Conservation genetic study of a mangrove plant genus *Bruguiera*

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Junya Ono

Graduate School of Science

CHIBA UNIVERSITY

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（マングローブ植物オヒルギ属の保全遺伝学的研究）

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小野　潤哉

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ABSTRACT

Mangroves are an intertidal forest ecosystem distributed in the subtropical and tropical regions of the world. Mangroves provide critical ecosystem services such as coastal protection, carbon sequestration and as a breeding ground for marine animals. However, the ecosystem as a whole is endangered due to anthropogenic disturbances and, therefore, needs urgent conservation. Conservation genetic research can help us plan conservation strategies, however, there is no clear consensus on conservation genetics of mangroves because most species still retain their broad distribution range as compared to other threatened species. Furthermore, knowledge of the genetic diversity of mangroves has not been well understood and not used for actual conservation actions. This study focuses on a mangrove genus *Bruguiera* as a model system of conservation genetics for mangrove plants. Firstly, a phylogenetic study of all species in the genus, including a critically endangered species *B. hainesii*, was conducted with both nuclear and chloroplast DNA markers. The results indicated that *B. hainesii* is a hybrid between *B. gymnorhiza* and *B. cylindrica*, thus undermining its current conservation status. Secondly, the phylogeographic study on the most widely distributed mangrove species, *B. gymnorhiza*, was conducted using nuclear microsatellite and chloroplast DNA markers, using samples obtained across its distribution range. The genetic structure detected in this study was not only across the Malay Peninsula but also within oceanic regions. In conclusion, the author made two recommendations for conservation of mangroves based on the findings of this study. First, species identity of other threatened mangrove species should be confirmed via phylogenetic analyses and conservation effort should be allocated appropriately. Second, according to the range-wide genetic study of *B. gymnorhiza*, a clearer structure was found than

previously thought, which can be considered as conservation units. Conservation units of other mangrove species should be determined by conducting phylogeographic analyses covering their entire distribution ranges.

GENERAL INTRODUCTION

Why mangroves are important and subject to conservation

Mangroves are an intertidal forest ecosystem distributed in the subtropical to tropical regions of the world. The major components of mangroves are woody plant species belonging to several unrelated angiosperm families. The global distribution of mangrove species is divided into two main regions, the Indo-West Pacific (IWP) and the Atlantic-East Pacific (AEP), without any species commonly distributed between the regions except for a fern species, *Acrostichum aureum*. Most species within each region has a wide distribution range (Tomlinson 1986). They have uniquely adapted characteristics to cope with environmental conditions in estuarine and coastal habitats. For example, mangroves have the ability to tolerate high salinity (Parida & Jha 2010). Their dispersal system is also adapted for sea dispersal, as all true mangrove species have propagules (fruit, seed, or seedling) that are buoyant in fresh, brackish and/or sea water (Tomlinson 1986).

Mangrove plants are important regarding the ecosystem services that they provide (Tomlinson 1986). Mangrove forests are nurseries for various marine organisms (Robertson & Duke 1987, Primavera 1998). They can protect inland areas from storms and tsunamis (Fosberg 1971, Dahdouh-Guebas et al. 2005). Also, mangroves sequester up to 25.5 million tonnes of carbon per year (Ong 1993).

However, the ecosystem as a whole is endangered due to anthropogenic disturbances and, therefore, is in urgent need for conservation (Polidoro et al. 2010). Mangroves are threatened by drastic land use change e.g. conversion into aquaculture ponds (Alongi 2002), over-exploitation for fuels and timbers (Valiela et al. 2001), and industrial and urban development (Field 1998). Recent reports warned that about 20 to 35 % of world

mangrove area has been lost in the last two decades (Valiela et al. 2001, FAO 2007). This critical loss of mangrove area may increase the risk of extinction for mangrove species. Thus, these threats to mangroves are a great concern for conservation.

The need for conservation genetics in mangroves

Conservation genetics is fundamental for conservation to reduce the extinction risk of threatened species. Conservation genetics uses genetic tools and concepts to provide practical solutions to conservation problems (Hedrick & Miller 1992). For example, phylogenetic information can help in prioritizing the species to be protected (Faith 1992). Genetic diversity parameters that can detect inbreeding, loss of genetic diversity and population fragmentation can be used to inform conservation strategies to minimize these adverse effects (Frankham et al. 2010). On the other hand, isolated populations could have unique local adaptations and may cause a case in which maladapted genes are introduced to restored populations and may undermine the success of conservation activities (Mckay et al. 2005). Therefore, genetic tools can facilitate the detection of populations vulnerable to not only genetic diversity loss but also genetic pollution.

There are fundamental differences between mangroves and other threatened species when we think about the application of genetics to conservation. Endangered species have small population size (Beissinger & Westphal 1998). In contrast, most mangrove species have a wide distribution range despite the rapid decline of their habitats and fragmentation of populations. The effects of habitat loss on individual mangrove species are not well known (Polidoro et al. 2010). Widespread mangrove species such as *Avicennia* produces an enormous number of sea-dispersed propagules and, therefore, the species may be genetically panmictic (Duke et al. 1998). If this is a general case for

mangroves, habitat loss may not have a significant influence on mangrove species on a global scale. However, this idea has not to be proven yet.

A significant knowledge gap in conservation genetics of mangroves is the genetic structure of a species across its entire distribution range. Recently population genetic studies have increased our understanding of the genetic patterns in mangroves (Triest 2008); however most of these studies had limited sampling coverage. Studies covering wide distribution range are rather frequent in the AEP (*Rhizophora*; Takayama et al. 2013, *Avicennia*; Mori et al. 2015a). Although the species richness of mangroves is much higher in the IWP, few studies with broad sampling scheme have been conducted (see Chapter 2). Thus, there is still no clear consensus on population genetic study of mangroves.

The genus Bruguiera as a model system for conservation genetics of mangroves

Bruguiera is a widespread genus in the IWP (Tomlison 1986, Duke & Ge 2011). This genus consists of six species, *Bruguiera gymnnorhiza* (L.) Lamk., *Bruguiera sexangula* (Lour.) Poir., *Bruguiera exaristata* Ding Hou, *Bruguiera hainesii* C. G. Rogers, *Bruguiera cylindrica* (L.) Bl., and *Bruguiera parviflora* Wight and Arnold ex Griffith (Tomlinson 1986).

In this study, the author focused on *Bruguiera* as a model system for conservation genetic studies of mangroves because of the following reasons. First, the genus has a critically endangered mangrove species *B. hainesii*, which was concerned as closest to extinction among all mangrove species (Polidoro et al. 2010). This species can be an appropriate model species to understand the genetic diversity of threatened mangrove species, but no genetic study has been conducted for the species. Second, *Bruguiera* has

the most widely distributed mangrove species *B. gymnorhiza* in the IWP and all mangrove plants, covering almost the whole IWP region (Tomlinson 1986). Therefore, *B. gymnorhiza* can be a suitable model species to understand the genetic diversity of widespread mangrove species.

Overview of this study

This dissertation has two main objectives. First, the author aimed to clarify the phylogenetic relationships between critically endangered species *Bruguiera hainesii* and its close relatives. Second, the author investigated the genetic structure of the most widely distributed species *B. gymnorhiza* over the IWP. By combining the findings from these two chapters, the author aimed to provide practical suggestions on the conservation strategies for *Bruguiera*, and for mangroves as a whole.

CHAPTER 1: PHYLOGENETIC STUDY ON A CRITICALLY ENDANGERED SPECIES *BRUGUIERA HAINESII* AND ITS RELATED SPECIES

Introduction

Phylogenetic analysis of rare species and their relatives gives us essential information for conservation management. For the species with unclear systematic position, phylogenetic analyses will provide its phylogenetic relationships with other related species and help us to determine the systematic status of the species. Systematic information obtained in this way can aid in setting priorities which species should be protected (Andreasen 2005). Phylogenetic analysis can also identify the occurrence and extent of introgression through hybridization between rare species and widespread congeners (Soltis & Gitzendanner 1999). Hybridization with other species may raise the risk of extinction of the rare species because hybridization may cause reduction of the ability of reproduction, competition, and interaction with disease-causing agents and predators, and, therefore, limit the growth of the populations (Levin et al. 1996). For these reasons, phylogenetic analysis is a first study which should be conducted when we plan conservation of an endangered plant species.

Bruguiera hainesii C. G. Rogers is one of the two mangrove species classified under the category "Critically Endangered (CR)" within the IUCN Red List of Threatened Species (Duke et al. 2010c). The 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 as 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 individuals of *B. hainesii* as well as carrying out further research to determine minimum viable population size.

Although *Bruguiera hainesii* is a highly prioritized mangrove species for conservation, understanding its phylogenetic position that would be useful for implementing scientific-based conservation strategies has not been well understood. Schwarzbach & Ricklefs (2000) provided the most comprehensive molecular phylogenetic study for the genus *Bruguiera* to date, but *B. hainesii* was not included in the study. Furthermore, phylogenetic relationships within the various species of *Bruguiera* were based on morphological characters. There are two groups of *Bruguiera* recognized by morphological traits, one with the large, solitary-flowered group (*B. gymnorhiza*, *B. sexangula* and *B. exaristata*), and the other with small, many-flowered group (*B. cylindrica* and *B. parviflora*) (Hou 1957, 1958). *B. hainesii*, which has large flowers in multiple-flowered inflorescences, was considered at an intermediate position between the two groups. Thus, phylogenetic and conservation statuses of *B. hainesii* remain poorly understood.

In this chapter, the author conducted molecular phylogenetic analyses for all six *Bruguiera* species including *B. hainesii* to determine the phylogenetic position of the critically endangered species and to evaluate the genetic diversity of the species for further understanding and designing a science-based conservation strategy. the author used chloroplast DNA (cpDNA) and two single-copy nuclear DNA markers to elucidate a clear evolutionary history of the species.

Materials and Methods

Plant Materials

Leaf samples collected for this study are as followings: nine individuals of *Bruguiera hainesii* from five populations in Malaysia and Singapore, 14 individuals of *B. gymnorhiza* from 12 populations in Mozambique, India, Myanmar, Malaysia, Vietnam, Philippines, Japan, Australia and Fiji, three individuals of *B. sexangula* from three populations in Myanmar, Malaysia and Vietnam, two individuals of *B. exaristata* from two populations in Australia, six individuals of *B. cylindrica* from four populations in India, Malaysia, Singapore and Philippines and one individual of *B. parviflora* from one population in Vietnam (Table 1-1). I used one individual of *Rhizophora stylosa* as an outgroup. Leaf samples were dried by 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 the CTAB extraction method (Doyle & 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

All samples were genotyped with two nuclear gene; Cellulose synthase (*Ces*A) was amplified by the primer pair of Cronn et al. (1999), and *UNK* by that of Urashi et al. (2013), respectively. To obtain improved sequencing, a new forward internal primer, *Ces*A-1150F (5'-CCACCTGAGCAGCAGATGGAAG-3'), was designed for *Ces*A according

to draft sequence results obtained using the PCR primers. The samples were also sequenced at three cpDNA regions, *trnL-trn*F, *trn*S-*trn*G and *atp*B-*rbc*L intergenic spacers (IGSs), by the primer pairs of Taberlet et al. (1991), Hamilton (1999) and Savolainen et al. (1994), respectively.

PCR amplifications were carried out with *ExTaq* polymerase (TaKaRa Bio Inc.). Total reaction volume was 10 μL of which total DNA was 0.5 μL (10-100 ng). The protocol was as follows: an initial denaturation step (95 °C for 1 min) followed by 30-35 cycles of denaturation, annealing, and elongation steps (95 °C for 45 sec, *Tm* for 45 sec, and 72 °C for 1 min) and a final elongation step (72 °C for 10 min), in which the *Tm*s (annealing temperatures) were 61 °C for *Ces*A and 58 °C for *trn*L-*trn*F and *trn*S-*trn*G IGSs. For *UNK* and *atp*B-*rbc*L IGS, a Touchdown PCR procedure was performed with a *Tm* decrease of 0.5 °C per cycle (from 55 °C to 50 °C) during the first 10 cycles. The PCR products were purified with Exo-Star kit (GE-Healthcare) and then sequenced using the BigDye Terminator cycle sequencing kit v3.1 (Applied Biosystems) with an ABI 3500 automated sequencer (Applied Biosystems). Whenever the sequencing results of nuclear gene regions exhibited double peaks at more than one site (suggesting heterozygosity), 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, I re-amplified the DNA obtained and performed direct sequencing following the method described above.

DNA sequences were aligned and manually corrected by using MEGA6 (Tamura et al. 2013) and aligned using the Clustal W algorithm (Thompson et al. 1994) included in the software. For *R. stylosa* samples, sequences of *trn*S-*trn*G IGS could not be completely determined due to poly-A site located at about 300 bp from the *trn*G gene.

Thus, only 300-bp from the *trn*G end of the sequence was used for subsequent phylogenetic analyses. The resulting nucleotide sequences were deposited in DDBJ as accessions LC076503 to LC076548 for *Ces*A, LC076391 to LC076437 for *UNK*, LC075996 to LC076031 for *trn*L-*trn*F IGS, LC076032 to LC076067 for *trn*S-*trn*G IGS and LC076068 to LC076103 for *atp*B-*rbc*L IGS.

Data analysis

I concatenated sequences of all three cpDNA regions (*trn*L-*trn*F, *trn*S-*trn*G and *atp*B-*rbc*L IGSs). Only one representative sequence of each haplotype was used for subsequent phylogenetic analyses. The nuclear gene sequences and the concatenated cpDNA sequence were analyzed separately using Bayesian Markov Chain Monte Carlo method implemented in MrBayes 3.2 (Ronquist et al. 2012) and maximum parsimony (MP) method implemented in PAUP* version 4.0b10 (Swofford 2002). For Bayesian method, nucleotide substitution model was determined for each two nuclear loci and three cpDNA regions by the Bayesian Information Criterion (BIC) method implemented in the program jModeltest2 (Darriba et al. 2012). The best fitting models were used as priors in MrBayes: HKY for nuclear *Ces*A gene and cpDNA *trn*L-*trn*F IGS, K80 for nuclear *UNK* gene and F81 for *trn*S-*trn*G and *atp*B-*rbc*L IGSs. Two independent runs with one cold and three heated Metropolis-Coupled Monte-Carlo Markov chains (MCMCMC) were conducted simultaneously for 10 million generations, in which trees were sampled every 100 generations. The first 25% of the trees were discarded as burn-in and the remaining trees were used to calculate a majority rule consensus tree. Default conditions and priors were used in all cases. Stationarity of the output parameters were examined by using the program Tracer v. 1.6 (Rambaut & Drummond

2013). For the MP method, a heuristic search was performed with 100 random addition sequence replicates involving tree-bisection-reconnection (TBR) branch swapping. Bootstrap analysis (Felsenstein 1985) was performed using 10,000 replicates with TBR branch swapping and the simple addition of sequences.

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.

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 in length, 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 1-1 and Figs. 1-1a, b).

Bruguiera hainesii did not have species-specific alleles at both *Ces*A and *UNK* genes (Table 1-1). 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 one 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 were with the allele *Ces*A03. As for *UNK* gene, the allele *UNK*3 was found only in *B. 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*.

The MP and Bayesian methods yielded mostly identical tree topologies (Fig. 1-2 for *Ces*A gene and Fig. 1-3 for *UNK* gene). When *Rhizophora stylosa* was used as an outgroup, *B. parviflora* was a sister to other four *Bruguiera* species in the tree of *Ces*A. Alleles of *B. sexangula* and *B. exaristata* were reciprocally monophyletic, respectively (Fig. 1-2). On the other hand, four alleles found in *B. gymnorhiza* were paraphyletic

even when the alleles of *B. hainesii* showing heterozygous genotype were ignored. For *UNK* gene, even though the resolution was low, monophyly of *B. cylindrica* alleles was suggested by both MP and Bayesian methods (Fig. 1-3).

Chloroplast DNA sequencing

The length of nucleotide sequences determined were 277–295 bp for *trn*L-*trn*F, 572–1180 bp for *trn*S-*trn*G and 692–744 bp for *atp*B-*rbc*L 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 1-1, Figs. 1-1c and 1-4). The two haplotypes found in *B. hainesii* (cp11 and cp12) were shared by *B. cylindrica*. No other haplotypes were shared among species.

The Bayesian tree with MP bootstrap values and Bayesian posterior probabilities are shown in Fig. 1-4. Because the MP and Bayesian methods gave similar topology, I showed on the Bayesian tree. Consistent with the result of nuclear *Ces*A gene, *B. parviflora* was found to be the sister to the clade of the other four *Bruguiera* species, within which two haplotypes found in *B. hainesii* and *B. cylindrica* (cp11 and cp12) were grouped together with strong supports (Fig. 1-4). Haplotypes found in *B. gymnorhiza* and *B. sexngula* (cp01 - cp06 for *B. gymnorhiza* and cp07 - cp09 for *B. sexangula*) also form a clade, but with weak BP bootstrap support (63%). Phylogenetic relationships among three groups, *B. gymnorhiza* - *B. sexangula* (cp01 - cp09), *B. exaristata* (cp10), and *B. hainesii* - *B. cylindrica* (cp11 - cp12), remained unresolved.

Discussion

Hybrid origin of Bruguiera hainesii

The phylogenetic analyses clearly suggest that *B. hainesii* originated through hybridization between *B. gymnorhiza* and *B. cylindrica*. There was no specific haplotype of *B. hainesii*. All nine samples of *B. hainesii* shared one nuclear haplotype with *B. cylindrica* (*Ces*A09, *UNK*6) and the other ones with *B. gymnorhiza* (*Ces*A01, *Ces*A03; *UNK*1, *UNK*3) at both loci (Table 1-1 and Figs. 1-1a, b), indicating hybrid origin of *B. hainesii*. For cpDNA, *B. hainesii* samples showed haplotypes, which were shared only with *B. cylindrica* (cp11 and cp12 in Table 1-1, Figs. 1-1c and 1-2). Since chloroplast DNA is maternally inherited in most angiosperms (Birky 1995, Mogensen 1996), *B. cylindrica* can be the putative maternal species of *B. hainesii* while *B. gymnorhiza* may serve as the paternal one. Furthermore, the distribution of the two different chloroplast haplotypes of *B. hainesii* (cp11, cp12) was equivalent to the ones of *B. cylindrica* in Malaysia and Singapore (Table 1-1), which suggests that *B. hainesii* may be formed at multiple locations where the distribution ranges of the parental species met.

The hybrid status of *Bruguiera hainesii* is also supported by the morphological features of the species. The genus *Bruguiera* consists of two groups. The group with larger leaves and larger solitary-flowered inflorescences includes *B. gymnorhiza*, *B. sexangula* and *B. exaristata*. The group with smaller leaves, smaller and multiple-flowered inflorescences of relatively small size includes *B. cylindrica* and *B. parviflora* (Tomlinson 1986, Sheue et al. 2005, Duke & Ge 2011). Although some authors placed it in the multiple-flowered group (Duke & Ge 2011), *B. hainesii* exhibits the intermediate state for these traits because it has larger flowers in multiple-flowered inflorescences (Hou 1957, 1958). Additionally, calyx lobe number of *B. hainesii* (9-11) is

also intermediate between the former and the latter groups (Tomlinson 1986, Duke & Ge 2011).

The distribution range of *B. hainesii* overlaps with both of putative parents, *B. gymnorhiza* and *B. cylindrica*. The putative parental species are known as common taxa in the Indo-West Pacific region (Tomlinson 1986, Kochummen 1989, Sheue et al. 2005). *B. gymnorhiza*, the putative paternal species, is the most widely distributed mangrove plant, with a longitudinal range covering from East Africa to Micronesia, Polynesia and Samoa, and latitudinal range from subtropical Australia to Ryuku Islands of Southern Japan (Tomlinson 1986, Allen & Duke 2006). While, putative maternal species, *B. cylindrica* is distributed from India and Sri Lanka throughout South-East Asian countries to northern Queensland in Australia (Faridah Hanum & van der Maesen 1997). Therefore, previously reported distribution range of *B. hainesii*, from Myanmar to Papua New Guinea (Tomlinson 1986), falls within the putative parental species' ranges. In mangrove forests, both putative parental species *B. gymnorhiza* and *B. cylindrica* tend to be found in downstream and intermediate zones of mid-intertidal regions (Duke et al. 2010a, b). Moreover, many instances of sympatry of the two species have been recorded (Putz & Chan 1986, White et al. 1989, Imai et al. 2006, Sun & Lo 2011). However, although the parental species sometimes coexist, *B. hainesii* is considered as the rarest mangrove species (Kochummen 1989, Sheue et al. 2005, Polidoro et al. 2010).

The infrequent occurrence of *B. hainesii* may be attributed to the different pollinators serving the two putative parental species. The two morphologically diverse groups of the genus *Bruguiera* use different pollinators along with their flower characters. The group with larger solitary-flowered inflorescences (including *B.*

gymnorhiza, *B. sexangula* and *B. exaristata*) is thought to be bird-pollinated (Tomlinson 1986, Kondo et al. 1987, Kondo et al. 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 facilitated by insects (Tomlinson 1986). Hybridization between them may not frequently occur because of the likely premating isolation measure due to the different types of pollinators.

Bruguiera hainesii may be an F1 hybrid affected by postmating isolation. *B. 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 between the two parental species. All individual samples of *B. hainesii* used in this study were heterozygous at both nuclear loci, which suggest all the samples of *B. hainesii* were F1 hybrids. It is contrasting with another hybrid species in the genus *Bruguiera*, *B.* × *rhynchopetala*, because the hybrid species has fertile seed sets, and can backcrosses with the parental species: *B. gymnorhiza* or *B. sexangula* (Sun & Lo 2011). These characteristics can be attributed to less reproductive isolation because both parental species are sister species weakly suggested by cpDNA phylogeny (Fig. 1-4), in the group with larger solitary-flowered inflorescences, and both may use birds as pollinators (Tomlinson 1986, Duke & Ge 2011).

Phylogenetic relationships among the genus Bruguiera

This study provided the most comprehensive phylogenetic relationship of genus *Bruguiera* to date. According to Tomlinson (1986), species of *Bruguiera* were morphologically divided into two groups as mentioned above. In contrast, molecular data of cpDNA and nuclear ribosomal DNA from Schwarzbach & Ricklefs (2000)

suggested that a small, many-flowered species *B. cylindrica* form a monophyletic group with three species belonging to a different morphological group. The resultant haplotype networks of nuclear *Ces*A gene and combined cpDNA haplotypes also supported the monophyly of *B. cylindrica* and the three large, solitary-flowered species (Figs. 1-1a, c and 1-2).

Unfortunately, however, phylogenetic relationship among *B. cylindrica*, *B. gymnorhiza*, *B. sexangula* and *B. exaristata* remains uncertain. Morphological similarity between *B. gymnorhiza* and *B. sexangula* has been well-documented (Tomlinson 1986, Allen & Duke 2006, Duke & Ge 2011). In the cpDNA tree (Fig. 1-2), the sister relationship of *B. gymnorhiza* and *B. sexangula* were weakly supported: 0.67 for MP bootstrap support and 0.99 for Bayesian posterior probability. As for nuclear CesA gene (Figs. 1-1a and 1-3), *B. gymnorhiza* alleles were highly variable and paraphyletic to the alleles found in *B. cylindrica*, *B. sexangula* and *B. exaristata*. The non-monophyly of *B. gymnorhiza* alleles could be due to incomplete lineage sorting or interspecific introgressive hybridization (Syring et al. 2007). Therefore, phylogenetic relationships between *B. gymnorhiza* and others cannot be determined by using this locus. The sequences determined for *UNK* gene were relatively short, and species relationship was not resolved due to low phylogenetic information (Figs. 1-1b and 1-4).

Conservation implication of Bruguiera hainesii

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. 2010c) should be re-considered. This study indicated that *B. hainesii* originated through 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). Further studies to determine the conservation status of *B. hainesii* are needed.

Apart from the delisting of *B. hainesii* from IUCN Red List, should we protect this putative hybrid from the perspective of mangrove conservation? The issue whether hybrids merit protection in conservation strategies is still controversial (Ellstrand et al. 2010). For example, hybridization with or without introgression may threaten a rare species' existence (Rhymer & Simberloff 1996). On the other hand, it is well understood that hybridization has been of importance for adaptation and speciation, especially in plants (Arnold 1992). As for the case of *B. hainesii* individuals, they may be F1 generation hybrids, so far examined. This type of hybridization would be detrimental because hybridization leads to wasted reproductive effort of parental species (Allendorf et al. 2001). “Fortunately”, *B. hainesii* is very “rare”, and thus, its detrimental effect would be negligible. It can be recommended that any conservation effort should not be paid to solitary *B. hainesii* individuals if this species is merely a hybrid.

Tables and Figures

Table 1-1. List of species, sampling localities, sample IDs and haplotypes.

Taxon	Country	Locality	Latitude	Longitude	Sample ID	*Ces* A haplotypes	*UNK* haplotypes	cpDNA haplotypes
Bruguiera hainesii	Malaysia	Merbok	5.6549	100.3719	BhMYS1	03/09	1/6	11
			5.6549	100.3719	BhMYS2	03/09	1/6	11
			5.6549	100.3719	BhMYS3	03/09	1/6	11
		Klang	2.9729	101.3617	BhMYS4	01/09	1/6	11
			2.9128	101.3124	BhMYS5	03/09	1/6	11
		Pulau Kukup	1.3264	103.4331	BhMYS6	01/09	3/6	11
			1.3233	103.4299	BhMYS7	01/09	3/6	11
	Singapore	Sungai Loyang	1.3810	103.9660	BhSGP1	01/09	1/6	12
		Pulau Ubin	1.4182	103.9636	BhSGP2	01/09	1/6	12
B. gymnorhiza	Mozambique	Maputo	-25.8501	32.6957	BgMOZ1	01	1	01
	India	Kerala	9.9860	76.2320	BgIND1	01	1	02
	Myanmar	Byonmwe I.	15.9760	95.2674	BgMMR1	02	1	03
	Malaysia	Merbok	5.6549	100.3719	BgMYS1	01	1	03
			5.6549	100.3719	BgMYS2	03	1	03
		Klang	2.9729	101.3617	BgMYS3	01/03	1	03
			2.9729	101.3617	BgMYS4	01	1	03
		Sabah	5.9389	118.0529	BgMYS5	01	2	01
	Vietnam	Dong Rui	21.2478	107.3905	BgVNM1	01	3	01
	Philippines	Panay I.	11.8126	122.1418	BgPHL1	01	3	04
	Japan	Iriomote I.	24.3134	123.9064	BgJPN1	01	3	01
		Ishigaki I.	24.4005	124.1454	BgJPN2	01	3	05
	Australia	Cairns	-29.4437	153.2087	BgAUS1	04	1	01
	Fiji	Viti Levu I.	-18.1560	178.4459	BgFJI1	04	1/4	06
B. sexangula	Myanmar	Byonmwe I.	15.9760	95.2674	BsMMR1	05	2	07
	Malaysia	Sabah	5.8536	116.0423	BsMYS1	06	2	08
	Vietnam	Ca Mau	8.6138	104.7322	BsVNM1	06	2	09
B. exaristata	Australia	Northern Territory	-12.4084	130.8324	BeAUS1	07	5	10
		Queensland	-16.2791	145.4394	BeAUS2	08	5	10
B. cylindrica	India	Mumbai	19.3301	72.8147	BcIND1	09	6	11
			19.3301	72.8147	BcIND2	09	6	11
	Malaysia	Klang	2.9128	101.3124	BcMYS1	09	6	11
	Singapore	Sungai Loyang	1.3810	103.9660	BcSGP1	09	6/7	12
	Philippines	Luzon I.	13.9700	120.6259	BcPHL1	09	6	11
			13.9700	120.6259	BcPHL2	09	6	11
B. parviflora	Vietnam	Ca Mau	8.6138	104.7322	BpVNM1	10	8	13
Rhizophora stylosa	Japan	Iriomote I.	24.3946	123.8222	RsJPN1	11	9	14

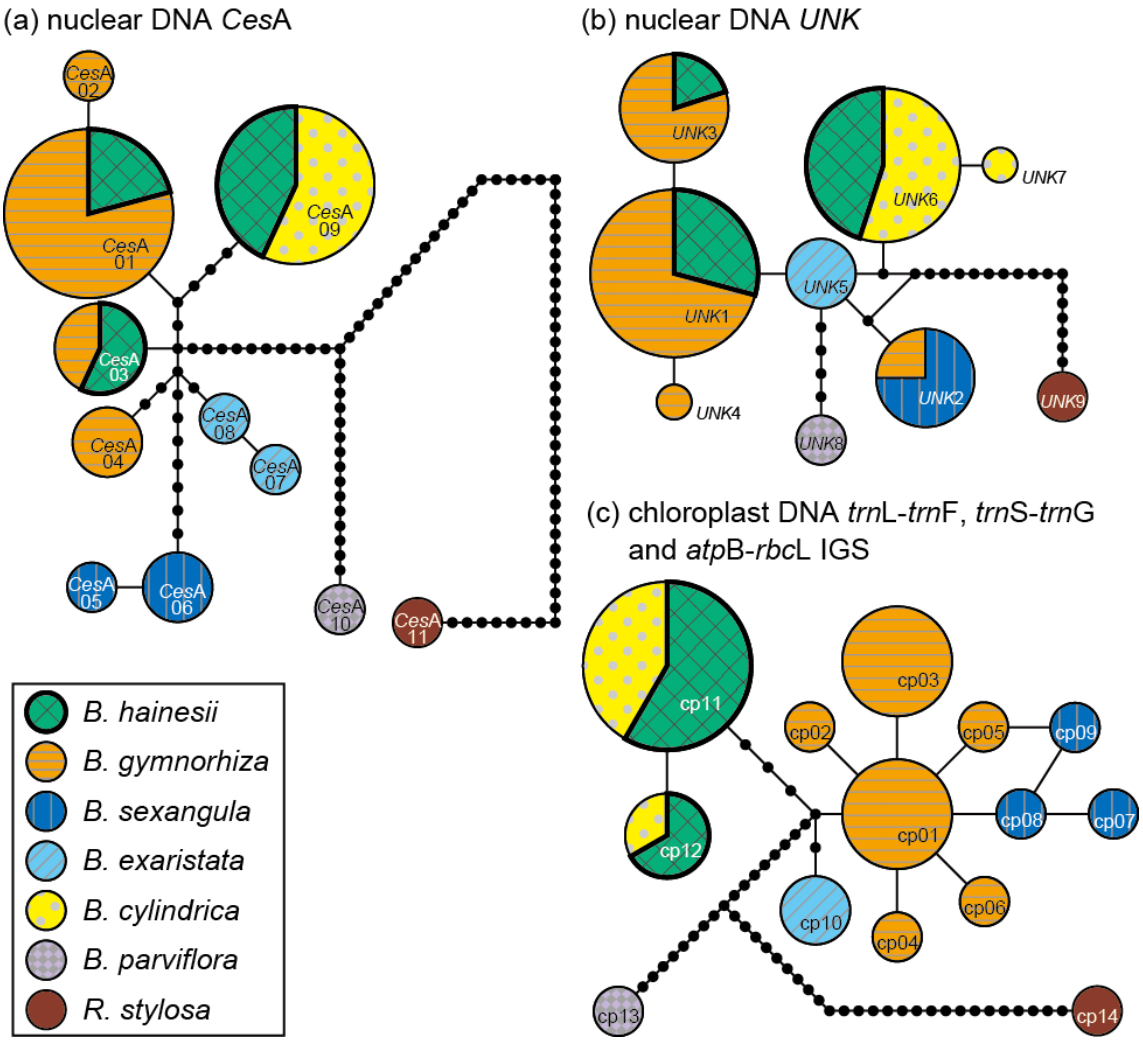

Fig. 1-1. Haplotype networks. (a) Nuclear DNA *Ces*A gene. (b) Nuclear DNA *UNK* gene. (c) Combined regions of chloroplast DNA *trn*L-*trn*F, *trn*S-*trn*G and *atp*B-*rbc*L intergenic spacers (IGSs). Each species is shown as distinct color and pattern, *Bruguiera hainesii*: green with grid lines, *B. 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).

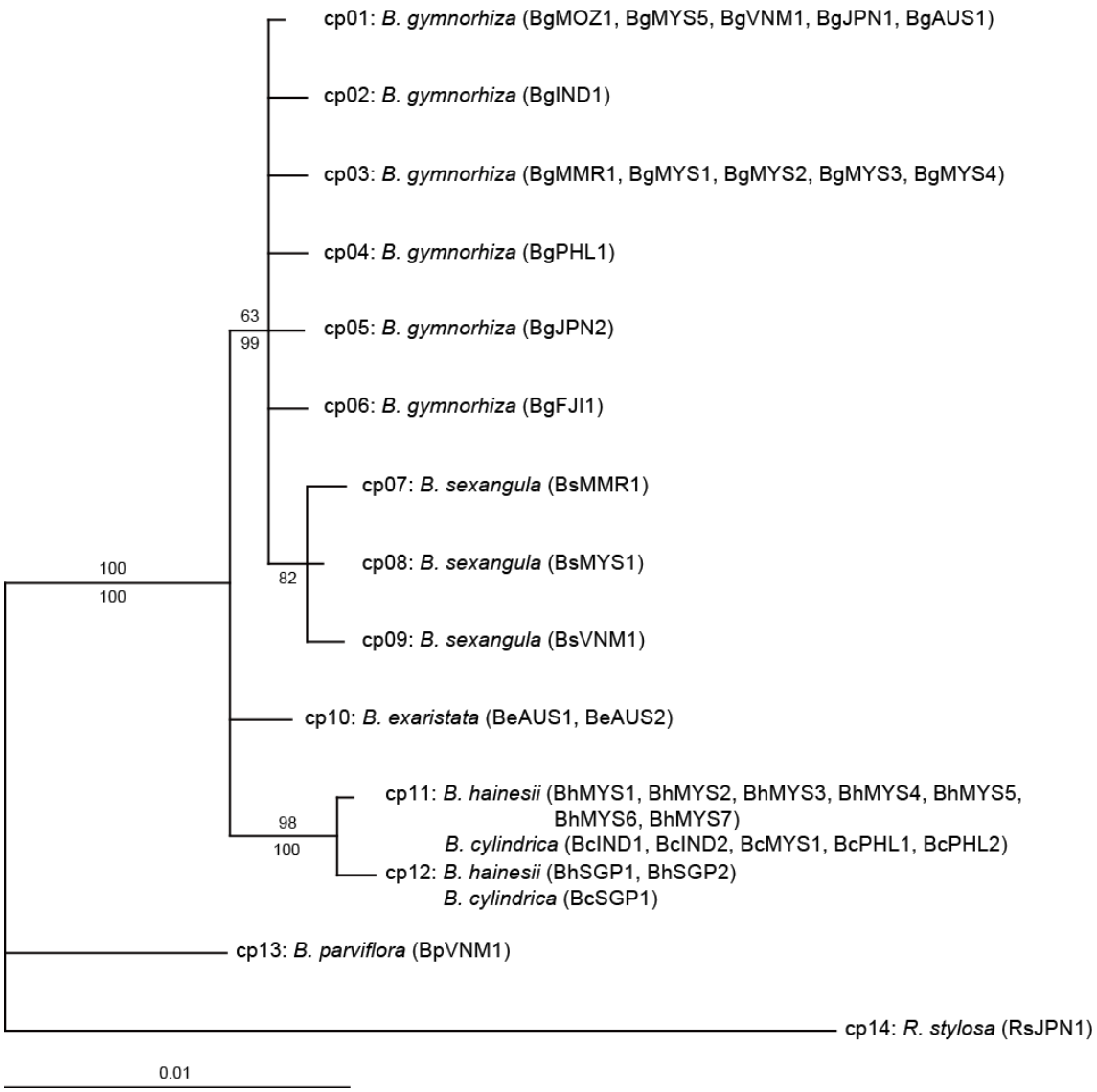

Fig. 1-2. A Bayesian phylogenetic tree of combined chloroplast regions of chloroplast DNA *trn*L-*trn*F, *trn*S-*trn*G and *atp*B-*rbc*L IGSs. Maximum parsimony bootstrap supports were shown above and Bayesian posterior probabilities were below. Sample IDs are as in Table 1-1.

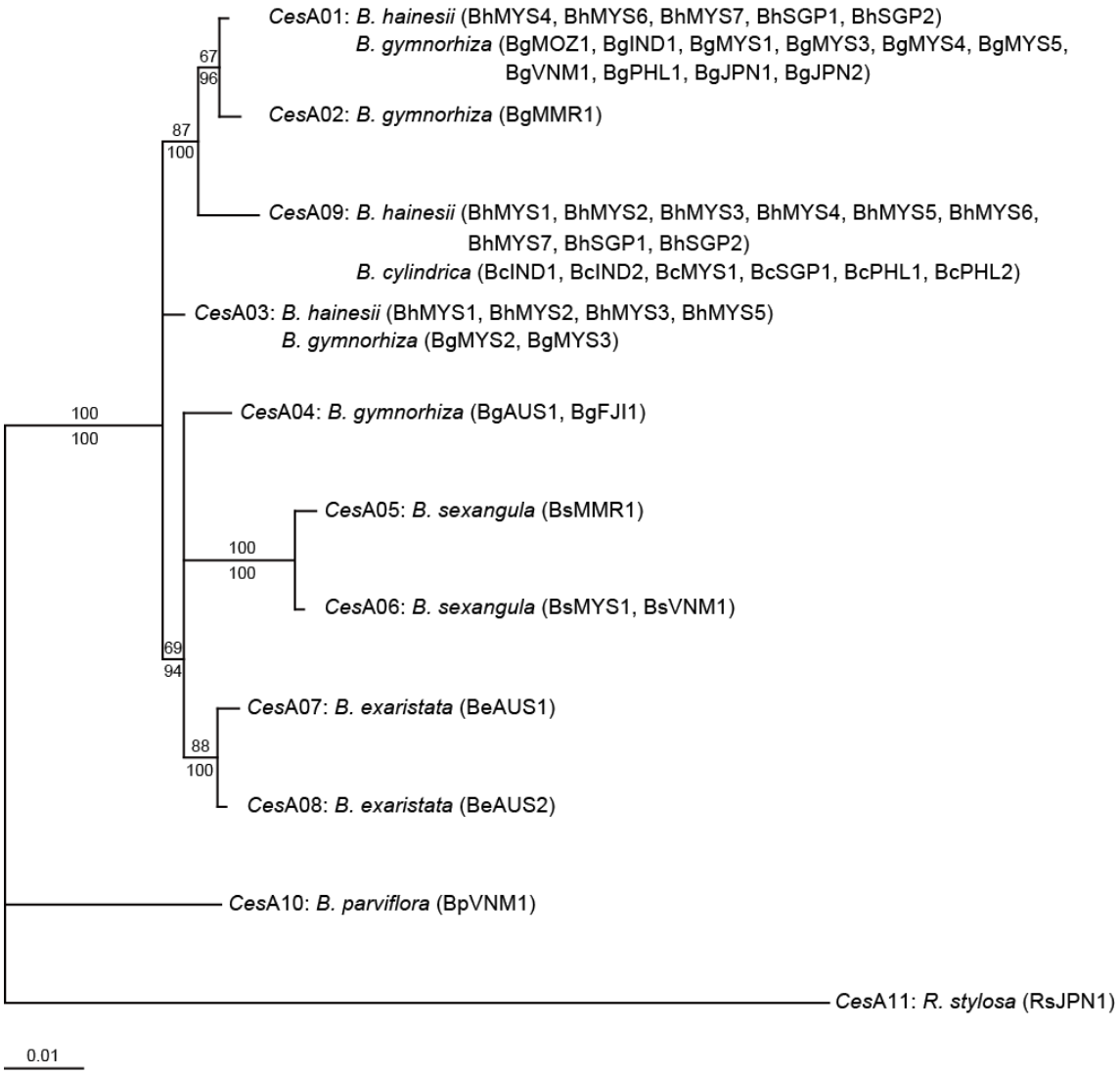

Fig. 1-3. A Bayesian phylogenetic tree of nuclear DNA *Ces*A gene. Maximum parsimony bootstrap supports were shown above and Bayesian posterior probabilities were below. Sample IDs are as in Table 1-1.

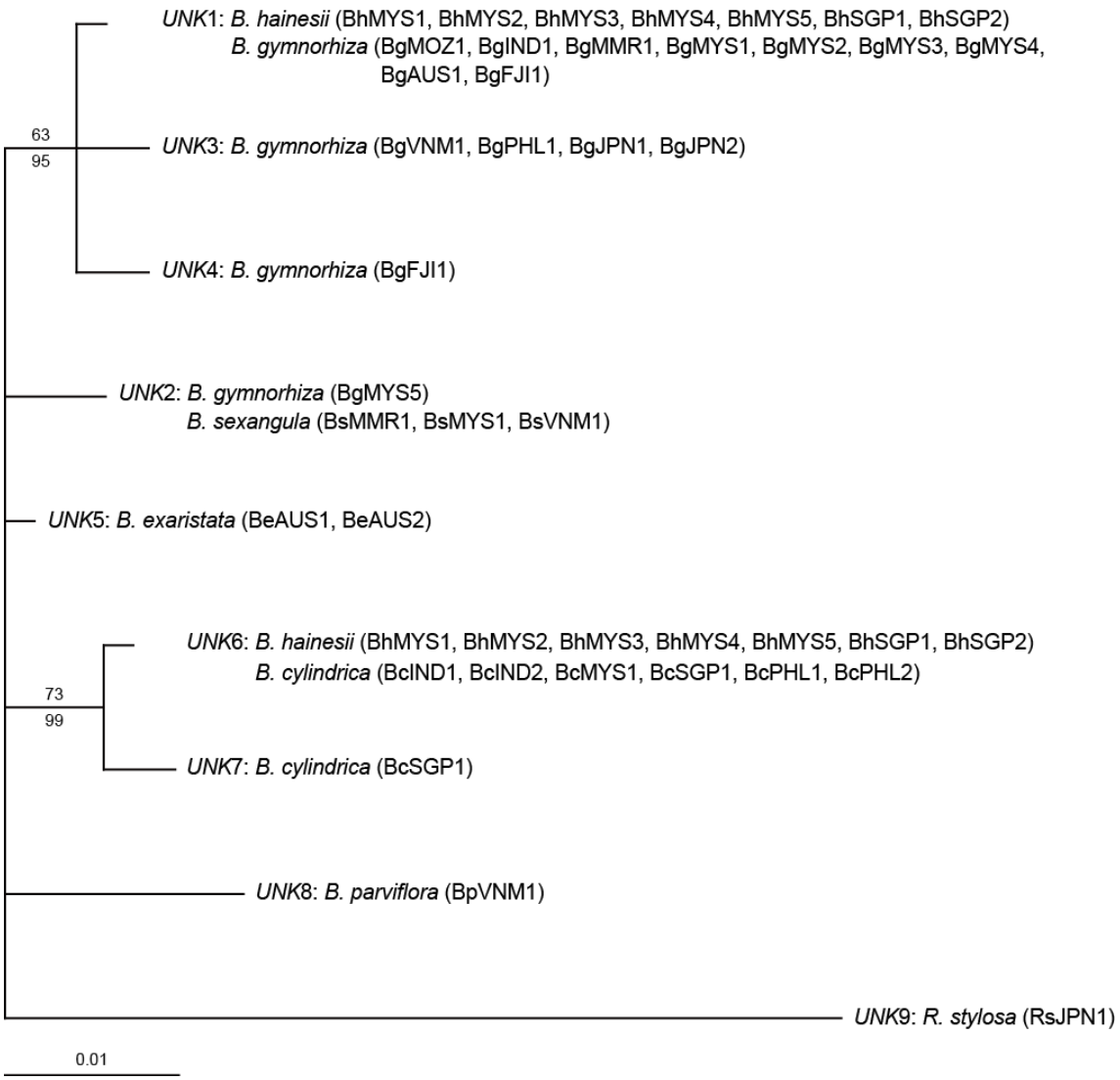

Fig. 1-4. A Bayesian phylogenetic tree of nuclear DNA *UNK* gene. Maximum parsimony bootstrap supports were shown above and Bayesian posterior probabilities were below. Sample IDs are as in Table 1.

CHAPTER 2: GLOBAL GENETIC STRUCTURE OF *BRUGUIERA GYMNORHIZA*

Introduction

Conservation Units (CUs), which are population units identified within species, are essential to help guide management and conservation effort to protect certain populations within species. CUs are populations that are considered as distinct units to be conserved, including Evolutionarily Significant Units (ESUs) and Management Units (MUs). An ESU in conservation genetics is a genetically differentiated unit composed of populations that warrant management as a separate unit (Frankham et al. 2010). Although several different definitions of ESU have been proposed (Ryder 1986, Waples 1991, Dizon et al. 1992, Moritz 1994, Avise 1994, Vogler & Desalle 1994, Crandall et al. 2000, Fraser & Bernatchez 2001), the major definition of ESU is based on its genetic and ecological distinctiveness (Funk et al. 2012). Identification and maintenance of ESUs are important because conservation of ESUs can maximize the potential to adapt to environmental changes (Funk et al. 2012). On the other hand, an MU is a unit defined as populations that are demographically independent and characterized as the significant divergence of alleles (Moritz 1994, Palsbøll et al. 2007). MUs are mostly smaller than ESUs and, therefore, several MUs may be within an ESU (Hanski & Gilpin 1997). Identifying CUs, regarding ESUs and MUs, is an important first step for conservation because understanding the boundary of the target population units is necessary for managements and for making conservation policies (Funk et al. 2012).

Studying the genetic structure of a species is essential to identify CUs. Neutral loci are suitable to estimate the levels of gene flow among populations and, therefore, can detect demographically isolated population units as MUs (Moritz 1994) or genetically differentiated units as ESUs (Frankham et al. 2010). Although recent studies focused more on adaptive differences for the recognition of ESUs, both neutral and adaptive loci should be still used for delineating CUs (Funk et al. 2012). Neutral loci solely also give important implication for adaptation because gene flow does not restrict adaptive divergence if migration is low (Slatkin 1985).

For mangroves, CUs have been recognized extensively as genetically differentiated populations in the AEP region. Clear genetic structures across the American Continents, which was discussed regarding genetic barrier formed by the closure of the Panama Isthmus, have been found in *Rhizophora* mangle, *R. racemosa* (Takayama et al. 2013) and *Avicennia germinans* (Nettel & Dodd 2007). In Brazil, genetic discontinuity has also been known between northern and southern populations of *R. mangle* (Pil et al. 2011), *A. germinans* and *A. schaueriana* (Mori et al. 2015b) which may be caused by the South Equatorial Current that acted as a genetic barrier. In these studies, *R. mangle* and *A. germinans* were surveyed as model species to discuss CUs of mangroves in the AEP and wide range sampling was performed, because both of them are widely distributed species across the AEP (Tomlinson 1986).

Although there are higher species diversity of mangrove plants in the IWP than the AEP region (Tomlinson 1986, Polidoro et al. 2010), studies on genetic structures for widely distributed mangrove species using broad sampling scheme were still limited. In a similar fashion to the Panama Isthmus in the AEP region, the Malay Peninsula has been recognized as a significant land barrier to several mangrove species in the IWP

region. Some studies on the species distributed across the peninsula have been conducted, e. g. *Ceriops tagal* (Liao et al. 2007), *Rhizophora apiculata* (Inomata et al. 2009, Ng et al. 2015), *C. zippeliana* and *C. decandra* (Sheue et al. 2009), *Bruguiera gymnorhiza* (Minobe et al. 2010, Urashi et al. 2013), and *R. mucronata* (Wee et al. 2015). In contrast to these studies, Wee et al. (2015) revealed the genetic structure of a widespread mangrove species within an oceanic region. They suggested that the populations of *R. stylosa* were genetically divided into two clusters, South and East China Sea, and the Southwest Pacific Ocean. Similarly, populations of *Lumnitzera racemosa* showed deep splits among the East Indian Ocean, the South China Sea, and the North Australia (Su et al. 2006). These results indicate that there are genetic discontinuities other than the Malay Peninsula in the IWP.

To understand the genetic structure of the widespread mangrove species in the IWP region as a whole, comprehensive sampling scheme is essential to detect genetic cohesion of the species that distributes across the IWP with broad sampling scheme. *Bruguiera gymnorhiza* (L.) Lam. is a suitable model species to understand the genetic diversity of widespread mangrove species in the IWP region. *B. gymnorhiza* has the broadest distribution range among all mangrove species in the IWP. The distribution range is from East Africa to Micronesia and Polynesia (Samoa) longitudinally and from subtropical Australia to Ryuku Islands of Southern Japan latitudinally. Viviparous seedling, so called propagule, of this species that has a cylindrically elongated hypocotyl, is buoyant (Tomplinson 1986, Allen & Duke 2006), and hence, can be dispersed in the ocean.

Previous phylogeographic analysis on *B. gymnorhiza* revealed distinct genetic structure over the Malay Peninsula (Minobe et al. 2010, Urashi et al. 2013). In Minobe

et al. (2010) using a limited number of population samples from both sides of the Malay Peninsula reported clear genetic differentiation across the peninsula using cpDNA and nucDNA markers. A recent study also confirmed higher levels of genetic differentiation among populations across the peninsular using numbers of nuclear loci (Urashi et al. 2013). Contrary to other sea-dispersal species such as *Hibiscus tiliaceus* (Takayama et al. 2006, Takayama et al. 2008) and *Ipomoea pes-caprae* (Miryeganeh et al. 2014), these results indicated restricted gene flow across the Malay Peninsula. No clear suggestion of genetic structure within oceanic regions has been suggested to date for *B. gymnorhiza* because of fewer population samples across the whole distribution range of the species.

In this chapter, the author studied the genetic structure of *B. gymnorhiza* to detect genetic cohesion and discontinuities and to determine CUs within the wide distribution range. The author conducted detail sampling covering entire the distribution range and genetic analysis by using eight nuclear microsatellite markers and chloroplast *trn*L-*trn*F and *trn*S-*trn*G IGS regions. Another species, *Bruguiera sexangula* (Lour.) Pour., was also included in the study. The species is morphologically similar to *B. gymnorhiza*, and these two species may produce a hybrid species *Bruguiera* × *rhynchopetala* (Ko) X. J. Ge et N. C. Duke (Ge 2001). Performing analyses including the two species, we confirmed that hybridization and the following introgression may not have caused complicated genetic structure in *B. gymnorhiza*.

Materials and Methods

Plant materials

I collected leaf samples from 1020 individuals of *B. gymnorhiza* from 40 populations across the IWP; 20 populations in the Indian Ocean and 20 populations in the Pacific Ocean (Table 2-1). *B. sexangula* samples were collected from 119 individuals in six populations; two in the Indian Ocean and four from the Pacific Ocean. Inflorescence characters used in previous publications were applied for species identification (Tomlinson 1986, Sheue et al. 2005). When either *B. gymnorhiza* or *B. sexangula* was the only species existing in populations, several DNA vouchers without inflorescences were collected. At sites where the two species were present, only DNA vouchers with inflorescences were collected. Except for two populations in Philippines, 33 samples which had intermediate inflorescence characters between the two species or did not have inflorescence were treated as *B.* sp. Collected leaf samples were put in Ziploc plastic bags and dried with silica gel.

DNA extraction

Total DNA was extracted from dried leaf material using CTAB extraction method (Doyle & Doyle 1987). Further purification was conducted on some specimens by using a GENECLEAN III Kit (MP Biomedicals).

Microsatellite analyses

Eight microsatellite markers developed by Takayama et al. (2011) were chosen for analyses based upon the polymorphism level. Primers for these loci were multiplexed by grouping them into sets of three: (a) BG118, BG147, BG165; (b) BG129, BG162; (c)

BG114, BG140, BG146. PCR amplifications were conducted by using the Qiagen Multiplex PCR Kit (Qiagen) according to the manufacturer's protocol with the annealing temperature at 57 °C in a final volume of 4 μL. The amplified DNA samples and GeneScan 600 LIZ size standards (Applied Biosystems) were electrophoresed using ABI 3500 automated sequencer (Applied Biosystems), ABI 3130xl Genetic Analyzer (Applied Biosystems) and ABI 3730xl Genetic Analyzer (Applied Biosystems) using standard settings. The program GENEMAPPER 4.1 (Applied Biosystems) was used to assign fragment length.

Chloroplast DNA amplification and sequencing

PCR amplifications were conducted for representative samples from each population using universal cpDNA primer pairs *trn*L-*trn*F IGS (Taberlet et al. 1991) and the *trn*S-*trn*G IGS (Hamilton 1999) with TaKaRa Ex Taq polymerase (TaKaRa Bio Inc.). A partial sequence of *trn*S-*trn*G IGS was used for all samples from 27_Philippines (*B. gymnorhiza*), 46_Philippines (*B. sexangula*), 47_Philippines (*B.* sp) and 48_Philippines (*B.* sp) to confirm the species identity validation and check out the intermediate characteristics of *B.* sp since *trn*S-*trn*G IGS region contains a mutation site which can identify *B. gymnorhiza* from *B. sexangula* (Zhou et al. 2008). Total reaction volume was 10 μL of which total DNA was 0.5 μL (10-100 ng). The protocol was as follows: an initial denaturation step (95 °C for 1 min) followed by 30-35 cycles of denaturation, annealing, and elongation steps (95 °C for 45 sec, 58 °C for 45 sec, and 72 °C for 1 min) and a final elongation step (72 °C for 10 min). The PCR products were purified with Exo-Star kit (GE-Healthcare) and electrophoresed using ABI 3500 automated sequencer (Applied Biosystems), ABI 3130xl Genetic Analyzer (Applied Biosystems) and ABI 3730xl

Genetic Analyzer (Applied Biosystems) using standard settings.

Data analyses

For nuclear microsatellite analysis, linkage disequilibrium of all pairs of microsatellite loci in each population was analyzed with 26320 permutations in FSTAT 2.9.3.2 (Goudet 2001). The software was also used to estimate allelic richness, expected heterozygosity (H_E), observed heterozygosity (H_O) and the inbreeding coefficient (F_{IS}) with significant departure from zero at $P < 0.05$. Null allele frequencies of each locus and population were estimated with FREENA (Chapuis & Estoup 2007), using the expectation maximization algorithm of Dempster et al. (1977).

To infer the genetic clusters of populations, I implemented the Bayesian clustering method, STRUCTURE ver. 2.3.3 (Pritchard et al. 2000). I tested cluster numbers (K) from one to 10. To confirm the convergence of Markov chain Monte Carlo (MCMC) chains, 20 independent runs were performed for each number of clusters under admixture model. 100,000 MCMC interactions were performed after burn-in period of 200,000 interactions for each run. I applied ΔK proposed by Evanno et al. (2005) to estimate the most probable number of clusters implemented in STRUCTURE HARVESTER (Earl & von Holdt 2012). I applied four sample sets for this analysis; (a) using all individuals of both *B. gymnorhiza* and *B. sexangula* to confirm species identity validation, (b) using populations of 1_South Africa–6_France (*B. gymnorhiza*), 41_Myanmar–46_Philippines (*B. sexangula*), and 47_Philippines–48_Philippines (*B.* sp) to test the species identities following the results of above sample set, (c) using populations of 27_Philippines (*B. gymnorhiza*), 46_Philippines (*B. sexangula*), 47_Philippines (*B.* sp), 48_Philippines (*B.* sp), 26_Malaysia (*B. gymnorhiza*) and

45_Malaysia (*B. sexangula*) to detect intermediate status of *B.* sp, in which 26_Malaysia is a typical population of *B. gymnorhiza* near populations where *B.* sp were taken and 45_Malaysia for *B. sexangula*, (d) using all individuals of *B. gymnorhiza* to clarify the genetic structure of the species. A neighbor-joining (NJ) tree was generated to estimate the genetic relationships among populations based on the genetic distance D_A (Nei et al. 1983) using the program Populations 1.2.30 (Langella 1999). Populations which containing individuals were less than 10 were excluded from this analysis (Takayama *et al.* 2013). I estimated the significance of the best topology with 1000 bootstrap replicates. The pattern of individual-based genetic differentiation was visualized via the Principal Coordinate Analysis (PCoA). The PCoA was performed using the Microsoft Excel macro program GenAlEx (Peakall & Smouse 2006) based on the mean genotypic distance between all individual pairs of both species. *F*-statistics of the pair wise θ (Weir & Cockerham 1984) between populations was estimated using the program FSTAT 2.9.3.2 (Goudet 2001). To confirm the significance of the correlation between pair wise $\theta/(1-\theta)$ estimates and log-transformed geographic distances between paired populations (Rousset 1997), a Mantel test (Mantel 1967) was performed with 9999 random permutations using GenAlEx (Peakall & Smouse 2006).

The cpDNA sequences were aligned and manually corrected using MEGA6 (Tamura et al. 2013). Gaps or indels were treated as fifth state mutations. Length polymorphisms in mononucleotide polyT repeat units found in *trn*L-*trn*F IGS were assigned numbers and treated as mutations. To visualize the relationships among haplotypes, a statistical parsimony network was constructed using TCS v1.21 (Clement et al. 2000).

Results

Microsatellite analyses

The genetic diversity parameters for eight microsatellite loci calculated for each species and each population are shown in Table 2-2. The highest allelic richness value (3.29) was shown in 48_Philippines in *B. sexangula*. In *B. gymnorhiza*, allelic richness value was high in 37_Australia (3.06), 16_Malaysia (2.92), and 20_Indonesia (2.92). Test of linkage disequilibrium showed no significant association between locus pairs ($P < 0.05$). The presence of null alleles (defined as null allele frequency > 0.10) was detected at one to five loci in several populations (Table 2-3).

The highest ΔK value was returned at K = 2 by the analysis using all populations of both *B. gymnorhiza* and *B. sexangula* implemented by STRUCTURE (Fig. 2-1a). Two species were divided into different clusters except for African populations of *B. gymnorhiza*, 1_South Africa, 2_Mozambique, and 3_Mozambique showed the same character as *B. sexangula* and 4_Tanzania, 5_France, and 6_France showed admixed clusters of both species (Fig. 2-2). The second highest ΔK value was returned at K = 5 (Fig. 2-1a), in which two species were almost completely divided into different clusters (Fig. 2-2). In *B. gymnorhiza*, component clusters were different not only over the Malay Peninsula but also within each side of the peninsula, in which populations were separated into the Indian Ocean (Africa) (1_South Africa–6_France), the Indian Ocean (Asia) (7_India–20_Indonesia), the North Pacific Ocean (21_Malaysia–36_Japan), and the South Pacific Ocean (37_Australia–40_Samoa). All results at K > 2 showed that *B. sexangula* had different character(s) from *B. gymnorhiza*. Most individuals of *B.* sp showed the same character as *B. sexangula*, however, a few samples showed admixed characters among clusters at K = 2 to 10.

Cluster number K = 2 was strongly indicated by ΔK values using populations of African *B. gymnorhiza* (1_South Africa–6_France), *B. sexangula* (41_Myanmar–46_Philippines), and *B.* sp (47_Philippines–48_Philippines) (Fig. 2-1b), in which *B. gymnorhiza* populations were almost completely divided into a different cluster from *B. sexangula* and *B.* sp (Fig. 2-3). Only one sample of *B.* sp showed the same characters as *B. gymnorhiza*. In a similar fashion to the results using all populations in Fig. 2-2, a few samples showed admixed characters of both species.

Cluster number K = 2 was strongly indicated by ΔK values using populations of 26_Malaysia, 27_Philippines, 45_Malaysia, 46_Philippines, 47_Philippines and 48_Philippines to investigate intermediate status of *B.* sp (Fig. 2-1c), in which *B. gymnorhiza* populations (26_Malaysia and 27_Philippines) and *B. sexangula* populations (45_Malaysia and 46_Philippines) were almost completely divided into different clusters (Fig. 2-4). Most individuals of *B.* sp (47_Philippines and 48_Philippines) showed the same characters as *B. sexangula* in both STRUCTURE analysis and cpDNA sequences. Only one sample of *B.* sp showed the same characters as *B. gymnorhiza* in both results. Two individuals of *B.* sp showed admixed characters between the two species, in which more than 80 percent of the cluster was occupied by the character of *B. gymnorhiza* in STRUCTURE analysis and cpDNA sequence showed the character of *B. sexangula*. According to these results, I treated the one individual of *B.* sp as a sample of *B. gymnorhiza* in 27_Philippines and other *B.* sp samples as *B. sexangula*, therefore I combined 46_Philippines with 47_Philippines.

The highest ΔK value was returned at K = 4 using only *B. gymnorhiza* populations; 1_South Africa–40_Samoa (Fig. 2-1d), in which geographic pattern similar to the result using both *B. gymnorhiza* and *B. sexangula* at K = 5 written above was obtained (Fig.

2-5). All results at K > 4 had the same genetic discontinuities as shown at K = 4.

The NJ tree result showed that *B. sexangula* populations composed one different cluster from *B. gymnorhiza* populations (Fig. 2-6). *B. gymnorhiza* populations composed distinct clusters along with geographical cohesion, in which *B. gymnorhiza* populations were divided into four clusters; the Indian Ocean (Africa) (2_Mozambique – 6_France), the Indian Ocean (Asia) (7_India – 20_Indonesia), the North Pacific Ocean (21_Malaysia – 36_ Japan) and the South Pacific Ocean (37_Australia – 40_Samoa).

The PCoA result described a clear genetic differentiation between *B. gymnorhiza* and *B. sexangula*, except for two samples of *B. sexangula* in Philippines which showed admixed characters of both species in STRUCTURE analyses written above (Fig. 2-7).

Results of Mantel test significantly supported positive correlation between genetic distance and geographic distance among populations within both the Indian Ocean and the Pacific Ocean (Fig. 2-8a). I divided *B. gymnorhiza* populations into four geographic groups according to the STRUCTURE result and conducted Mantel test again. Positive correlation between genetic and geographic distance was significantly supported in three groups (the Indian Ocean (Asia), the North Pacific and the South Pacific), significance was not supported only in the Indian Ocean (Africa) but positive straight line was shown on the graph (Fig. 2-8b).

Chloroplast DNA analyses

12 haplotypes were produced from two cpDNA regions including indel information of *B. gymnorhiza* and *B. sexangula* across the broad IWP range. The haplotype network was shown in Fig. 2-9. No haplotypes were shared between the two species. The geographical distribution and composition of haplotypes of *B. gymnorhiza* was plotted in

Fig. 2-10 and of *B. sexangula* was in Fig. 2-11. In *B. gymnorhiza*, haplotype 1 – 3 were found within the Indian Ocean. Haplotype 6 – 9 were found within the Pacific Ocean. Haplotype 4 and 5 were found mostly within the Pacific Ocean and some were among border parts of two oceanic regions. In all African populations (1_South Africa – 6_France), observed haplotype was only haplotype 1. In populations in the South Pacific Ocean (38_New Caledonia – 40_Samoa), only haplotype 9 was observed and it was not found in other populations. In similar fashions, only haplotype 2 was found in 7_India, haplotype 7 was found in 27_Philippines, haplotype 8 was found in 31_Australia and these haplotypes were not found in other populations.

Discussion

Population genetic structure of B. gymnorhiza

Clear genetic structures of *B. gymnorhiza* were observed not only across the Malay Peninsula but also within each of the Indian and the Pacific Oceans. The STRUCTURE result of *B. gymnorhiza* based on nuclear SSR markers suggested the genetic discontinuities, in which four geographic groups were recognized; the Indian Ocean (Africa), the Indian Ocean (Asia), the North Pacific Ocean and the South Pacific Ocean (K = 4, Fig. 2-5). Since the clustering patterns were geographically congruent with the distribution of cpDNA haplotypes (Fig. 2-10), the existence of genetic structure within each the Indian Ocean and the Pacific Ocean was supported.

Genetic discontinuity of *B. gymnorhiza* across the Malay Peninsula is concordant with previous findings (Minobe et al. 2010, Urashi et al. 2013). In the Last Glacial Maximum (LGM), sea-level was 100 – 120m below present and the Malay Peninsula, Sumatra, Java, and Borneo was connected as a single land mass (Voris 2000), separating the Indian and the Pacific Oceans. The vicariance may have resulted in different genotypes between both coasts of the Malay Peninsula (Triest 2008). The observation of two genetic clusters across the Malay Peninsula indicates that it is an effective barrier to propagule dispersal of *B. gymnorhiza*, and the effects of the previous vicariance were still detectable in the present day.

Admixed DNA components from east and west of the Malay Peninsula were found in several populations. Admixtures of clusters in STRUCTURE were detected in 16_Malaysia, 17_Singapore, and 20_Indonesia (Fig. 2-5). Results of cpDNA analysis showed a similar pattern in four populations; 14_Malaysia, 17 Singapore, 19_Indonesia, and 20_Indonesia had haplotypes from east and west of the peninsula (Fig. 2-10). These

populations face both the Pacific and the Indian Ocean. Therefore, gene flow among populations on both sides of the Malay Peninsula can happen. The results of this study showed that even though the Malay Peninsula has been an effective barrier from the past to present, gene flow might currently have occurred among boundary populations between two oceanic regions.

Within each the Pacific and the Indian Ocean, cause for restricted propagule dispersal may differ across oceanic regions. In the Pacific Ocean, the Sahul Shelf, located between New Guinea and Australia, may be a land barrier to propagule dispersal of *B. gymnorhiza* during the LGM (Voris 2000). Similarly, genetic discontinuities resulting from this land barrier were also found in various marine animals, including barramundi (*Latescalcarifer*; Chenoweth et al. 1998), green turtle (*Cheloniamydas*; Dethmers et al. 2006) and invertebrates (Benzie 1999). Although most of the Sahul Shelf is submerged at present, ocean current might have acted as barriers to gene flow for *B. gymnorhiza*. Analysis of ocean circulation (Stammer et al. 2002) showed that an upper part of ocean current (at 27.5m depth) goes from Southeast Asia to the west of Australian continent. Therefore, the current does not flow into the South Pacific Ocean. Also, around Australia-New Guinea, ocean currents flow westward across the Torres Strait and are either interrupted by other current from Southeast Asia to northwestern Australia or flow eastward via northern New Guinea. Therefore, these currents do not flow into the Southeast Asia. Such ocean currents may inhibit dispersal of *B. gymnorhiza* and contribute the present-day genetic discontinuity.

On the other hand, within the Indian Ocean, the genetic structure between Africa and Asia, without an apparent land barrier was revealed. One explanation could simply be the vastness of the Indian Ocean, which acts as a barrier to dispersal in *B.*

gymnorhiza. Long-distance propagule dispersal of *B. gymnorhiza* might not occur between populations at both longitudinal ends of the Indian Ocean. In this study, cpDNA result showed only one haplotype in Africa. On the other hand, many haplotypes including African one were found in the west side of Sunda Islands. Given two regions have a source-sink relationship, ancestors of African population may have emigrated from Southeast Asian populations, via major ocean currents from western Java Island to the African continent (Stammer et al. 2002). The source-sink relationship between Asia and African were also used to explain the higher species richness of mangrove species in Southeast Asia as compared to Africa (Tomlinson 1986).

Geohistory may also play a role in shaping the observed genetic discontinuity. During the LGM, temperature around equatorial zone in the Indian Ocean was suspected to be higher than 23 degrees Celsius even in the coolest month (Barrows & Juggins 2005). Therefore mangroves could survive around this region. In the LGM, more and larger islands were present across the Indian Ocean (Peltier 1994). There may have been a chain of islands between Seychelles, Mascarenes and India, highly reducing the distance of the open ocean (Warren et al. 2010). Thus, *B. gymnorhiza* might have dispersed via a stepping-stone manner through these islands. In the present, gene flow across this region could have become more difficult for *B. gymnorhiza* because of more distant land connections.

The results of this study indicate that long-distance dispersal of *B. gymnorhiza* does not frequently occur among populations. Tests of isolation by distance showed a positive correlation, although only one result was not significant (Fig. 2-8). Arnaud-Haond et al. (2006) also reported a correlation between geographic and genetic distances in *Avicennia marina* from China, Malaysia, and Australia, though they attributed the

significant relationship to perforated sampling scheme. Since this study has a more comprehensive sampling scheme, the correlation between geographic distance and genetic distance of *B. gymnorhiza* are unlikely to be affected by this confounding factor. Previously seedling dispersal of *B. gymnorhiza* had been suspected to occur over a broad range within an ocean (Minobe et al. 2010). However, the results of the present study suggest that distance limits propagule dispersal, and that geologic history, ocean currents, and adaptation may lead to a strong genetic structure in *B. gymnorhiza*.

Genetic distinctiveness of B. gymnorhiza and B. sexangula

This study indicated that *B. gymnorhiza* and *B. sexangula* are distinct two different species. Both nuclear SSR and cpDNA results showed clear genetic differentiation between the two species (Figs. 2-2, 2-3 and 2-4). In STRUCTURE analysis, the result at the highest ΔK did not show clear genetic differentiation between *B. sexangula* and several populations of *B. gymnorhiza* (Fig. 2-2). This result may be caused because STRUCTURE is very sensitive to the number of sampled individuals and partial sampling of individuals leads to a lower ΔK at the true K as Evanno et al. (2005) explained. Relatively small number of *B. sexangula* samples used in this study could have led to failure in detecting its specific signal. The PCoA result obviously supported the distinctiveness of each species (Fig. 2-7). In cpDNA analysis, the two species had no shared haplotype (Figs. 2-9, 2-10 and 2-11).

This study also provided a clear evidence of hybridization between *B. gymnorhiza* and *B. sexangula*. *B.* × *rhynchopetala*. Two individuals from 48_Philippines had admixed structure in STRUCTURE analyses (Figs. 2-2, 2-3 and 2-4), which suggest hybrid formation. The hybrid between *B. gymnorhiza* and *B. sexangula* is taxonomically

recognized as *B.* × *rhynchopetala* (Ge 2001) and has been reported in China, Indonesia and Australia (Zhou et al. 2008, Sun & Lo 2011). This result may be the first report from the Philippines. Both putative hybrid individuals found in this study shared the same cpDNA haplotype as *B. sexangula* individuals. However, the inferred ancestry of their nuclear genotype was largely (> 80 percent) similar to *B. gymnorhiza*. This result indicates that the putative hybrid individuals may result from introgression events over several generations. Sun & Lo (2011) suggested that *B.* × *rhynchopetala* occurs only within the parental habitat. Since the distribution range of *B. sexangula* overlaps with *B. gymnorhiza* from India to Australia longitudinally (Duke & Ge 2011), hybridization and introgression between the two species can happen in the overlapped areas. Although, F1 and F2 hybrids were reported from Hainan Island, North Sulawesi and northeastern Australia (Sun & Lo 2011), my results showed that the two species were genetically almost completely differentiated within the range. Thus, the hybridization event of *B.* × *rhynchopetala* might not have affected the general genetic structure of *B. gymnorhiza*.

Recognition of CUs in Bruguiera gymnorhiza

Genetically discrete population units of *B. gymnorhiza* found in this study can be treated as individual CUs. This study suggested populations of *B. gymnorhiza* were divided into four units, the Indian Ocean (Africa), the Indian Ocean (Asia), the North Pacific Ocean, and the South Pacific Ocean. According to Moritz (1994), populations with significant divergence in allelic frequency are recognized as MUs even though the populations are not reciprocally monophyletic. Although only a few populations shared chloroplast haplotypes and/or clusters in STRUCTURE analyses across regions, most

populations in a region obviously retained their distinctiveness (Fig. 2-5). Thus, the author recommends that genetically and geographically discrete four population units of *B. gymnorhiza* should be recognized as individual CUs such as MUs, and should be managed and conserved separately.

Because these units have possibly adapted to local environments, adaptive differentiation should be tested via further analyses using nuclear coding loci. Detecting adaptive differences among CUs is important according to two reasons. Firstly, the most adaptively differentiated populations should have the highest priority to be protected (de Guia & Saitoh 2007). Secondly, knowledge of the patterns of adaptive differentiation is helpful to avoid translocation between populations adapted to different environments that cause outbreeding depression (Moritz 1999). Thus, understanding of adaptive differences can contribute to future success in conservation activity.

Tables and Figures

Table 2-1. List of samples of *Bruguiera* species used in this study. N_N and N_C are population sizes used for chloroplast analysis and microsatellite analysis, respectively.

Taxon	Region	Locality	Population	N_N	N_C
B. gymnorhiza	Indian Ocean	South Africa: Kuazulu-Natal	1_South Africa	8	5
		Mozambique: Maputo	2_Mozambique	39	5
		Mozambique: Zalala Beach	3_Mozambique	18	5
		Tanzania: Bagamoyo	4_Tanzania	20	5
		France: Europa I.	5_France	21	5
		France: Mayotte I.	6_France	30	5
		India: Kerala	7_India	11	5
		Myanmar: Byonmwe I.	8_Myanmar	18	5
		Myanmar: Myeik	9_Myanmar	32	5
		Thailand: Krabi	10_Thailand	33	
		Thailand: Kantang	11_Thailand	24	5
		Thailand: Palian	12_Thailand	24	
		Malaysia: Sungai Merbok	13_Malaysia	30	
		Malaysia: Klang	14_Malaysia	20	5
		Malaysia: Linggi	15_Malaysia	30	
		Malaysia: Benut	16_Malaysia	22	5
		Singapore: Sungei Buloh	17_Singapore	36	5
		Indonesia: Banda Aceh	18_Indonesia	34	5
		Indonesia: Cilacap	19_Indonesia	29	5
		Indonesia: Bali I.	20_Indonesia	30	5
	Pacific Ocean	Malaysia: Rompin	21_Malaysia	18	5
		Malaysia: Tanjung Lumpur	22_Malaysia	38	
		Malaysia: Paka	23_Malaysia	16	5
		Thailand: Trat	24_Thailand	28	5
		Vietnam: Ca Mau	25_Vietnam	31	5
		Malaysia: Sabah	26_Malaysia	36	5
		Philippines: Fish pond, Panay I.	27_Philippines	3	3
		Palau: Ngaremlengui	28_Palau	23	
		Palau: Airai	29_Palau	32	5
		Japan: Mairabashi, Iriomote I.	30_Japan	29	5
		Japan: Urauchibashi, Iriomote I.	31_Japan	28	
		Japan: Nagura Amparu, Ishigaki I.	32_Japan	24	5
		Japan: Fukidogawa, Ishigaki I.	33_Japan	24	
		Japan: Miyaragawa, Ishigaki I.	34_Japan	28	
		Japan: Kin, Okinawa I.	35_Japan	29	5
		Japan: Gesashi, Okinawa I.	36_Japan	28	
		Australia: Cairns	37_Australia	28	5
		New Caledonia: Canala	38_New Caledonia	29	5
		Fiji: Viti Levu I.	39_Fiji	21	5
		Samoa: Saanapu	40_Samoa	18	5
B. sexangula	Indian Ocean	Myanmar: Byonmwe I.	41_Myanmar	24	5
		Myanmar: Myeik	42_Myanmar	26	5
	Pacific Ocean	Malaysia: Paka	43_Malaysia	28	5
		Vietnam: Ca Mau	44_Vietnam	7	5
		Malaysia: Sabah	45_Malaysia	29	5
		Philippines: Ibajay Mangrove Eco-Park, Panay I.	46_Philippines	5	
B. sp	Pacific Ocean	Philippines: Ibajay Mangrove Eco-Park, Panay I.	47_Philippines	14	5
		Philippines: Fish pond, Panay I.	48_Philippines	19	6
Total				1172	184

Table 2-2. Descriptive statistics of genetic diversity over all loci for each population of *Bruguiera gymnorhiza* and *B. sexangula* obtained by microsatellite analysis. Allelic richness, allelic richness standardized for 11 individuals; H_E, the expected proportion of heterozygotes; H_O, the observed proportion of heterozygotes; F_{IS}, inbreeding coefficient, with asterisks indicating significant departure from zero at $P < 0.05$.

Taxon	Population	Allelic richness	H_E	H_O	F_{IS}	
B. gymnorhiza	1_South Africa	–	0.106	0.078	0.263	
	2_Mozambique	1.42	0.050	0.026	0.480	*
	3_Mozambique	1.86	0.148	0.097	0.342	
	4_Tanzania	2.44	0.283	0.156	0.448	*
	5_France	1.86	0.307	0.351	−0.146	
	6_France	2.07	0.217	0.167	0.231	
	7_India	1.25	0.066	0.011	0.828	
	8_Myanmar	2.38	0.242	0.222	0.080	
	9_Myanmar	2.76	0.327	0.273	0.164	
	10_Thailand	1.63	0.150	0.155	−0.037	
	11_Thailand	2.49	0.315	0.208	0.340	*
	12_Thailand	2.15	0.276	0.182	0.339	*
	13_Malaysia	2.70	0.332	0.217	0.348	*
	14_Malaysia	2.53	0.325	0.256	0.212	
	15_Malaysia	2.38	0.244	0.221	0.095	
	16_Malaysia	2.92	0.414	0.347	0.162	
	17_Singapore	2.56	0.423	0.326	0.229	*
	18_Indonesia	2.40	0.359	0.198	0.447	
	19_Indonesia	2.38	0.252	0.237	0.060	
	20_Indonesia	2.92	0.467	0.304	0.348	*
	21_Malaysia	2.56	0.339	0.271	0.202	
	22_Malaysia	2.28	0.286	0.201	0.298	*
	23_Malaysia	1.83	0.244	0.187	0.232	
	24_Thailand	2.32	0.197	0.129	0.341	*
	25_Vietnam	1.12	0.012	0.012	−0.011	
	26_Malaysia	2.30	0.292	0.295	−0.011	
	27_Philippines	–	0.146	0.094	0.357	
	28_Palau	2.25	0.363	0.272	0.252	
	29_Palau	2.09	0.310	0.246	0.205	
	30_Japan	1.97	0.148	0.125	0.156	
	31_Japan	1.73	0.182	0.152	0.165	
	32_Japan	1.82	0.192	0.135	0.294	
	33_Japan	1.58	0.200	0.125	0.374	
	34_Japan	1.92	0.243	0.147	0.394	*
	35_Japan	1.69	0.205	0.147	0.284	
	36_Japan	1.80	0.205	0.121	0.413	*
	37_Australia	3.06	0.299	0.196	0.343	*
	38_New Caledonia	2.25	0.373	0.198	0.469	*
	39_Fiji	1.98	0.159	0.083	0.476	*
	40_Samoa	1.74	0.170	0.090	0.469	*
B. sexangula	41_Myanmar	2.54	0.293	0.198	0.325	*
	42_Myanmar	2.18	0.265	0.168	0.366	*
	43_Malaysia	2.46	0.223	0.116	0.480	*
	44_Vietnam	–	0.220	0.179	0.189	
	45_Malaysia	1.98	0.228	0.194	0.149	
	46, 47_Philippines	2.35	0.329	0.171	0.480	*
	48_Philippines	3.29	0.487	0.278	0.430	*

Table 2-3. Null allele frequency estimated by FREENA for each population-locus comparison.

Taxon	Population	*N*	Estimate of null allele frequency							
			Locus 1	Locus 2	Locus 3	Locus 4	Locus 5	Locus 6	Locus 7	Locus 8
B. gymnorhiza	1_South Africa	8	0.001	0.001	0.183	0.001	0.000	0.001	0.001	0.001
	2_Mozambique	39	0.000	0.000	0.167	0.000	0.098	0.001	0.001	0.001
	3_Mozambique	18	0.073	0.116	0.157	0.001	0.045	0.000	0.001	0.001
	4_Tanzania	20	0.167	0.088	0.267	0.158	0.029	0.000	0.001	0.001
	5_France	21	0.000	0.001	0.000	0.000	0.098	0.009	0.001	0.001
	6_France	30	0.044	0.133	0.000	0.130	0.000	0.001	0.001	0.001
	7_India	11	0.001	0.001	0.298	0.001	0.000	0.001	0.001	0.001
	8_Myanmar	18	0.053	0.056	0.000	0.001	0.000	0.001	0.001	0.001
	9_Myanmar	32	0.074	0.067	0.093	0.063	0.107	0.000	0.000	0.001
	10_Thailand	33	0.001	0.000	0.000	0.001	0.001	0.000	0.001	0.001
	11_Thailand	24	0.202	0.217	0.118	0.000	0.000	0.119	0.001	0.031
	12_Thailand	24	0.086	0.208	0.049	0.093	0.075	0.000	0.001	0.001
	13_Malaysia	30	0.212	0.058	0.141	0.123	0.000	0.001	0.170	0.000
	14_Malaysia	20	0.082	0.090	0.116	0.108	0.000	0.001	0.001	0.204
	15_Malaysia	30	0.000	0.000	0.000	0.092	0.143	0.001	0.001	0.001
	16_Malaysia	22	0.000	0.000	0.064	0.030	0.043	0.001	0.001	0.237
	17_Singapore	36	0.029	0.038	0.044	0.082	0.046	0.001	0.001	0.277
	18_Indonesia	34	0.120	0.098	0.216	0.112	0.167	0.138	0.001	0.001
	19_Indonesia	29	0.000	0.016	0.148	0.001	0.000	0.000	0.000	0.001
	20_Indonesia	30	0.069	0.097	0.240	0.022	0.104	0.120	0.001	0.218
	21_Malaysia	18	0.051	0.000	0.064	0.000	0.010	0.001	0.001	0.204
	22_Malaysia	38	0.036	0.026	0.123	0.000	0.024	0.001	0.001	0.190
	23_Malaysia	16	0.001	0.000	0.163	0.001	0.220	0.000	0.001	0.055
	24_Thailand	28	0.168	0.000	0.107	0.001	0.038	0.000	0.001	0.165
	25_Vietnam	31	0.000	0.001	0.000	0.001	0.001	0.001	0.001	0.001
	26_Malaysia	36	0.000	0.000	0.062	0.001	0.000	0.001	0.001	0.113
	27_Philippines	4	0.153	0.001	0.001	0.001	0.001	0.001	0.001	0.001
	28_Palau	23	0.012	0.034	0.148	0.000	0.058	0.001	0.001	0.277
	29_Palau	32	0.000	0.030	0.127	0.001	0.000	0.000	0.001	0.317
	30_Japan	29	0.000	0.098	0.047	0.001	0.101	0.001	0.001	0.126
	31_Japan	28	0.084	0.064	0.000	0.001	0.000	0.001	0.001	0.001
	32_Japan	24	0.049	0.004	0.167	0.001	0.031	0.000	0.001	0.137
	33_Japan	24	0.047	0.126	0.104	0.001	0.001	0.001	0.001	0.188
	34_Japan	28	0.053	0.138	0.187	0.001	0.059	0.001	0.001	0.236
	35_Japan	29	0.059	0.009	0.001	0.001	0.000	0.116	0.001	0.207
	36_Japan	28	0.032	0.000	0.264	0.001	0.038	0.001	0.001	0.236
	37_Australia	28	0.279	0.250	0.088	0.073	0.033	0.000	0.000	0.000
	38_New Caledonia	29	0.081	0.017	0.365	0.287	0.094	0.001	0.001	0.050
	39_Fiji	21	0.112	0.085	0.246	0.001	0.001	0.001	0.001	0.000
	40_Samoa	18	0.221	0.001	0.185	0.000	0.001	0.001	0.000	0.000
B. sexangula	41_Myanmar	24	0.000	0.000	0.242	0.001	0.000	0.304	0.000	0.001
	42_Myanmar	26	0.147	0.008	0.155	0.000	0.035	0.215	0.001	0.001
	43_Malaysia	28	0.117	0.029	0.295	0.117	0.000	0.090	0.001	0.117
	44_Vietnam	7	0.001	0.001	0.000	0.001	0.001	0.001	0.001	0.236
	45_Malaysia	29	0.000	0.011	0.161	0.001	0.001	0.000	0.001	0.002
	46, 47_Philippines	18	0.000	0.000	0.419	0.060	0.001	0.126	0.001	0.178
	48_Philippines	19	0.242	0.000	0.286	0.163	0.000	0.196	0.213	0.033

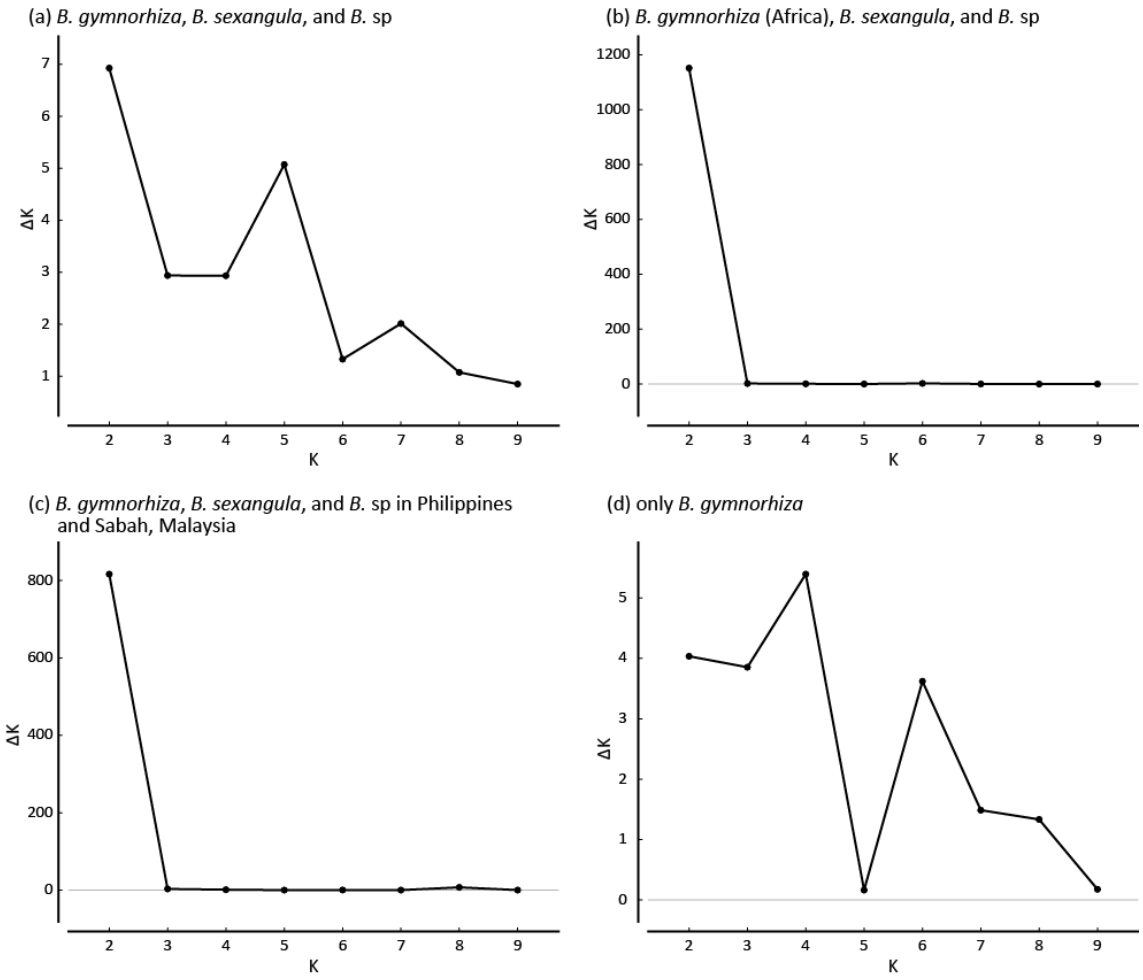

Fig. 2-1. ΔK by Bayesian clustering (STRUCTURE, Pritchard et al. 2000) shown in four different population settings. (a) All populations of *Bruguiera gymnorhiza*, *B. sexangula*, and *B.* sp. (b) Populations of African *B. gymnorhiza* (1_South Africa - 6_France), *B. sexangula*, and *B.* sp. (c) Populations of *B. gymnorhiza*, *B. sexangula*, and *B.* sp in Philippines and Sabah, Malaysia (26_Malaysia, 27_Philippines, 45_Malaysia - 48_Philippines). (d) All populations of *B. gymnorhiza*.

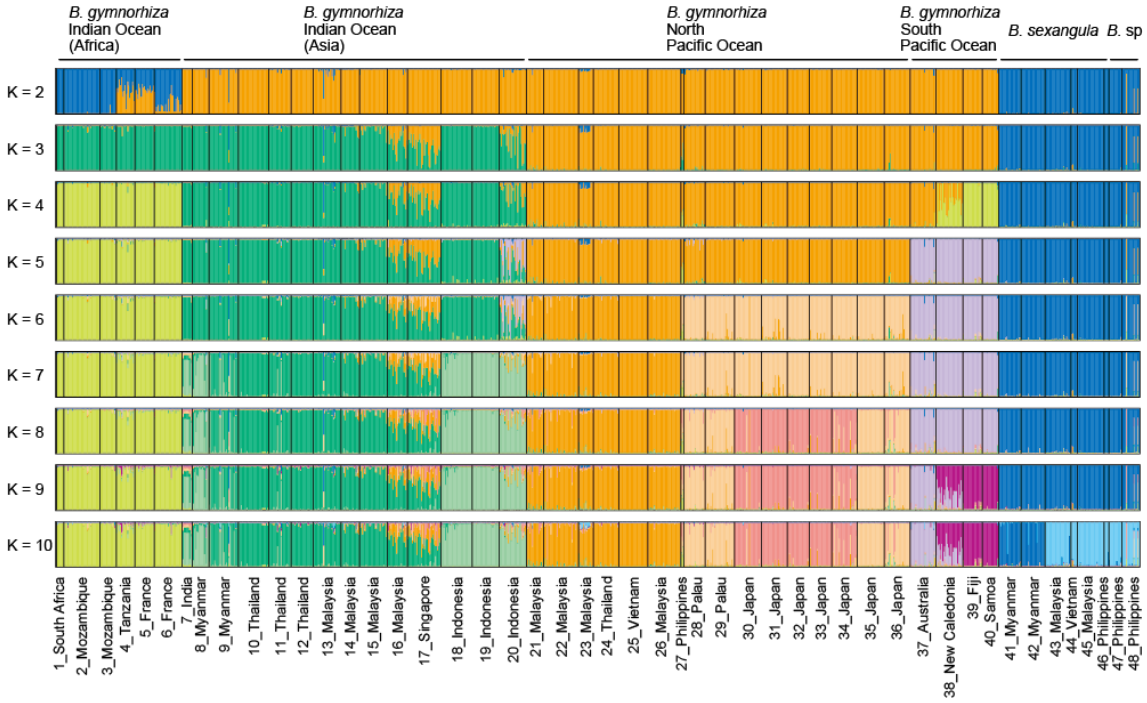

Fig. 2-2. Results of Bayesian clustering (STRUCTURE, Pritchard et al. 2000) of *Bruguiera gymnorhiza*, *B. sexangula* and *B.* sp. Vertical columns represent single individuals, and the height of bars represents the proportion of cluster memberships.

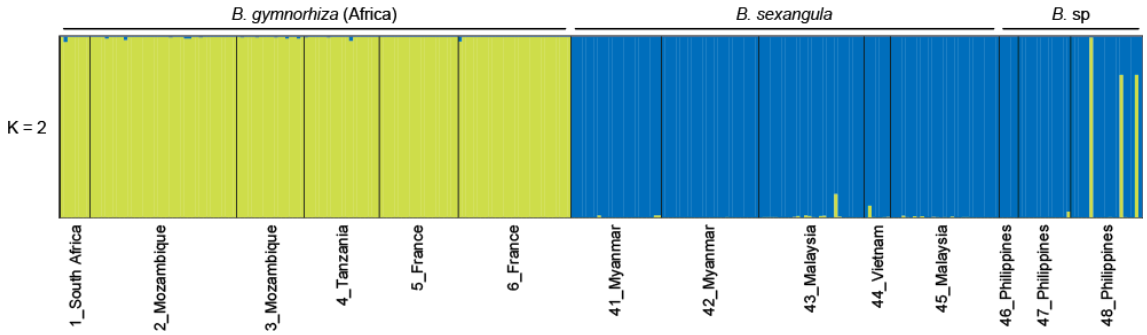

Fig. 2-3. Result of Bayesian clustering (STRUCTURE, Pritchard et al. 2000) of African *Bruguiera gymnorhiza*, *B. sexangula*, and *B.* sp. Vertical columns represent single individuals, and the height of bars represents the proportion of cluster memberships.

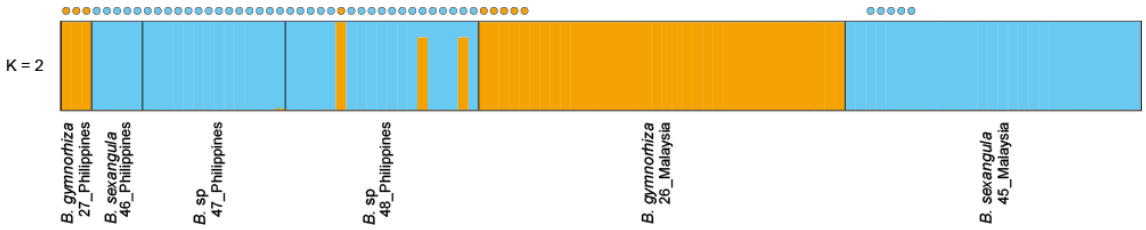

Fig. 2-4. Result of Bayesian clustering (STRUCTURE, Pritchard et al. 2000) of *Bruguiera gymnorhiza*, *B. sexangula* and *B.* sp in Philippines and Malaysia. Vertical columns represent single individuals, and the height of bars represents the proportion of cluster memberships. Circles on the histogram represent a nucleotide substitution at site 184 on the *trn*S-*trn*G intergenic spacer region (A; orange circle, C; light blue circle).

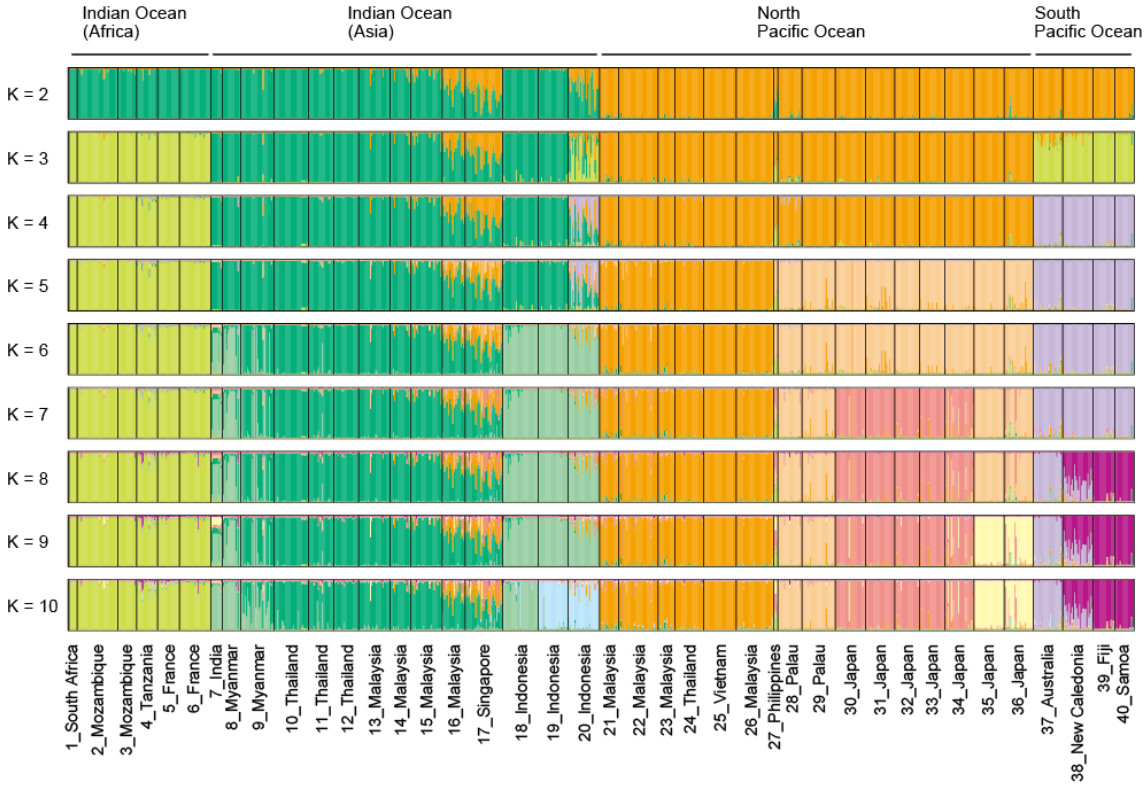

Fig. 2-5. Results of Bayesian clustering (STRUCTURE, Pritchard et al. 2000) of *Bruguiera gymnorhiza*. Vertical columns represent single individuals, and the height of bars represents the proportion of cluster memberships.

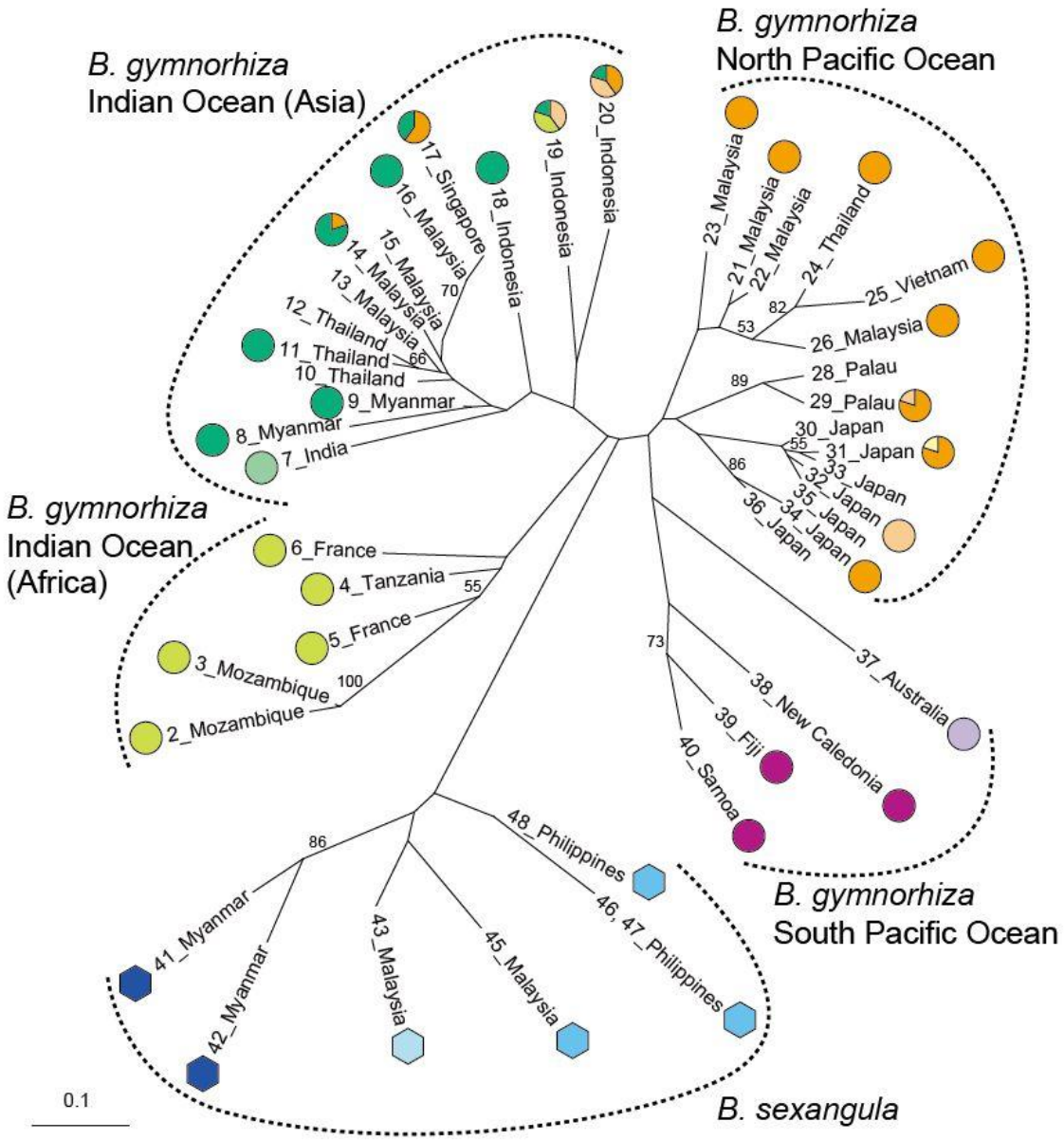

Fig. 2-6. Neighbor-joining tree based on D_A distance for the 44 populations *Bruguiera gymnorhiza* or *B. sexangula* with more than 10 individuals calculated by microsatellites data. Bootstrap probabilities larger than 50% are shown above the branches. Circle or hexagonal graphs on the end of population names represent chloroplast DNA haplotypes shown in the population.

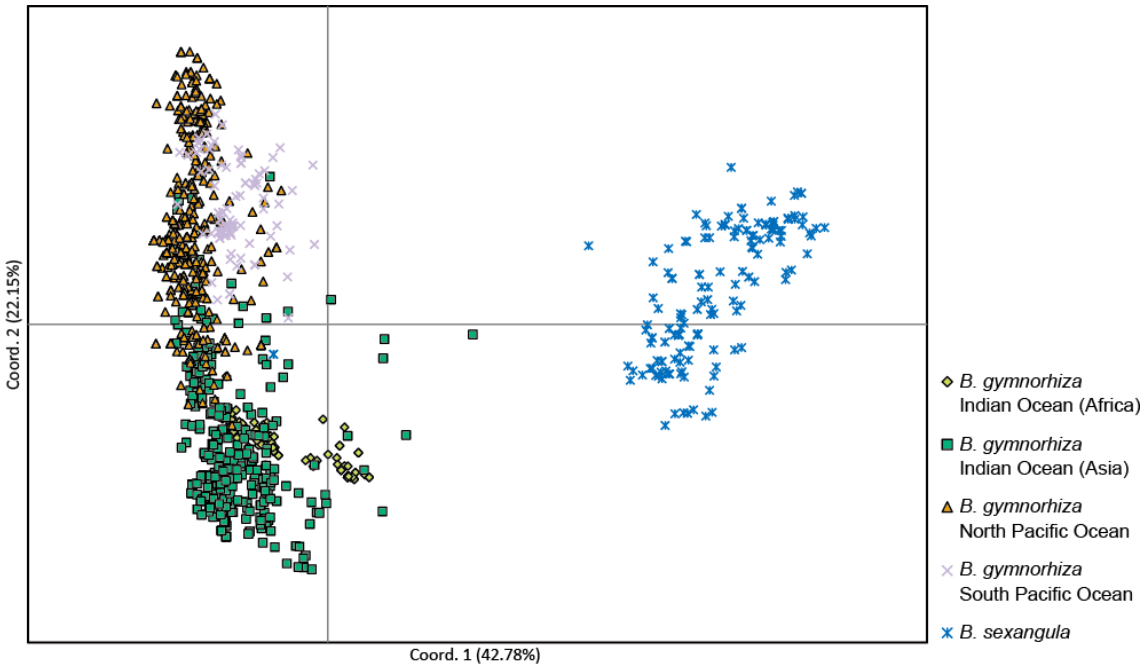

Fig. 7. Principal Codominant Analysis (PCoA) of *Bruguiera gymnorhiza* and *B. sexangula*. The PCoA plots were based on the mean genotypic distance between all individual pairs of both species calculated by microsatellites data.

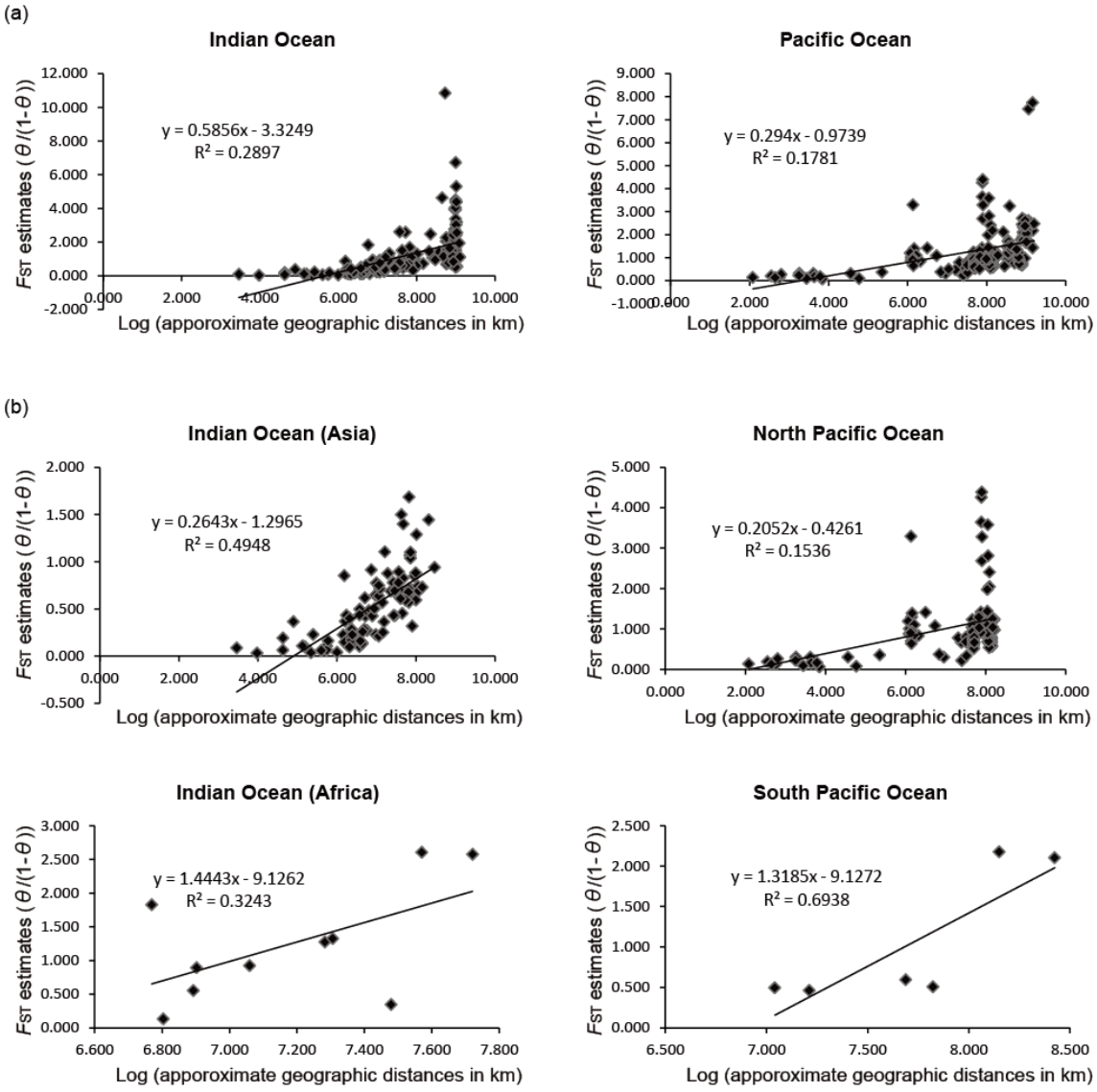

Fig. 2-8. Correlation between genetic and geographic distances among populations of *Bruguiera gymnorhiza* with more than 10 individuals. (a) Populations were divided into the Indian Ocean and the Pacific Ocean. *P* values were $P < 0.0001$ and $P < 0.01$, respectively. (b) Populations were divided into four regions, the Indian Ocean (Africa), the Indian Ocean (Asia), the North Pacific Ocean, and the South Pacific Ocean. *P* values were $P < 0.0902$, $P < 0.0001$, $P < 0.01$, and $P < 0.02$, respectively.

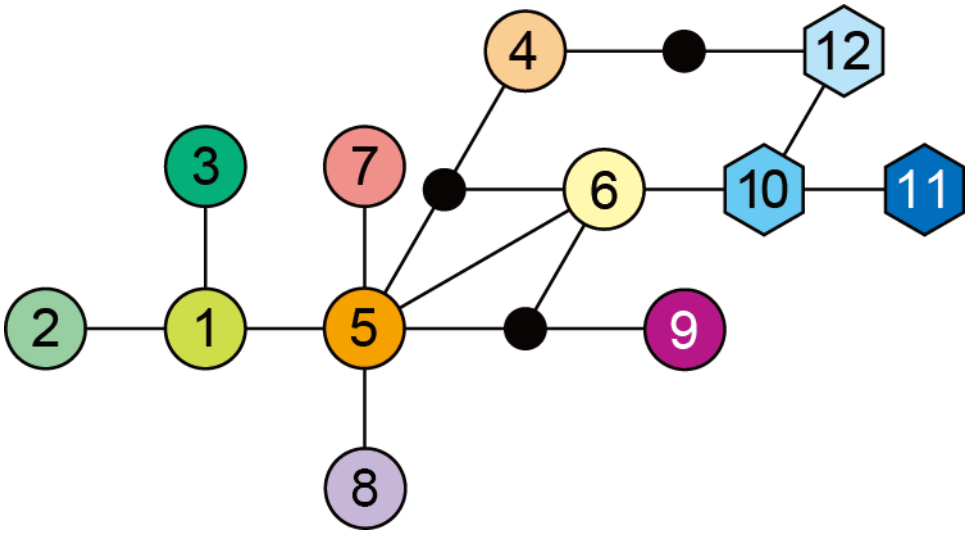

Fig. 2-9. Haplotype network of *Bruguiera gymnorhiza* and *B. sexangula* obtained by concatenated sequence of *trn*L-*trn*F and *trn*S-*trn*G IGSs. Circles on the haplotype network represent haplotypes found in *B. gymnorhiza*, hexagons in *B. sexangula*, and filled dot shows nucleotide substitution.

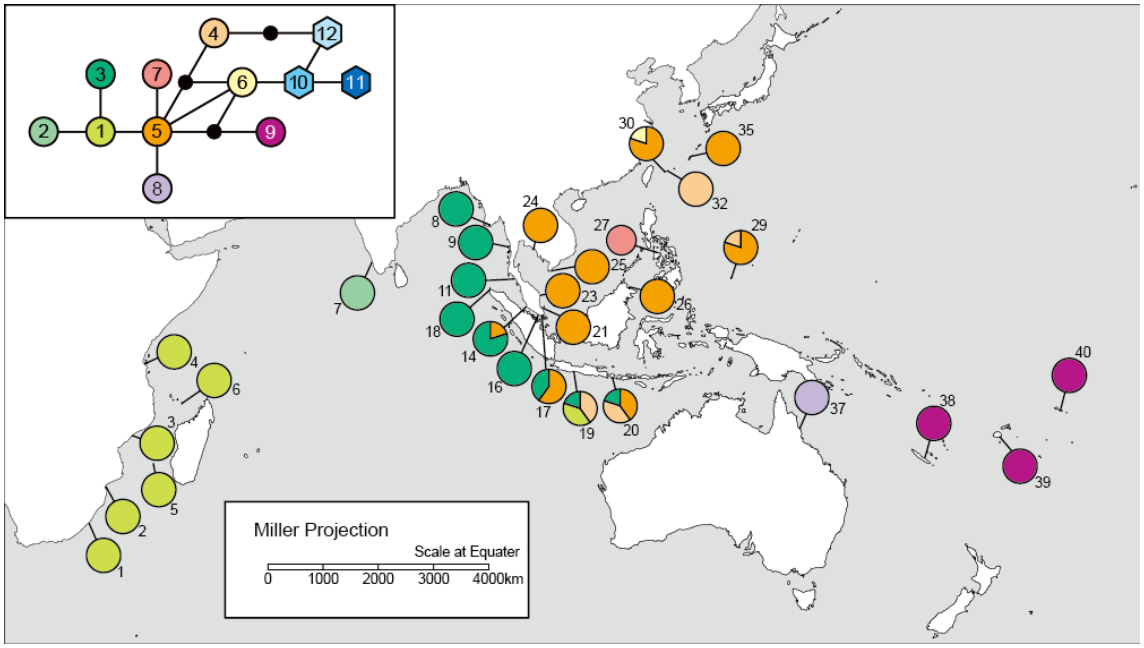

Fig. 2-10. Geographic distribution of chloroplast haplotypes of *Bruguiera gymnorhiza* obtained by concatenated sequence of *trn*L-*trn*F and *trn*S-*trn*G IGSs. Number represents abbreviated population name shown in Table 2-1. To help understanding of the readers, the haplotype network is shown on the map.

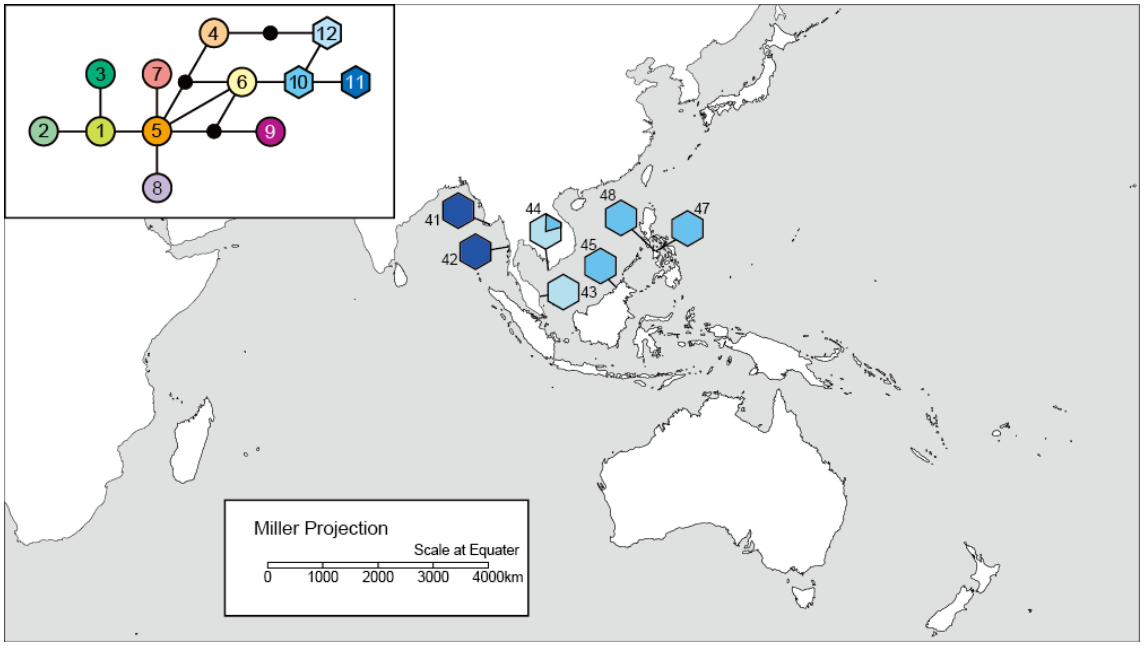

Fig. 2-11. Geographic distribution of chloroplast haplotypes of *Bruguiera sexangula* obtained by concatenated sequence of *trn*L-*trn*F and *trn*S-*trn*G IGSs. Number represents abbreviated population name shown in Table 2-1. To help understanding of the readers, the haplotype network is shown on the map.

GENERAL DISCUSSION

This study employed genetic markers to clarify the species status of a critically endangered mangrove species, *B. hainesii*, and to delimitate conservation units in the most widespread mangrove species, *B. gymnorhiza*. Phylogenetic analysis of *B. hainesii* and its related species using both chloroplast and nuclear markers suggests that the endangered species is a hybrid between two other common species in the same genus. This finding provides an important suggestion for the conservation of mangroves. Although the finding looks providing unrelated data to conservation, in fact, it helps us to plan effective conservation management. Based on the result, we can reconsider or reduce conservation efforts that have been paid to *B. hainesii* and allocate limited resources to other genuinely vulnerable mangrove species.

The finding of hybrid status of a critically endangered species also provides valuable insight that other endangered mangrove species might also be hybrids between common species. Hybridization seems a common phenomenon for mangrove plant species. Many hybrid mangrove species have been reported: e.g. *Bruguiera* × *rhynchopetala* (Ge 2001), *Rhizophora* × *annamalayana* (Kathiresan 1995, 1999), *Rhizophora* × *lamarckii* (Tomlinson & Womersley 1976), *Rhizophora* × *selala* (Tomlinson 1978, Duke 2010), *Sonneratia* × *gulngai* (Duke 1984), and *Sonneratia* × *hainanensis* (Wang et al. 1999). Possible reason that various hybrid species exist in mangrove plants can be the overlapping distribution ranges. 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 in the ocean (Tomlinson 1986), distribution ranges of closely related species could overlap easier

than terrestrial plants. The sympatric distribution of congeners may increase chances of hybridization for mangrove plants.

Phylogenetic analysis of other threatened mangrove species should be conducted to confirm whether they are not hybrids. There are 11 threatened mangrove species globally (Polidoro et al. 2010). Several phylogenetic analyses on endangered mangrove species have been conducted (*Sonneratia griffithii*; Yang et al. 2015, *Avicennia rumphiana*; Huang et al. 2014), however, for the most species, phylogenetic information has not been obtained. Rare mangrove species may have possibilities of hybrids. Since budget available to conservation is limited, the species identity of threatened mangrove species should be confirmed via phylogenetic analyses using both nuclear and chloroplast DNA sequencings, to prioritize the species to be protected.

The delimitation of CUs in the distribution range of *B. gymnorhiza* provided another valuable insight for conservation genetics of mangroves. Although some widespread sea-dispersal species are capable of long-distance dispersal to maintain frequent gene flow and retain species cohesion (Takayama et al. 2008), this study showed clear genetic structure across the distribution range of the most widely distributed mangrove species *B. gymnorniza*, and suggests that the four distinct geographic units of the species should be treated as separate CUs. One of the factors that delimited the geographic distribution of the four units is the Malay Peninsula as reported in previous studies (Minobe et al. 2010, Urashi et al. 2013) and in other mangrove species (*Lumnitzera racemosa*; Su et al. 2006, *Ceriops tagal*; Liao et al. 2007, *Rhizophora apiculata*; Inomata et al. 2009, Ng et al. 2015, *Rhizophora mucronata*; Wee et al. 2015). The Malay Peninsula indeed acted as a land barrier for gene flow between populations by preventing sea-dispersal of propagules of the mangrove species.

In addition to the land barrier, this study also provided clear evidence that mangrove populations are genetically structured even in an oceanic region that could act as a corridor of dispersal of propagules by ocean currents within both the Indian and Pacific Oceans. Genetic structure across the Indian Ocean may be a new finding in mangrove species. This finding suggests that even though there is no land barrier, the vast ocean can act as a barrier to gene flow among populations by preventing dispersal of propagules. Indeed species richness of major mangrove species is different between Africa (8 species) and Asia (19-31 species) (Tomlinson 1986), but the geographic structure of the Indian Ocean has not been reported. The large difference in species richness indicates that the Indian Ocean may have inhibited seed dispersal of many mangrove species and historically acted as a boundary of species distribution. Adaptation to the local environment in Africa and Asia may be another possible factor. Genetic structure of the Pacific Ocean has been reported in some studies. For example, *Rhizophora stylosa* has genetically differentiated units in the North Pacific Ocean and the South Pacific Ocean (Wee et al. 2015), also in the two closely related *Ceriops* species, *C. pseudodecandra* and *C. zippeliana* (Sheue et al. 2010), unique haplotypes were found in each of these regions from several species in northern Australia (*Lumnitzera racemosa*; Su et al. 2006, *Ceriops tagal*; Huang et al. 2012), and species richness is different between the west and east coasts of Australia (Tomlinson 1986). Because effect to restrict seed dispersal has been found in multiple mangrove species, a historical barrier such as the Sahul Shelf and ocean circulation patterns in the Pacific Ocean mentioned in Chapter 2 may act as a barrier to gene flow.

Contrary to a conventional belief that widespread species have continual gene flow among populations, this study showed that even the most widely distributed mangrove

species *B. gymnorhiza* has restricted gene flow within oceanic regions. Also, similar reports were from other mangrove species in the Pacific Ocean. These findings may indicate that oceanic regions are also common barriers to gene flow in mangrove species. Even though the ocean acted as a vector or corridor for the expansion of distribution through sea dispersal of propagules, historical geological changes or simply the distance of the ocean among population might have prevented gene flow among regional populations and shaped the present genetic structures. Therefore, although mangrove species have buoyant diaspores and can disperse across the ocean, appropriate CUs of each mangrove species should be determined based on the genetic diversity by conducting phylogeographic analyses covering their entire distribution ranges.

CONCLUSION

In this dissertation, the author makes two recommendations for mangrove conservation based on the findings of this study. First, as this study revealed that a critically endangered species is merely a hybrid, species identity of other threatened mangrove species should be confirmed via phylogenetic analyses, and conservation effort should be allocated appropriately. Second, as this study suggested clear genetic structures within oceanic regions for the most widely distributed mangrove species in the IWP region, conservation units of other mangrove species should be considered by conducting phylogeographic analyses that cover their entire distribution ranges.

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