BMC
Evolutionary Biology

RESEARCH ARTICLE

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Genetic differentiation and phylogeography of partially sympatric species complex *Rhizophora mucronata* Lam. and *R. stylosa* Griff. using SSR markers

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Abstract

Background: Mangrove forests are ecologically important but globally threatened intertidal plant communities. Effective mangrove conservation requires the determination of species identity, management units, and genetic structure. Here, we investigate the genetic distinctiveness and genetic structure of an iconic but yet taxonomically confusing species complex *Rhizophora mucronata* and *R. stylosa* across their distributional range, by employing a suite of 20 informative nuclear SSR markers.

Results: Our results demonstrated the general genetic distinctiveness of *R. mucronata* and *R. stylosa*, and potential hybridization or introgression between them. We investigated the population genetics of each species without the putative hybrids, and found strong genetic structure between oceanic regions in both *R. mucronata* and *R. stylosa*. In *R. mucronata*, a strong divergence was detected between populations from the Indian Ocean region (Indian Ocean and Andaman Sea) and the Pacific Ocean region (Malacca Strait, South China Sea and Northwest Pacific Ocean). In *R. stylosa*, the genetic break was located more eastward, between populations from South and East China Sea and populations from the Southwest Pacific Ocean. The location of these genetic breaks coincided with the boundaries of oceanic currents, thus suggesting that oceanic circulation patterns might have acted as a cryptic barrier to gene flow.

Conclusions: Our findings have important implications on the conservation of mangroves, especially relating to replanting efforts and the definition of evolutionary significant units in *Rhizophora* species. We outlined the genetic structure and identified geographical areas that require further investigations for both *R. mucronata* and *R. stylosa*. These results serve as the foundation for the conservation genetics of *R. mucronata* and *R. stylosa* and highlighted the need to recognize the genetic distinctiveness of closely-related species, determine their respective genetic structure, and avoid artificially promoting hybridization in mangrove restoration programmes.

Keywords: Biogeography, Coastal plants, Gene flow, Genetic diversity, Genetic structure, Indo-West Pacific, Mangrove, Microsatellite, Nuclear DNA, Population genetics

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Background

Mangrove forests are ecologically important but globally threatened intertidal plant communities [1]. Despite their crucial roles as sediment filter [2], carbon sink [3], breeding ground for coastal fauna [4] and coastal defense against storm surges [5] and tsunami [6], mangroves are facing global habitat loss—mainly due to land conversion—that surpasses those for other terrestrial ecosystems [7]. Increased awareness of mangrove loss has led to a surge in mangrove conservation worldwide, especially following the deadly tsunami in 2004 [8]. Effective mangrove conservation depends upon contemporary knowledge on taxonomy and phylogeography to clarify species identity, define management units, identify genetic structure and understand population connectivity [9,10]. In the long term, these should serve to preserve the evolutionary potential of mangroves via the identification and subsequent conservation of evolutionary significant units (ESUs). In this regard, genetic studies have contributed substantially by resolving taxonomical uncertainties [11,12] and identifying genetic stocks [13,14].

One of the most pressing species identity issues in mangroves concerns the iconic genus *Rhizophora*. In the Indo-West-Pacific (IWP), *Rhizophora* consists of three endemic species (*R. apiculata*, *R. mucronata* and *R. stylosa*), a variant of *R. mangle* from the Atlantic-East Pacific (AEP) that colonized the IWP (*R. samoensis*) [12], and two hybrids *R. × annamalayana* (*R. apiculata × R. mucronata*) and *R. × lamarckii* (*R. apiculata × R. stylosa*) [15-18]. *Rhizophora* is the most popular genus for mangrove restoration in the IWP [19-21], yet the distinction between the two major IWP *Rhizophora* species *R. mucronata* and *R. stylosa*, has remained elusive. Whereas *Rhizophora apiculata* is morphologically [15,22] and genetically [12,23] distinct from *R. mucronata* and *R. stylosa*, the latter two species are morphologically and genetically similar, and were even suggested to be variants of the same species [22]. *Rhizophora mucronata* and *R. stylosa* are dominant in the west and east IWP, respectively [24,25], with overlaps in distribution in Southeast Asia, Northwest Pacific Ocean and northern Australia (Duke et al. [22]). The diagnostic characteristics of these two species—the leaf morphology and the length of the style and propagule—were observed to have substantial intra-species variation and inter-species overlaps [18,22,26]. These possibly lead to local taxonomic confusion. For example, both *R. mucronata* and *R. stylosa* were reported to be dominant in Japan, even though only one common morphotype was observed [24,27,28]. The wide distribution range, large variation in morphological diagnostic characters, and the putative occurrence of hybridization thus undermines a clear distinction between *R. mucronata* and *R. stylosa*, presenting practical challenges to effective conservation.

Recent molecular studies are yet to resolve the taxonomic confusion between the two species. Phylogenetic analyses across the IWP with chloroplast DNA (cpDNA) and nuclear ITS sequence data did not support monophyly for either species, suggesting that they are genetically proximate and/or have experienced gene flow via introgressive hybridization [24,29]. This is further supported by evidence of a proposed natural hybrid between *R. mucronata* and *R. stylosa* in Malaysia [18]. Nevertheless, population genetics based on nuclear inter simple sequence repeat (ISSR) data [24] and nuclear genes [18] were able to discriminate the two species in sympatric populations, suggesting a certain level of reproductive isolation and genetic distinctiveness between them. However, whether the genetic distinctiveness can be found across the distributional range of the sibling species remain to be confirmed.
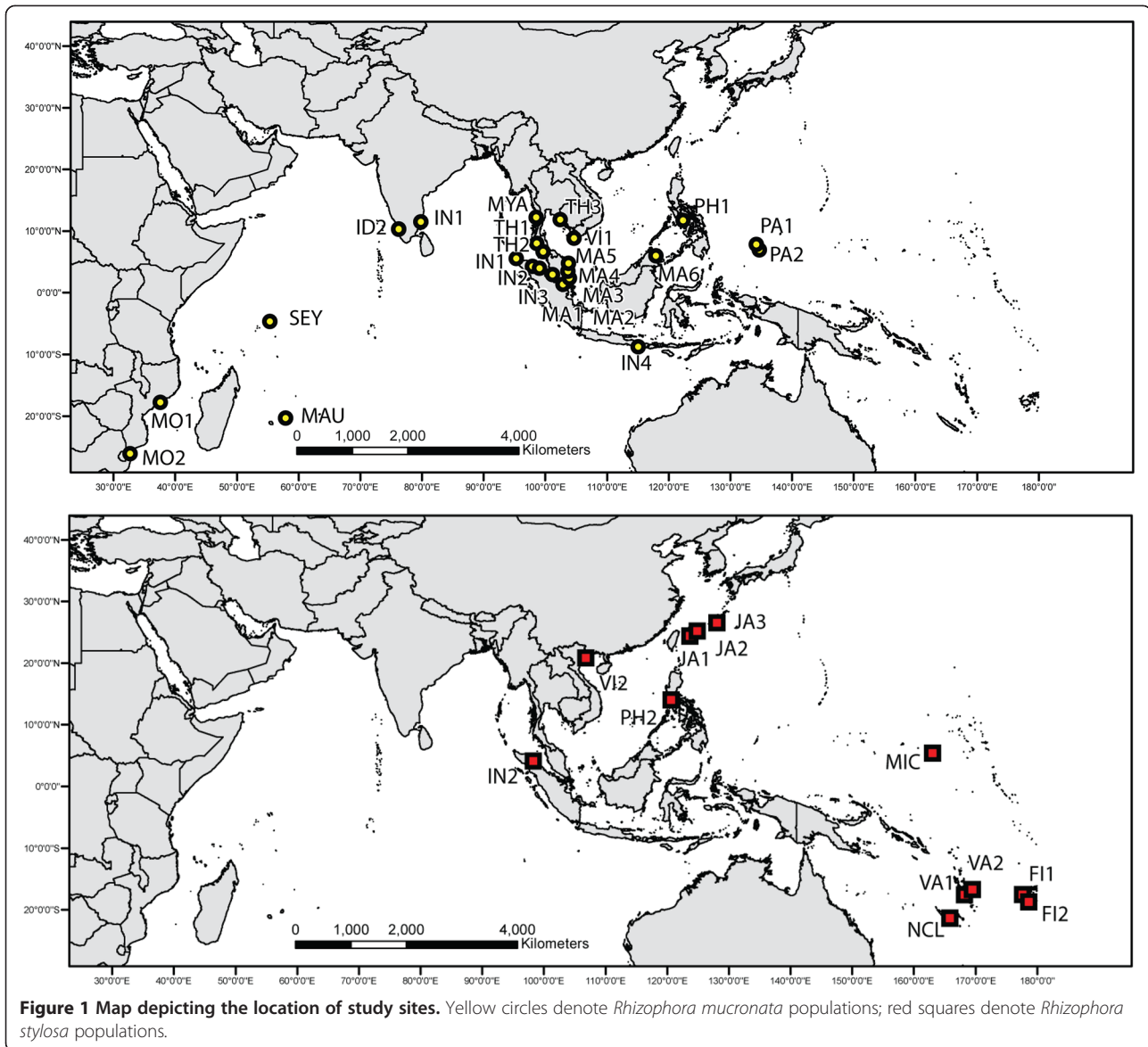
To resolve these problems on the genetic distinctiveness between *R. mucronata* and *R. stylosa*, we collected both species from their entire distribution range (Figure 1, Table 1) and genotyped them with 20 rapidly-mutating nuclear microsatellite (SSR) loci. The sample collection was conducted through an unprecedented level of international collaboration among mangrove scientists, whereby field identification and sampling in every site involved both local and international representatives of the cooperative network, using a standardized sampling protocol across a large geographical area. Specifically, we aim to determine the degree of genetic distinctiveness between these two species and their respective genetic structure.

Results

Genetic diversity

All loci were polymorphic, with the total number of alleles ranging from six to 16 per locus (mean = 10.25 alleles per locus) (see Additional file 1: Table S1 for genetic diversity parameters by locus). Low genetic diversity was detected in both species. The average observed heterozygosity (H_O) across all populations was 0.108 and 0.097 for *R. mucronata* and *R. stylosa*, respectively (see Additional file 1: Table S2 for genetic diversity parameters by population). Levels of observed heterozygosity, expected heterozygosity and allelic richness were not significantly different between the two species (Unpaired *t* test, p > 0.05 for all comparisons).

A general heterozygote deficit was detected in both species; a significant level of inbreeding (F_{IS}) was found in 85% and 67% of *R. mucronata* and *R. stylosa* populations, respectively (Additional file 1: Table S2). Deviation from Hardy-Weinberg Equilibrium (HWE) was significant in 235 out of 720 population–locus comparisons; seven of those were due to heterozygote excesses and the rest were associated with heterozygote deficits. All loci with heterozygote excesses were from the *R. mucronata* population in

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Figure 1 Map depicting the location of study sites. Yellow circles denote *Rhizophora mucronata* populations; red squares denote *Rhizophora stylosa* populations.

PA1. One quarter (25.8%) of the detected heterozygote deficits were associated with a particular population: 19 loci were from the *R. mucronata* population in PH1, and 18 and 15 loci were from the *R. stylosa* populations in MIC and FI2, respectively. Based on the null allele frequency estimated by FREENA, null alleles were potentially implicated (defined as null allele frequency > 0.10) in 30.1% of all population-locus combinations (see Additional file 1: Table S3 for null allele frequencies). Except for the four populations with heterozygote excess/deficit described above, the detection of potential null alleles was not associated with any locus or population.

Inter-species genetic differentiation

The genetic diversity detected by the 20 loci employed in this study was sufficiently informative to reflect the genetic distinction between species. All loci harboured alleles that were unique to either one or both species. These species-specific alleles made up 57.6% of the total number of alleles in our data set. The proportion of species-specific alleles harboured by each locus ranged from 16.7% in RM107 to 75% in RM110 (see Additional file 1: Table S1).

The partitioning of genetic variation, as revealed by Analysis of Molecular Variance (AMOVA), was comparable when categorizing populations by species or by oceanic regions (Table 2). Between species, most of the genetic variation was partitioned among populations within species. Among regions, most of the genetic variation was partitioned among populations within oceanic regions.

We detected an overall strong genetic structure across all populations, with significant genetic differentiation

Table 1 Location information for all populations

Location	Country	Oceanic region	Pop. code	N	Latitude	Longitude	Voucher
R. mucronata							
Quelimane	Mozambique	West Indian Ocean	MO1	34	17°52′58″S	36°51′40″E	OR10012802
Maupto	Mozambique	West Indian Ocean	MO2	37	25°51′00″S	32°41′44″E	TK11050101
Mahebourg	Mauritius	West Indian Ocean	MAU	32	20°25′41″S	57°43′34″E	OBY01012401
Mahe	Seychelles	West Indian Ocean	SEY	21	04°39′21″S	55°24′29″E	TK07101101
Cochin	India	Arabian Sea	ID1	27	09°59′29″N	76°14′07″E	TK07101201
Tamil Nadu	India	Bay of Bengal	ID2	32	11°25′52 N	79°47′38″E	TK07101401
Myeik	Myanmar	Andaman sea	MYA	30	12°23′53″N	98°34′11″E	TK12121401
Banda Aceh	Indonesia	Andaman sea*	IN1	33	05°34′33″N	95°19′08″E	TK07092705
Brandan	Indonesia	Andaman sea*	IN2	31	03°59′58″N	98°14′56″E	KT10073105
Jaring Halus	Indonesia	Strait of Malacca	IN3	27	03°56′22″N	98°33′46″E	KT10080201
Phuket	Thailand	Strait of Malacca	TH1	37	08°24′28″N	98°30′42″E	KT09012903
Palian	Thailand	Strait of Malacca	TH2	31	07°07′53″N	99°42′33″E	KT09012607
Klang	Thailand	Strait of Malacca	MA1	35	03°00′01″N	101°16′39″E	OR10012802
Benut	Malaysia	Strait of Malacca	MA2	39	01°36′22″N	103°16′17″E	TK11050101
Rompin	Malaysia	South China Sea	MA3	34	02°45′34″N	103°30′54″E	OBY01012401
Tanjung Lumpur	Malaysia	South China Sea	MA4	33	03°48′10″N	103°19′52″E	TK07101101
Paka	Malaysia	South China Sea	MA5	31	04°39′33″N	103°26′22″E	TK07101201
Nam Chiao	Thailand	South China Sea	TH3	32	12°09′57″N	102°28′36″E	TK07101401
Ca Mau	Vietnam	South China Sea	VI1	38	08°44′29″N	104°52′34″E	TK12121401
Sabah	Malaysia	South China Sea	MA6	30	05°56′19″N	118°03′10″E	TK07092705
Panay	Phillipines	South China Sea	PH1	35	11°47′28″N	122°13′41″E	KT10073105
Bali	Indonesia	Bali Sea	IN4	30	08°44′01″S	115°11′48″E	KT10080201
Airai	Palau	West Pacific Ocean	PA1	34	07°22′04″N	134°34′34″E	KT09012903
Karamadoo	Palau	West Pacific Ocean	PA2	23	07°30′12″N	134°32′09″E	KT09012607
R. stylosa							
Brandan	Indonesia	Strait of Malacca	IN2	22	03°59′58″N	98°14′56″E	OR10012802
Dong Rui	Vietnam	South China Sea	VI2	47	21°13′33″N	107°22′30″E	TK11050101
Lian Batangas	Phillipines	South China Sea	PH2	32	13°58′11″N	120°37′33″E	OBY01012401
Iriomote	Japan	East China Sea	JA1	30	24°24′09″N	123°46′28″E	TK07101101
Ishigaki	Japan	East China Sea	JA2	29	24°24′06″N	124°08′44″E	TK07101201
Okinawa	Japan	East China Sea	JA3	30	26°36′21″N	128°08′35″E	TK07101401
Kosrae	Micronesia	Northwest Pacific Ocean	MIC	31	05°20′55″N	163°01′09″E	TK12121401
Canala	New Caledonia	Southwest Pacific Ocean	NCL	28	21°30′26″S	165°58′09″E	TK07092705
Malatie	Vanuatu	Southwest Pacific Ocean	VA1	30	17°33′00″S	168°20′26″E	KT10073105
Maolapa	Vanuatu	Southwest Pacific Ocean	VA2	19	17°31′46″S	168°24′53″E	KT10080201
Lautoka	Fiji	Southwest Pacific Ocean	FI1	16	17°30′46″S	177°33′06″E	KT09012903
Mannikau	Fiji	Southwest Pacific Ocean	FI2	27	18°09′21″S	178°26′45″E	KT09012607

All specimens were identified by the collectors (one of the authors) designated by the initial at the first some letters of voucher information. Identification was confirmed by AWKS and TK.

*Although both Banda Aceh and Brandan are geographically located within the Strait of Malacca, Wee et al. [40] showed that these R. mucronata populations genetically clustered with the Andaman Sea populations. Hence we classified them under "Andaman Sea".

N, number of individual per population.

(F_{ST}) estimated at 0.737 averaging all loci and populations (p < 0.001). All pairwise population genetic differentiation was significant at the p < 0.001 level, except between adjacent *R. mucronata* populations MA1 and MA2 (pairwise F_{ST} = 0.013, p > 0.05), and *R. stylosa* populations VA1 and VA2 (pairwise F_{ST} = 0.044, p > 0.05).

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Table 2 AMOVA analysis comparing the genetic variation between species and among regions

	df	Variance component	Percentage variation	Φ-statistics
Between species				
Between species	1	2.675	31.02	0.310***
Among populations within species	34	4.098	47.53	0.689***
Within populations	2175.4	1.850	21.45	0.785***
Among oceanic regions				
Among regions	6	2.414	31.96	0.320***
Among populations within regions	29	3.256	43.11	0.634***
Within populations	2175.4	1.883	24.93	0.751***
Within *R. mucronata*				
Among regions	4	2.477	40.48	0.405***
Among populations within regions	18	1.899	31.04	0.522***
Within populations	1395.5	1.743	28.48	0.715***
Within *R. stylosa*				
Among regions	3	3.051	45.82	0.458***
Among populations within regions	7	2.004	30.11	0.556***
Within populations	605.8	1.602	24.07	0.759***

df; degree of freedom, ***; significant at the P < 0.001 level.

Pairwise F_{ST} estimates are listed in Additional file 1: Table S4. The PCoA results demonstrated a clear genetic differentiation between *R. mucronata* and *R. stylosa*, as well as among oceanic region in each species (Figure 2). There was no overlap between species, except for (1) several *R. mucronata* individuals from the South China Sea region observed in the *R. stylosa* clusters, and (2) an overlap of *R. mucronata* individuals from Bali Sea (IN4) with *R. stylosa* individuals from northwest Pacific Ocean (MIC). Model-based individual assignment via *STRUCTURE* was in agreement with the PCoA results. We found strong support for two genetic clusters among our samples that generally corresponded to the respective species (Figure 3). All *R. mucronata* individuals had > 90% of inferred ancestry from the same genetic cluster except for several individuals in populations SEY, IN2, PH1 and IN4. *R. mucronata* individuals from PA1 had more than 50% inferred ancestry from the *R. stylosa* genetic cluster, hence may represent putative hybrids between the two species. Similarly, mixed inferred ancestry was also found in *R. stylosa* individuals from MIC.

The relationships between populations in the NJ tree supported the findings from PCoA and *STRUCTURE*. Genetic clustering of populations was in concordance with their respective species and oceanic region (Figure 4). With the exception of *R. mucronata* population PA1 and *R. stylosa* population MIC, of which individuals were estimated to have mixed ancestry by *STRUCTURE*, the NJ tree supported a genetic distinction between *R. mucronata* and *R. stylosa*.
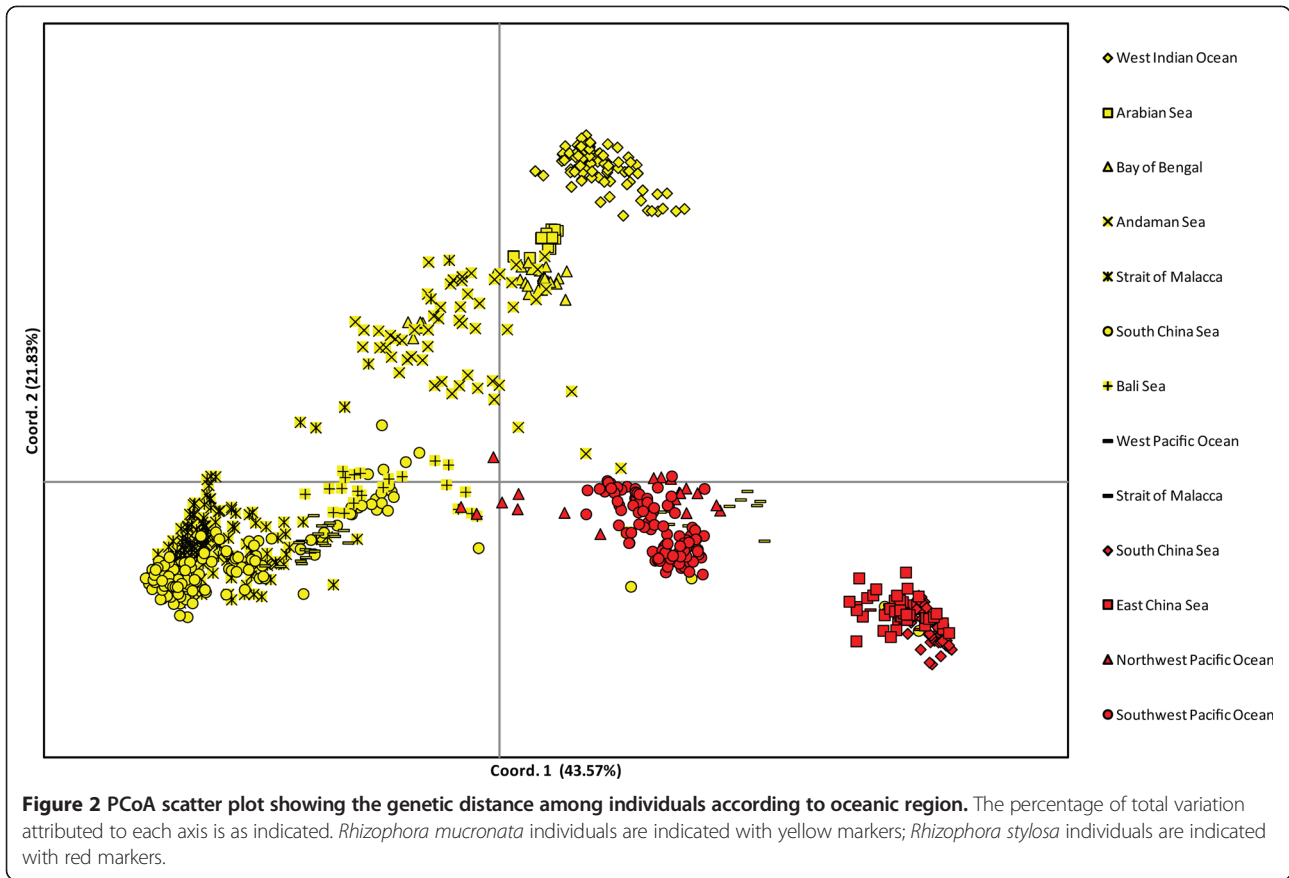
Intra-species genetic differentiation

STRUCTURE analysis with pure individuals (after removing both putative hybrids and possibly misidentified individuals) supported two genetic clusters (K = 2) for both *R. mucronata* and *R. stylosa* (Figure 5). In *R. mucronata*, the two genetic clusters were: (1) populations from West Indian Ocean, Arabian Sea, Bay of Bengal and Andaman Sea, and (2) populations from Malacca Strait, South China Sea, Bali Sea and West Pacific Ocean (Figure 6A). Low level of admixture was detected in *R. mucronata* populations from the Andaman Sea and Malacca Strait. Two genetic clusters were also detected in *R. stylosa*; the clustering pattern was more conspicuous than that of *R. mucronata*. A strong genetic break separated populations in the South China Sea and East China Sea from populations in the Southwest Pacific Ocean (Figure 6B). The genetic breaks were constantly supported with increasing number of clusters in STRUCTURE analyses. AMOVA analysis revealed that within each species, most of the genetic variation was partitioned among regions (40.48% and 45.82% for *R. mucronata* and *R. stylosa*, respectively) (Table 2).

Discussion

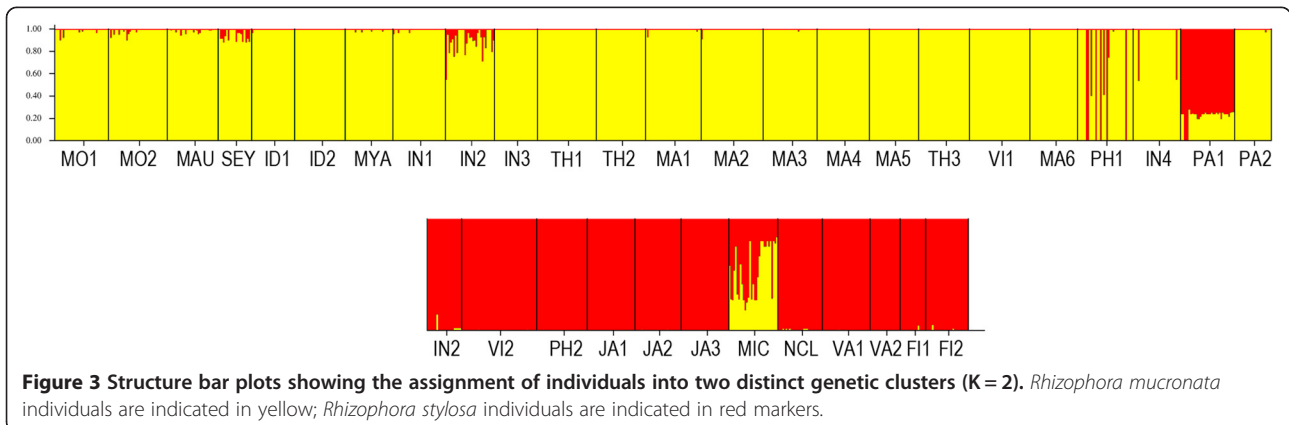### Genetic distinctiveness between *R. mucronata* and *R. stylosa*

Our study provides strong evidence of the genetic distinctiveness between *R. mucronata* and *R. stylosa*. Even though the genetic proximity of these two species is unquestionable—as all 20 SSR loci were successfully amplified in both species—we are confident of the genetic
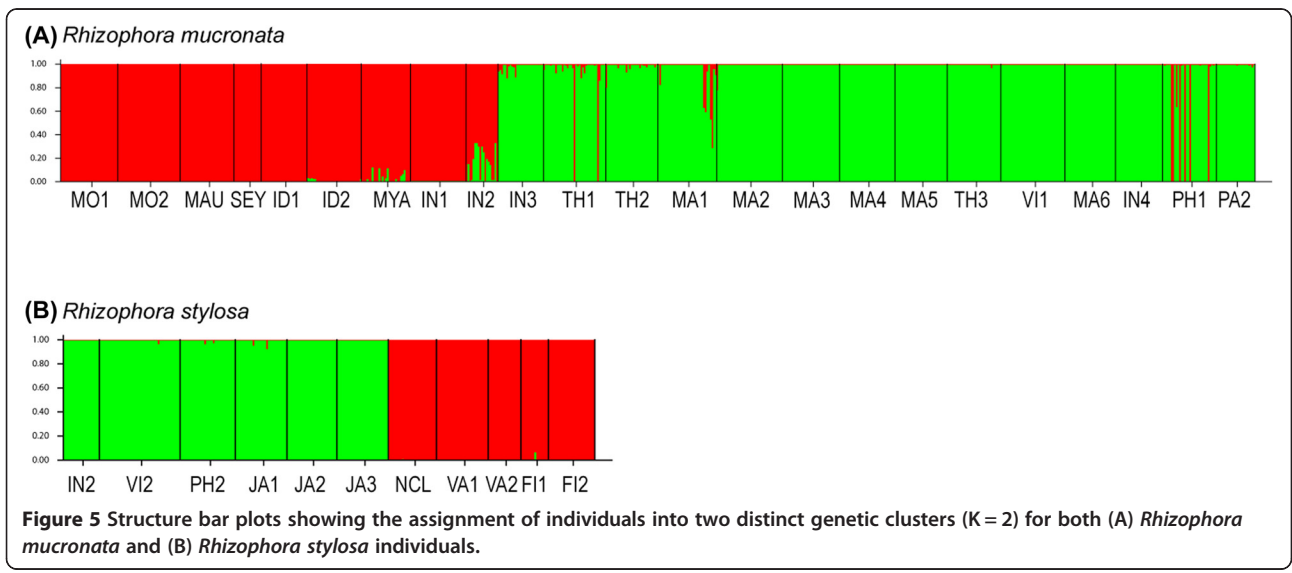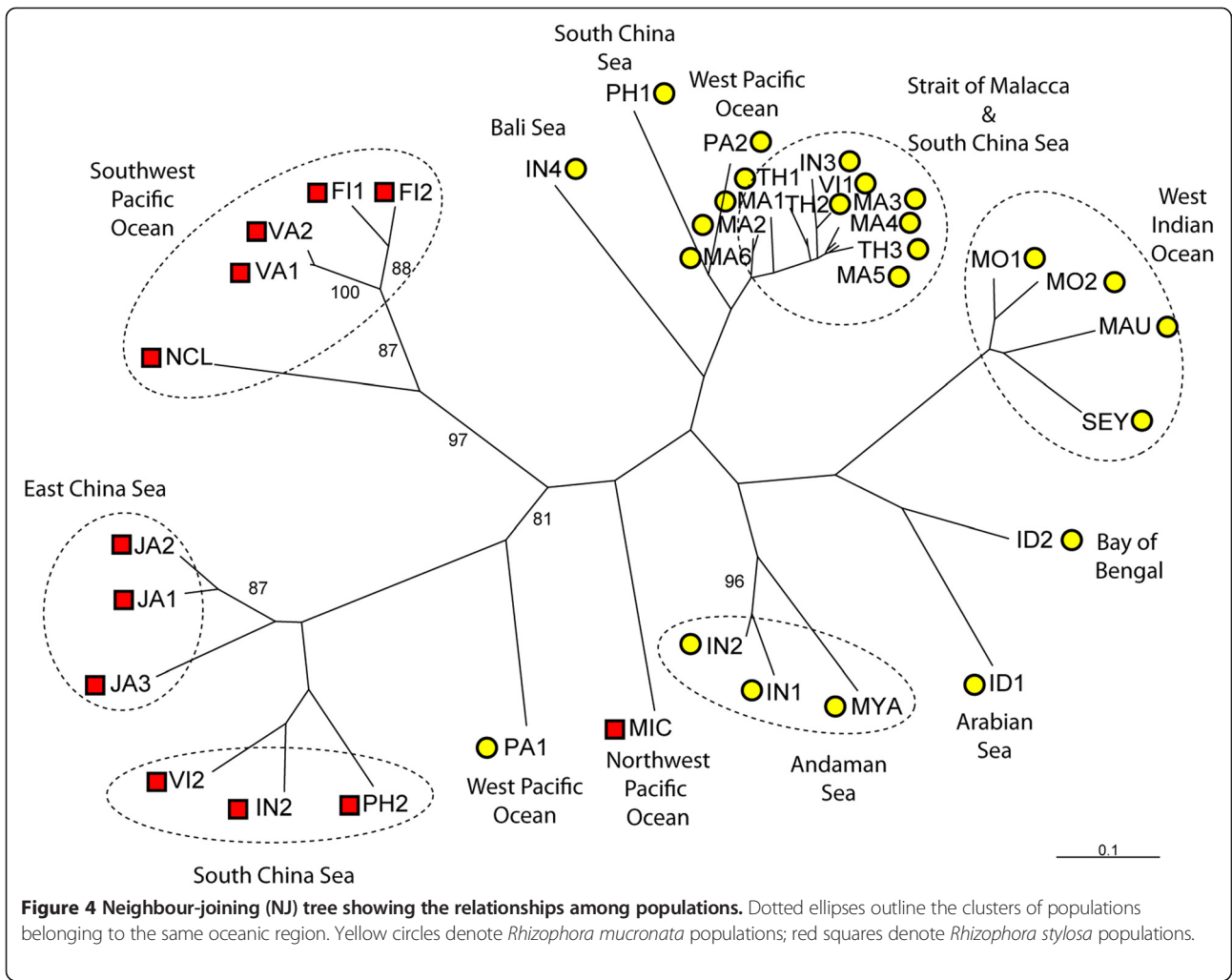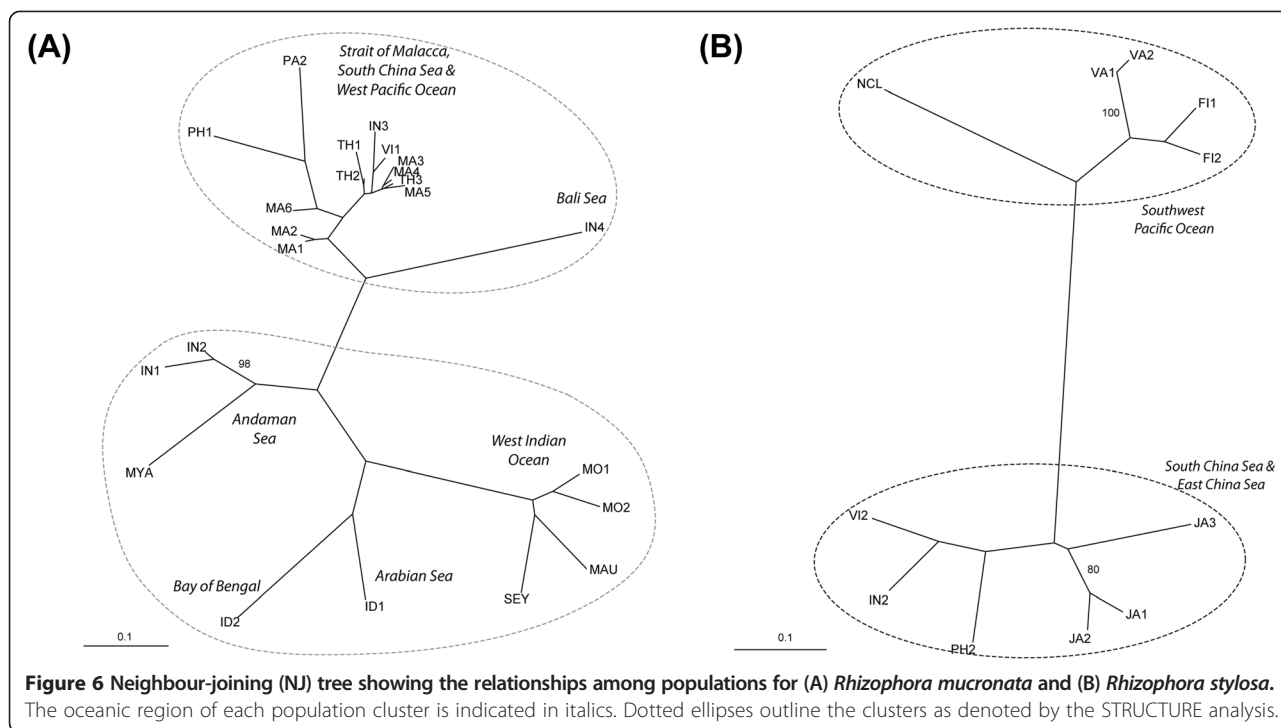
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Figure 2 PCoA scatter plot showing the genetic distance among individuals according to oceanic region. The percentage of total variation attributed to each axis is as indicated. *Rhizophora mucronata* individuals are indicated with yellow markers; *Rhizophora stylosa* individuals are indicated with red markers.

distinctiveness between both species based on two lines of evidences. First, a large proportion of alleles detected in our study were unique to each species. This indicated that the detected genetic divergence was not merely a difference in allele frequency, which may be prone to the effects of population processes such as bottlenecks and genetic drift [30]. Second, the genetic assignment of individuals by species was consistent with our field identification, even in populations from Southeast Asia where the distribution ranges of both species overlap (e.g. in

IN2 where both *R. mucronata* and *R. stylosa* were collected). Therefore, these two species remained as distinct genetic entities even in close geographical proximity, either via reproductive isolation or the fixation of alleles resulting from historical vicariance.

Due to the fine resolution afforded by polymorphic SSR markers, our study also detected admixed genotypes in *R. mucronata* population PA1 and *R. stylosa* population MIC despite a clear inter-species genetic divergence in all other populations. As most of the individuals in

Figure 3 Structure bar plots showing the assignment of individuals into two distinct genetic clusters (K = 2). *Rhizophora mucronata* individuals are indicated in yellow; *Rhizophora stylosa* individuals are indicated in red markers.

Figure 4 Neighbour-joining (NJ) tree showing the relationships among populations. Dotted ellipses outline the clusters of populations belonging to the same oceanic region. Yellow circles denote *Rhizophora mucronata* populations; red squares denote *Rhizophora stylosa* populations.

Figure 5 Structure bar plots showing the assignment of individuals into two distinct genetic clusters (K = 2) for both (A) *Rhizophora mucronata* **and (B)** *Rhizophora stylosa* **individuals.**

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Figure 6 Neighbour-joining (NJ) tree showing the relationships among populations for (A) *Rhizophora mucronata* and (B) *Rhizophora stylosa*. The oceanic region of each population cluster is indicated in italics. Dotted ellipses outline the clusters as denoted by the STRUCTURE analysis.

these two populations tend to have admixed genetic characteristics despite the clear morphological assignment to one species, we inferred that hybridization and continuous introgression might be occurring in these two populations. Genetic evidence recently confirmed the presence of hybrids between *R. mucronata* and *R. stylosa* [18,31], even though morphological intermediates and the lack of complete ecological reproductive isolation (in flowering phenology and niche specialization) between the two species have long suggested it [22]. Therefore, the presence of potentially hybrid-derived lineages between *R. mucronata* and *R. stylosa*, though few, raise doubts on the integrity of these two species and the most appropriate species boundary applicable to them.

Hybridization or introgression between two genetically distinct species is not uncommon among coastal organisms and is usually attributed to a recent overlap of distributional ranges following historical geographical separation [32,33]. The substantial geological age of the genus *Rhizophora* (estimated at 50 million years) [22] and its coastal distribution presented opportunities for repeated population contraction and expansion—and consequently reproductive isolation and introgression—among its species during the glacial-interglacial cycles. If indeed the dispersal centers of ancestral *R. mucronata* and *R. stylosa* were as postulated in East Africa and Australasia, respectively [22], then the present interglacial period would have brought these two species into contact. Hence, hybridization or introgression in sympatric populations would be possible. Previous studies on coral

reef fishes reported a marine hybrid hotspot at Christmas and Cocos Islands, located at the Indo-Pacific biogeographic border [34]. Our data, coupled with that of Ng *et al.* [18], suggested that the hybrid zones for *R. mucronata* and *R. stylosa* might be much wider and located further eastward, between Southeast Asia and Micronesia.

Phylogeography of *R. mucronata* and *R. stylosa*

By excluding genetically mixed individuals from the analysis, our study was able to provide a more definitive representation of the intra-species genetic structure. The phylogeography of *R. mucronata* and *R. stylosa*, investigated independently after removing putative hybrids, demonstrated a close association between the genetic structure and oceanic region. This supports the general genetic patterns in marine and coastal species whereby ocean currents act to maintain gene flow within an oceanic region and prevent gene flow between oceanic regions [35].

In *Rhizophora mucronata*, a strong divergence was detected between populations from the Indian Ocean region (Indian Ocean and Andaman Sea) and the Pacific Ocean region (Malacca Strait, South China Sea and Northwest Pacific Ocean). The dichotomous genetic differentiation into Indian and Pacific Ocean lineages have been reported in other mangrove species [36,37] and coastal fauna [38,39], and was attributed to the role of Sundaland as a land barrier during past glaciations periods. *R. mucronata* population IN4, located at the boundary between the two oceanic regions, was an exception to this dichotomous division. Our results showed that IN4 was included in the

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Pacific Ocean genetic cluster, thus indicating that it either shared the same ancestry as, or had maintained frequent gene flow with, the other populations from the Pacific Ocean. The genetic break we detected at the northern boundary of the Malacca Strait concurred with an earlier study involving a smaller geographical coverage of Southeast Asia [40]. An analysis of regional oceanic circulation patterns suggested that contemporary oceanic currents may act as a cryptic barrier to gene flow that prevents admixture across this genetic boundary [40].

A dichotomous divergence was also observed in *R. stylosa*, though the genetic break was located more eastward, between populations from South and East China Sea and populations from the Southwest Pacific Ocean. This was supported by a previous study which found a genetic disjunction between populations from the Malay Peninsula and Japan [41]. The location of the genetic break coincided with complex surface currents at the western equatorial Pacific Ocean—aptly described as a "water mass crossroads" [42]—that may have prevented gene flow between the two oceanic regions. Similar north–south genetic divergence observed around the equator was also observed in the phylogeography of Atlantic coral fishes, indicating that oceanography has a substantial influence on the genetic structure of sea-dispersed organisms [43]. The detection of this genetic break together with the presence of putative hybrids in the MIC (Kosrae) population calls for more detailed investigation on the gene flow among *R. stylosa* populations from the Pacific islands located around the equator, as well as cross-comparisons with other sympatric mangroves, such as *Bruguiera gymnorhiza*.

Our findings differed from that of a previous investigation using chloroplast DNA and nuclear ribosomal DNA, which showed that *R. apiculata*, *R. mucronata* and *R. stylosa* shared similar genetic structure [24]. Lo *et al.* [24] reported that a common genetic break—separating the populations into one cluster from Southeast Asia and Sri Lanka and another from Africa, Australia and the Pacific—was found in all three species. As both studies employed molecular markers with different mutation properties, it is possible that the data sets represent genetic patterns across different ecological and evolutionary time scales. Our findings, generated by nuclear SSR markers, tend to demonstrate more contemporary gene flow while those from Lo *et al.* [24] represented historical gene flow that may have dated back to the Oligocene-Miocene boundary circa 29–24 Ma. Future studies, with finer-resolution molecular dating and additional sampling at the genetic boundaries will be able to fill in the gap between both studies and further elucidate the colonization pathways and species boundaries between *R. mucronata* and *R. stylosa*.

The genetic clusters we identified in *R. mucronata* and *R. stylosa* provide a basis for the definition of ESUs in these species. Even though the exact levels of molecular phylogenetic distinctiveness required for the definition of ESUs are still debatable [44,45], the two genetic clusters found in each species were geographically discrete, suggesting prolonged genetic and physical isolation. Hence, populations in these two clusters should be considered as distinct ESUs. Our genetic data can be further combined with morphological measurements [22] to verify the adaptive significance of the observed divergence in allele frequencies. We recommend that these genetic clusters should be managed separately and care should be taken to avoid artificial transplantation of individuals from a different cluster.

Low genetic diversity and heterozygosity
In this study, low levels of genetic diversity and heterozygosity were widespread in *R. mucronata* and *R. stylosa* populations. Excessive homozygosity has been shown in *Rhizophora* [41], and is common in mangroves and mangrove associates, and may be attributable to low genetic diversity at range limits [46,47], the presence of null alleles [48,49] or inbreeding [50-52]. Since the studied populations were from the entire species distribution range, the observed heterozygote deficit is unlikely to result from low genetic diversity at the range limit. Our data indicated that null alleles might be present at several loci (frequency < 0.2) and may have led to heterozygote deficit. However, null allele frequencies can be overestimated in inbred populations that are not under HWE [53]. Inbreeding and self-compatibility are expected to be higher in mangroves than in other tropical plants because these are traits that facilitate the colonization of distant locations [54]. Indeed, *Rhizophora* species have been shown to be self-compatible [27,55,56], possessing flowers that are mainly wind-pollinated but with facultative pollination by small insects [27,57]. For example, pollinator limitation is common in *R. stylosa*, which exhibits a typical fertilization rate of only 3-4% under natural conditions [57,58]. Thus, pollinator limitation, which often leads to selfing [59], may be widespread in *Rhizophora*. Inbreeding in *R. mucronata* and *R. stylosa* could be similarly expected in naturally fragmented mangrove habitats, as fragmentation reduces pollen availability and the number of pollen donor in wind-pollinated plants [60,61]. Since the excess of homozygotes is in concordance with the biology of *Rhizophora*, we interpret this as a result of inbreeding rather than the presence of null alleles.

Conclusions
Our study represents the first population genetic studies covering the entire distributional range of the species complex *R. mucronata* and *R. stylosa*. By employing a suite of 20 informative nuclear SSR markers, we demonstrated the general genetic distinctiveness of *R. mucronata*

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and *R. stylosa*, and potential hybridization or introgression between them. Since inter-species gene flow was implicated, we investigated the population genetics of each species without the putative hybrids, and found strong genetic structure between oceanic regions in both *R. mucronata* and *R. stylosa*. Both species showed a dichotomous genetic divergence among their respective populations. Even though the locations of the genetic break were different for each species, they coincided with the boundaries of oceanic currents, thus suggesting that oceanic circulation patterns might have acted as a cryptic barrier to gene flow.

Our findings have important implications on the conservation of mangroves, especially relating to replanting efforts and the definition of ESUs in *Rhizophora* species. Previous studies on Californian seagrass revealed that unintentional anthropogenic mixing of two genetically distinct species in a transplantation effort might have promoted recent hybridization and introgression between them [62]. This highlighted the need to recognize the genetic distinctiveness of closely-related species, determine their respective genetic structure, and avoid artificially promoting hybridization in mangrove restoration programmes. Hence, our results serve as the foundation for the conservation genetics of *R. mucronata* and *R. stylosa* by outlining their respective genetic structure and identifying geographical areas that require further investigations.

Methods

Population sampling and genotyping

From 2008 to 2012, *R. mucronata* and *R. stylosa* samples were collected from a total of 24 and 12 populations, respectively, from the entire Indo-West Pacific region (16–47 individuals per population) (Table 1). Sample collection was conducted under the Research Network for Conservation Genetics of Mangrove. To ensure consistency, species identification was performed by one local and one international representative of the network according to morphological characteristics that were previously reported to be useful in distinguishing both species [15,22] and local knowledge of species distribution. Voucher specimens used for the identification were deposited in URO (the University of Ryukyus). A leaf sample was collected from each individual and dried in silica gel. Genomic DNA was extracted using a modified CTAB method [63].

Twenty SSR loci were employed in this study. Twelve were developed for *R. mucronata*: RM102, RM103, RM107, RM110, RM111, RM112, RM114, RM116, RM121 [64], RMu21, RMu35 and RMu54 [65]; and eight were developed for *R. stylosa*: Rhst01, Rhst02, Rhst11, Rhst13, Rhst15 [66], RS19, RS59 and RS78 [67]. All loci were genotyped using fluorescent-labeled primers with the following dye-primer combinations – 6-FAM: RM102, RM107, RM110,

Rhst01, Rhst02, Rhst11, and Rhst15; VIC: RM103, RM114, RM116, RMu35 and RS19; NED: RM121, RMu21 and RMu54; PET: RM111, RM112, Rhst13, RS59 and RS78. The polymerase chain reaction (PCR) conditions were: initial denaturation 5 min at 94℃; 35 cycles of 45 s at 95℃, 45 s at 50℃, 45 s at 72℃; final elongation of 10 min at 72℃. Total reaction volume was 10 μL, of which 1.5 μL was DNA. PCR was conducted with iTaq DNA polymerase (i-DNA Biotechnology, Singapore). PCR products were run on an ABI 3130xl automated sequencer with the GeneScan-600 LIZ size standard and analysed using Genemapper 4.1 (Applied Biosystems, Grand Island, NY, USA).

Genetic diversity and heterozygote deficiency

To estimate the genetic diversity within population, expected heterozygosity (H_E) and observed heterozygosity (H_O) were calculated using GenAlEx 6.5 software [68]. The software fstat 2.9.3.2 [69] was used to compute the allelic richness (A_R) and heterozygote deficiency (estimated by F_{IS} and assessed at the P < 0.05 significance level). Allelic richness was rarefied to the minimum sample size of 16 individuals.

Null allele frequencies were estimated for each locus and population with FREENA [70], using the expectation maximization algorithm of [71]. Deviations from HWE were tested for each locus and population by an exact test using Genepop 3.4 [72].

Inter-species genetic differentiation

The pattern for genetic differentiation was visualized via one individual-based analysis, the Principal Coordinate Analysis (PCoA), and two population-based analyses, an unrooted consensus neighbor-joining (NJ) tree and a Bayesian model-based clustering method implemented in the software STRUCTURE [73]. PCoA was performed using GenAlEx v6.5 software [67] based on the mean genotypic distance between all individual pairs of both species. The NJ tree was generated with POPULATIONS v.1.2.31 [74] using Nei et al.'s (1983) D_A as an estimator for the genetic distance between populations [75]. The STRUCTURE clustering analysis was conducted by employing the admixture model, with 20 runs for each number of subpopulations (K), from K = 1 to K = 15. Each run consisted of 10^6 replicates of the Markov chain Monte Carlo (MCMC) after a burn-in of 10^5 replicates. The most likely number of population clusters was estimated by the ΔK parameter [76] using the Structure Harvester online program [77].

AMOVA analysis was conducted to determine the partitioning of genetic variation under two scenarios: (1) populations were grouped according to species; and (2) populations were grouped according to oceanic region regardless of species (refer to Table 1 for oceanic region

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categories). The AMOVA was conducted in Arlequin with a 10,000 permutations [78].

Genetic differentiation within-species

For each species, population differentiation (F_{ST}) averaged across all loci, and pairwise F_{ST} estimates between all population pairs [79] were calculated using fstat 2.9.3.2. [69].

Based on results from inter-species STRUCTURE analysis, putative hybrids (genetically mixed individuals) between two species were identified and removed from the following analysis. The hybrids were defined as individuals with < 90% of its genotype having an inferred ancestry from either species when K = 2 for the inter-species STRUCTURE analysis. The genetic clusters were determined using STRUCTURE [73] with the same run parameters as the inter-species analysis.

Availability of supporting data

The data set supporting the results of this article is available in the Dryad repository [80], http://datadryad.org/resource/doi:10.5061/dryad.42711/1.

Additional file

Additional file 1: Table S1. Genetic diversity parameters of the twenty microsatellite loci employed in this study. **Table S2.** Genetic diversity parameters of sampled populations. **Table S3.** Null allele frequency as estimated by FREENA for each population-locus comparison. **Table S4.** Pairwise FST estimates between all population pairs.

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