i>)</b> ; AG005 (CL wet collection), ex Flinders University, SA (MELU103820); AB159 (CL wet collection), ex Maranoa Gardens, Camberwell, Vic.; CANB412315; PERTH1377221, 1377752, 5018870, 5810795, 6502156
<i>E. eudesmioides</i> F.Muell.	<b>Steane <i>et al.</i> (2002) AF390468 (ITS2); CCA DN279, ex Bunjil, WA; FJ654416 (ETS) FJ654346 (<i>psbA-trnH</i>)</b> ; AB144 (CL wet collection), ex The Points Arboretum, Hamilton, Vic.; CANB412315; PERTH2250497, 5625971, 5796717, 6070248, 1380435
<i>E. extrica</i> D.Nicolle	<b>CCA DN1092, ex Cape Arid, WA; FJ654392 (ITS1), FJ654376 (ITS2) &amp; FJ654343 (<i>psbA-trnH</i>)</b> ; AD105855, 96922191; PERTH1455494, 1441779, 1443313, 5160944
<i>E. gamophylla</i> F.Muell.	<b>MELU104216 Grown from seed, King s.n., ex near Utopia, now known as Urapuntja, NT; FJ654395 (ITS1), FJ654372 (ITS2), FJ654415 (ETS) &amp; FJ654360 (<i>psbA-trnH</i>)</b> ; seedlings grown from RBG Syd. Seedbank, seed parent 951394, ex north of Beyondie Homestead, WA; CCA, location unknown; MEL272670, 702822, 711268, 1530685, 2117879, 2201104
<i>E. gigantangion</i> L.A.S.Johnson & K.D.Hill	<b>Grown from DN2483 (AD), ex Twin Falls escarpment, NT; FJ654413 (ITS1), FJ654390 (ITS2) &amp; FJ654365 (<i>psbA-trnH</i>)</b> ; CANB415734, 435968; DNA24713, 30587, 55319, 57083, 145009
<i>E. gittinsii</i> Brooker & Blaxell subsp. <i>gittinsii</i>	<b>CCA DN1170, ex Murchison River, WA; FJ654401 (ITS1) &amp; FJ654344 (<i>psbA-trnH</i>)</b> ; CANB412344, 447243, 460178, 513678.1; CBG7908949–50; MEL1610348
<i>E. gittinsii</i> subsp. <i>illucida</i> D.Nicolle	<b>CCA DN252, ex Mt Misery, WA; FJ654375 (ITS2) &amp; FJ654345 (<i>psbA-trnH</i>)</b> ; CANB447240, 460184, 530803, 610761; CBG7901929, 7901936
<i>E. gongylocarpa</i> Blakely	<b>CCA DN519, ex Mulga Rockhole, WA; FJ654398 (ITS1), FJ654370 (ITS2), FJ654423 (ETS) &amp; FJ654355 (<i>psbA-trnH</i>)</b> ; seedlings grown from CCA 200, ex Camel Well, WA; seedlings grown from CSIRO seed 17079, ex Lake Amadeus, NT; B2412 (FRI wet collection); MEL1610424, 1610426; PERTH1172425, 1250531, 1281445, 4159586

## Appendix 1. (continued)

Taxon	Source
<i>E. jucunda</i> C.A.Gardner	CCA DN275, ex Mullewa northern wheatbelt, WA; FJ654404 (ITS1), FJ654379 (ITS2), FJ654420 (ETS) & FJ654356 ( <i>psbA-trnH</i> ); seedlings grown from RBG Syd. Seedbank, seed parent 910297, ex 30 km on Coolcalalaya Rd off North Coastal Hwy, near Binu, WA; PERTH1411519, 1411616, 5054338, 5530822, 5546206, 5837162
<i>E. lirata</i> W.Fitzg. ex Maiden	Grown from RBG Syd. Seedbank, seed parent 841844, ex 79 km south-west of Pentecost River crossing on Gibb River Rd, WA; FJ654396 (ITS1), FJ654385 (ITS2), FJ654424 (ETS) & FJ654361 ( <i>psbA-trnH</i> ); B4282 (FRI wet collection); CANB414680; MEL1611019; PERTH1282239, 1282182, 1282700, 1283235
<i>E. miniata</i> A.Cunn. ex Schauer	Manbulloo Station, NT, King s.n. (MELU104213); FJ654411 (ITS1), FJ654388 (ITS2), FJ654426 (ETS) & FJ654363 ( <i>psbA-trnH</i> ); seedlings grown from CCA DN1903, Derby, WA (plains form); seedlings grown from DN4224 (CANB) (scarp form), ex Arnhem Land, WA; AG027 (CL wet collection), ex near Bells Gorge, WA; CANB414774, 507083; CBG8003470, 8309293; DNA30582, 154953; NSW231330
<i>E. odontocarpa</i> F.Muell.	Grown from seed, MELU104217, King s.n., ex Tennant Creek, NT; FJ654394 (ITS1), FJ654371 (ITS2), FJ654418 (ETS) & FJ654354 ( <i>psbA-trnH</i> ); MEL278530, 230689, 563165, 703571, 709981, 1614334
<i>E. pallida</i> L.A.S.Johnson & K.D.Hill	CCA DN2207, ex North of Nerren Nerren Station, WA; FJ654373 (ITS2), FJ654414 (ETS) & FJ654347 ( <i>psbA-trnH</i> ); seedlings grown from RBG Syd. Seedbank, seed parent 872194, ex Talisker Station, WA; PERTH1380419, 5277620, 5309948, 5546273; NSW242695, 341124
<i>E. phoenicea</i> F.Muell.	MELU104215 Grove Hill, NT, King s.n.; FJ654391 (ITS2), FJ654427 (ETS) & FJ654366 ( <i>psbA-trnH</i> ); seedlings grown from CSIRO seed 19158, ex Battle Camp, Qld; AQ109039, 620796; DNA3887, 15727, 19878, 133820, 162101
<i>E. pleurocarpa</i> Schauer	CCA DN154, ex Grass patch, eastern wheatbelt, WA; FJ654393 (ITS1), FJ654377 (ITS2) & FJ654349 ( <i>psbA-trnH</i> ); Parra-O. <i>et al.</i> (2006) DQ352535 (ETS); CCA 65, ex Cape Arid, WA; PERTH1455524, 1470329, 2941325, 4357477, 5024587, 5236762
<i>E. roycei</i> S.G.M.Carr, D.J.Carr & A.S.George	CCA DN1176, ex Coburn Station, west coast, WA; FJ654405 (ITS1); AND472 (MELU), Kings Park Arboretum, WA; FJ654357 ( <i>psbA-trnH</i> )
<i>E. selachiana</i> L.A.S.Johnson & K.D.Hill	CCA DN1176, ex Coburn Station, west coast, WA; FJ654400 (ITS1), FJ654374 (ITS2), FJ654417 (ETS) & FJ654348 ( <i>psbA-trnH</i> ); CANB445001; PERTH1353551, 5483689, 1380494; NSW242679, 341174
<i>E. similis</i> Maiden	Grown from RBG Syd. Seedbank, seed parent 854070, ex 48.4 km from Aramac towards 'Eastmere', Qld; FJ654409 (ITS1), FJ654386 (ITS2) & FJ654359 ( <i>psbA-trnH</i> ); Drinnan and Ladiges (1989c), A.R. Bean 314; AQ133070, 457476, 541366, 599673, 652439, 657933
<i>E. tetradonta</i> F.Muell.	MELU104214 Manbulloo Station, NT, King s.n.; FJ654410 (ITS1), FJ654387 (ITS2), FJ654428 (ETS) & FJ654362 ( <i>psbA-trnH</i> ); seedling grown from CSIRO seed 13673, ex 11 km north of Laura, Qld; MEL703418, 706055, 262659, 1558302, 1601291, 1614764
<b>Subgenus <i>Acerosae</i></b>	
<i>E. curtisii</i> Blakely & C.T.White	Steane <i>et al.</i> (2002) AF390524 (ITS2); Grown from RBG Syd. Seedbank, seed parent 950722, ex Ballon, Qld (seed lot 106); FJ654342 ( <i>psbA-trnH</i> ); Parra-O. <i>et al.</i> (2006) DQ352530 (ETS); AB068 (CL wet collection), ex Maranoa Gardens, Camberwell, Vic.; AQ92731; CANB30490, 432505, 544961, 7901894; CBG7901894; MEL713756, 1610192, 1609015; NSW307251
<b>Subgenus <i>Cuboidea</i></b>	
<i>E. tenuipes</i> (Maiden & Blakely) Blakely & C.T.White	Grown from RBG Syd. Seedbank, seed parent 961716, ex Isla Gorge National Park, Qld; FJ654397 (ITS1), FJ654368 (ITS2) & FJ654340 ( <i>psbA-trnH</i> ); PYL857 (CL wet collection), ex Yeluba-Serat road, Qld; AQ133268, 490871, 650188; NSW221028, 227787, 304692
<b>Subgenus <i>Idiogenes</i></b>	
<i>E. cloeziana</i> F.Muell.	Grown from RBG Syd. Seedbank, seed parent 861779, ex 26 km south-west of Monto, Qld (CSIRO seed lot 13543); FJ654369 (ITS2) & FJ654341 ( <i>psbA-trnH</i> ); Parra-O. <i>et al.</i> (2006) DQ352529 (ETS); AB119 (CL wet collection), ex Maranoa Gardens, Camberwell, Vic.; CANB413264-5, 413269, 413280-3, 522831, 558256