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Molecular phylogeny and evidence for natural hybridization and historical introgression between *Ceriops* species (Rhizophoraceae)

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ABSTRACT

Ceriops (Rhizophoraceae) is a genus comprised of five species of mangroves distributed in tropical and subtropical coastal regions. In this study, sequences from nuclear ribosomal ITS and the plastid *trn*L intron are used to construct molecular phylogenies of this genus revealing two species complexes, the *C. tagal* complex (*C. tagal* and *C. australis*), and the *C. decandra* complex (*C. decandra*, *C. pseudodecandra* and *C. zippeliana*), each forming a distinct clade. All five species, including the newly designated species *C. pseudodecandra*, are well supported. However, natural hybridization and historical introgression between *Ceriops* species are also demonstrated. The ITS sequences of *Ceriops* species, in contrast to their plastid *trn*L intron sequences, show a great amount of homoplasy during evolution. Historical introgression originating from natural hybridization was demonstrated based on the additivity of ITS sequences from putative parents. Of the five *Ceriops* species, *C. pseudodecandra* is a relatively isolated species. *Ceriops decandra* and *C. zippeliana* show mutual introgression in most populations. According to both the nuclear ITS sequences and the plastid *trn*L intron, an intermediate form from Darwin is likely a natural hybrid, with *C. tagal* and *C. australis* respectively the maternal and paternal parents.

Keywords: Ceriops, historical introgression, ITS, mangrove, natural hybridization, *trn*L

1. Introduction

'Mangroves' are the intertidal vascular plants, mostly trees and shrubs, distributed in regions of estuaries, deltas and riverbanks or along the coastlines of tropical and subtropical areas (Tomlinson, 1986; Saenger, 2002). *Ceriops* Arn. is one of four mangrove genera in the Rhizophoraceae (Tomlinson, 1986). *Ceriops* has a widespread geographical range from eastern Africa, throughout tropical Asia, northern Australia to Melanesia, Micronesia and southern China (Hou, 1958; Tomlinson, 1986; Duke, 2006). *Ceriops* species are typically constituents of the inner mangroves, often forming pure stands on better drained sites, and stunted stands in exposed and highly saline sites within the reach of occasional tides (Hou, 1958).

In revising the genus *Ceriops*, Hou (1958) recognized just two species, *C. decandra* and *C. tagal*. White (1926) and Tomlinson (1986) recognized an additional variety *C. tagal* (Perr.) C. B. Rob. var. *australis* C. T. White, originally reported from Australia and Papua New Guinea. Based on polyacrylamide gel electrophoresis analysis of isozymes from the populations of *Ceriops* in northern Australia, Ballment et al. (1988) raised this variety to specific rank as *C. australis*, which was later supported by both morphological and molecular evidence (Sheue et al., 2009b).

Of these three species of the genus *Ceriops, C. tagal* is the most widely distributed

(Tomlinson, 1986). The species *C. decandra* was also reported as widely distributed across India, Indochina, the Malay Peninsula, Indonesia and northern Australia (Duke, 1992; Tomlinson, 1986). However, the highly genetically divergent populations reported as *C. decandra* (Tan et al., 2005) were divided into three species, including *C. decandra*, *C. zippeliana* Blume, and a new species *C. pseudodecandra* Sheue, Liu, Tasi & Yang by Sheue et al. (2009a, 2010), based on morphological, palynological and DNA evidence. Thus, the genus *Ceriops* is now regarded as having five extant species, *C. australis, C.decandra, C. pseudodecandra, C. tagal and C. zippeliana.*

Despite clear differentiation between *C. tagal* and *C. australis* at five sympatric locations in north Queensland, Australia, as determined by isozyme markers (Ballment et al., 1988) and DNA evidence (Sheue et al., 2009b), there are anecdotal accounts of intermediate forms (see Ballment et al., 1988). Moreover, an intermediate form between *C. tagal* and *C. australis* can be found in Darwin (Sheue et al., 2009b), and elsewhere in the Northern Territory, Australia (Sheue, personal observation), suggesting that some hybridization may have occurred in nature.

To construct phylogenies and obtain evidence on historical introgression and natural hybridization of *Ceriops*, we used both plastid and nuclear ribosomal DNA. Because these two forms of DNA are inherited differently, they provide a good means for

detecting reticulate evolution in plants by comparing nuclear and plastid phylogenies. Maternal inheritance has been shown to occur for plastid DNA (cpDNA) in most flowering plants (Derepas and Dulieu, 1992). Nuclear ribosomal DNA (nrDNA) is inherited biparentally. It has repeated sequences organized into families in tandem arrays in the nucleolar organizer regions of chromosomes in all eukaryotes (Rogers and Bendich, 1987). Copy numbers of nrDNA vary in different species from a few hundred to several thousand. Each repeat unit consists of a non-transcribed spacer known as an intergenic spacer (IGS) and a transcription unit coding for the precursor of rRNA. Ribosomal repeat sequences are usually identical due to concerted evolution via unequal crossing over (Schlotterer and Tautz, 1994) and biased gene conversion (Hillis et al., 1991; Linares et al., 1994). Incongruence between loci from the plastid and nrDNA often indicates gene flow (Soltis and Kuzoff, 1995), allowing the detection of historical introgression or natural hybridization.

Natural hybridization and subsequent introgression have been characterized based on molecular evidence in several plant taxa (e.g., Mao et al., 1995; Brar and Khush, 1997; Rieseberg et al., 1999; Tsai et al., 2006). Hybrid species were identified by full or partial ITS sequence additivity of the putative parental species based on direct sequencing of PCR products (Sang et al., 1995; Campbell et al., 1997; Hugall et al.,

1999; Andreasen and Baldwin, 2003; Chiang et al., 2001b; Tsai et al., 2006; Soltis et al., 2008). Partial sequence additivity was suggested to come from partial homogenization of parental ITS repeat sequences via gradients of gene conversion (Sang et al., 1995). In contrast, the complete homogenization of parental repeat sequences in certain hybrid species could not be detected by ITS sequences (Wendel et al., 1995). If a hybrid species shows ITS sequences from one parent via gene conversion, but cpDNA inherited from the other parent, incongruencies between the ITS and cpDNA phylogenies will be shown in the hybrid species (e.g., Soltis and Kuzoff, 1995; Lihova et al., 2006; Kim and Donoghue, 2008).

To construct a molecular phylogeny and understand the natural hybridization and historical introgression between *Ceriops* species, we analyzed the sequences of the nuclear ribosomal ITS and plastid *trn*L introns for the five species of *Ceriops* from a broad geographical range, widely representing these species. Because these two DNA markers represent biparental and maternal inheritance, respectively, in most flowering plants (see Derepas and Dulieu, 1992; Sang et al., 1995; Wendel et al., 1995), the direction of introgression also can be assessed in this study.

2. Materials and methods

2.1. Plant materials

During 2000 to 2007, we collected 38 specimens from the five *Ceriops* species (*C. australis*, *C. tagal*, *C. decandra*, *C. zippeliana* and *C. pseudodecandra*) from India to Australia (Table 1, Fig. 1). *Ceriops* distribution ranges (Fig. 1) were obtained from herbarium records and field surveys over the period 2000-2011. In addition, we collected specimens from five outgroup species representing three other genera of the tribe Rhizophoreae, and one outgroup species from the tribe Gynotrocheae. All voucher specimens of this study were deposited in the Herbarium of the Department of Life Sciences (TCB) of National Chung Hsing University.

2.2. DNA extraction

Using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987), total DNA was extracted from fresh etiolated leaves. Ethanol-precipitated DNA was dissolved in TE (Tris-EDTA) buffer and stored at –20 ºC. Qiagen (Valencia, CA,

USA) columns were used to clean the DNA of samples that were difficult to amplify by PCR. The approximate DNA yields were then determined using a spectrophotometer (model U-2001, Hitachi).

2.3. PCR amplification and electrophoresis

We used a 50-µl mixture containing 40 mM Tricine-KOH (pH 8.7), 15 mM KOAc, 3.5 mM $Mg(OAc)_2$, 3.75 µg/ml BSA, 0.005% Tween-20, 0.005% Nonidet-P40, four dNTPs (0.2 mM each), primers (0.5 µM each), 2.5 units of Advantage 2 DNA polymerase (Clontech Laboratories, Inc., California, USA), 10 ng genomic DNA, and a 50-µl volume of mineral oil. The amplification reactions were completed in a dry-block with two-step thermal cycles (Biometra, Germany). The universal primers for amplifying the *trn*L intron of chloroplast DNA were also referenced from Taberlet et al. (1991). The PCR reaction conditions for the *trn*L intron are as follows: the mixture was incubated at 94 ºC for 3 min, then underwent 10 cycles of denaturation at 94 ºC for 30 s, annealing at 68 ºC for 10 s, and extension at 72 ºC for 45 s. Then, the sample underwent 30 cycles of denaturation at 94 ºC for 30 s, annealing at 66 ºC for 10 s, and extension at 72 ºC for 45 s, with a final extension for 5 min at 72 ºC. The ITS primers were designed

from conserved regions at the 3’ end of the 18S rRNA gene and at the 5’end of the 26S rRNA gene using sequences from different species present in GenBank (primer sequences used were 5’ TCGTAACAAGGTTTCCGTAGGT 3’ and 5’ GTAAGTTTCTTCTCCTCCGCT 3’, respectively). The PCR reaction conditions for the ITS region are as follows: the mixture was incubated at 94 ºC for 3 min, then it underwent 10 cycles of denaturation at 94 ºC for 45 s, annealing at 58 ºC for 45 s, and extension at 72 ºC for 1 min. Then the samples underwent 30 cycles of denaturation at 94 ºC for 45 s, annealing at 54 ºC for 45 s, extension at 72 ºC for 1 min, with a final extension for 10 min at 72 ºC. The PCR products were detected by agarose gel electrophoresis (1.0%, w/v in TBE), stained with 0.5 µg/ml ethidium bromide, and were finally photographed under UV light.

2.4. DNA recovery and sequencing

The PCR products in this study were recovered using glassmilk (BIO 101, California). The DNA was directly sequenced following the method of dideoxy chain-termination using an ABI3730 automated sequencer with the Ready Reaction Kit (PE Biosystems, California, USA) of the BigDye™ Terminator Cycle Sequencing.

Sequencing primers were the same as those used for PCR. Each sample was sequenced two or three times to confirm the sequence. These reactions were performed as recommended by the manufacturers. Due to intra-individual variations in some samples, sequences of those samples showed additive polymorphisms by direct sequencing of PCR products. In order to clarify the intra-individual variation found in those samples, a PCR cloning method was used. Recovered PCR products were ligated into a T-vector (Promega Co., USA), and the resulting recombinants were transformed in *Escherichia coli*. Five to seven clones for each individual were randomly selected, and plasmid DNA was purified with Qiagen spin miniprep kits (Qiagen, Germany). Plasmid DNA was sequenced with vector-specific primers (SP6 and T7). These reactions were performed based on the recommendations of the manufacturer.

2.5. Data analysis

The sequence alignment was determined using the Clustal W multiple alignment program (Thompson et al., 1994) in BioEdit (Hall, 1999). The alignment was then checked, and apparent alignment errors were corrected manually. All the characters were given equal weights. Genetic relationships were then determined using the

program MEGA version 3.1 (Kumar et al., 2004). All positions containing indels (insertions/deletions) were eliminated from the dataset (Complete deletion option). Maximum parsimony (MP) analyses (Fitch, 1971) were done using code modified from the Close-Neighbor-Interchange (CNI) algorithm (Rzhetsky and Nei, 1992) in MEGA version 3.1 (Kumar et al., 2004). Bootstrapping (1000 replicates) was carried out to estimate the support for MP and ML topologies (Felsenstein, 1985; Hillis and Bull, 1993). Maximum likelihood analyses were conducted using the program PhyML version 3.0 (Guindon and Gascuel, 2003) and a phylogenetic tree was constructed using the program MEGA version 3.1 (Kumar et al., 2004). The aligned data matrix and tree files are available from the corresponding author (tsaicc@mail.kdais.gov.tw). The number of haplotypes was counted, including all polymorphic sites and indels, for all accessions of *Ceriops* studied. The haplotype diversity (*h*), nucleotide diversity (π) (Nei, 1987), recombination test (Hudson and Kaplan, 1985) and Tajima's D (Tajima, 1989) test for departure from neutrality on the total number of segregating sites were calculated using the DnaSP program version 2.0 (Rozas et al., 2003).

3. Results and Discussion

3.1. Sequence alignment, sequence variation and haplotype diversity

PCR products of the plastid *trn*L introns of each sample were directly sequenced (Table 1). All of the *trn*L intron sequences were aligned and resulted in 704 characters, with 40 variable sites and 20 potentially parsimony-informative sites. There were 20 singleton sites. Among 38 samples of *Ceriops*, there were 20 variable sites, 15 potentially parsimony-informative sites, and 5 singleton sites (Tables 2 and 3). Seven haplotypes were detected among the 38 accessions of *Ceriops* examined, with a haplotype diversity of 0.8240 and a nucleotide diversity value of 0.01214. Within species of *Ceriops*, *C. australis*, *C. decandra*, *C. pseudodecandra* and *C. tagal* were shown to each have a single unique haplotype. Three haplotypes were found among ten samples of *C. zippeliana* examined, with a haplotype diversity of 0.3780 and a nucleotide diversity value of 0.00160 (Tables 2 and 3). In addition, six indels (insertion/deletion) were found among species of *Ceriops* (Fig. 2).

The PCR products of ITS sequences for some specimens (18/37=48.6%) of the genus *Ceriops* were not homogeneous. The sequences of those species were finally

sequenced after T-vector cloning. Five separate clones were randomly selected for sequencing for an individual with heterogeneous ITS sequences (Table 1). All ITS sequences were aligned and resulted in 622 characters with 233 variable sites and 154 potentially parsimony-informative sites. There were also 79 singleton sites. Among 37 samples of *Ceriops*, there were 137 variable sites and 93 potentially parsimony-informative sites, along with 44 singleton sites (Table 3). A total of 53 ITS haplotypes were detected among the 37 specimens of *Ceriops* examined, with a haplotype diversity of 0.9651 and a nucleotide diversity value of 0.02916 (Table 3). Within species of *Ceriops*, *C. zippeliana* has the greatest number of haplotypes, 21, with a haplotype diversity of 0.8966 and a nucleotide diversity of 0.00830. By contrast, *C. australis* has a single unique haplotype (Table 3).

Based on the above results, the ITS sequences show more variable and parsimony-informative sites than do plastid DNA. Based on the molecular data from nuclear and plastid DNA, sequences of *C. zippeliana* show the highest genetic diversity among the five species of *Ceriops*.

3.2. Phylogeny reconstruction

Based on the MP method, the cpDNA (*trn*L intron) analysis yielded 424 equally parsimonious trees with a length of 47 steps, a consistency index (CI) of 0.936, and a retention index (RI) of 0.983. The strict consensus tree is shown in Fig. 3. The phylogenetic tree constructed following the ML method is shown in Fig. 4. The MP and ML trees constructed from the *trn*L intron sequences are mostly congruent. Within the genus *Ceriops*, two major clades are shown based on both MP and ML trees (Figs. 3 and 4). One major clade, the "*C. tagal* species complex," includes *C. tagal* and *C. australis*, is supported by a 70% bootstrap value in the MP tree and 92% in the ML tree. The other major clade, "the *C. decandra* species complex," includes *C. decandra*, *C. zippeliana* and *C. pseudodecandra*, and is supported by a 98% bootstrap value in the MP tree and 100% in the ML tree.

Each species of *Ceriops* is separated from the others based on cpDNA. *Ceriops tagal* collected from various locations is clearly separated from *C. australis* based on its MP and ML trees. This result is in agreement with an isozyme analysis (Ballment et al., 1988), DNA band markers (Lakshmi et al., 2002) and a recent reevaluation of the taxonomic status of *C. australis* based on both morphological and molecular evidence (Sheue et al., 2009b). Moreover, based on the MP and ML trees, *C. decandra* collected from the Indian coast is also clearly separated from its two similar species, *C.*

zippeliana, and the newly described species, *C. pseudodecandra*, occurring in eastern Indonesia, New Guinea, and Australia (Sheue et al., 2010). This result is in agreement with the ISSR DNA markers (Tan et al., 2005) and supports the idea that the current widely recognized population of *C. decandra* can be treated as three separate species, *C. decandra*, *C. zippeliana*, and *C. pseudodecandra*, as suggested by Sheue et al. (2007, 2009a, 2010).

These findings are consistent with the biogeography of *Ceriops* (Fig. 1) in which *C. decandra* occurs in India through the western Malay Peninsula of Thailand. In contrast, *C. zippeliana* is distributed on the west coast of the southern Malay Peninsula of Malaysia through Singapore, the Gulf of Thailand, Vietnam, Borneo, Indonesia and the Philippines (Sheue et al., 2009a). The other newly described species, *C. pseudodecandra*, ranges through Indonesia, Papua New Guinea and Australia (Sheue et al., 2010).

ITS repeat sequences in some accessions (18/37 = 48.6%) of *Ceriops* are shown to be heterogeneous based on additive polymorphisms by direct sequencing of PCR products (data not shown). The phylogenetic tree of ITS sequences used characters that were equally weighted. Based on the MP method, the analysis yielded 376 equally parsimonious trees with a length of 355 steps, a CI of 0.718, and an RI of 0.881. One of

the parsimonious trees is shown in Fig. 5. The phylogenetic tree constructed following the ML method is shown in Fig. 6. The MP and ML trees (Figs. 5 and 6) derived from ITS sequences do not clearly separate *Ceriops* species, with the exception of *C. pseudodecandra*. The same two major clades identified by the plastid *trn*L intron phylogenies (Figs. 3 and 4) appear also in the ML ITS tree. Although, the *C. tagal* complex is supported by a less than 50% bootstrap value in the ML ITS tree, the *C. decandra* complex is supported by a 96% bootstrap value in the ML ITS tree (Fig. 6).

The lower resolution of the *Ceriops* trees derived from ITS sequences than the *trn*L intron data is not due to slow rates of mutation and a lack of informative characters because, within species of *Ceriops*, the ratio of potentially parsimony-informative sites is higher in ITS sequences (93/622 = 15.0%) than in *trn*L introns (15/704 = 2.1%). The consistency index (CI) value in nuclear ITS (0.718) is lower than that of the plastid *trn*L intron (0.936). This result can be explained by noting that a low CI indicates a high level of homoplasy, resulting in an unresolved tree, as discussed by Mickevich (1978). The level of homoplasy is positively related to the level of recombination (Sanderson and Doyle, 1992). A recombination test of the ITS sequences was conducted revealing recombination to be common with and between *Ceriops* species (Table 4). This result can explain why the tree resolution for each species is lower in the ITS sequence than in

the *trn*L intron phylogeny.

3.3. Natural hybridization and historical introgression between Ceriops *species*

Different species within the *C. decandra* species complex (*C. decandra*, *C. pseudodecandra* and *C. zippeliana*) and within the *C. tagal* species complex (*C. tagal* and *C. australis*) cannot be separated clearly based on ITS sequences (Figs. 5 and 6). The result is not in accord with the *trn*L intron phylogeny in which species are well separated (Figs. 3 and 4). Because biparental inheritance and maternal inheritance are respectively shown to occur for nuclear and plastid DNA in most flowering plants (Derepas and Dulieu, 1992), we suggest that historical introgression has caused differences between a species' nuclear gene genealogy and its plastid DNA gene genealogy, making tree-based species delineation ambiguous in the nuclear ITS tree. Similar results can be found in several reports of different taxa (Campbell et al., 1997; Andreasen and Baldwin, 2003; Tsai et al., 2006; Soltis et al., 2008). Historical introgression between species of the *C. tagal* species complex, and between species of the *C. decandra* species complex, is indicated by these ITS data. Within the *C. tagal* complex, *C. australis* has homogenized sequences for either the plastid *trn*L intron or

nuclear ITS sequences within individuals. These results show no evidence of historical introgression from other *Ceriops* species into any accessions of *C. australis*. In *C. tagal*, all accessions showed the same type of plastid *trn*L intron, but two accessions (i.e., *C. tagal*-Rh-32 and *C. tagal*-Rh-66) showed heterogeneous ITS sequences in which some clones are closely related to those of *C. australis* (Figs. 5 and 6). The morphology of *C. tagal*-Rh-66, but not *C. tagal*-Rh-32, is intermediate between *C. tagal* and *C. australis* (Sheue et al., 2009b). This result suggests that *C. tagal*-Rh-66 is the result of a recent natural hybridization event between *C. tagal* and *C. australis*. In contrast, *C. tagal*-Rh-32 may be the result of a natural historical hybridization between *C. tagal* and *C. australis*, backcrossed to *C. tagal*.

Within the *C. decandra* species complex, *C. pseudodecandra* has homogenized sequences for both the plastid *trn*L intron and the nuclear ITS, within individuals. All accessions of *C. pseudodecandra* form a clade as evidenced by both the plastid *trn*L intron and nuclear ITS sequences (Figs. 3-6), again providing no evidence of historical introgression from other *Ceriops* species. Excepting the four accessions *C. decandra*-Rh-26, Rh-29 and Rh-35 and *C. zippeliana*-Rh-43, historical introgression can be found between most *C. decandra* and *C. zippeliana* based on nuclear ITS sequences, indicating natural hybridization and backcrossing. However, accessions of

both *C. decandra* and *C. zippeliana* in the study do not show intermediate morphology (C.-R. Sheue pers. obs.).

As mentioned above, some individuals of three studied taxa (*C. tagal*, *C. decandra* and *C. zippeliana*), with historical introgression, do not show forms intermediate between maternal and paternal parents. Similar results have been reported in some hybrid progenies between cultivated and wild sunflowers (*Helianthus annuus*) (Rieseberg et al., 1995). Foreign genotypes of both plastid and nuclear DNA can be diluted within main local populations by multiple generations of back crossing. However, an entirely neutral process does not explain why ITS intron DNA from introgression can still be detected. A possible explanation is that historical changes in selection regimes have led to loss of foreign plastid DNA and convergence of morphology by natural selection, while ITS intron DNA is selectively neutral and is retained.

3.4. Ceriops species evaluation

Based on the *trn*L intron phylogeny, five species of *Ceriops* can be separated clearly. This supports previous investigations of *C. pseudodecandra* (Sheue et al., 2010), *C.*

australis (Sheue et al., 2009b), and *C. zippeliana* (Sheue et al., 2009a). Some individuals of *C. tagal* located in Darwin, Australia have a narrower and more oblong calyx lobe, a longer clavate appendage on the petal apex, and a longer style than those of the same taxon from other populations (Sheue et al., 2009b). In this study, the intermediate form *C. tagal*-Rh-66, collected from Darwin, Australia, was shown to be a natural hybrid based on ITS sequences. Based on the *trn*L intron phylogeny, *C. tagal*-Rh-66 forms a clade with other *C. tagal* varieties and is separated from populations of *C. australis*. As plastid DNA is inherited maternally in most flowering plants (Derepas and Dulieu, 1992), *C. tagal* and *C. australis* are inferred to be the maternal and paternal parents respectively in these hybridizations.

Some ITS sequences of *C. tagal*, including Rh-66-clone 1, Rh-85-clone 1 and Rh-86-clone 1, are unique sequences showing a long internal branch in the ML tree (Fig. 6). In those ITS sequences, unique nucleotides occur throughout the ITS region, even the 5.8S rRNA gene, but those unique nucleotides are not found in other ITS sequences of *Ceriops* species or the outgroups. A partial sequence alignment of ITS region, ITS1, between *Ceriops* species is shown in Fig. 7. Similar long internal branches and unique nucleotides also have been shown in natural and artificial hybrids, *Begonia* × *taipeiensis* (Chiang et al., 2001b) and natural hybrids, *Phalaenopsis* × *intermedia* (Tsai et al., 2006).

In addition, the above three clones derived from three accessions of *C. tagal* might have lost their biological function as a result of the 5.8S rRNA gene having 15 substitutions (data not shown). Thus, we suggest that these three clones belong to pseudogenes of the 5.8S rRNA of *C. tagal*. Based on ITS sequences (and also plastid *trn*L intron) *C. pseudodecandra* is relatively isolated among *Ceriops* species. However, ITS sequences show that *C. decandra* and *C. zippeliana* have mutual historical introgression for most populations.

In summation, the DNA evidence revealed in this study supports the current five described species of the genus *Ceriops* and shows two species complexes, strongly in accordance with morphological diagnostic features, such as inflorescence structure and petal features (Sheue et al., 2009a, b; 2010). The morphological and DNA data provide independent evidence supporting the division of this genus into two subgenera. Historical introgression and natural hybridization are implicated in three *Ceriops* species. It is noteworthy that interspecific gene flow was evident only within the species complexes. In addition, the intermediate form of *C. tagal*, identified here as a hybrid, occurs sympatrically with *C. australis* in the Nothern Territory, Australia and Port Moresby, Papua New Guinea in the absence of the typical form of *C. tagal* (Fig. 1). The typical form of *C. tagal* occurs sympatrically with *C. australis* in northern Queensland,

Australia. Interestingly, the three members of the *C. decandra* complex are currently allopatrically distributed (Fig. 1), raising the question of how historical introgression between the *C. decandra* complex found in this study occurred. We suggest two possible explanations. First, long distance dispersal of some fraction of *Ceriops* propagules is likely, even though most propagules may not disperse far (McGuinness, 1997). Second, the distributions of *Ceriops* may have differed historically due to climate change and geological events. In this regard, it is noteworthy that a *Ceriops* fossil has been recently discovered in Tasmania (Carpenter et al., 2012) well outside the current distribution of the genus.

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Figure captions

Fig. 1. Map showing the distribution of *Ceriops*, and the samples collected in this study. Sample localities are 1: Moreton Bay; 2: Cairns; 3: Cape York; 4: Darwin; 5: Singapore; 6: West Sundarbans; 7: Pichavaram.

Fig. 2. Polymorphic sites and indels (insertion/deletion) of the *trn*L intron in the genus *Ceriops*.

Fig. 3. The strict consensus parsimony tree based on the plastid *trn*L intron sequence from the 38 specimens of the genus *Ceriops* and six outgroup species from closely related genera. Bootstrap values > 50% are shown on each branch.

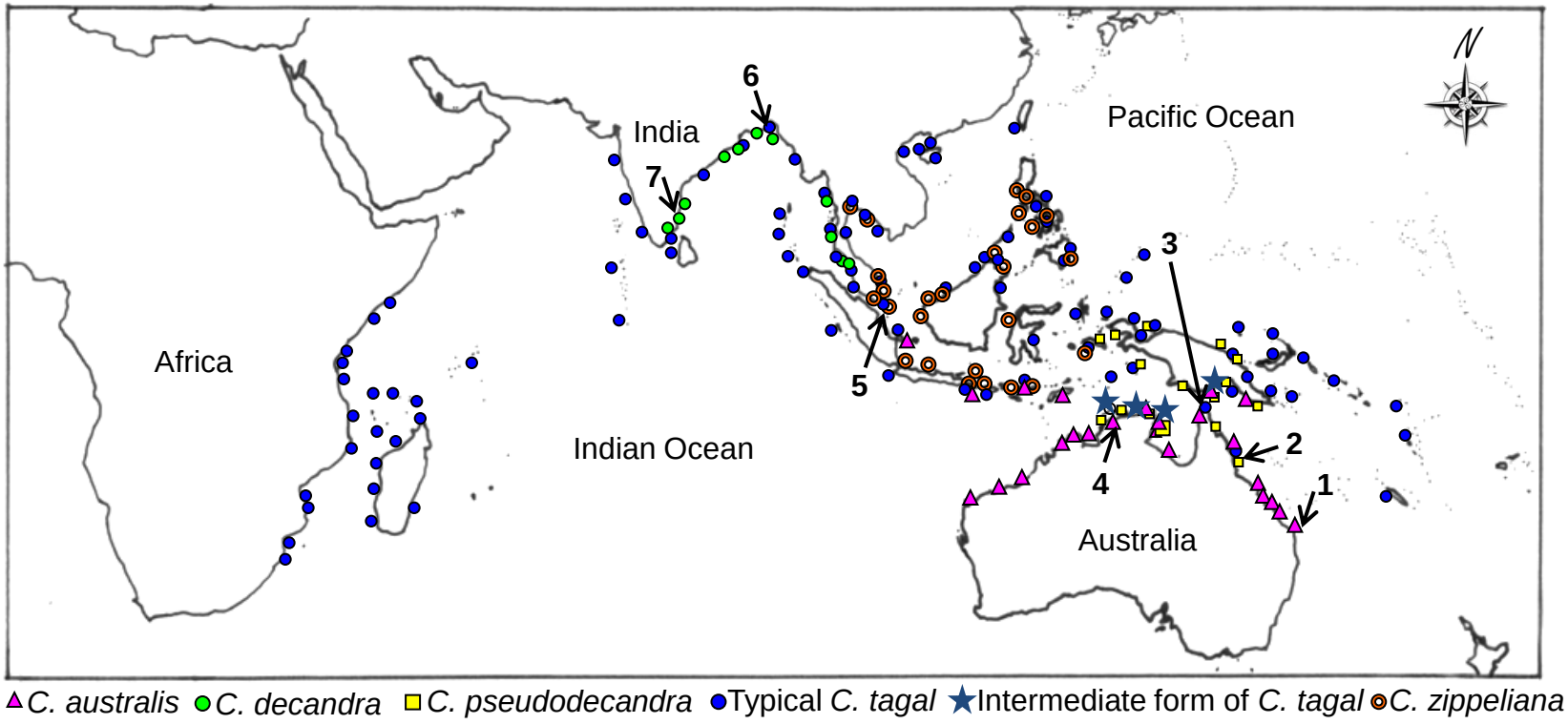
Fig. 4. The maximum likelihood tree derived from the plastid *trn*L intron sequence of the 38 specimens of genus *Ceriops* and six outgroup species from closely related genera. Bootstrap values > 50% are shown on each branch.

Fig. 5. One of the parsimony trees derived from the ITS sequence of 37 specimens of the genus *Ceriops* and six outgroup species from closely related genera. Bootstrap values > 50% are shown on each branch.

Fig. 6. The maximum likelihood tree derived from ITS sequences of 37 specimens of the genus *Ceriops* and six outgroup species from closely related genera. Bootstrap values > 50% are shown on each branch.

Fig. 7. Partial sequence alignment of the ITS1 region in the genus *Ceriops*.

Fig. 1



1: Moreton Bay, 2: Cairns, 3: Cape York, 4: Darwin, 5: Singapore, 6: West Sundarbans, 7: Pichavaram

Figure 2

Fig. 2

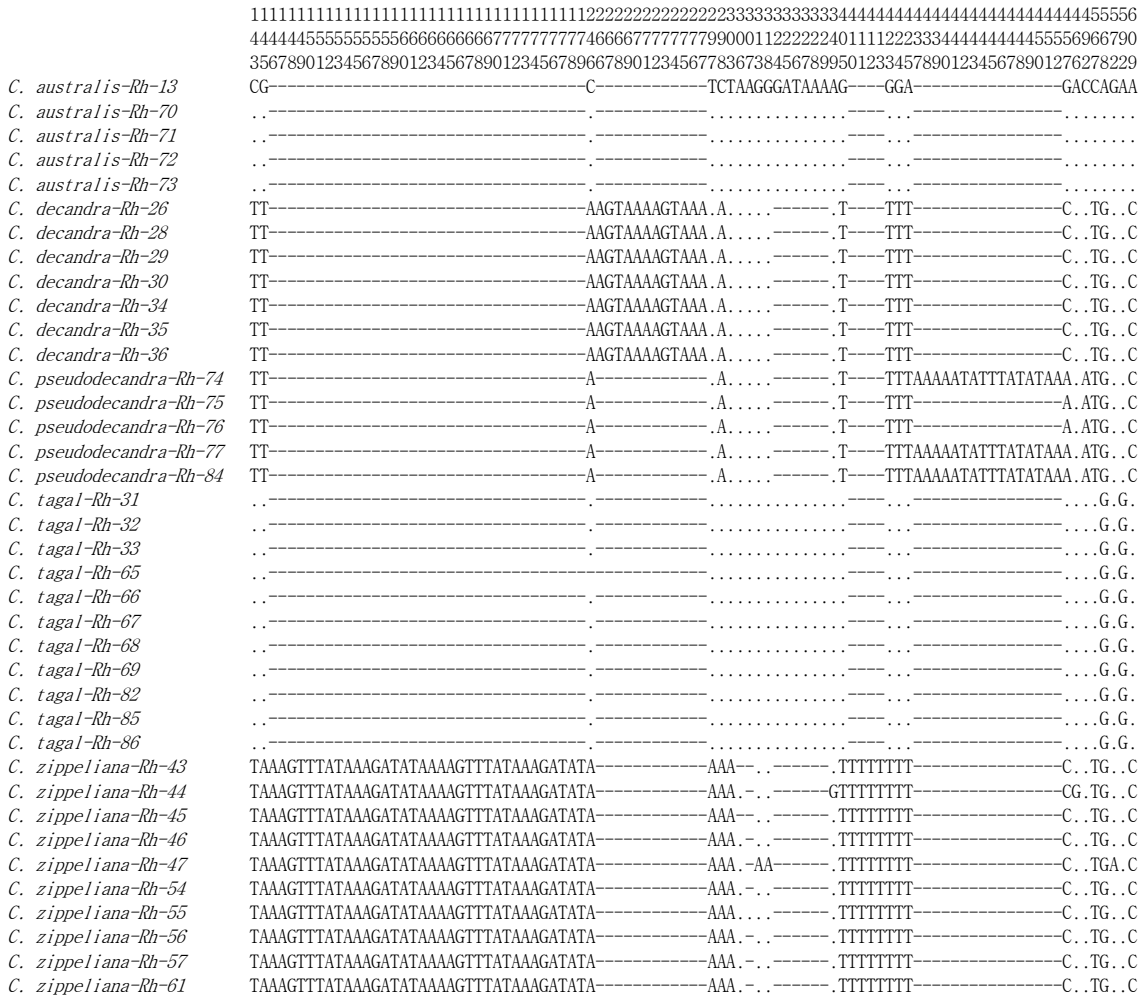


Figure 3

Fig. 3

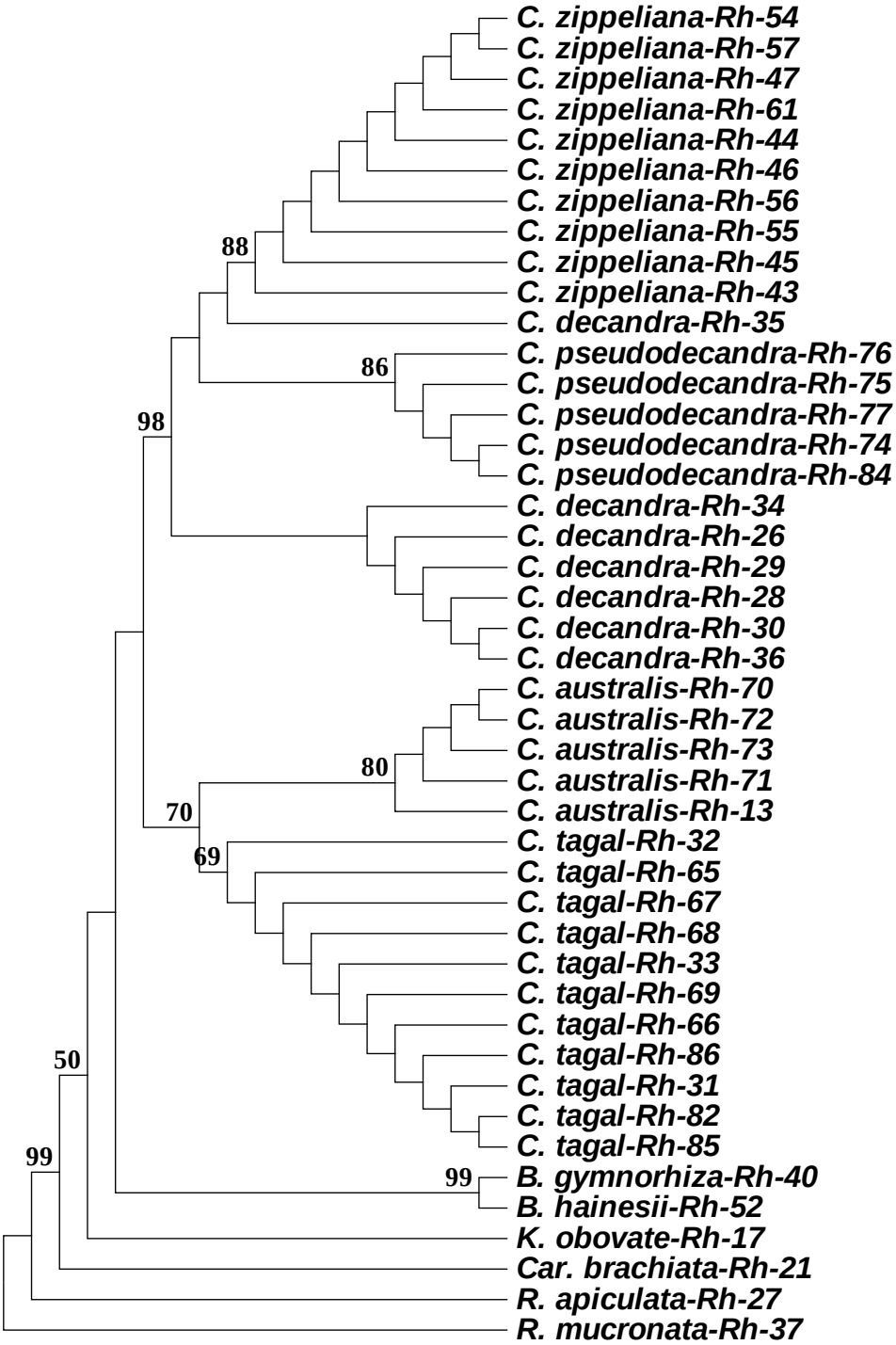


Figure 4

Fig. 4

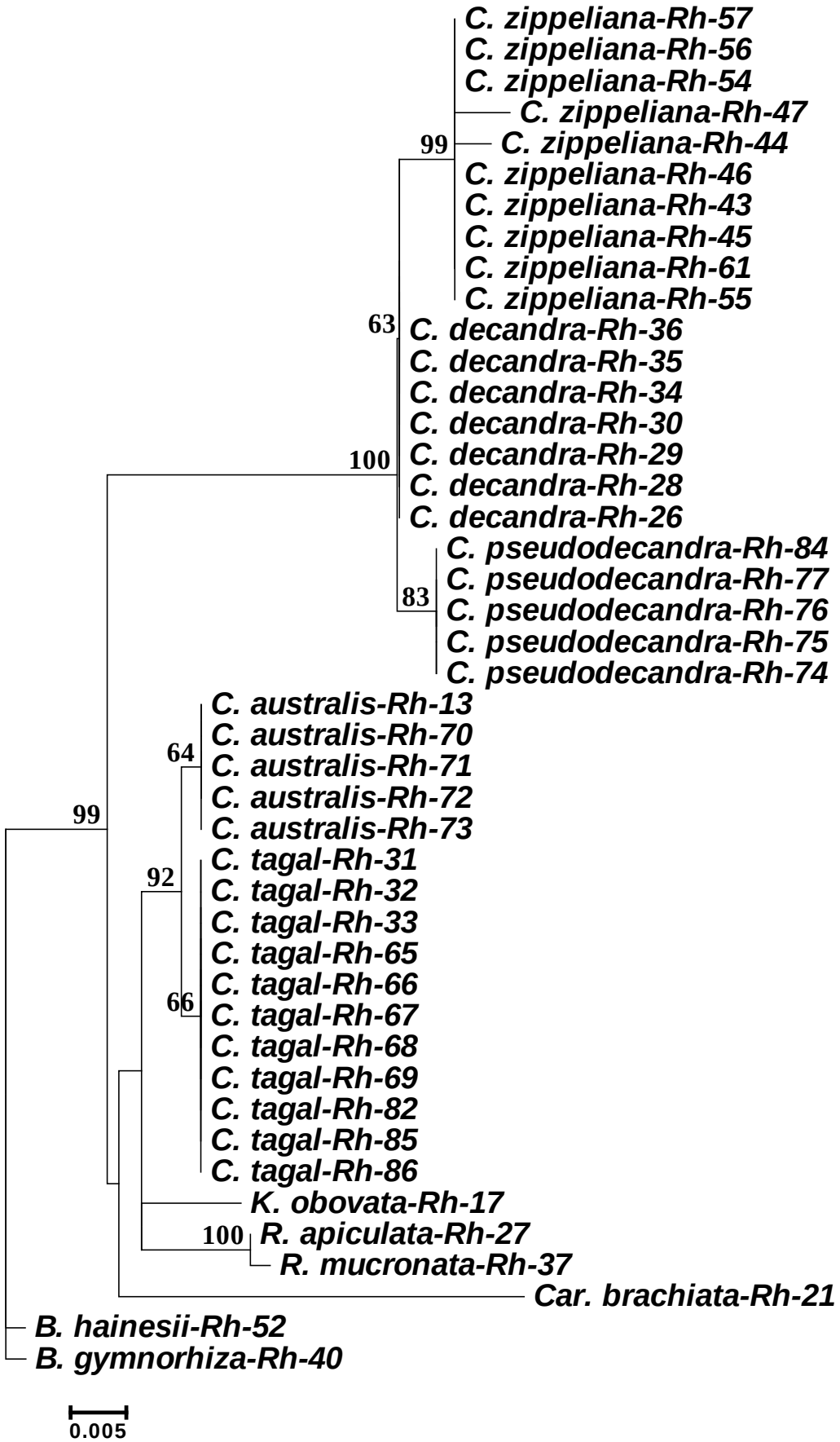


Figure 5

Fig. 5

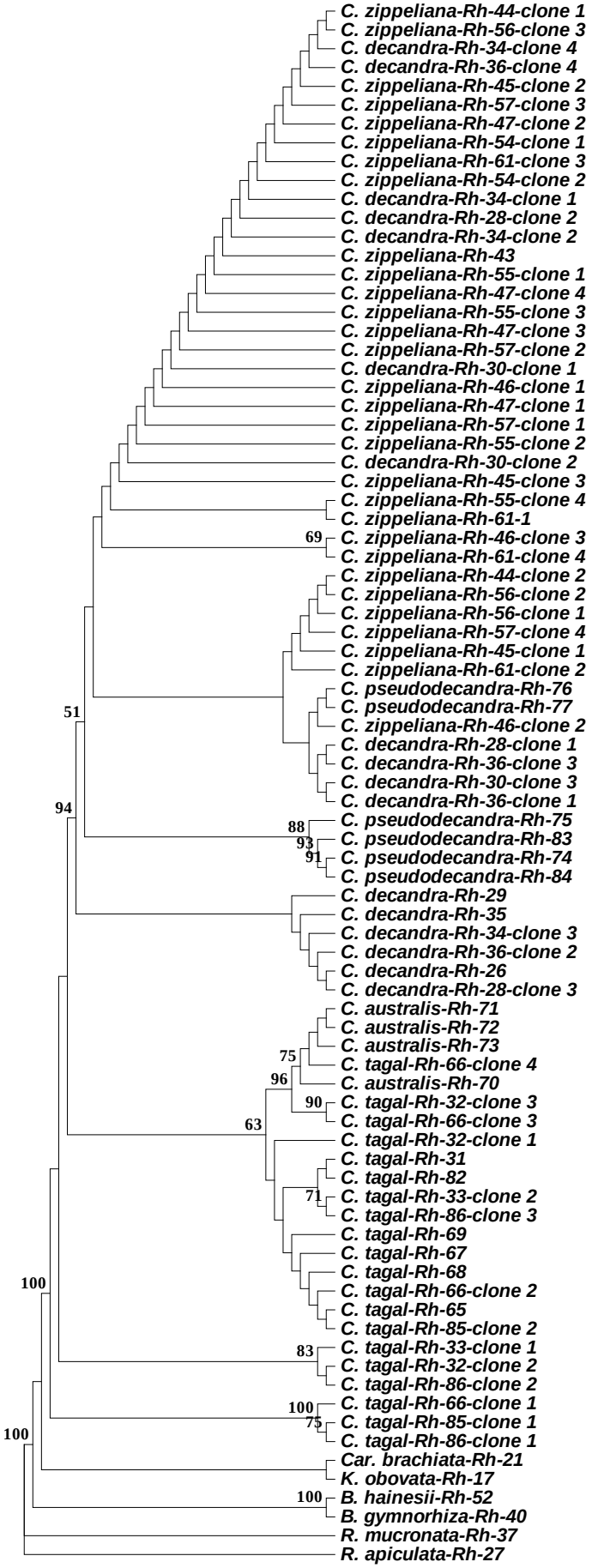


Figure 6

Fig. 6

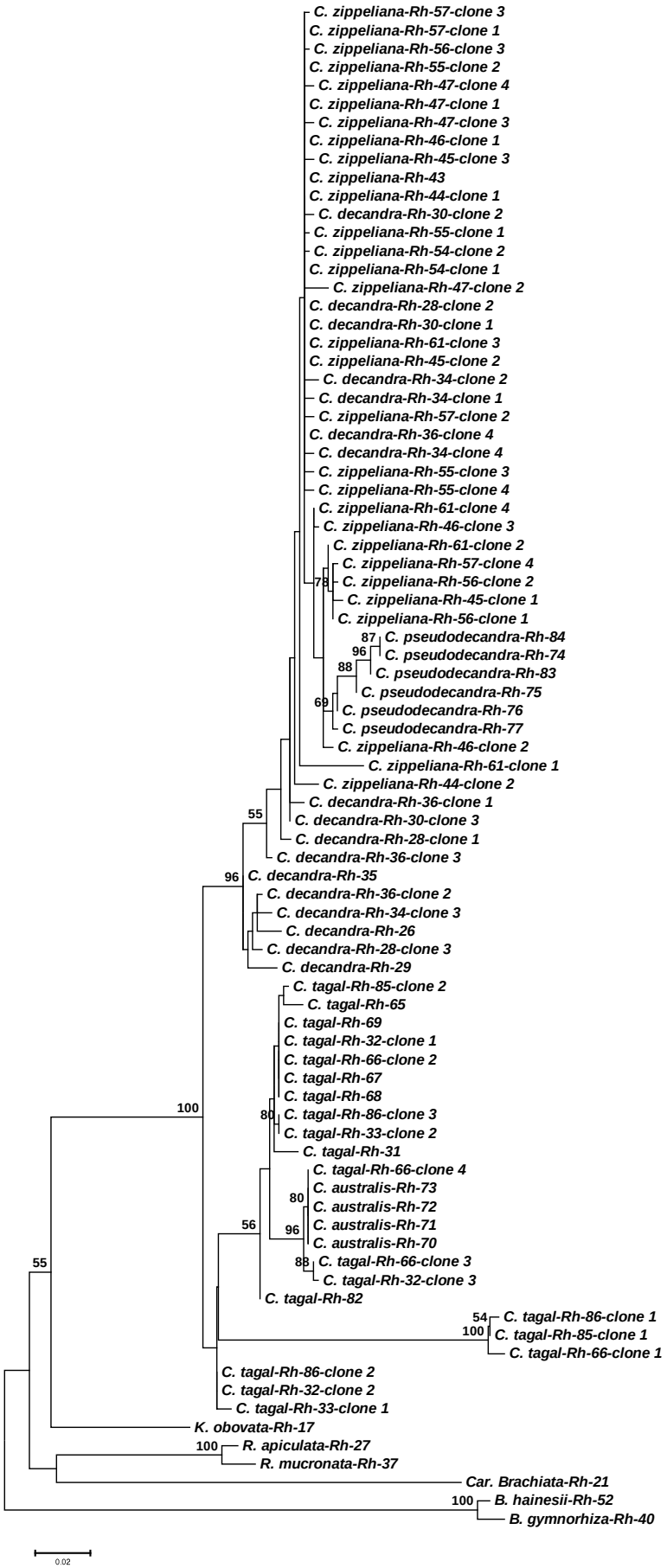


Figure 7

Fig. 7

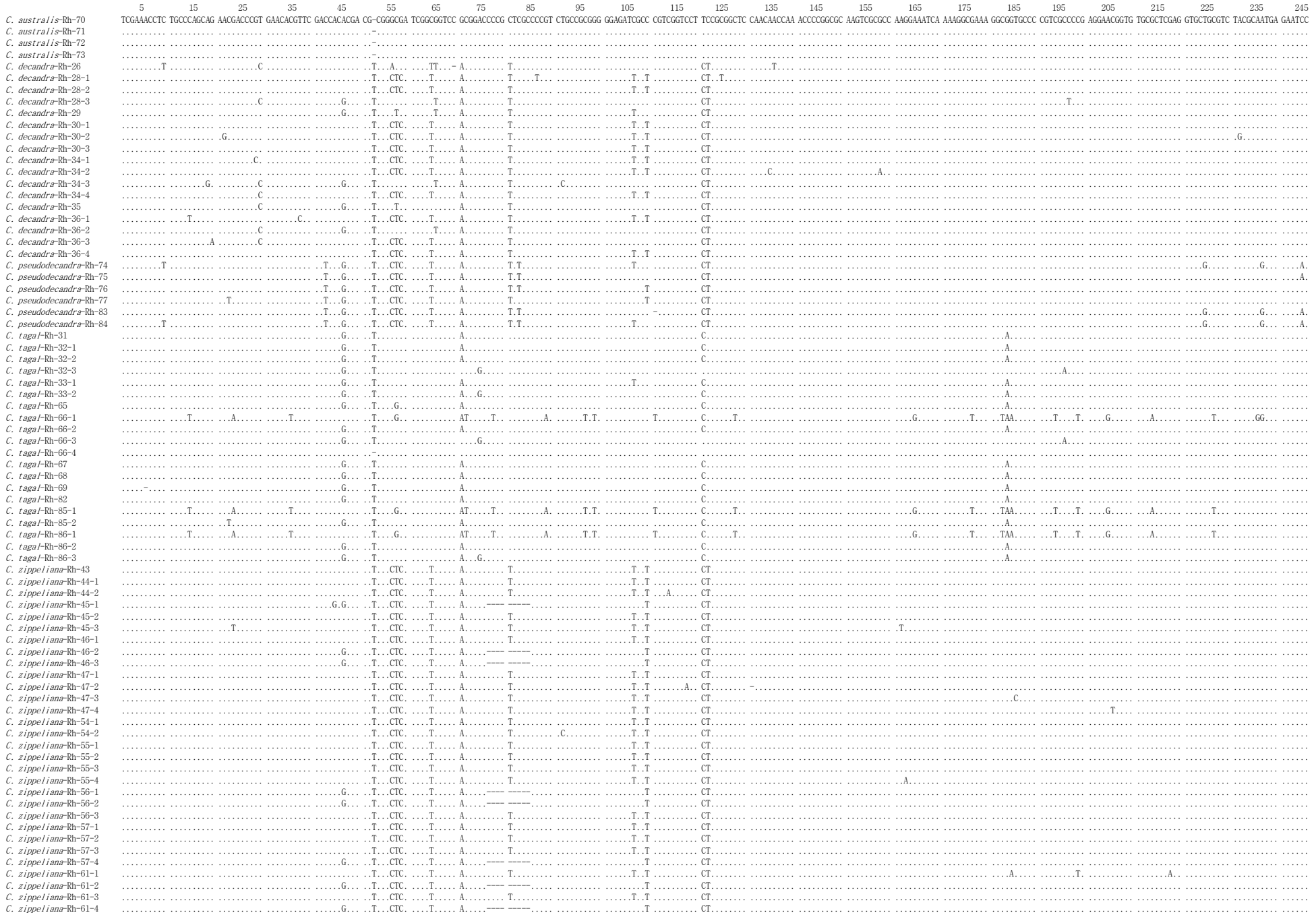


Table 1
Details of molecular studies of the 38 specimens of genus *Ceriops* and ten outgroup species of closely related genera and their collection locations.

Abb.	Taxon	Location	Accession No.	
			*trn*L intron	ITS
	Tribe Rhizophoreae			
	Genus *Ceriops*			
Rh-13	*C. australis*	Moreton Bay, Australia (AU)	EF118948	
Rh-70	*C. australis*	Cairns, Australia (AU)	EF118971	EF118995
Rh-71	*C. australis*	Darwin, Australia (AU)	EF118949	EF118996
Rh-72	*C. australis*	Darwin, Australia (AU)	EF118950	EF118997
Rh-73	*C. australis*	Darwin, Australia (AU)	EF118951	EF118998
Rh-26	*C. decandra*	Pichavaram, India (IN)	EF118952	EF119002
Rh-28	*C. decandra*	West Sundarbans, India (IN)	EF118953	EF118999
				EF119000
				EF119001
Rh-29	*C. decandra*	West Sundarbans, India (IN)	EF118954	EF119003
Rh-30	*C. decandra*	West Sundarbans, India (IN)	EF118955	EF119004
				EF119005
				EF119006
Rh-34	*C. decandra*	West Sundarbans, India (IN)	EF118956	EF119007
				EF119008
				EF119009
				EF119010
Rh-35	*C. decandra*	West Sundarbans, India (IN)	EF118957	EF119011
Rh-36	*C. decandra*	West Sundarbans, India (IN)	EF118958	EF119012
				EF119013
				EF119014
				EF119015
Rh-31	*C. tagal*	West Sundarbans, India (IN)	EF118987	EF119021
Rh-32	*C. tagal*	West Sundarbans, India (IN)	EF118964	EF119022
				EF119023
				EF119024
Rh-33	*C. tagal*	West Sundarbans, India (IN)	EF118965	EF119025
				EF119026
Rh-65	*C. tagal*	Cairns, Australia (AU)	EF118966	EF119027
Rh-85	*C. tagal*	Cairns, Australia (AU)	EF118986	EF119036
				EF119037
Rh-86	*C. tagal*	Cairns, Australia (AU)	EF118988	EF119038
				EF119039
				EF119040
Rh-66	*C. tagal*	Darwin, Australia (AU)	EF118967	EF119028
				EF119029
				EF119030
				EF119031
Rh-67	*C. tagal*	Darwin, Australia (AU)	EF118968	EF119032
Rh-68	*C. tagal*	Cape York, Australia (AU)	EF118969	EF119033
Rh-69	*C. tagal*	Cape York, Australia (AU)	EF118970	EF119034
Rh-82	*C. tagal*	Pulau Ubin, Singapore (SING)	EF118972	EF119035
Rh-43	*C. zippeliana*	Pasir Ris Nature Park, Singapore (SING)	EF118973	EF119041
Rh-44	*C. zippeliana*	Pasir Ris Nature Park, Singapore (SING)	EF118974	EF119042
				EF119043
Rh-45	*C. zippeliana*	Pasir Ris Nature Park, Singapore (SING)	EF118975	EF119044
				EF119045
				EF119046
Rh-46	*C. zippeliana*	Pasir Ris Nature Park, Singapore (SING)	EF118976	EF119047
				EF119048
				EF119049

Rh-47	*C. zippeliana*	Pasir Ris Nature Park, Singapore (SING)	EF118977	EF119050
				EF119051
				EF119052
				EF119053
Rh-54	*C. zippeliana*	Pasir Ris Nature Park, Singapore (SING)	EF118979	EF119054
				EF119055
Rh-55	*C. zippeliana*	Pasir Ris Nature Park, Singapore (SING)	EF118980	EF119056
				EF119057
				EF119058
				EF119059
Rh-56	*C. zippeliana*	Pasir Ris Nature Park, Singapore (SING)	EF118982	EF119060
				EF119061
				EF119062
Rh-57	*C. zippeliana*	Pasir Ris Nature Park, Singapore (SING)	EF118983	EF119063
				EF119064
				EF119065
				EF119066
Rh-61	*C. zippeliana*	Pasir Ris Nature Park, Singapore (SING)	EF118984	EF119067
				EF119068
				EF119069
				EF119070
Rh-74	*C. pseudodecandra*	Cairns, Australia (AU)	EF118959	EF119016
Rh-84	*C. pseudodecandra*	Cairns, Australia (AU)	EF118963	EF119020
Rh-75	*C. pseudodecandra*	Darwin, Australia (AU)	EF118960	EF119017
Rh-76	*C. pseudodecandra*	Darwin, Australia (AU)	EF118961	EF119018
Rh-77	*C. pseudodecandra*	Darwin, Australia (AU)	EF118962	EF119019
	Genus *Bruguiera*			
Rh-40	*B. gymnorhiza*	Pasir Ris Nature Park, Singapore (SING)	EF118992	EU000398
Rh-52	*B. hainesii*	Loyang, Singapore (SING)	EF118993	EU000399
	Genus *Kandelia*			
Rh-17	*K. obovata*	Tungshih, Taiwan (TW)	EF118989	EU000400
	Genus *Rhizophora*			
Rh-37	*R. mucronata*	West Sunderbans, India (IN)	EF118991	EF119073
Rh-27	*R. apiculata*	Benut, Malaysia (MA)	EF118990	EF119074
	Tribe Gynotrocheae			
	Genus *Carallia*			
Rh-21	*Car. brachiata*	Singapore Botanic Gardens, Singapore (SING)	EF118994	EF119072

Table 2
Plastid sequence analysis for five *Ceriops* species..

Species	**No. of samples**	**No. of haplotypes**	**Polymorphic sites**	**Parsimony-informative sites**	**haplotype diversity (*h*)**	**nucleotide diversity (π)**	**Tajima's *D***
Genus *Ceriops*	38	7	20	15	0.8240	0.01214	1.39721
C. australis	5	1	0	0	0.0000	0.00000	-
C. decandra	7	1	0	0	0.0000	0.00000	-
C. pseudodecandra	5	1	0	0	0.0000	0.00000	-
C. tagal	11	1	0	0	0.0000	0.00000	-
C. zippeliana	10	3	5	0	0.3780	0.00160	-1.74110[a]

[a] indicates $P < 0.05$

Table 3
ITS sequence analysis for five *Ceriops* species.

Species	No. of samples	No. of haplotypes	Polymorphic sites	Parsimony-informative sites	haplotype diversity (*h*)	nucleotide diversity (π)	Tajima's *D*
Genus *Ceriops*	37	53	137	93	0.9651	0.02916	-1.53494
C. australis	4	1	0	0	0.0000	0.00000	-
C. decandra	7	15	35	14	0.9779	0.01507	-0.76278
C. pseudodecandra	5	5	11	9	0.9333	0.00929	1.06601
C. tagal	11	14	79	69	0.9368	0.03651	-0.16159
C. zippeliana	10	21	43	8	0.8966	0.00830	-2.08748[a]

[a] indicates $P < 0.05$.

Table 4
The minimum number of recombination events (Rm) between the ITS sequences of five *Ceriops* species.

	C. australis	*C. decandra*	*C. pseudodecandra*	*C. tagal*	*C. zippeliana*
C. australis	0				
C. decandra	0	6			
C. pseudodecandra	0	4	0		
C. tagal	0	0	0	8	
C. zippeliana	0	2	3	0	2