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The taxonomic relationships within the genus *Excoecaria* L. (Euphorbiaceae) based on leaf morphology and rDNA sequence data.

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Abstract

The tropical Indo-Pacific genus *Excoecaria* L. (Euphorbiaceae) has several closely related species in Australia whose taxonomic relationships are unclear. The most widely reported species in Australia is the mangrove species *Excoecaria agallocha* L., (type species) whose taxonomic and geographic limits are difficult to define from its closely related species or sub-species. Two additional taxa have also been described but not clearly differentiated from the type species: *Excoecaria dallachyana* Baillon and *Excoecaria ovalis* Endl. This project aimed to determine the taxonomic relationships of the Australian *Excoecaria* species using both leaf morphological data and DNA sequence data from the internal transcribed spacer (ITS) region of ribosomal genes. The nucleotide differences in the examined ITS1 region show that *E. agallocha* from eastern Australia and *E. ovalis* from Western Australia respectively, are genetically uniform within species but differ from each other consistently, thus supporting species status. The leaf morphological data also support this view: single factor analysis of variance consistently separated *E. ovalis* from *E. agallocha* on the basis of leaf width, leaf length and length of petiole. In contrast, *E. ovalis* from the Gulf of Carpentaria differs only slightly from *E. ovalis* in Western Australia, but no evidence was found to suggest any leaf morphological differentiation within this species. The analysis also suggests that *E. dallachyana* is not closely related to either mangrove species *E. agallocha* or *E. ovalis,* despite superficial morphological similarity.

Introduction

The tropical Indo-Pacific genus *Excoecaria* L., (Euphorbiaceae) with approximately 40 species in tropical Africa and Asia to the Western Pacific, has several closely related species in Australia whose taxonomic relationships are unclear.

The most widely reported species in Australia is the mangrove species *Excoecaria agallocha* L., (type species) which also occurs from East Africa, India and Sri Lanka, to China and Japan and extends south-east via Malesia to Papua New Guinea and into the Pacific as far as Samoa. In several parts of its range (e.g. Tanzania, India, Pakistan, Bangladesh) this species forms the basis of commercial forestry utilisation. In Australia, this species is recorded from the Hastings River in NSW northwards (Saenger and Moverley, 1985) around the Australian coastline and down as far south in Western Australia as Thangoo Station (18°16'S) (Semeniuk et al., 1978).

Throughout its range, *E. agallocha* is distinguished by its simple, alternate leaves, dioecious condition, flowers lacking petals and an abundance of white latex throughout all parts of the plant. However, the habit (growth form) of the plant and the morphology of the leaves are extremely variable (Tomlinson, 1986). Due to its morphological plasticity its geographic limits are difficult to define from its closely related species or sub-species by morphology alone.

In Australia, two additional taxa have been described but not clearly differentiated from the type species *E. agallocha.* Firstly, *Excoecaria agallocha* var. *dallachyana* Baill., was described by Baillon (1866) from southern Queensland and northern NSW and distinguished mainly by its inland habitat in depauperate rainforest of the Macpherson and Sandiland Ranges (Pax and Hoffman, 1912; Stanley and Ross, 1983; Floyd, 1989). It was then elevated by Bentham (1873) to species level as *E. dallachyana* (Baill.) Benth., although its status as a separate species was questioned by Tomlinson (1986).

A second species, *Excoecaria ovalis* Endl., was described by Endlicher (1833) from material collected by Ferdinand Bauer on the shores of the Gulf of Carpentaria in 1803. However, it was reduced to varietal status by Mueller (1866) as *E. agallocha* var. *ovalis* (Endl.) Muell.-Arg. This species has since been reduced to synonymy under the type species by Airy-Shaw (1975) and by Semeniuk et al. (1978). In contrast, F. Mueller used the unpublished binomial of *E. sphaerosperma* for material that he collected as part of the Northern Australia Expedition of 1855-6 and, more recently, Wightman (1989) re-established *E. ovalis* to species rank.

Comparisons of morphological characters are most often used to develop phylogenies and these inferences relate to the evolutionary connection of particular taxa and show the possible natural historic development of families, genera and species. More recently, techniques for reconstructing plant phylogenies using DNA sequence data have become well established and there are several reviews on the topic (Hillis and Dixon, 1991).

Ribosomal DNA (rDNA) sequences have been used to infer phylogenetic histories across a very broad spectrum, from families to relationships among closely related species and populations. The reasons for the systematic versatility of rDNA include: the numerous rates of evolution among the different regions of rDNA; the presence of multiple copies per genome; and the pattern of concerted evolution that occurs among related copies. These features facilitate the study of rDNA by direct DNA sequencing methods. The internal transcribed spacer (ITS) regions of ribosomal genes have been used successfully at the generic and species level in many plants (Baldwin et al., 1995, Sun et al., 1994, Bain and Jansen, 1994, Liden et al., 1995), and most plant ITS surveys suggest little variation within species (Baldwin et al., 1995). ITS has several advantages that make it an ideal region to sequence. Its rate of evolution is appropriate at the species and generic levels, it is phylogenetically interpretable (ie., the sequences are relatively easy to align because there tends to be little length variation at the generic level in flowering plants), it is large enough to offer potentially enough characters for phylogenetic reconstruction, and it is flanked by

regions that are highly conserved within genera making polymerase chain reaction (PCR) amplification and sequencing straightforward.

The aim of this project was to determine the taxonomic relationships of the Australian species of the genus *Excoecaria* using both leaf morphological data and ITS sequence data. Given the poorly defined limits of the type species *E. agallocha*, based on morphology and distribution, it is hoped that through DNA sequence analysis this can be more fully resolved.

Materials and Methods

Plant material

Plant material was collected from the following sites: *E. dallachyana,* Mallanganee Flora Reserve, NSW, 31/3/96; *E. agallocha,* banks of North Creek, a tributary of the Richmond River, at Ballina, NSW, 18/3/98; *E. ovalis,* Cambridge Gulf, WA, 1/8/97; the eastern population of this species was collected from the type locality, Sweers Island, in the south-eastern Gulf of Carpentaria, Qld., 17/10/97.

Excoecaria parvifolia J. Muell., a distinctive species from the wet-dry savannah of northern Australia, was used as the out-group for sequence analysis; material of this species was collected from Sweers Island, Qld., 17/10/97.

Leaf morphological analysis

In order to be able to distinguish the various taxa from herbarium specimens, an analysis of leaf morphological characteristics was carried out in conjunction with rDNA analysis. For leaf morphological analysis, representative samples of leaves were collected from each of the above localities. At each locality, ten trees were sampled and five randomly selected leaves

were taken from each tree for the following quantitative parameters: leaf length, leaf width and petiole length. Qualitative data on leaf margins and apex shape were also recorded.

One-way analysis of variance (ANOVA) was carried out on these data using Statview™. Multivariate analysis was used to investigate the taxonomic distance between the taxa using the PRIMER software package (Plymouth Marine Laboratories, U.K.). The multivariate cluster analyses were based on normalised Euclidian distance without data transformation. These analyses were followed by non-metric multidimensional scaling ordination (Kruskal and Wish, 1978).

DNA sequence analysis

For DNA sequence analysis leaf samples were collected and stored frozen at -70°C until DNA was extracted. Genomic DNA was isolated using the procedure described by Maguire et al., (1994). DNA was then subjected to gel electrophoresis on a 1.6% agarose gel in TBE buffer (Sambrook et al., 1989) and stained with ethidium bromide. DNA concentration was estimated by visual assessment of band intensities compared to salmon sperm genomic DNA standards.

Based on the published sequence of rice ribosomal DNA (Takaiwa et al., 1985) conserved regions in the 17S and 26S ribosomal genes were chosen as primer sites for PCR amplification of the ITS1 region. The primer sequences were as follows:

(Forward) 5'CGCGAGAAGTTCATTGAACC3';
(Reverse) 5'GATATCCGTTGCCGAGAGTCG3'.

The ITS1 fragment amplified using these two PCR primers was approximately 350 bp long. The PCR amplification conditions were: 1X reaction buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.3, Boehringer Mannheim), 1.5 mM $MgCl_2$, 200 uM each dNTP, 1 uM each

primer, 1 unit Taq polymerase (Boehringer Mannheim), and 10 - 50 ng genomic DNA, in a total volume of 25 uL. After an initial heating step at 94˚C for 3 mins, samples were incubated for 35 cycles of 94°C for 1 min, 60˚C for 1 min, and 72˚C for 2 min. The reactions were completed by incubating at 72˚C for 5 min and held at 4˚C. Cycling conditions were performed using a Perkin Elmer Cetus 9700 Thermal Cycler.

Following amplification, products were purified using a Promega Wizard PCR Preparation kit. Direct PCR sequencing of the purified fragments was carried out using standard conditions in the DyeDeoxy Terminator Sequencing Kit of Applied Biosystems, then automatically sequenced on an Applied Biosystems Model 373A sequencer. Both directions of the ITS1 region were sequenced. Resulting sequence data were visualised and edited using the computer program SEQED (Perkin Elmer Applied Biosystems) and a consensus sequence was determined from both the forward and reverse sequence data for each sample.

Multiple alignments of the ITS1 consensus sequences were performed using the computer program CLUSTAL W (1.7) (Thompson et al., 1994) and manually checked. Phylogenetic relationships between the Australian members of *Excoecaria,* using *E. parvifolia* as the out-group, were analysed using the computer program PAUP 3.1.1 (Swofford, 1990). The phylogeny was assessed using the exhaustive search method of PAUP. The resulting trees were then bootstrapped for 1,000 replications to test the robustness of the tree. Distance analysis was performed using the computer program MEGA (Kumar et al., 1993) and the proportion of nucleotide differences was used to construct a neighbour joining tree.

Results

DNA sequence analysis

Double stranded DNA amplification products were obtained for all samples. The aligned consensus sequences of the ITS1 region for all samples had a size of 263 bp (Table 1). There was no intraspecific variation between the three individuals of *E. ovalis* (WA) and the three individuals of *E. agallocha.*

A distance matrix of the number of different nucleotides (Table 2) showed that there are no differences within *E. ovalis* from WA (samples 1, 2 and 3 respectively), and no differences within *E. agallocha* (samples 5, 6, and 7) indicating no intraspecific sequence variation. There are 3 differences between *E. ovalis* WA (1, 2 and 3) and *E. ovalis* from Sweers Island (sample 4). There are 6 differences between *E. ovalis* WA (1,2 and 3) and *E. agallocha* (5, 6 and 7), and 7 differences between *E. ovalis* from Sweers Island (4) and *E. agallocha* (5, 6, 7). There are 13 differences between *E. ovalis* WA (1, 2 and 3) and *E. dallachyana* (9), 14 differences between *E. ovalis* from Sweers Island (4) and *E. dallachyana* (9), 13 differences between *E. agallocha* (5, 6 and 7) and *E. dallachyana* (9), and 6 differences between *E. parvifolia* (out-group 8) and *E. dallachyana* (9). The out-group *E. parvifolia* (8) has 9 differences to *E. ovalis* WA (1,2 and 3) and *E. agallocha* (5, 6 and 7), and 10 differences to *E. ovalis* from Sweers Island (4).

Leaf morphological analysis

The leaf morphological data are presented in Table 3 together with the results of the ANOVA. All three species can be distinguished on the basis of the leaf morphology, although the populations of *E. ovalis* WA could not be distinguished from *E. ovalis* Sweers Island. These results are supported by the non-metric multidimensional scaling ordination (Fig. 1) which shows the proximity of the *E. ovalis* populations from Sweers Island and Western Australia.

From data in Table 3, *E. agallocha* has the largest leaf with a proportionately short petiole relative to leaf length. The leaf blade is characterised as ovate to elliptic (length:width 1.75)

with an acute apex amd crenate leaf margins. *E. ovalis* has the smallest leaf with a proportionately long petiole relative to leaf length. It leaf blade is characterised as obovate to elliptic (length:width 1.55-1.61) with a blunt apex and entire margins. *E. dallachyana* has a leaf size intermediate between the other two species with a proportionately long petiole relative to leaf length. The leaf blade is ovate to lanceolate (length:width 1.79) with an acuminate apex and crenulate to serrate leaf margins.

Photographs of each of the above species are shown in Plate 1.

Discussion

Given the number of nucleotide differences, the following hypotheses can be evaluated: ie., (i) All samples in the in-group are of the same species; (ii) Two species comprise the in-group; and (iii) There are three species in the in-group.

From the available literature, it has been generally suggested that ITS regions can have 0 - 3 bp difference within a species (Liden et al., 1995; Sun et al., 1994), and > 3 differences between species (Sun et al., 1994). Clearly, the nucleotide differences (Table 2) support the third hypothesis ie., three species are present in the in-group, as there were 9 - 10 differences between the in-group species and the designated out-group *E. parvifolia,* and 6 - 14 bp differences between the ingroup taxa.

The phylogenetic relationships of the taxa were then assessed using parsimony. The single most parsimonious tree found using PAUP had a consistency index of 1.0, tree length of 19, and a retention index of 1.0. The branch support for this tree was determined by bootstrap analysis (Fig. 2), and a neighbour joining tree was constructed using MEGA based on the distance matrix of the proportion of nucleotide differences (Fig. 3).

The nucleotide differences in the ITS1 region show that *E. agallocha* from eastern Australia and *E. ovalis* from Western Australia are each genetically homogeneous but differ from each other consistently by 6 bp, thus supporting specific status. The differences in nucleotides in the ITS1 region are clearly supported by the analysis of leaf morphology which indicated that *E. ovalis* could be consistently distinguished from *E. agallocha* on the basis of leaf length and width, and length of petiole. On the other hand, *E. ovalis* from the type locality in the Gulf of Carpentaria differs slightly (by 3 bp difference) from *E. ovalis* in Western Australia, and this species may be in the process of differentiating genetically at the extremes of its geographical distribution. However, no evidence to suggest any leaf morphological differentiation in this species was found. Thus, the minor difference in nucleotides between populations of *E. ovalis* from Western Australia and Sweers Island in the Gulf of Carpentaria is supported by the absence of statistical differences in the leaf morphological parameters examined, and by the proximity of these populations in the MDS ordination (Fig. 1).

The phylogenetic and leaf morphological analysis also suggests that *E. dallachyana* is not closely related to either mangrove species *E. agallocha* or *E. ovalis,* despite some superficial morphological similarity, and this is supported by its distinctly different habitat requirements. Rather, the nucleotide similarity between *E. dallachyana* to the out-group suggests a closer relationship with *E. parvifolia* than with either of the mangrove species.

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Figure 1.

Clustering of leaf morphological data (upper) obtained from PRIMER using normalised Euclidean distance of four *Excoecaria* populations. The multi-dimensional scaling ordination (lower) shows the proximity of the *E. ovalis* populations from Sweers Island and Western Australia.

Figure 2.

Bootstrap (50% majority-rule) consensus tree obtained from PAUP with 1000 replicates using maximum parsimony. Of the 263 total characters, all characters were un-ordered and of equal weight. 244 characters were constant, 8 variable characters were parsimony-uninformative, and 11 characters were parsimony-informative. Gaps were treated as missing. Taxa represent the Australian members of the genus *Excoecaria,* with *E. parvifolia* as the designated out-group in the analysis.

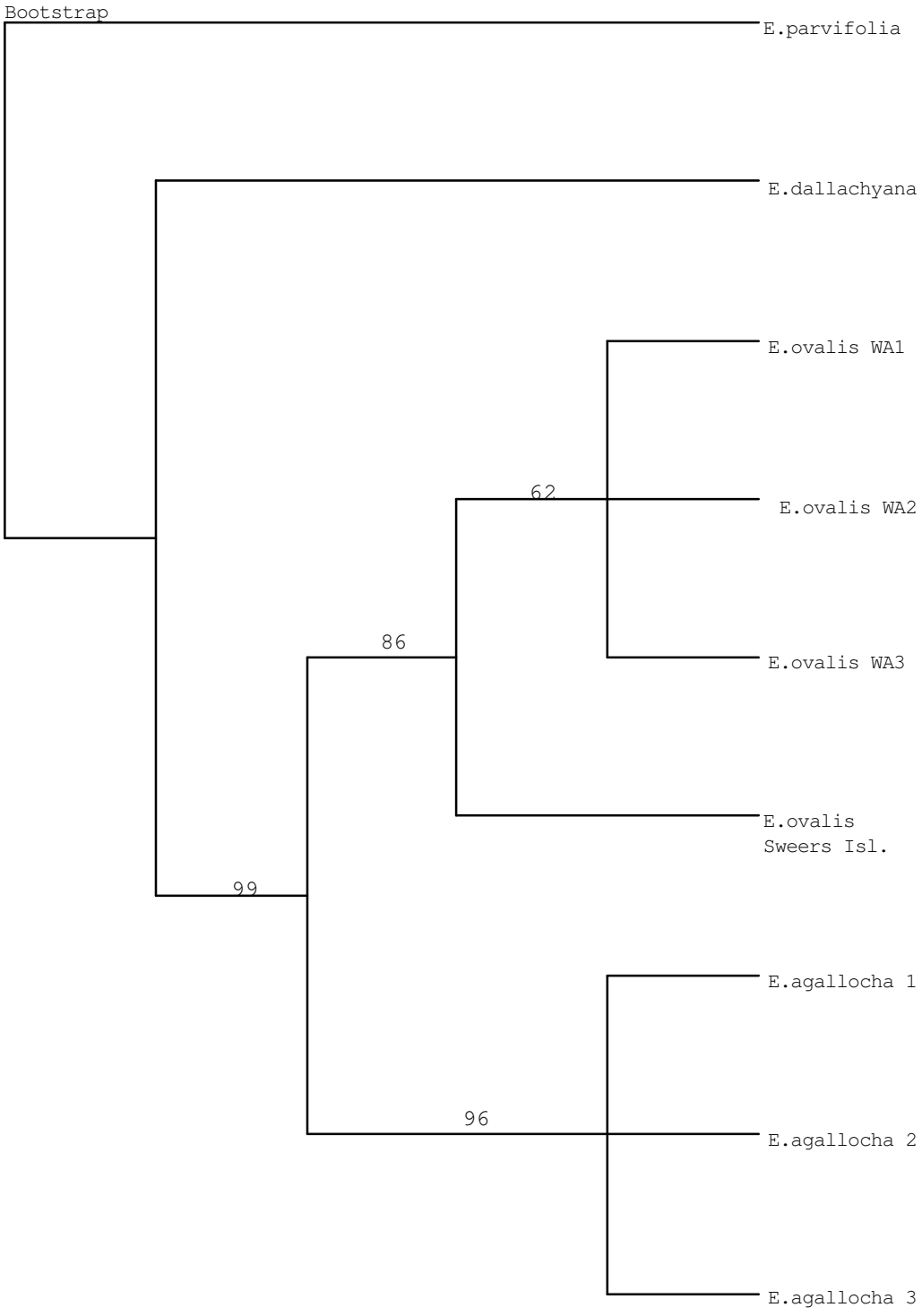
Bootstrap
E.parvifolia
E.dallachyana
E.ovalis WA1
62
E.ovalis WA2
86
E.ovalis WA3
E.ovalis
Sweers Isl.
99
E.agallocha 1
96
E.agallocha 2
E.agallocha 3

Figure 3.

Neighbour-joining (NJ) tree. The distance measure used was the proportion of nucleotide differences calculated using MEGA. Taxa represent the Australian members of the genus *Excoecaria,* with *E. parvifolia* as the designated out-group in the analysis.

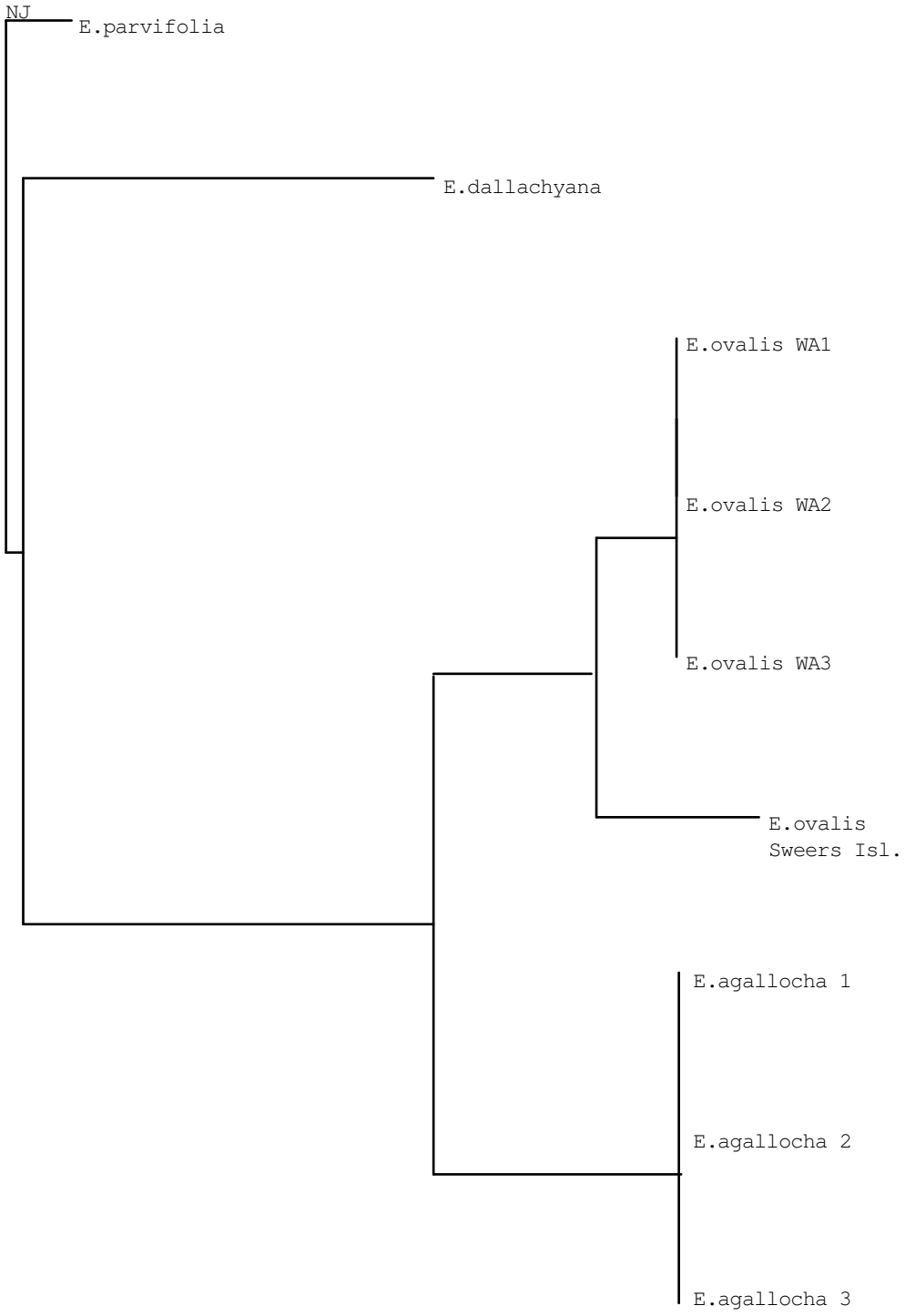
NJ
E.parvifolia
E.dallachyana
E.ovalis WA1
E.ovalis WA2
E.ovalis WA3
E.ovalis
Sweers Isl.
E.agallocha 1
E.agallocha 2
E.agallocha 3

Plate 1.

Photographs of leaves of each of the species *E. ovalis, E. agallocha* and *E. dallachyana*. (a) Leaves and male inflorescences of *E. ovalis* collected at Cambridge Gulf, WA. (b) Leaves and mature fruits of *E. agallocha* collected on the banks of North Creek, a tributary of the Richmond River, at Ballina, NSW. (c) Leaves of *E. ovalis* collected from Sweers Island, in the south-eastern Gulf of Carpentaria, Qld. (d) Leaves and mature fruits of *E. dallachyana* collected from Mallanganee Flora Reserve, NSW.

Table 1.

Complete nucleotide sequence of the aligned ITS1 consensus data (263 bp) for each sample, amplified by the following polymerase chain reaction (PCR) primers (Forward) 5'CGCGAGAAGTTCATTGAACC3' and (Reverse) 5'GATATCCGTTGCCGAGAGTCG3'), designed in conserved regions of ribosomal DNA (rDNA). Dots represent identical nucleotide bases and "-" represents missing data.

```
                  1       10        20        30        40        50
E.ovalis WA1        TGCGGAAGGA TCATTGTCGA AACCTGCAGT AGCAGAACGA CCCGTGAACA
E.ovalis WA2        .......... .......... .......... .......... ..........
E.ovalis WA3        .......... .......... .......... .......... ..........
E.ovalis Sweers     .......... .......... .......... .......... ..........
E.agallocha 1       .......... .......... .......... .......T.. ..........
E.agallocha 2       .......... .......... .......... .......T.. ..........
E.agallocha 3       .......... .......... .......... .......T.. ..........
E.parvifolia        .......... .......... ........A. .......... ..........
E.dallachyana       .......... .......... ........A. .......... ..........

                 51      60        70        80        90       100
E.ovalis WA1        TGTCATCAAA CTCATAGGGT CGCGGGGGCC TTCGATCCTC GTCGATCCCG
E.ovalis WA2        .......... .......... .......... .......... ..........
E.ovalis WA3        .......... .......... .......... .......... ..........
E.ovalis Sweers     .......... .......... .......... .......... ..........
E.agallocha 1       .......G.. .......... .......... .......... ..........
E.agallocha 2       .......G.. .......... .......... .......... ..........
E.agallocha 3       .......G.. .......... .......... .......... ..........
E.parvifolia        .......G.. .......... .......... .......... ..........
E.dallachyana       .......G.. ...G.C.... .......... .......... ..........

                101    110       120       130       140       150
E.ovalis WA1        TAGGCCAGGG GGAGGGATGC CGATCGTGGG CTTTGGCCCT CATCGCTCTC
E.ovalis WA2        .......... .......... .......... .......... ..........
E.ovalis WA3        .......... .......... .......... .......... ..........
E.ovalis Sweers     .......... .......... .......... .......... ..........
E.agallocha 1       ......G... .......... .......... .......... ..........
E.agallocha 2       ......G... .......... .......... .......... ..........
E.agallocha 3       ......G... .......... .......... .......... ..........
E.parvifolia        ......G... .........T ..G....... .......... ..........
E.dallachyana       ......G... .........T ..G....... .......... ..........

                151    160       170       180       190       200
E.ovalis WA1        CCTCGCTCTG GCTTTCTAAC CAACCCCGGC GCGACACGCG CCAAGGAATT
E.ovalis WA2        .......... .......... .......... .......... ..........
E.ovalis WA3        .......... .......... .......... .......... ..........
E.ovalis Sweers     .......... .......... .......... ..A....... ..........
E.agallocha 1       .......... .......... .......... .......... ..........
E.agallocha 2       .......... .......... .......... .......... ..........
E.agallocha 3       .......... .......... .......... .......... ..........
E.parvifolia        .......... ...C...... .......... .......... ..........
E.dallachyana       ....C.C... .......... .......... .......... ..........

                201    210       220       230       240       250
E.ovalis WA1        TTAAATGCAA AGGGATAACT CCGGCGAGCC TCGGAGATGA TGTGCTCGCA
E.ovalis WA2        .......... .......... .......... .......... ..........
E.ovalis WA3        .......... .......... .......... .......... ..........
E.ovalis Sweers     .A........ .......G.. .......... .......... ..........
E.agallocha 1       .......... .......G.. .......... ...AT..... ..........
E.agallocha 2       .......... .......G.. .......... ...AT..... ..........
E.agallocha 3       .......... .......G.. .......... ...AT..... ..........
E.parvifolia        .......... .......G.. ..A....... .......C.. ..........
E.dallachyana       .......... .......G.. ..AC...... .......C.. ..........

                251    260 263
E.ovalis WA1        G-GATGCTCT GTC
E.ovalis WA2        .......... ...
E.ovalis WA3        .......... ...
E.ovalis Sweers     .......... ...
E.agallocha 1       ....-.-..- ---
E.agallocha 2       .......... ...
E.agallocha 3       .......... ...
E.parvifolia        .......... ...
E.dallachyana       .......... ...
```

Table 2.

Lower-left distance matrix output of the number of different nucleotides using PAUP. OTU (operational taxonomic unit) numbers represent the following taxa: (1) *E. ovalis* WA1, (2) *E. ovalis* WA2, (3) *E. ovalis* WA3, (4) *E. ovalis* Sweers Island, (5) *E. agallocha* 1, (6) *E. agallocha* 2, (7) *E. agallocha* 3, (8) *E. parvifolia* (out-group), and (9) *E. dallachyana.*

OTUs	1	2	3	4	5	6	7	8	9
1									
2	0								
3	0	0							
4	3	3	3						
5	6	6	6	7					
6	6	6	6	7	0				
7	6	6	6	7	0	0			
8	9	9	9	10	9	9	9		
9	13	13	13	14	13	13	13	6	

Table 3.

Leaf morphological data comparison for *Excoecaria agallocha, E. ovalis* and *E. dallachyana.* Means and standard deviations are given; all measurements in mm. Values in columns followed by the same letters are not significantly different ($P > 0.05$ with $n = 50$).

Species/Location	Length	Width	Petiole	Margin	Apex
E. ovalis WA	39.3±2.1[a]	25.3±2.5[a]	11.3±1.2[a]	entire	blunt
E. ovalis Sweers Is.	49.8±7.0[a]	30.9±5.4[a]	12.2±1.6[a]	entire	blunt
E. dallachyana	59.0±13.0[b]	32.9±7.8[ab]	15.7±4.4[b]	serrate	acuminate
E. agallocha	71.2±10.0[c]	40.7±7.7[b]	14.6±2.9[b]	crenate	blunt - acute