

Identifying conservation units of mangrove *Rhizophora apiculata* in the Indo West Pacific region

Introduction

The increasing rate of natural habitat destruction, fragmentation and other anthropogenic activities coupled with global climate change are leading to unprecedented animal and plant species extinction rates (Ceballos et al., 2015; Thomas, 2015; Gray, 2018). In order to address the global loss of biodiversity, genomic tools can fill in gaps in our knowledge of a species' evolutionary processes (e.g. genetic diversity, genetic drift, mutation, selection, migration, inbreeding) and allows to prediction of how the effective population sizes of populations might affect future genetic diversity and evolvability (Edwards, 2015; Carvalho et al., 2017). By combining the assessment of the phylogenetic diversity, population distribution and connectivity of a species, individual populations can be prioritised for conservation management to lower the risk of extinction, increase genetic diversity and population sizes (Frankham, 2010; Buerki et al., 2011). Separate lineages of a species might form clusters, termed 'Evolutionarily Significant Units' (ESUs) using a large set of nuclear DNA (nDNA) sequences (Coates et al., 2018) while using chloroplast DNA (cpDNA) regions, we use the term 'Conservation Units' (CU) for distinguishing such clusters (Crandall et al., 2000). While to date, 27,514 plant species are listed on the IUCN Red List of Threatened Species (IUCN, 2018), the total number of plant species is estimated to be 450,000 (Pimm and Joppa, 2015; Christenhusz and Byng, 2016). In 2015, the extinction rate of IUCN plant species showed that more than one species is threatened with extinction per year while tropical plant species are twice as likely to be threatened compared to temperate regions (Callmender et al., 2005; Brummit et al., 2015).

Mangroves are salt-tolerant, intertidal evergreen trees which grow in tropical and subtropical regions. These forests occupy approximately 152,000 km² globally, out of which 39% is found in Asia (Sandilyan and Kathiresan, 2012; Takayama et al., 2013; Máñez et al., 2014) (Figure 1). There are approximately 70 mangrove species, out of which 11 species are at elevated risk of extinction based on IUCN Red List Criteria (Polidoro et al., 2010). Mangroves show unique adaptations by having aerial roots, salt-excretion physiology and mainly reproduce via vivipary (seedling dispersal). The propagules grow on the parent tree for 10-12 months and are dispersed into the sea where ocean surface currents disperse them (Rabinowitz, 1978; Tomlinson, 2000). The hypocotyl is rod-like, and its anatomy greatly influences each species' dispersal range and survival (Clarke et al., 2001; Sousa et al., 2007; Pickens and Hester, 2011). Mangroves are estimated to live up to 80-100 years (Verheyden et al., 2007), reaching reproductive age after 2-5 years, but as it is difficult to determine the age of mangroves, there are many unknowns about their life history (Robert et al., 2011).

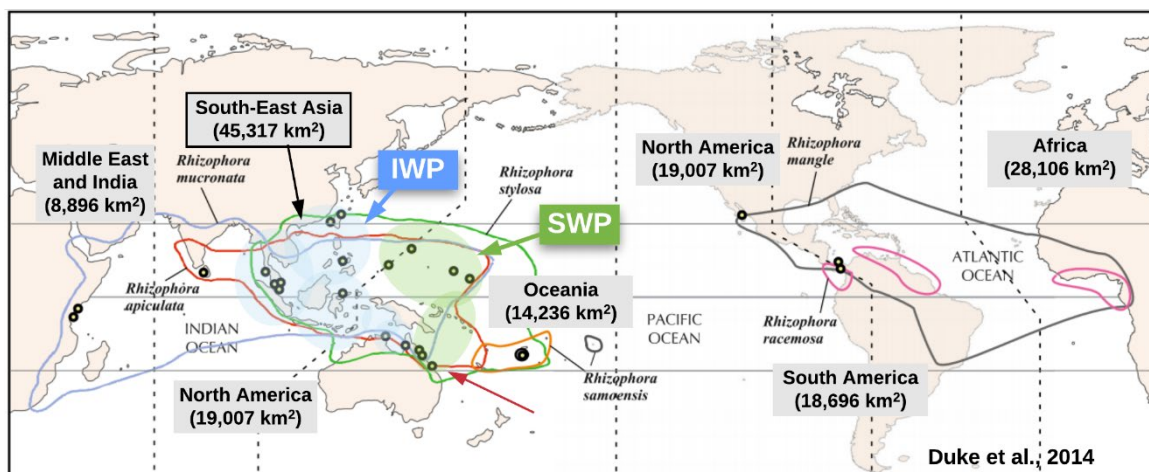


Figure 1. The worldwide distribution of mangroves (regional area cover, km²) and distribution of mangrove species in genus *Rhizophora* (areas circulated) (modified from Duke et al., 2014). The two major groups are expected to be the Indo West Pacific (IWP) and the South West Pacific (SWP) (Duke et al., 2002 and 2014).

Since 1980, over 35% of global mangrove forest area (> 3 million ha) has been lost, mostly in Southeast Asia (Thomas et al., 2017). Its value of ecosystem services (e.g. water purification, fish catch, coast protection, Barbier, 2016) is estimated to be USD 200,000-900,000 ha⁻¹ or USD 1.6 billion annually (Gilman et al., 2008; Polidoro et al., 2010). The rapid decline in mangroves is mainly due to shrimp farming, deforestation, agriculture and urbanisation (Valiela et al., 2001; Gilman et al., 2008; Románach et al., 2018). The future threats caused by climate change include sea-level rises, more frequent storms, uneven rainfall distributions, phenological shifts (Van der Stocker et al., 2017) and inability to follow global warming patterns (Hickey et al., 2017). The disappearance of the area of mangrove habitats is estimated to be 2-8% per year, and without radical change, these unique habitats might functionally disappear within 80-100 years (Duke et al., 2007; Lovelock et al., 2015).

Mangrove evolution is dated back to the mid Cretaceous era (100 Mya) and its distributions followed the continental drifts (Duke, 2007), while their speciation reflects signs of vicariance caused by land barriers and ocean currents and also cyclic events of extinctions and re-associations due to sea-level changes (Duke et al., 2002; Wee et al., 2014; Guo et al., 2017; Ngeve et al., 2017). The Rhizophoraceae tribe consists of the most abundant, fast-growing mangrove species and have been in the focus of mangrove phylogenetics. Whole-genome duplication (WGD) during the extreme global warming event (Paleocene-Eocene Thermal Maximum, 55.5 Mya) is likely to have provided most of the genetic material (Xu et al., 2017) followed by Genome Size (GS) reduction when mangroves moved into intertidal habitats and became a 'founder species' (Lyu et al., 2018). Both vivipary and salt-exclusion adaptations are likely to have evolved multiple times (Shi et al., 2005), reflecting mangrove convergent evolution (Sahu et al., 2016; Li, 2017).

Within the mangrove diversity hotspots in Asia (Polidoro et al., 2010), the genus *Rhizophora* has shown varying genetic diversity patterns in the Indo West Pacific (IWP) and the South West Pacific (SWP) regions (Duke et al., 2002; Takayama et al., 2013; Lo et al., 2014; Ng et al., 2015), following land barriers and oceanic currents. Coincidentally, these areas face the most habitat destruction globally (Polidoro et al., 2010) and some countries have a bad track record of implementing mangrove conservation plans (i.e. rehabilitation, protection) (Dharmawan et al., 2016; Peng et al., 2016; Kodikara et al., 2017; Granado et al., 2018).



The ambitious goal of the Global Mangrove Alliance is to increase mangrove cover by 20% by 2030 (The Mangrove Alliance, 2018a) but a conservation plan is crucial in advance. In this study, we focus on *R. apiculata* mangrove populations from IWP and SWP regions (Figure 2) to i) understand their phylogeny (Wee et al., 2018), ii) understand the distribution of haplotypes and gene flow between populations, and iii) identify conservation units and efficient management practices to protect their future genetic diversity.

Figure 2. The propagules (a), aerial roots (b) and brown spots (cork warts) on the leaf abaxial side (c) of *R. apiculata*. The cork warts are absent in southern Papua and Australian populations (Guo et al., 2016) and are useful for species identification (Samadder and Jayakumar, 2015).

Methods

The total of 51, *trnS-trnG* chloroplast DNA regions were obtained from GenBank (Takayama et al., 2013; Lo et al., 2014). A total of 46 individual *R. apiculata* were used from 18 natural populations: 14 from Australia, nine from Malaysia, seven from Micronesia, three from Thailand, the Philippines, Indonesia and Sri Lanka. Individual sequences were from Japan and New Caledonia. Satellite imagery was used to investigate local habitats for the main sample sites (Appendix 1). As outliers, *R. mucronata*, *R. stylosa* and *Avicennia marina* were used from Australia and the Philippines (Table 1).

Sequences were aligned in BioEdit software (Hall, 2016) using ClustalW algorithm, which were then used for Maximum Likelihood (Tamura model) phylogenetic tree construction with 1,000 permutations in MEGA7 (Kumar et al., 2016). The genetic diversity of *R. apiculata* from each country was analysed in DnaSP6 (Librado and Rozas, 2009). Tajima's D test with 10,000 permutations was used to investigate whether the three major populations (Malaysia, Australia and 'Others') evolving randomly or showed directional or balancing selection. The weighted average for estimated haplotype diversities (H_s) was calculated as a pairwise comparison between the different countries. The estimate of the haplotype diversity of the total populations (H_{ST}), Wright's (1949) fixation index (F_{ST}) and the number of migrants were also calculated for the total population (Hudson et al., 1992).

Analysis of molecular variance (AMOVA) was carried out in Arlequin 3.5.1 (Excoffier and Lischer, 2010), dividing the 18 populations into three major groups, calculating the distance matrix and testing it with 1,000 permutations. Haplotype frequencies were also counted for each country.

Results

The aligned sequences of the cpDNA region was 541 bp long, and within *R. apiculata* populations, two polymorphic sites were found in Malaysia and Australia while only one was found in all the other countries (Table 2). The nucleotide diversity (π) ranged between 0.00116 - 0.00197 and was highest in Australian while the population mutation rate (Θ) ranged between 0.0006 - 0.0013 and was the highest in Malaysia (Figure 2). Tajima's D were all positive and was the highest in Australia and the lowest in Malaysia. The genetic differentiation between different countries showed that H_{ST} was statistically significant ($df = 28$, $P < 0.0001$) and the allele frequencies also differed in between different countries ($F_{ST} = 0.846$) despite having very few migrants ($N_m = 0.05$).

Table 1. The mangrove species, the locality and the number of individual sequences used in this study.

Species	Locality	N
<i>Rhizophora apiculata</i>	Australia - Cato River	3
	Australia - Danitree River	5
	Australia - Embley River	3
	Australia - Trinity Islet	3
	Malaysia - Blue Lagoon	3
	Malaysia - Pulau Babi	1
	Malaysia - Sementa Klang	2
	Malaysia - Pulau Burong	3
	Thailand - Phang Nga Bay	3
	Philippines - Panay Island	3
	Micronesia - Chuuk	3
	Micronesia - Kosrae	2
	Micronesia - Yap	2
	Indonesia - North Sulawesi	3
	Guam - Unknown	2
	Japan - Iriomote Island	1
	Sri Lanka - West Coast	3
	New Caledonia - Canala	1
<i>Rhizophora mucronata</i>	Australia - Danitree River	1
	Philippines - Panay Island	1
<i>Rhizophora stylosa</i>	Australia - Embley River	1
	Australia - Shoalwater Bay	1
<i>Avicennia marina</i>	Australia - Swartzbach	1

The ML phylogenetic analysis showed four distinct clusters (Figure 3), where the two largest groups both consisted of 7 different populations (IWP and SWP, BS 65% and 57%). Sri Lanka formed another distinct group (SriL, BS 100%) as well as the Blue Lagoon with Pulau Babi, and Sementa Klang populations in Malaysia (Malay, BS 59% and 65%). The four sequences from *R. mucronata* and *R.*

stylosa clustered into a distinct group (BS 98%), while *Avicennia marina* was the most distantly related species (BS 100%).

The pairwise H_s ranged between 0.000 - 0.722 and was the largest between Malaysia and Guam, followed by Malaysia and Indonesia, Philippines, Thailand and Sri Lanka (Table 3). Due to shared haplotype between Malaysia and Australia (Table 4), the haplotype diversity was 0.618. While Haplotypes 1 and 3 were only found in Malaysia, Haplotype 2 was found in Malaysia, Japan, Indonesia, Philippines, Thailand and Australia. Haplotype 4 was found in Australia, Guam and Micronesia, Haplotype 5 in New Caledonia and Haplotype 6 was in Sri Lanka.

The AMOVA test showed that the distribution of genetic variation showed that 55.9% of the total variation occurred among the three groups (df = 4, $P < 0.0001$), 42.7% among the different countries (df = 5, $P < 0.0001$), while only 1.44% variation occurred among the different countries within the three groups but was also statistically significant (df = 35, $P < 0.0001$) (Table 5).

Table 2. Due to low genetic diversity within countries, the genetic diversity of the three major groups of *R. apiculata* is presented. Tajima's D tests were not significant.

	Malaysia	Australia	Others
N	9	12	24
Polymorphic sites	2	2	1
Mutations	2	2	1
Haplotypes	3	2	2
Haplotype diversity	0.722	0.545	0.518
Pi (π)	0.00167	0.00197	0.00116
Theta-W (θ)	0.0013	0.0012	0.0006
Tajima D	0.9751	1.8912	1.5734

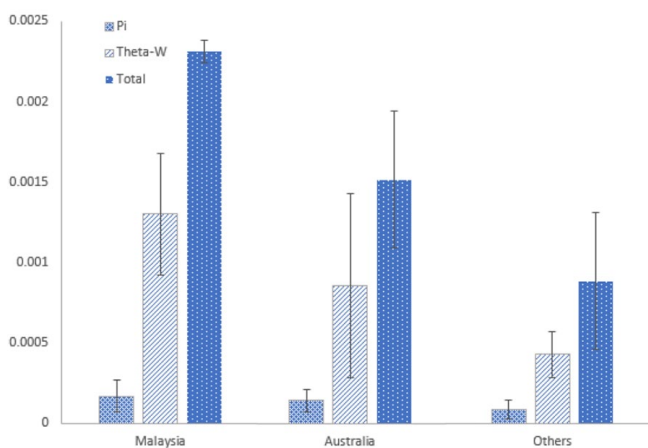


Figure 2. Genetic diversity of the cpDNA region of *R. apiculata* showing the nucleotide diversity (Pi) and the number of segregating sites (Theta W) with standard deviations.

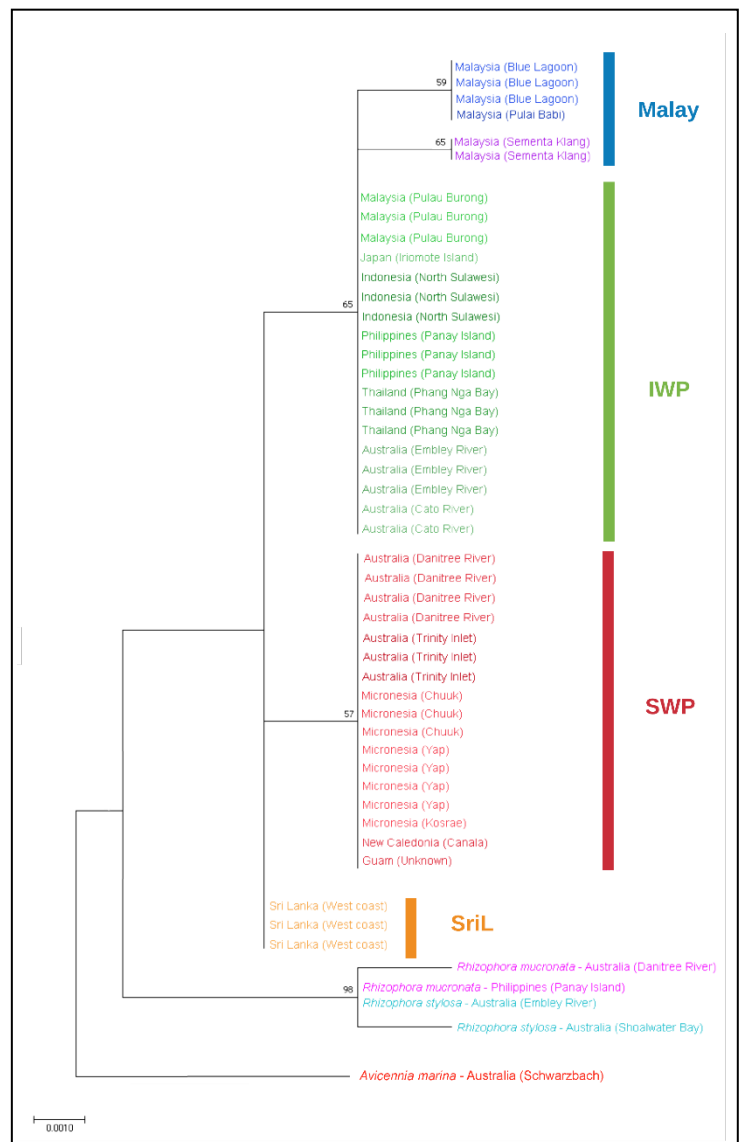


Figure 3. ML phylogenetic tree reveals two major groups (IWP and SWP), with separate groups from Sri Lanka (SriL) and Malaysia (Malay).

Table 3. Pairwise estimated haplotype diversity (H_s) in individual countries showing that mostly the Malaysian, and Australian haplotypes differed from other populations.

Population 1	Population 2	H_s
Malaysia	Indonesia	0.632
Malaysia	Philippines	0.632
Malaysia	Thailand	0.632
Malaysia	Australia	0.618
Malaysia	Guam	0.722
Malaysia	Micronesia	0.389
Malaysia	Sri Lanka	0.632
Indonesia	Philippines	0.000
Indonesia	Thailand	0.000
Indonesia	Australia	0.496
Indonesia	Guam	0.000
Indonesia	Micronesia	0.000
Indonesia	Sri Lanka	0.000
Philippines	Thailand	0.000
Philippines	Australia	0.496
Philippines	Guam	0.000
Philippines	Micronesia	0.000
Philippines	Sri Lanka	0.000
Thailand	Australia	0.496
Thailand	Guam	0.000
Thailand	Micronesia	0.000
Thailand	Sri Lanka	0.000
Australia	Guam	0.545
Australia	Micronesia	0.341
Australia	Sri Lanka	0.496
Guam	Micronesia	0.000
Guam	Sri Lanka	0.000
Micronesia	Sri Lanka	0.000

Table 4. Haplotype estimation in each country by counting. Malaysia and Australia were the only two countries with two or more different haplotypes while in other countries, only one haplotype was found.

	Mal*	Jap	Indo	Phil	Thai	Aus	Guam	Micro	NC	SriL
Haplotype 1	4	0	0	0	0	0	0	0	0	0
Haplotype 2	3	1	3	3	3	6	0	0	0	0
Haplotype 3	2	0	0	0	0	0	0	0	0	0
Haplotype 4	0	0	0	0	0	6	2	8	0	0
Haplotype 5	0	0	0	0	0	0	0	0	1	0
Haplotype 6	0	0	0	0	0	0	0	0	0	3

* Mal = Malaysia, Jap = Japan, Indo = Indonesia, Phil = Philippines, Thai = Thailand, Aus = Australia, Micro = Micronesia, NC = New Caledonia, SriL = Sri Lanka

Table 5. Population structure investigated by AMOVA test of *R. apiculata* within the three major groups and within countries.

	Df	Sum of squares	Variance component	% total	P-value
Among groups	4	8.153	0.22053	55.9	< 0.0001
Among populations within groups	5	0.909	0.00569	1.44	< 0.0001
Within populations	35	5.889	0.16825	42.65	< 0.0001

Discussion

Overall, the 18 *R. apiculata* natural populations showed very low genetic diversity ($\pi = 0.00116 - 0.00197$) even though the allele frequencies greatly differed between the three major groups ($F_{ST} = 0.846$). In total, six cpDNA haplotypes were found and interestingly, out of 12 samples, six Australian haplotypes were shared with the common Malaysian haplotype while the other half consisted of a distinct haplotype, shared with populations in Micronesia and Guam. Haplotype diversity ($H_d = 0.518 - 0.722$) was similar to haplotype diversity detected by five nuclear loci in the same regions for *R. apiculata* (Ng et al., 2015). Genetic differentiation was previously described between the West and East coasts ($F_{CT} = 0.6911$) and the Western and South parts ($F_{CT} = 0.5597$) of the Malay Peninsula (Ng et al., 2015) which explains why the greatest number of different haplotypes were found in Malaysia.

The ML phylogenetic analysis showed four distinct clusters (IWP, SWP, Malay and SriL) which is consistent with previous studies (Figure 4) (Duke et al., 2002; Wee et al., 2014; Guo et al., 2017; Ngeve et al., 2017). Interestingly, populations from Embley and Cato Rivers in Australia showed divergence from the Danitree River and Trinity Islets samples. This can be explained by the major ocean currents despite having a very few migrants ($N_m = 0.05$). A strong directional asymmetry of gene flow in

adjacent populations was previously reported in Australia (Yan et al., 2016). The distinct cluster consisting of *R. mucronata* and *R. stylosa* was a sign that no hybrids were included within the *R. apiculata* sequences within our analyses (Ng et al., 2013; Chen et al., 2015). Sri Lanka formed a separate group, however another mangrove genus, *Xylocarpus*, shared haplotypes between the west coast of the Malay Peninsula and Sri Lanka and suggested the possibility of a sympatric distribution (Guo et al., 2017) and therefore, further sampling would be beneficial in Sri Lanka (Amarasignhe and Perera, 2017) to investigate possible gene flow.

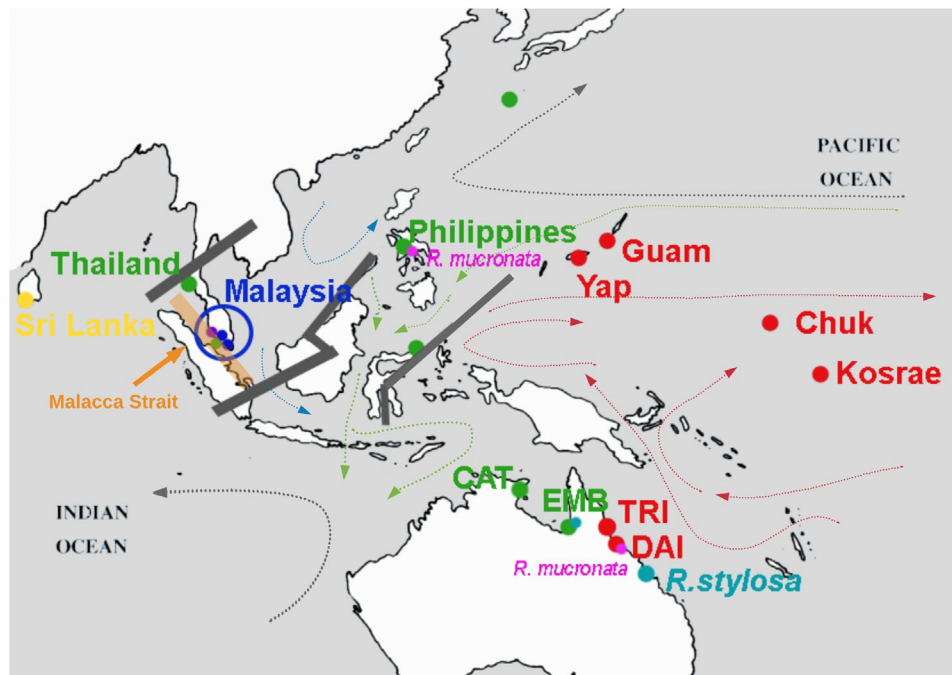


Figure 4. This study showed that there are four distinct conservation units based on ML phylogenetic analysis (Green, Red, Blue and Yellow in Figure 3). The major ocean currents are coloured according to which group they influence. Main land and oceanic barriers are incorporated in the map (Wee et al., 2014; Yan et al., 2016).

While no bottleneck effect was revealed in this study, Guo et al. (2016) found reduced effective population sizes in the Philippines (-3.26) and in Northern Australia in *R. apiculata* populations. Using microsatellites in 11 populations, Guo et al (2016) also revealed similar nucleotide diversity ($\pi = 0.00024 - 0.001327$) but higher nucleotide polymorphism ($\theta = 0.00224 - 0.00895$) compared to our results. They predicted asymmetric gene flow through the Malacca Strait with the isolation-with-migration model with higher number of migrants ($N_m = 0.328 - 2.134$) compared to our results ($N_m = 0.05$). They propose that the Sahul-land acted as a land barrier and blocked migrations of propagules. In *R. mucronata*, the gene flow did not appear to be restricted by geographical distance (Wee et al., 2014), so the study proposed that ocean surface circulation led to genetic discontinuities which are a sign of marine vicariance. Similar patterns of vicariance and bottleneck were detected in Micronesia and in New Caledonia (Wee et al., 2017). Yan et al. (2016) reported similar genetic diversity within and among populations from the Greater Sunda Islands of Indonesia ($F_S = 0.030 - 0.653$; $F_{ST} = 0.381$), however the AMOVA test showed that most variation occurred within populations (67%) as opposed among populations (33%) while our findings showed that more variation (60%) occurred among major groups than among the different countries (43%). A 'cryptic barrier' was identified between the South China Sea and Oceania in 18 populations of *Xylocarpus granatum* which were divided into three conservation units (Tomizawa et al., 2017). This study also divides *R. apiculata* populations into three conservation units (Malaysian, IWP and SWP).

High deficiency of heterozygosity and the presence of informative private alleles, null alleles and the possibility of Wahlund effect have been previously reported in mangroves (Arnaud-Haond et al., 2006; Wee et al., 2014; Ng et al., 2015; Guo et al., 2017). The high outcrossing rate, low gene flow, and inbreeding within subpopulations in previous studies reflect the capability of self-fertilisation (Coupland et al., 2006; Yahya et al., 2014; Mori et al., 2015). These suggest that mangroves are at high risk to continue to lose genetic diversity along with their rapid habitat destruction and fragmentation. Fine-scale genetic structure (FSGS, at 30 m) (Ngeve et al., 2017) in *R. racemosa* from the Cameroon Estuary complex (CEC), where historical effective migration rates greatly varied ($N_{em} = 0 - 167.61$), showed an inverse relationship between genetic differentiation (F_{ST}) and effective population size (N_e) but a positive relationship with disturbance. These populations might have consisted of hidden founders due to human impacts which expanded by local recruitment.

Future conservation management

This study proposes the management of three conservation units of *R. apiculata* (IWP, SWP and Malay). While the IUCN Red List status of this species is 'Least Concern', we highlighted the evolutionary processes which increase the rate of future genetic loss. By combining our findings with the Mangrove Restoration Map (The Mangrove Alliance, 2018b), we identified six focus areas, approximately 20,000 – 30,000 ha land in total, in Malaysia, Philippines, Papua New Guinea and Australia (Figure 5). Firstly, as deforestation and shrimp farming are the greatest threats to mangrove habitats, we suggest the protection of all mangrove habitats with the help of policies and the private sector (Friess et al., 2016). As these forests have a large capacity to store large volumes of carbon and can sequester 22.8 million metric tons of carbon annually (Sandilyan and Kathiresan, 2012; Sanderman et al., 2018), they can receive funding from REDD (Reduced Emissions from Deforestation and Degradation) programs (Jardine and Siikamaki, 2014; Aziz et al., 2015). Secondly, we recommend 'gapping-up' mangrove fragments as a 10-60 km habitat discontinuity can disrupt gene flow (Arnaud-Haond et al., 2006; Brinks et al., 2017). Thirdly, additional buffer zones between mangrove forests and development sites are needed to allow the delay of mangrove submergence in the future (Guo et al., 2017). While ex-situ plant conservation is cheaper (Li and Pritchard, 2009), due to the viviparous nature of mangroves, it is not a possibility.

For rehabilitation sites in Malaysia and the Philippines, local propagules should be used, following Brancalion and Chazdon's (2017) suggestions. As the SWP group consists of numerous small islands, exchanging propagules at a higher rate ($N_m = 1$) than the current number of migrants ($N_m = 0.05$) could help reduce the level of within-population inbreeding depression between New Caledonia, Guam and SWP side of Australia. Translocating propagules needs further research (Ellison, 2000) as the survival rate of saplings decreases with changing salinity (Aziz and Khan, 2001; Jayatissa et al., 2008), light availability (Farnsworth et al., 1996) and other abiotic and biotic factors (Krauss et al., 2008; Perry and Berkeley, 2009). In Sri Lanka, a mangrove plantation project cost USD 13 million and 54% of the planted saplings died within five years (Kodikara et al., 2017) while in Columbia 60% of 51,000 ha area died back due to human-induced hydrology changes. Careful planning is therefore crucial (Kamali and Hashim, 2011) with a focus on habitat heterogeneity (Farnsworth et al., 1998; Bosire et al., 2008), exclusion of invasive species (López et al., 2016) as well as continued monitoring of genetic diversity within the restored habitat (Granado et al., 2018). Successful restoration projects have managed to establish 2,500 seedlings/ha within three years at the cost of USD 1,300/ha (Wetlands International, 2018). If all potential mangrove restoration areas would be rehabilitated, the 'Gordian knot' of mangrove conservation would be loosened (Máñez et al., 2014), lowering the risk of the extinction of these unique habitats.

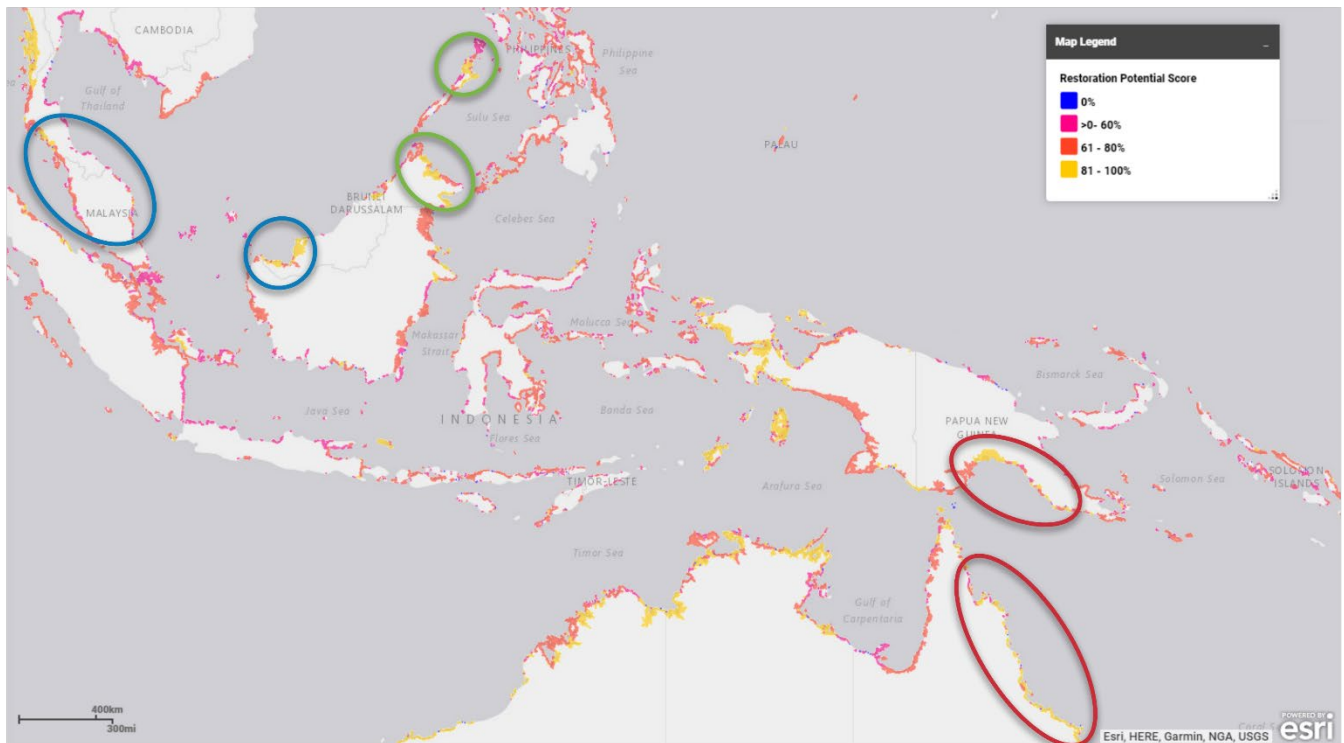


Figure 5. Using the Mangrove Restoration Map (The Mangrove Alliance, 2018b), the following circulated areas are identified as conservation focus areas with the corresponding colours to the conservation units identified in this study. These areas received high (> 75) restoration scores based on: sediment erosion, the tidal range recent sea level rise, projected future sea level rise, recent change in sediments, time since loss, average size of loss patches, and the proximity of loss areas to remaining mangroves.

Final word count (excluding within-text references): 1987

Appendix 1. Satellite images (Landsat 8, 2018) showing different habitat compositions and pressures of the main sample collection sites. In Australia, the Australian National Map (2018) was used to highlight in dark green the mangrove habitats. The picture frame colour reflects the three conservation units identified in this study.



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