Bruguiera hainesii, a critically endangered mangrove species, is a hybrid between B. cylindrica and B. gymnorhiza (Rhizophoraceae)

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RESEARCH ARTICLE

Bruguiera hainesii, a critically endangered mangrove species, is a hybrid between *B. cylindrica* and *B. gymnorhiza* (Rhizophoraceae)

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Abstract *Bruguiera hainesii* (Rhizophoraceae) is one of the two Critically Endangered mangrove species listed in the IUCN Red List of Threatened Species. Although the species is vulnerable to extinction, its genetic diversity and the evolutionary relationships with other *Bruguiera* species are not well understood. Also, intermediate morphological characters imply that the species might be of hybrid origin. To clarify the genetic relationship between *B. hainesii* and other *Bruguiera* species, we conducted molecular analyses including all six *Bruguiera* species using DNA sequences of two nuclear genes (*Ces*A and *UNK*) and three chloroplast regions (intergenic spacer regions of *trnL-trnF*, *trnS-trnG* and *atpB-rbcL*). For nuclear DNA markers, all nine *B. hainesii* samples from five populations were heterozygous at both loci, with one allele was shared with *B. cylindrica*, and the other with *B. gymnorhiza*. For chloroplast DNA markers, the two haplotypes found in *B. hainesii* were shared only by *B. cylindrica*. These results suggested that *B. hainesii* is a hybrid between *B. cylindrica* as the maternal parent and *B. gymnorhiza* as the paternal one. Furthermore, chloroplast DNA haplotypes found in *B. hainesii* suggest that hybridization has occurred independently in regions where the distribution ranges of the parental species meet. As the IUCN Red List of Threatened Species currently excludes hybrids (except for apomictic plant hybrids), the conservation status of *B. hainesii* should be reconsidered.

Keywords Mangrove · Hybridization · Endangered species · Genetics

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Introduction

Mangroves are an intertidal forest ecosystem distributed in subtropical to tropical regions of the world (Tomlinson 1986; Polidoro et al. 2010; Spalding et al. 2010). The major constituents of mangroves are woody plants with unique characteristics, such as aerial roots, viviparous propagules and high tolerance to salinity, which are adapted to intertidal environment. 73 species and hybrids from 20 families are known as "true mangroves" (Spalding et al. 2010), and they form mangrove forests at coastal zones in 123 countries and territories of the world. Despite the important ecological services provided by the mangroves (Fosberg 1971; Robertson and Duke 1987; Ong 1993; Primavera 1998; Dahdouh-Guebas et al. 2005), they are threatened by land use change (Field 1998; Valiela et al. 2001; Alongi 2002). Several reports warn that 20–35 % of world's mangrove area has been lost in the last two decades (Valiela et al. 2001; FAO 2007; Polidoro et al. 2010). This critical loss of mangrove area may increase the extinction risk of mangrove species. Although the majority of mangrove species are widespread and not considered to be threatened with extinction, 16 % (11 species) of the 73 true mangroves are categorized as threatened by extinction (Polidoro et al. 2010).

Bruguiera hainesii C. G. Rogers (Fig. 1) is one of the two mangrove species classified as "Critically Endangered (CR)" within the IUCN Red List of Threatened Species (Duke et al. 2010). This species has a wide geographic distribution extending from Myanmar and Thailand through the Malay Archipelago to Papua New Guinea (Tomlinson 1986; Sheue et al. 2005). However, fewer than 250 mature individuals are currently known, and the species is considered to be the rarest mangrove species (Kochummen 1989; Sheue et al. 2005; Polidoro et al. 2010). Polidoro et al. (2010) suggested that urgent protection is needed for the remaining *B. hainesii* individuals as well as carrying out further research to determine minimum viable population size.

Although *B. hainesii* is a highly prioritized mangrove species for conservation, its genetic background and the evolutionary relationships with other *Bruguiera* species are not well understood. Schwarzbach and Ricklefs (2000) provided the most comprehensive molecular phylogenetic study for the genus *Bruguiera* to date, but *B. hainesii* was not included. In studies that discussed phylogenetic relationships within the genus based on morphological features of the species, the genus *Brugueira* is generally divided into two groups (Tomlinson 1986; Sheue et al. 2005; Duke and Ge 2011). One group has larger leaves and larger solitary-flowered inflorescences and includes *B. gymnorhiza* (L.) Lamk., *B. sexangula* (Lour.) Poir. and *B. exaristata* Ding Hou. The other group has smaller leaves, smaller and multiple-flowered inflorescences of relatively small size and includes *B. cylindrica* (L.) Bl. and *B. parviflora* Wight & Arnold ex Griffith. Although some authors have placed *B. hainesii* in the multiple-flowered group (Duke and Ge 2011), this species exhibits an intermediate state for these traits having larger flowers in multiple-flowered inflorescences (Hou 1957, 1958) (Fig. 1). The intermediate morphological features of *B. hainesii* may imply the species is of hybrid origin.

In this study, we used molecular markers to analyse genetic relationships between *B. hainesii and* all other *Bruguiera* species, especially in terms of hybrid origin. To understand the genetic background of the critically endangered species is crucially important because hybrid taxa are not to be included on the IUCN Red List of Threatened Species (IUCN 2015). We used chloroplast DNA (cpDNA) and two single-copy nuclear DNA markers to elucidate clear genetic relationships of the species.

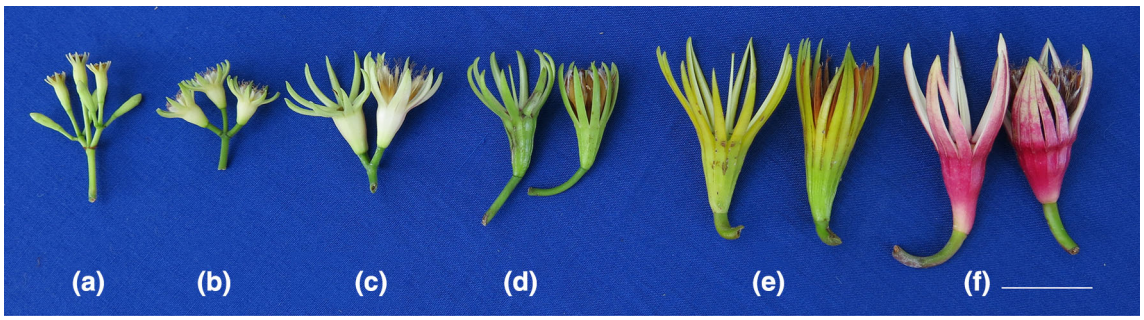

Fig. 1 Flowers of six species of genus *Bruguiera*. **a** *B. parviflora,* **b** *B. cylindrica,* **c** *B. hainesii,* **d** *B. exaristata,* **e** *B. sexangula,* **f** *B. gymnorhiza. Scale bar* = 2 cm

Materials and methods

Plant materials

We collected leaf samples of *B. hainesii*, and other five species of genus *Bruguiera* from the localities listed in Table 1. We used *Rhizophora stylosa* Griff. as an outgroup. Leaf samples were dried using silica gel powder and kept in plastic bags for subsequent DNA extraction.

DNA extraction

Total genomic DNA was extracted from the dried leaf material using a modified CTAB extraction method (Doyle and Doyle 1987). All samples were purified using GENECLEAN III Kit (MP Biomedicals). The extracted DNA was used for nuclear and chloroplast DNA analyses.

DNA amplification and sequencing

We performed PCR for two nuclear DNA and three cpDNA markers using PCR primers reported by previous studies. For nuclear DNA markers, we employed primers of Cellulose synthase (*Ces*A) reported by Cronn et al. (1999) and *UNK* by Urashi et al. (2013). To obtain improved results for *Ces*A, a new forward internal primer, *Ces*A-1150F (5′-CCACCTGAGCAGCAGATGGAAG-3′), was designed according to draft sequence results obtained using the PCR primers of Cronn et al. (1999). For cpDNA markers, we employed primers of *trnL-trnF*, *trnS-trnG* and *atpB-rbcL* intergenic spacers (IGSs) reported by Taberlet et al. (1991), Hamilton (1999) and Savolainen et al. (1994), respectively.

PCR amplifications were carried out according to Miryeganeh et al. (2014) with scaling down the total reaction volume to 10 μL using optimized annealing temperatures for markers: 55 °C for *UNK* and *atpB-rbcL* IGS, 58 °C for *trnL-trnF* and *trnS-trnG* IGSs and 61 °C for *Ces*A. When enough PCR products were not obtained for *atpB-rbcL* IGS and *UNK*,, touchdown PCR was alternatively performed with an annealing temperature decrease of 0.5 °C per cycle (from 55 to 50 °C) during the first 10 cycles. PCR products were purified with Exo-Star kit (GE-Healthcare), cycle-sequenced using the BigDye Terminator cycle sequencing kit v3.1 (Applied Biosystems), purified by the ethanol precipitation method, and then sequenced with an ABI 3500 automated sequencer (Applied Biosystems). Whenever the sequencing results of nuclear gene regions exhibited double peaks (suggesting heterozygosity) at more than one site, single-strand conformation polymorphism of PCR products (PCR-SSCP) was performed to separate allelic DNA fragments following the method of Jaruwattanaphan et al. (2013). After separating each DNA band, we re-amplified the obtained DNA and performed direct sequencing following the method described above.

DNA sequences were aligned in MEGA6 (Tamura et al. 2013) using the Clustal W algorithm (Thompson et al. 1994) included in the software, and alignments were subsequently manually corrected. For *R. stylosa* samples, sequences of *trnS-trnG* IGS could not be completely determined due to poly-A site located at about 300 bp from the *trnG* gene. Thus, only 300-bp from the *trnG* end of the sequence was used for subsequent analyses. The resulting nucleotide sequences were deposited in DDBJ with accession numbers LC076503–LC076548 for *Ces*A, LC076391 to LC076437 for *UNK*, LC075996–LC076031 for *trn*L-*trn*F IGS, LC076032–LC076067 for *trn*S-*trn*G IGS and LC076068–LC076103 for *atp*B-*rbc*L IGS (Table S1).

Data analysis

Statistical parsimony networks were constructed using TCS 1.21 (Clement et al. 2000) to visualize the relationships among alleles for the two nuclear genes and among cpDNA haplotypes. For the analysis, we concatenated sequences of all three cpDNA regions (*trnL-trnF*, *trnS-trnG* and *atpB-rbcL* IGSs).

Results

Nuclear DNA sequencing

The nucleotide sequence length determined were 594–597 bp for nuclear *Ces*A and 398 bp for nuclear *UNK*. The aligned sequences of *Ces*A and *UNK*, in which all gap sites were excluded, were 594 bp and 398 bp, respectively. Among six *Bruguiera* species and *Rhizophora stylosa*, a total of 11 and nine alleles were detected from nuclear *Ces*A and *UNK* genes, respectively (Table 1; Fig. 2a, b).

Bruguiera hainesii did not have species–specific alleles at either *Ces*A or *UNK* genes (Table 1). All nine *B. hainesii* samples from five populations were heterozygous at both nuclear loci, in which one haplotype was shared with *B. gymnorhiza* (*Ces*A01 or *Ces*A03, and *UNK*1 or *UNK*3), and the other with *B. cylindrica* (*Ces*A09 and *UNK*6). Furthermore, alleles shared with *B. gymnorhiza* were different among individuals of *B. hainesii*. One of the two individuals of *B. hainesii* from Klang and all individuals from Pulau Kukup and Singapore, had the allele *Ces*A01, whereas all three *B. hainesii* individuals from Merbok and another individual from Klang carried the allele *Ces*A03. As for *UNK* gene, the allele *UNK*3 was found only in *B.*

Table 1 Species, sample ID, sampling localities, coordinate, genotype and voucher information used in this study

Taxon/ID	Country	Locality	Lat.	Long.	*Ces*A	*UNK*	Cp	Voucher
Bruguiera hainesii								
BhMYS1	Malaysia	Merbok	5.655	100.372	03/09	1/6	11	LZA2013082101 (UPM)
BhMYS2			5.655	100.372	03/09	1/6	11	LZA2013082102 (UPM)
BhMYS3			5.655	100.372	03/09	1/6	11	LZA2013082103 (UPM)
BhMYS4		Klang	2.973	101.362	01/09	1/6	11	TK 11121408 (URO)
BhMYS5			2.913	101.312	03/09	1/6	11	JO2014111403 (URO)
BhMYS6		Pulau Kukup	1.326	103.433	01/09	3/6	11	JO2015022607 (URO)
BhMYS7			1.323	103.430	01/09	3/6	11	JO2015022604 (URO)
BhSGP1	Singapore	Sungai Loyang	1.381	103.966	01/09	1/6	12	JO2015022701 (URO)
BhSGP2		Pulau Ubin	1.418	103.964	01/09	1/6	12	JO2015022704 (URO)
B. gymnorhiza								
BgMOZ1	Mozambique	Maputo	−25.850	32.696	01	1	01	TK 10122701 (URO)
BgIND1	India	Kerala	9.986	76.232	01	1	02	TK 10112002 (URO)
BgMMR1	Myanmar	Byonmwe I.	15.976	95.267	02	1	03	TK 11100903 (URO)
BgMYS1	Malaysia	Merbok	5.655	100.372	01	1	03	AKS WBGMK01 (URO)
BgMYS2			5.655	100.372	03	1	03	AKS WBGMK05 (URO)
BgMYS3		Klang	2.973	101.362	01/03	1	03	TK 11121407164 (URO)
BgMYS4			2.973	101.362	01	1	03	TK 11121407166 (URO)
BgMYS5		Sabah	5.939	118.053	01	2	01	TK 11072203 (URO)
BgVNM1	Vietnam	Dong Rui	21.248	107.390	01	3	01	TK 10050102 (URO)
BgPHL1	Philippines	Panay I.	11.813	122.142	01	3	04	TK 11062402 (URO)
BgJPN1	Japan	Iriomote I.	24.313	123.906	01	3	01	TK 07101102 (URO)
BgJPN2		Ishigaki I.	24.401	124.145	01	3	05	TK 07101202 (URO)
BgAUS1	Australia	Cairns	−16.900	145.755	04	1	01	TK 04121203 (URO)
BgFJI1	Fiji	Viti Levu I.	−18.156	178.446	04	1/4	06	KT 09012615 (URO)
B. sexangula								
BsMMR1	Myanmar	Byonmwe I.	15.976	95.267	05	2	07	TK 11100908 (URO)
BsMYS1	Malaysia	Sabah	5.854	116.042	06	2	08	TK 11072401 (URO)
BsVNM1	Vietnam	Ca Mau	8.614	104.732	06	2	09	TK 10042903 (URO)
B. exaristata								
BeAUS1	Australia	Darwin	−12.408	130.832	07	5	10	KT13032201 (URO)
BeAUS2		Kimberley	−16.279	145.439	08	5	10	KT13032006 (URO)
B. cylindrica								
BcIND1	India	Mumbai	19.330	72.815	09	6	11	TK 1011181088 (URO)
BcIND2			19.330	72.815	09	6	11	TK 1011181089 (URO)
BcMYS1	Malaysia	Klang	2.913	101.312	09	6	11	JO 2014111401 (URO)
BcSGP1	Singapore	Sungai Loyang	1.381	103.966	09	6/7	12	JO 2015022703 (URO)
BcPHL1	Philippines	Luzon I.	13.970	120.626	09	6	11	OBY PHL6-1229(URO)
BcPHL2			13.970	120.626	09	6	11	OBY PHL7-1230(URO)
B. parviflora								
BpVNM1	Vietnam	Ca Mau	8.614	104.732	10	8	13	TK 10042904 (URO)
Rhizophora stylosa								
RsJPN1	Japan	Iriomote I.	24.395	123.822	11	9	14	TK 07101101 (URO)

For voucher information, collectors are designated by their initials. Herbaria are specified by their acronyms

LZA Latifah Zainal Abidin , *JO* Junya Ono, *TK* Tadashi Kajita, *AKSW* Alison Kim Shan Wee, *KT* Koji Takayama, *OBY* Orlex Baylen Yllano, *Lat* Latitude, Long longitude, *Cp* CpDNA haplotype, *UPM* Universiti Putra Malaysia, *URO* University of the Ryukyus, Faculty of Education

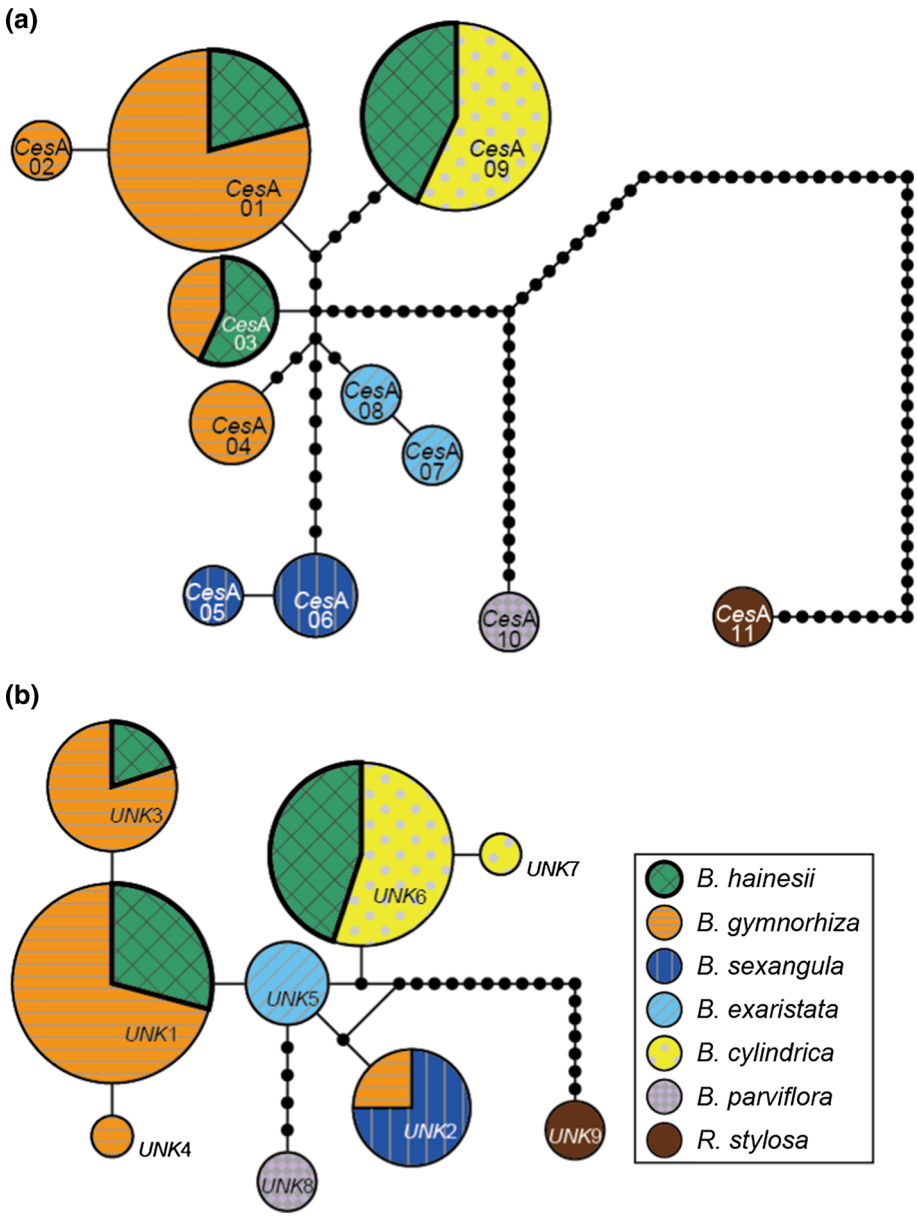

Fig. 2 Haplotype networks. **a** Nuclear DNA *Ces*A gene. **b** Nuclear DNA *UNK* gene. Each species is shown as distinct color and pattern, *Bruguiera hainesii*: *green* with *grid lines*, *B. gymnorhiza*: *orange* with *horizontal lines*, *B. sexangula*: *deep blue* with *vertical lines*, *B. exaristata*: *light blue* with *diagonal lines*, *B. cylindrica*: *yellow* with *polka-dots*, *B. parviflora*: *purple* with *square dots*, *Rhizophora stylosa*: brown. The size of *circles* is relative to the haplotype frequency. Haplotypes segregated by a *single line* are one mutation apart and *black dots* are missing haplotypes (ancestral or un-sampled haplotypes)

hainesii individuals from Pulau Kukup. The other *UNK* alleles were not shared between species, except for *UNK*2, which was shared between one *B. gymnorhiza* individual (BgMYS5) and *B. sexangula*.

Chloroplast DNA sequencing

The length of nucleotide sequences determined were 277–295 bp for *trnL-trnF*, 572–1180 bp for *trnS-trnG* and 692–744 bp for *atpB-rbcL* IGSs. The aligned concatenated sequences without all gap sites were 1494 bp in length. A total of 14 haplotypes were recognized from the three cpDNA regions of six *Bruguiera* species and *Rhizophora stylosa*. (Table 1; Fig. 3). The two haplotypes found in *B. hainesii* (cp11 and cp12) were shared by *B. cylindrica*. No other haplotypes were shared among species.

Discussion

Hybrid origin of *Bruguiera hainesii*

This study clearly suggests that *B. hainesii* originated through hybridization between *B. gymnorhiza* and *B. cylindrica*. There was no specific allele of *B. hainesii* and all nine samples of *B. hainesii* shared one nuclear allele with *B. cylindrica* (*Ces*A09, *UNK*6) and the other ones with *B. gymnorhiza* (*Ces*A01 *Ces*A03; *UNK*1, *UNK*3) at

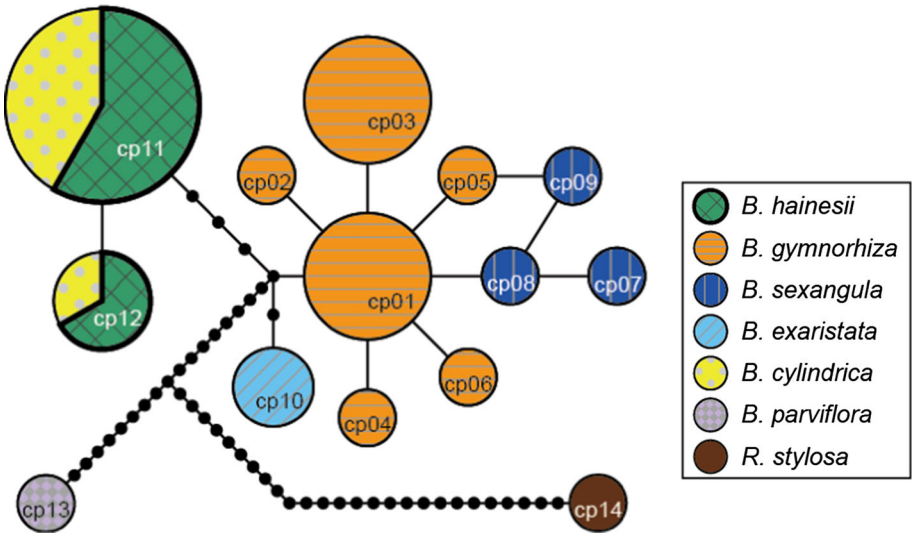

Fig. 3 Haplotype networks of combined regions of chloroplast DNA *trnL-trnF*, *trnS-trnG* and *atpB-rbcL* intergenic spacers (IGSs). Each species is shown as distinct color and pattern, *Bruguiera hainesii*: *green* with *grid lines*, *B. gymnorhiza*: *orange* with *horizontal lines*, *B. sexangula*: *deep blue* with *vertical lines*, *B. exaristata*: *light blue* with *diagonal lines*, *B. cylindrica*: *yellow* with *polka-dots*, *B. parviflora*: *purple* with *square dots*, *Rhizophora stylosa*: *brown*. The size of *circles* is relative to the haplotype frequency. Haplotypes segregated by a *single line* are one mutation apart and *black dots* are missing haplotypes (ancestral or un-sampled haplotypes)

both loci (Table 1; Fig. 2a, b), indicating hybrid origin of *B. hainesii*. For cpDNA of *B. hainesii* samples only haplotypes shared with *B. cylindrica* were observed (cp11 and cp12 in Table 1; Fig. 3). Since chloroplast DNA is primarily maternally inherited in angiosperms (Birky 1995; Mogensen 1996), *B. cylindrica* is likely to be the putative maternal species of *B. hainesii* while *B. gymnorhiza* may serve as the paternal one. Furthermore, different haplotypes were shared between *B. hainesii* and *B. cylindrica* in different locations (cp11 in Malaysia and cp12 in Singapore) (Table 1), which suggests multiple origin of *B. hainesii* in different places where the distribution ranges of the parental species meet.

Bruguiera hainesii is considered to be the rarest mangrove species (Kochummen 1989; Polidoro et al. 2010; Sheue et al. 2005), and the infrequent occurrence may be attributed to the dissimilar pollinators serving the two putative parental species. The two morphologically dissimilar groups of the genus *Bruguiera* have different pollinators associated with their floral characters. The larger solitary-flowered inflorescence group (including *B. gymnorhiza*, *B. sexangula* and *B. exaristata*) is thought to be bird-pollinated (Tomlinson 1986; Kondo et al. 1987, 1991; Noske 1993; Wee et al. 2014). On the other hand, the group with smaller and multiple-flowered inflorescences (including *B. cylindrica* and *B. parviflora*) is thought to be pollinated by insects (Tomlinson 1986). Although the putative parental species are common species in the Indo-West Pacific region (Tomlinson 1986; Kochummen 1989; Sheue et al. 2005) and have been recorded in sympatry (Putz and Chan 1986; White et al. 1989; Imai et al. 2006; Sun and Lo 2011), hybridization between them may not occur frequently because they use different types of pollinators.

Bruguiera hainesii may possibly be a F1 hybrid affected by postmating isolation. *Bruguiera hainesii* has been reported to have very low rates of propagation and low rates of germination (Polidoro et al. 2010), which may result from outbreeding depression. All individual samples of *B. hainesii* used in this study were heterozygous at both nuclear loci, which suggest all of our *B. hainesii* samples were F1 hybrids. This contrasts with another hybrid taxon in the genus *Bruguiera*, *Bruguiera* × *rhynchopetala* (Ko) X. J. Ge et N. C. Duke, where the hybrid taxon produces fertile seed, and can backcross with the putative parental species: *B. gymnorhiza* or *B. sexangula* (Sun and Lo 2011). These characteristics can be attributed to less reproductive isolation between the two putative parental species that use birds as pollinators (Tomlinson 1986; Duke and Ge 2011).

Conservation of *Bruguiera hainesii* and other threatened mangrove species

Because of the putative hybrid status of *B. hainesii* shown in this study, the IUCN red list category CR given to this species (Duke et al. 2010) should be re-considered. Our study indicates that *B. hainesii* originated through successful hybridization between *B. cylindrica* and *B. gymnorhiza*, and suggests that it may be a locally formed F1 hybrid. In the IUCN Red List of Threatened Species,

hybrids will be excluded if they are not apomictic plants (IUCN 2015).

This study implies that other critically endangered mangrove plants may also be of hybrid origin. Hybridization appears to be a common phenomenon for mangrove plant species, with numerous hybrid taxa have been reported e.g. *Bruguiera* × *rhynchopetala* (Ge 2001), *Rhizophora* × *annamalayana* Kathir. (Kathiresan 1995, 1999), *Rhizophora* × *lamarckii* Montr. (Tomlinson and Womersley 1976), *Rhizophora* × *selala* (Salvoza) (Tomlinson 1978; Duke 2010), *Sonneratia* × *gulngai* N.C. Duke (Duke 1984), and *Sonneratia* × *hainanensis* W.C. KO, E.Y. Chen & W.Y. Chen (Wang et al. 1999). A recent study also suggested hybridization between two closely related *Rhizophora* species (*R. mucronata* and *R. stylosa*) using DNA markers (Wee et al. 2015). A possible reason why various hybrid taxa exist in mangrove plants would be attributed to the distribution pattern. According to Tomlinson (1986), most mangrove species have wide distribution ranges and the geographic ranges overlap in many cases. Since propagules of mangrove species are buoyant and can be dispersed across the ocean (Tomlinson 1986), distribution ranges of closely related species could overlap more easily than in terrestrial plants. The sympatric distribution of congeners may increase chances of hybridization for mangrove plants.

Molecular studies of other threatened mangrove species should also be conducted. Polidoro et al. (2010) assessed there are 11 threatened mangrove species. Since budget available for conservation of mangrove species is limited, species identity of threatened mangrove species should be confirmed using DNA markers, which will help us to determine priorities for mangrove conservation.

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