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PRIMARY RESEARCH PAPER

Chloroplast phylogeography of a widely distributed mangrove species, *Excoecaria agallocha*, in the Indo-West Pacific region

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Abstract *Excoecaria agallocha* is one of the predominant mangrove species in the Indo-West Pacific (IWP) region with an extensive range of distribution. To infer the current geographical patterns of genetic variation and provide new insights on the historical population dynamics of mangrove species in the IWP region, we sampled *E. agallocha* across its distribution range and investigated the phylogeography of this species using four chloroplast DNA (cpDNA) fragments. Our results showed that *E. agallocha* possessed a high degree of species-level genetic diversity, while the average genetic diversity within populations was much lower. The presence of population genetic structure was supported by the estimates of genetic differentiation and the analysis of molecular variance (AMOVA). Of the ten haplotypes identified, no haplotypes were shared between the East Indian Ocean (EIO), the West Pacific Ocean (WPO), and the North Australian (NA) regions. Genealogy analysis, haplotype distribution patterns, and the principal coordinate analysis (PCoA) consistently suggested the existence of three haplotype groups distributed in distinct geographical locations. The genealogical breaks observed and further analysis of geographic/genetic barriers indicated that both land barriers and oceanic currents may have played important roles in the divergence and demography of *E. agallocha*.

Keywords Genetic diversity · Genetic structure · Genealogy · Chloroplast DNA · Phylogeographic subdivision

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Introduction

Mangroves constitute among the most dominant intertidal ecosystem along the tropical and subtropical coastlines. The distribution of mangrove populations is considered to be largely shaped by their responses to colder climate and arid conditions at the limit of their ranges, and have been expanding their ranges along changing coastal zones since the last glacial period (Duke et al., 1998; Saenger, 1998; Dodd et al., 2002; Saintilan et al., 2014). Most mangrove species have viviparous propagules that are buoyant and can be dispersed by ocean currents. These make mangrove species good candidates for the study of population genetic structures influenced by founder effects resulting from frequent local extinction, recolonization, and long-distance dispersal (LDD) of propagules by water currents along coastlines of different forms.

Moreover, the accurate assessment of genetic diversity and population genetic structure of mangroves is essential for the preservation and management of this unique ecosystem, and can also provide us a perspective of evolutionary mechanisms that shaped the current diversity and adaptability of coastal species (Urashi et al., 2013).

The geographical distribution of a species is a key determinant of population subdivision and microevolution. The spatial configuration of populations, combined with physical features of the landscape, can either restrict or facilitate gene flow by affecting the movement of individuals or gametes (i.e., pollen) (Slatkin, 1987). For mangrove species, population subdivision across a distribution range resulting from lack of gene flow can be explained by the effects of three factors: geographic distance, land barriers, and ocean currents. First, gene flow through propagule and pollen dispersal could be geographically restricted and genetic exchanges would be limited to neighboring populations, leading to genetic differentiation increasing with physical distance, a process termed isolation-by-distance (IBD; Kimura & Weiss, 1964). Inconsistent evidence on the presence of IBD has been observed in mangrove species (e.g., Dodd et al., 2002; Mori et al., 2015; Ngeve et al., 2016). Since species or region-specific characteristics could exist and resolving the impact of geographic distance on population subdivision in mangrove species will not likely result in a one-size-fits-all conclusion, extended efforts are needed to further explore the impacts of geographical distance on shaping the genetic structure of various mangrove populations. Second, as plants with sea-drifted seeds cannot migrate through land, gene flow among populations of a mangrove species can be blocked by land barriers (Wiley, 1988; Taberlet et al., 1998). For example, genetic discontinuities across the landscape have been examined for mangroves in the Indo-West Pacific (IWP) in several previous studies (e.g., Tan et al., 2005; Su et al., 2006, 2007; Liao et al., 2007), indicating that both the Malay Peninsula and Indonesian archipelago may have acted as land barriers that prevented gene flow among the East Indian Ocean (EIO), West Pacific Ocean (WPO), and North Australia (NA). Third, ocean currents may also be an important force promoting population subdivision thus affecting genetic structure of the extant mangrove populations. For example, ocean currents may act as a barrier to gene flow among geographically proximate marine populations (Ayre & Dufty, 1994; Waters, 2008; Wee et al., 2014). As genetic isolation of populations may result from a complex combination of multiple mechanisms, an explicit interpretation of phylogeography and genetic structure is necessary to clarify the evolutionary forces and historical population dynamics that a species is subjected to.

Excoecaria agallocha L. (Euphorbiaceae), also known as the "milk mangrove," is the most predominant mangrove species in the genus. This species grows on sandy soil or sandy mud near the terrestrial fringes of mangrove vegetation, and is widely distributed from east Africa to Samoa (Tomlinson, 1986). Besides being an important part of the specific costal ecosystem of mangroves, *E. agallocha* is also important for its latex, which contains powerful chemicals being used on sores and to treat marine stings. The leaves are also used for fishing, and the leaf sap is currently being tested for its medicinal properties (Das et al., 2011). *E. agallocha* is dioecious and produces small and buoyant seeds, which are about 3 mM in diameter, with an air space in the seed coat. Although the exact dispersal ability of these seeds (i.e., the dispersal unit in *E. agallocha*) has not been experimentally tested, their characteristics (i.e., small size and buoyant) are thought to help them float on water and, with the help of surface currents, disperse over long distances (Zhang et al., 2008; Das et al., 2011). Unlike most other mangrove species however, *E. agallocha* is non-viviparous, and thus represents a suitable model for studying the phylogeographic patterns and population dynamics of non-viviparous mangrove populations in the IWP. Although many studies (e.g., Tan et al., 2005; Su et al., 2006, 2007; Liao et al., 2007) have been conducted on the genetic variation, population structure, and demography of various mangrove species, to our knowledge, only three reports (i.e., Lakshmi et al., 2000; Zhang et al., 2008; Das et al., 2011) exist for *E. agallocha*. All these studies used dominant markers (i.e., RAPD and RFLP in Lakshmi et al., 2000; ISSR in Zhang et al., 2008; RAPD and ISSR in Das et al., 2011) and were restricted in terms of the sampling regime. Although dominant markers allow the interpretation of genetic diversities and genetic structuring of populations, the inference of spatial haplotype distribution is not possible. Maternally inherited genetic markers, i.e., chloroplast (cp) DNA, are considered especially

relevant to phylogeographic surveys (Triest et al., 2008). Most phylogeographic studies of plants have been based on cpDNA and have revealed genetic heterogeneity throughout the range of a species and allowed an inference of historical range shifts and recolonization routes (Bai et al., 2010). Besides, to draw a general picture of genetic structure and evolutionary history, a survey of populations covering most, if not all, areas of a species' distribution is necessary.

In the present study, we examined the phylogeographic patterns of *E. agallocha* through cpDNA sequence data and an extensive sampling across the entire range of the species. Fifty-six cpDNA fragments were initially screened, and four loci that showed polymorphism were further sequenced and analyzed for 419 individuals from 47 populations. Our major objectives were to (1) characterize the levels of genetic variation across the geographical range of *E. agallocha*; (2) determine the genetic structure among these populations; and (3) trace the historical range shift of *E. agallocha*, and interpret how historical, geographical, and ecological factors (i.e., climate oscillations, land barriers, and oceanic current barriers) may have influenced the present distribution pattern of this species.

Materials and methods

Sample collection

A total of 419 leaf samples from 47 natural populations of *E. agallocha* were collected across its entire distribution range in the IWP region, including 13 populations from the East Indian Ocean (EIO) region, 32 populations from the West Pacific Ocean (WPO) region, and two populations from the North Australian (NA) region. The sampling details are listed in Table 1. The sampled individuals within each population were located at least 5 m apart. Given that *E. agallocha* populations usually do not occur as monospecific stands and that the usual distance criterion for mangrove sampling is 3–10 m (Cerón-Souza et al. 2012; Ruan et al., 2013; Dasgupta et al., 2015; Ngeve et al., 2016), the minimum spacing of 5 m used here is adequate and familial sampling could largely be avoided under such a sampling scheme. Plant materials were stored with silica gel in zip-lock plastic bags until DNA isolation.

DNA extraction and PCR amplification

Genomic DNA of each individual was extracted using the CTAB method (Doyle & Doyle, 1990). A total of 56 published primer pairs for different cpDNA regions (Taberlet et al., 1991; Small et al., 1998; Hamilton, 1999; Shaw et al., 2005) were initially screened on a subset of five individuals per population randomly collected from six populations (i.e., ADR, STC, SSB, TPN, HBR, and NSL) that were the farthest apart. Only four fragments that showed polymorphism within or among populations (i.e., *trnV-trnM, petB-petD, rpoC1-rpoC2,* and *trnC-rpoB*) were subsequently analyzed for all samples.

DNA amplification was carried out in 30μl PCR reaction mixtures containing approximately 1 μg of total DNA, 5 pmol of each primer, 10 mM of Tris–HCl (pH 8.4), 1.5 mM of $MgCl_2$, 0.1 mM of dNTP, and 2 units of *Taq* polymerase (Shengong Inc., Shanghai, China). PCR reactions were performed under the following cycle profile: initial denaturation at 94°C for 5 min, followed by 45 s at 94°C, 45 s at 53°C, and 1.5 min at 72°C for 30 cycles, and 10 min at 72°C for final extension. The PCR products were separated on 1.0% agarose gel, stained with ethidium bromide and viewed under UV light. Purified DNA fragments were then sequenced for both strands on an ABI 3730XL DNA Analyzer (Applied Biosystems, Inc.).

Data analysis

The chloroplast DNA sequences were assembled and manually edited using SeqMan™ (DNASTAR). The sequences of *E. agallocha* generated in this study have been deposited in GenBank under the accession numbers MF381193–MF381206. The geographic distribution of cpDNA haplotypes was plotted on a map using GenGis v2.11 (Parks et al., 2009), and the relationship among haplotypes were inferred using the median-joining method implemented in NETWORK v4.6.1.2 (Bandelt et al., 1999). Continuous indels were treated as single mutational events in the analysis.

Haplotype diversity (*H*d) and nucleotide diversity (π) were calculated using DnaSP v5.10.1 (Librado & Rozas, 2009). Average gene diversity within

Table 1 Sampling locations, sample size (N), number of each chloroplast haplotype (C1–C10) per population, and estimates of haplotype diversity (*H*d) of *E. agallocha*

Region	Population	Code	ID	Latitude	Longitude	*N*	Haplotypes	*H*d
EIO	Negombo, Sri Lanka	NSL	44	N08°00′	E 79°50′	6	C2 (6)	0.00
EIO	Pambala, Sri Lanka	PSL	42	N08°00′	E 79°50′	5	C2 (5)	0.00
EIO	Rekawa, Sri Lanka	RSL	43	N06°04′	E 80°48′	6	C2 (6)	0.00
EIO	Harbaria, Sundarban, Bangladesh	HBR	40	N22°16′	E 89°36′	12	C1 (10), C2 (2)	0.30
EIO	Katka, Sundarban, Bangladesh	KKS	41	N21°51′	E89°46′	10	C1 (7), C2 (3)	0.47
EIO	La-un District, Ranong, Thailand	TLU	37	N10°10′	E98°43′	9	C1 (9)	0.00
EIO	Bang Ben, Kapoe, Thailand	TBBK	36	N09°35′	E98°29′	10	C1 (1), C2 (9)	0.20
EIO	Phang-Nga, Thailand	TPN	38	N08°19′	E98°25′	7	C2 (7)	0.00
EIO	Phuket, Thailand	TPK	39	N07°49′	E98°22′	8	C2 (8)	0.00
EIO	Langkawi, Malaysia	LAN	30	N06°25′	E99°52′	11	C2 (11)	0.00
EIO	Penang, Malaysia	PN	29	N05°24′	E100°13′	6	C2 (6)	0.00
EIO	Kuala Sepetang, Malaysia	KSP	28	N04°51′	E100°39′	10	C2 (10)	0.00
EIO	Kuala Selangor, Malaysia	KSL	31	N03°21′	E101°15′	8	C2 (8)	0.00
WPO	Samut Songkhram, Thailand	TBK	35	N13°34′	E100°26′	9	C3 (9)	0.00
WPO	Krong Kaeb, Cambodia	KEP	45	N10°29′	E104°20′	11	C3 (10), C6 (1)	0.18
WPO	ChaiYa, Thailand	TCY	33	N09°22′	E99°15′	10	C3 (8), C6 (2)	0.36
WPO	Khanom, Thailand	TKN	34	N09°13′	E99°49′	11	C3 (11)	0.00
WPO	Sungai Cherating, Malaysia	SgC	26	N04°08′	E103°24′	8	C3 (8)	0.00
WPO	Sungai Kuantan, Malaysia	SgK	25	N03°49′	E103°20′	8	C3 (6), C6 (2)	0.43
WPO	Sungai Balok, Malaysia	SgB	27	N03°57′	E103°22′	10	C3 (10)	0.00
WPO	Sungei Buloh Wetland Reserve, Singapore	SSB	32	N01°22′	E103°54′	10	C3 (6), C8 (1), C6 (3)	0.60
WPO	Kuching, Malaysia	KUC	21	N01°40′	E110°20′	11	C3 (8), C4 (3)	0.44
WPO	Sibu, Malaysia	SU	20	N02°06′	E111°21′	10	C3 (7), C4 (3)	0.47
WPO	Kuala Penyu, Malaysia	SBKP	18	N05°34′	E115°36′	11	C3 (11)	0.00
WPO	Sandakan, Malaysia	SDG	19	N05°55′	E118°02′	10	C3 (10)	0.00
WPO	Bali Island, Indonesia	BAL	24	S08°41′	E115°15′	10	C3 (10)	0.00
WPO	Tangang PutusRaja Ampat, Papua, Indonesia	TP	23	S00°49′	E130°42′	11	C3 (6), C6 (5)	0.55
WPO	Sawinggrai, Gam Island, Papua, Indonesia	SG	22	S00°24′	E130°02′	11	C3 (6), C6 (5)	0.55
WPO	Nacidoc, Palawan, the Philippines	PA	17	N09°58′	E118°49′	10	C3 (10)	0.00
WPO	Sabang, Palawan, the Philippines	SA	16	N10°13′	E118°54′	7	C3 (7)	0.00
WPO	Mactan Island, Cebu, the Philippines	PCM	13	N10°20′	E124°00′	6	C6 (6)	0.00
WPO	Kalibo, Aklan, the Philippines	KA	15	N11°43′	E122°24′	10	C3 (5), C6 (5)	0.56
WPO	Ibajay, Aklan, the Philippines	IB	14	N11°49′	E122°24′	8	C3 (1), C6 (7)	0.25
WPO	Nakama River, Iriomote, Japan	IRM	9	N24°17′	E123°52′	10	C6 (10)	0.00
WPO	Fukido, Ishigaki, Japan	ISG	10	N24°25′	E124°10′	6	C6 (6)	0.00
WPO	Tainan, Taiwan, China	TN	11	N23°02′	E120°02′	11	C3 (4), C6 (5), C5 (2)	0.69
WPO	Kaohsiung, Taiwan, China	GX	12	N22°04′	E120°16′	10	C3 (6), C6 (4)	0.53
WPO	Haifeng, Guangdong, China	HF	5	N22°48′	E115°01′	10	C6 (10)	0.00
WPO	Sanyachong, Hong Kong, China	STC	8	N22°30′	E114°16′	11	C6 (11)	0.00
WPO	Qi'aodao, Guangdong, China	QAD	6	N22°25′	E113°36′	6	C6 (6)	0.00
WPO	Suixi, Guangdong, China	SX	7	N21°20′	E109°49′	7	C6 (7)	0.00
WPO	Beihai, Guangxi, China	BH	1	N21°32′	E109°47′	10	C6 (10)	0.00
WPO	Fangchenggang, Guangxi, China	FC	2	N21°31′	E108°20′	7	C6 (7)	0.00
WPO	Dongzhaigang, Hainan, China	DZG	4	N19°55′	E110°35′	6	C6 (6)	0.00

Table 1 continued

Region	Population	Code	ID	Latitude	Longitude	*N*	Haplotypes	*Hd*
WPO	Sanya, Hainan, China	SY	3	N18°29′	E109°45′	9	C6 (9)	0.00
NA	Daintree River, Australia	ADR	46	S16°00′	E145°18′	9	C7 (9)	0.00
NA	Efate island, Vanuatu	VT	47	S17°42′	E168°16′	7	C10 (5), C9 (2)	0.48

populations (H_S) and total gene diversity (H_T) were estimated for all populations and for each region using PERMUT (Pons & Petit, 1996). Two parameters of population differentiation, G_{ST} and N_{ST}, were also calculated and compared through a permutation test with 1000 permutations to examine the presence of phylogeographic structure. While G_{ST} considers all haplotypes equally divergent and is dependent of haplotype frequencies, N_{ST} takes into account both haplotype frequencies and their sequence similarities (Pons & Petit, 1996). A significantly higher N_{ST} than G_{ST} could be an indication of the presence of phylogeographic structure.

To further characterize the population structure and genetic variation, multiple hierarchical analyses of molecular variance (AMOVAs) were performed with significance tests of variance components based on 1000 permutations using ARLEQUIN (Excoffier & Lischer, 2010), including three with a region-specific focus, one with an among-region focus, and one that considers all populations without partitioning. Pairwise F_{ST} values for all populations were also calculated to measure the levels of genetic differentiation between populations. Isolation-by-distance (IBD) was examined both across all regions combined and within each region separately by testing the relationship between pairwise F_{ST} and natural-log-transformed (Ln-transformed) geographical distances (Rousset, 1997) using the Mantel test implemented in GenAlEx v6.5 (Peakall & Smouse, 2012), with 1,000 random permutations. A principal coordinate analysis (PCoA) on the Kimura-2-parameter genetic distance of all populations was also performed using GenAlEx.

BARRIER v2.2 (Manni et al., 2004) was used to implement the Monmonier's maximum difference algorithm to identify biogeographical boundaries or areas exhibiting the largest genetic discontinuities between population pairs using the F_{ST} matrix as the input. The robustness of each barrier was assessed by bootstrapping over loci to generate 100 matrices of genetic differentiation and then tabulating the number of bootstraps that supported the barrier.

Mismatch distribution analysis was conducted using ARLEQUIN to test whether *E. agallocha* had undergone recent population expansion. An expected distribution of the number of differences between haplotypes under a model of sudden demographic expansion was generated. Goodness-of-fit was tested with the sum-of-squared deviations (SSD) between observed and expected mismatch distributions, and the raggedness index of Harpending (H_{Rag}; Harpending, 1994). We also performed the Tajima's *D* test (Tajima, 1989) and Fu's *Fs* test (Fu, 1997) to investigate the recent demographic expansions. Significant *D* values and large negative *Fs* values generally suggest rapid demographic expansions (Hudson, 1990).

Results

Chloroplast DNA variation and haplotype distribution

The low proportion (four out of 56 loci; 7.14%) of polymorphic cpDNA regions suggested that the chloroplast genome of *E. agallocha* is highly conserved. The alignment lengths of the four cpDNA fragments, *trnV-trnM, petB-petD, rpoC1-rpoC2*, and *trnC-rpoB*, were 843, 1,626, 1,793, and 1,159 bp, respectively. A total of 10 polymorphic sites were present in the total concatenated length of 5,421 bp, corresponding to 10 haplotypes (C1-C10; Table 1) in the 419 samples analyzed. Of these, two haplotypes (C1 and C2), five haplotypes (C3, C4, C5, C6, and C8), and three haplotypes (C7, C9, and C10) were confined to populations located in EIO, WPO, and NA, respectively, indicating substantial differentiation among these three regions at least during the recent past (Fig. 1). Despite the differences in geographic distribution, these haplotypes showed close

relationship with each other, with a maximum of three mutation steps (i.e., between haplotypes C6 and C10).

Distinct and non-homogeneous distribution of haplotypes in the three regions was observed. In EIO, haplotype C2 was widely shared by all populations except for the TLU population that was fixed for haplotype C1. Haplotype C1 was also present in two populations from the Bay of Bengal with high frequency and one population from the west Malay Peninsula with low frequency. In WPO, most populations from Southeast China, Japan, and the Philippines were fixed for haplotype C6, while populations from east Malay Peninsula and Borneo were dominated by haplotype C3. Differing by a single site with each other, haplotypes C3 and C6 coexisted in 10 populations located in east Malay Peninsula, Southeast China, the Philippines, and Indonesia. Haplotypes C4 and C5 were private to populations in Borneo and Taiwan, respectively, with low frequency. In NA, population ADR was fixed for haplotype C7, while haplotypes C9 and C10 were specific to population VT.

Genetic diversity and population structure

Haplotype diversity (*H*d) was estimated to be 0.723 for the total samples, and was in the range of 0.00–0.69 within the 47 populations (Tables 1, 2). The overall genetic diversity (haplotype diversity and nucleotide diversity) was higher in the WPO populations ($Hd = 0.525$; $\pi = 0.00010$) than in the EIO populations ($Hd = 0.379$; $\pi = 0.00007$) (Table 2). Total genetic diversity H_T (0.740) across all sampled populations was much higher than the average intra-

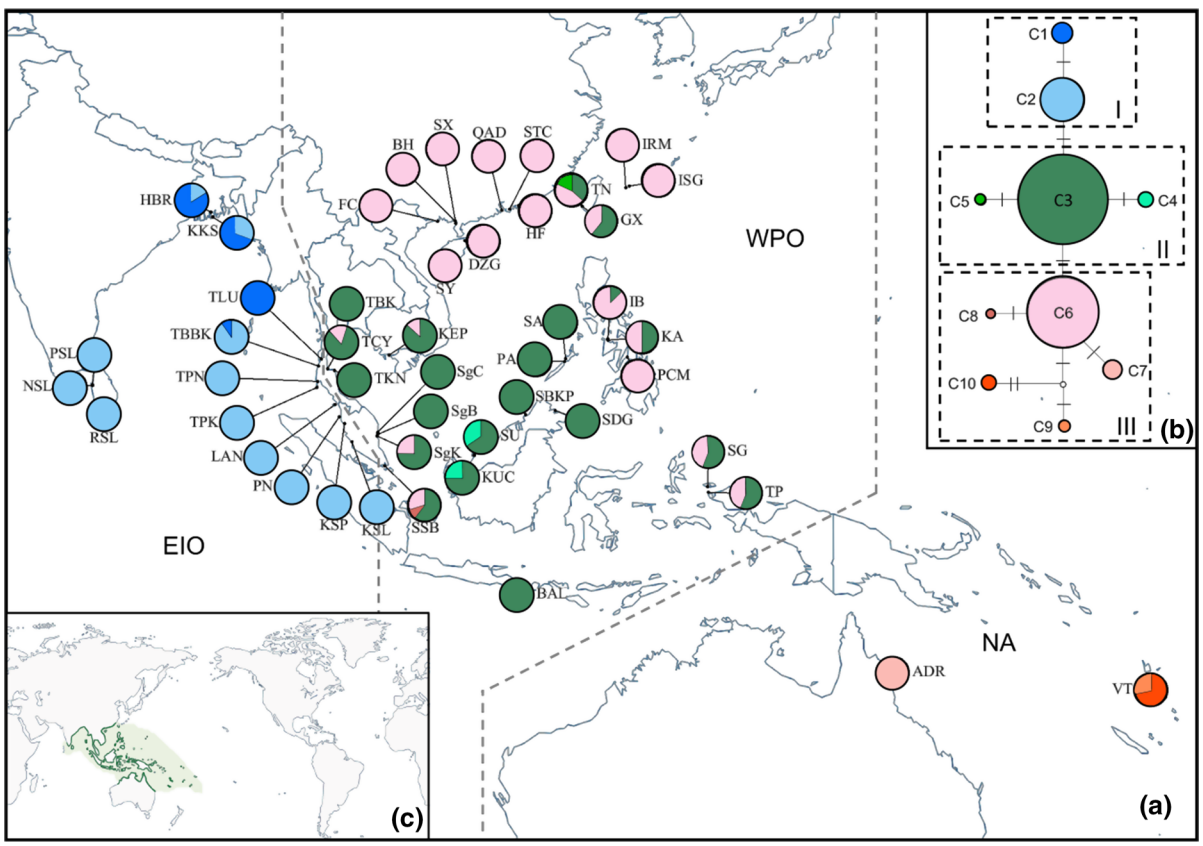

Fig. 1 Map of the sampling sites and the geographical distribution of cpDNA haplotypes in *E. agallocha*. (a) Sampling region and distribution of all inferred haplotypes; (b) median-joining network for the ten haplotypes detected. In the median-joining network, the size of the circle is proportional to the frequency of each sampled haplotype. The small white circle indicates median vectors (i.e., unsampled or extinct haplotypes). The black line on the branches indicates the number of steps separating adjacent haplotypes. Three hypothetical haplotype groups are indicated as I, II, and III. (c) The distribution of *E. agallocha* (*source* Duke, 2013)

Table 2 Results of genetic diversity analysis and Mantel test

Region	*Hd*	π	H_S	H_T	G_{ST}	N_{ST}	Mantel test *r* (*P*)
EIO	0.379	0.00007	0.075	0.344	0.783	0.783 (ns)	0.0463 (0.070)
WPO	0.525	0.00010	0.155	0.597	0.740	0.756 (ns)	0.1118 (0.001)
NA	0.608	0.00041	0.545	0.496	–	–	–
Total	0.723	0.00021	0.150	0.740	0.798	0.850 (ns)	0.0582 (0.001)

Hd overall haplotype diversity for all sampling locations within each region; π, nucleotide diversity, H_S average genetic diversity within populations, H_T total genetic diversity, G_{ST} interpopulation differentiation, N_{ST} the number of substitution types, *ns* not significant, indicating that N_{ST} is not significantly larger than G_{ST} ($P > 0.05$); *r*, correlation coefficient obtained from the appropriate matrix

population diversity H_S (0.150), suggesting that the majority of cpDNA diversity is distributed among populations. Compared with that of EIO ($H_S = 0.075$; $H_T = 0.344$), H_S and H_T were each remarkably higher for WPO ($H_S = 0.155$; $H_T = 0.597$).

Population differentiation was high ($G_{ST} = 0.798$; $N_{ST} = 0.850$) for the entire IWP. Compared with that of EIO ($G_{ST} = 0.783$; $N_{ST} = 0.783$), population differentiation and number of substitution types were each slightly lower for WPO ($G_{ST} = 0.740$; $N_{ST} = 0.756$). The permutation test showed that N_{ST} is not significantly greater than G_{ST} either for the entire IWP or for EIO and WPO separately ($P > 0.05$).

Hierarchical AMOVA analysis revealed that 56.88% of the variation can be attributed to the differentiation among EIO, WPO, and NA, while 26.02 and 17.11% of the variation are among populations within the regions and within populations, respectively, supporting the geographical divergence of *E. agallocha* among these regions. When performing the analysis on each region independently, more than half of the total genetic variation (74.48, 59.10, and 56.95%) was found among populations with high F_{ST} values (0.755, 0.591, and 0.570 for EIO, WPO, and NA, respectively). When all populations from the three regions were combined, there was more genetic variation among populations (75.03%) than within populations (24.97%), with a significant genetic differentiation coefficient of $F_{ST} = 0.750$ (Table 3). Mantel test revealed weak significant correlation between population differentiation (F_{ST}) and geographical distance for all sampled populations ($r^2 = 0.0582$, $P = 0.001$), while no significant correlation was observed when applied to EIO or WPO separately ($r^2 = 0.215$, $P = 0.070$ for EIO; $r^2 = 0.398$, $P = 0.001$ for WPO).

Phylogeographic subdivision and population demography

A genealogical network reflecting the relationship among populations and frequency of all cpDNA haplotypes was constructed (Fig. 1). The network showed that the centrally located nodes (i.e., haplotypes C2, C3, and C6) most likely represent hypothetical ancestral haplotypes, with C6 more closely related to C3 than to C2. The remaining haplotypes were linked to these central haplotypes by one to three steps in a star-like network, within which only haplotypes C9 and C10 were separated from C6 by unidentified haplotype(s). As shown in Fig. 1, haplotypes C2, C3, C6 and their derived haplotype(s) were named group I, group II, and group III hereafter, respectively.

Principal coordinate analysis (PCoA) resulted in a population grouping pattern generally consistent with the above haplotypes aggregation analysis (Fig. 2): Populations from EIO (fixed for group I haplotypes) were located close to each other; most populations from east Malay Peninsula, Borneo, and Bali (dominated by haplotype C3) formed a second group; and most populations from Southeast China, Japan, the Philippines, and Indonesia (dominated by haplotype C6) were clustered into a third group. In addition, population VT was placed relatively distant to population ADR, indicating barrier(s) of gene flow between these populations. Similar observation was also found in populations from the Bay of Bengal, which were placed relatively distant to other populations of west Malay Peninsula.

Table 3 Analysis of molecular variance (AMOVA) for *E. agallocha*

Source	*df*	SS	VC	PV (%)	*F* statistics
EIO					
Among populations	12	15.583	0.151	75.48	$F_{ST} = 0.755$*
Within populations	95	4.667	0.049	24.52	
Total	107	20.250	0.200		
WPO					
Among populations	31	106.806	0.348	59.10	$F_{ST} = 0.591$*
Within populations	263	63.350	0.241	40.90	
Total	294	170.156	0.589		
NA					
Among populations	1	4.661	0.540	56.95	$F_{ST} = 0.570$*
Within populations	14	5.714	0.408	43.05	
Total	15	10.375	0.948		
All populations					
Among populations	46	253.138	0.596	75.03	$F_{ST} = 0.750$*
Within populations	372	73.731	0.198	24.97	
Total	418	326.869	0.794		
EIO vs WPO vs NA					
Among regions	2	126.088	0.659	56.88	$F_{CT} = 0.569$*
Among populations within regions	44	127.050	0.301	26.02	$F_{SC} = 0.603$*
Within populations	372	73.731	0.198	17.11	$F_{ST} = 0.829$*
Total	418	326.869	0.159		

df degrees of freedom, SS sum of squares, VC variance components, PV percentage of variation, F_{CT} differentiation among regions within species, F_{SC} differentiation among populations within regions, F_{ST} differentiation within populations

*$P < 0.001$ (1000 permutations)

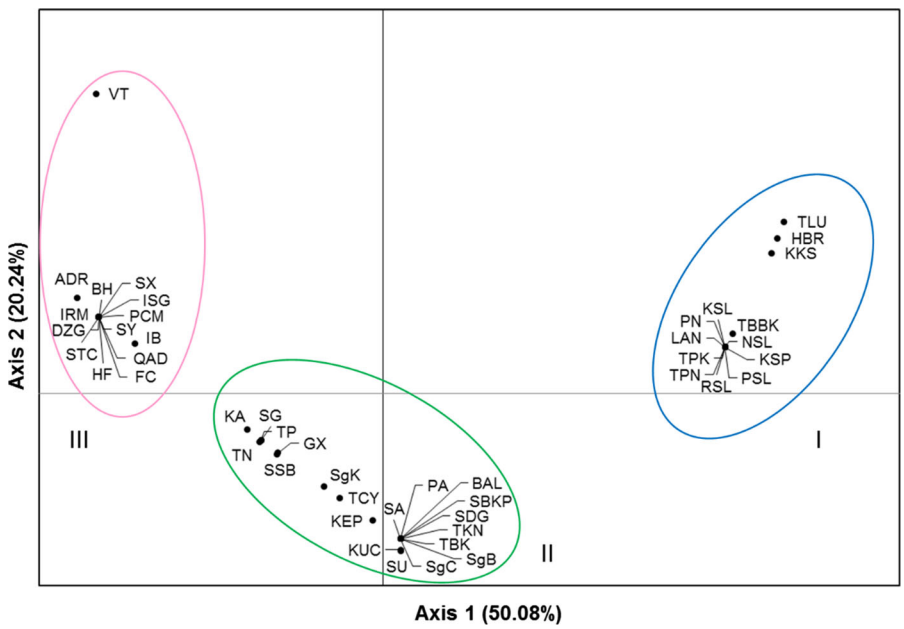

Fig. 2 Principal coordinate analysis (PCoA) for all sampled populations of *E. agallocha* from the Indo-West Pacific (IWP) region. The 47 populations were clustered into three groups (i.e., I, II, and III), generally consistent with the three haplotype groups indicated in Fig. 1

Further investigation based on the Monmonier's algorithm identified potential geographical barriers associated with the genetic abruption between EIO and WPO, and between WPO and NA (Fig. 3), reflecting significant genetic isolation between them. Besides, barriers between the Bay of Bengal and west

Malay Peninsula in EIO, between east Malay Peninsula and Southeast China in WPO, and between populations ADR and VT in NA were also identified, with the latter one having the highest bootstrap support of 96%.

Mismatch distribution analyses for both WPO and NA displayed multi-modal graphs (Fig. 4), which were significantly different from the expected sudden expansion model, as supported by the analyses of raggedness index ($H_{Rag} = 0.650$, $P = 0.019$ for WPO; $H_{Rag} = 0.719$, $P = 0.015$ for NA) although not by that of the sum-of-squared deviations ($SSD = 0.170$, $P = 0.089$ for WPO; $SSD = 0.197$, $P = 0.062$ for NA). Neutrality tests showed no significantly negative Fs and D values for samples from WPO and NA, thus no support for hypotheses of sudden population expansion in these two regions (Table 4). For EIO, although statistical tests for both SSD ($SSD = 0.006$, $P = 0.119$) and H_{Rag} ($H_{Rag} = 0.202$, $P = 0.155$) were not significant, the observed curve in the mismatch distribution analysis was not of a typical unimodal distribution. Neutrality tests of Tajima's D and Fu's Fs statistics also did not support the population expansion hypothesis in EIO. As for total samples from all locations, although the mismatch distribution showed a unimodal distribution, both SSD ($SSD = 0.117$, $P = 0.031$) and H_{Rag} ($H_{Rag} = 0.385$, $P = 0.000$) suggested that the observed distributions differed significantly from those expected under a sudden expansion model. Tajima's D and Fu's Fs tests were negative but not statistically significant, which also did not support the population expansion hypothesis. Collectively, no compelling evidence of recent expansion for the three

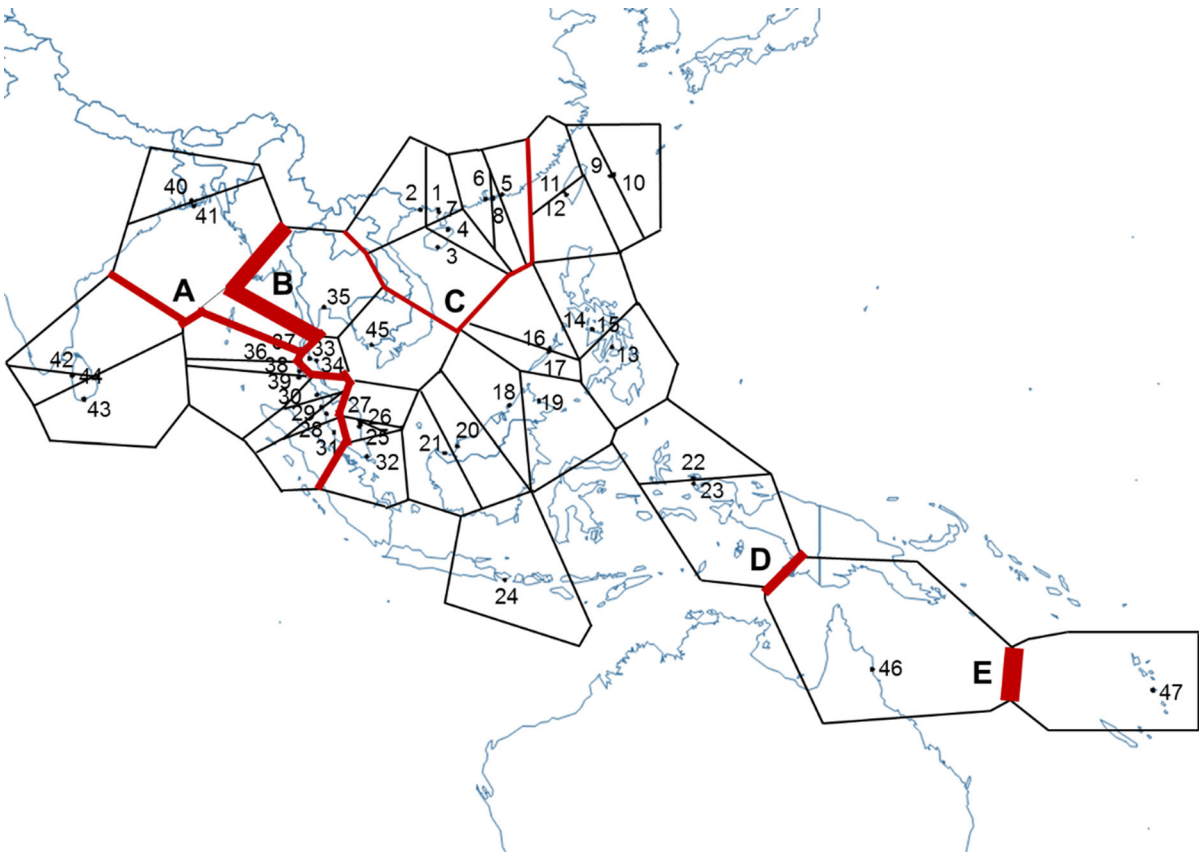

Fig. 3 Spatial separation and gene flow barriers of *E. agallocha* populations. Barriers (thick red lines) were detected and numbered A–E in the map. The thickness of the red line indicates the support of the barriers based on pairwise F_{ST} matrix with 100 permutations. Black dots with ID numbers indicate sampled populations

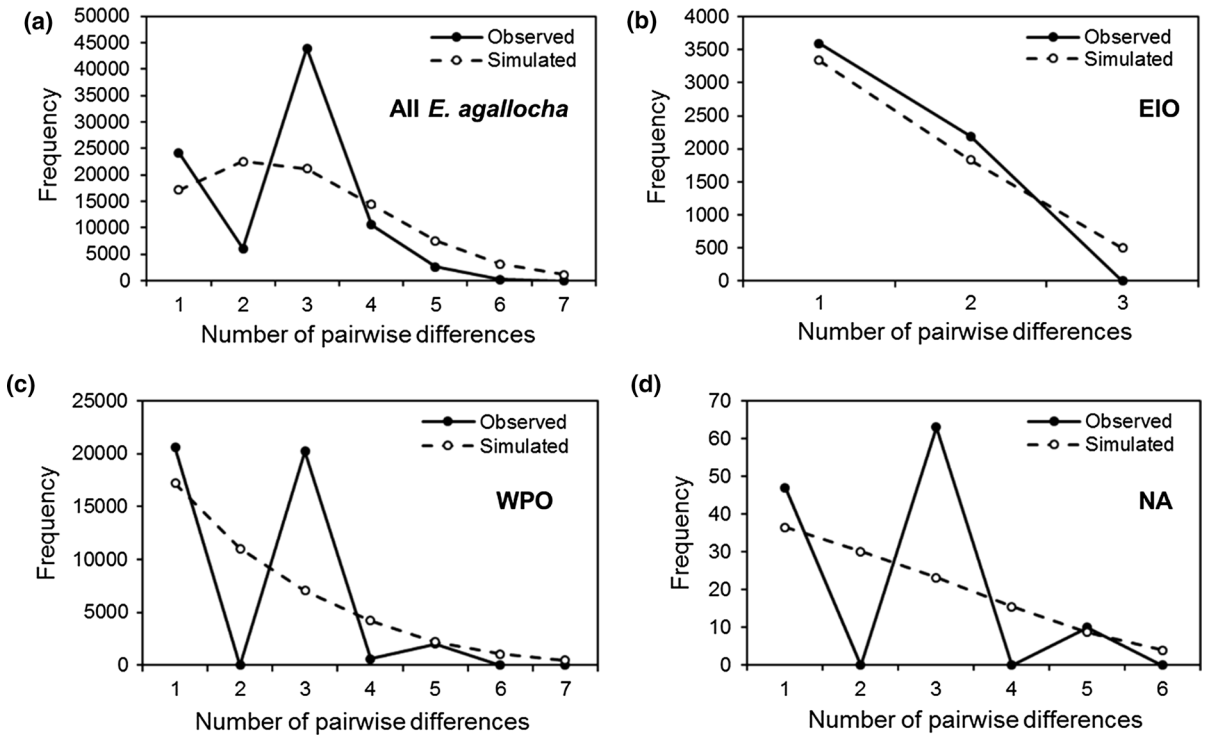

Fig. 4 Mismatch distribution analysis for (**a**) *E. agallocha* as a whole, (**b**) populations from EIO, (**c**) populations from WPO, and (**d**) populations from NA, respectively. The solid lines show observed distributions of pairwise differences among cpDNA haplotypes and the dashed lines represent the distributions expected for an expanding population

Table 4 Results of mismatch distributions analysis and neutrality test (Tajima's *D*, Fu's *Fs* tests)

Region	Mismatch distribution		Neutrality tests	
	SSD (*P* value)	H_{Rag} (*P* value)	Fu's *Fs* (P value)	Tajima's *D* (*P* value)
EIO	0.006 (0.119)	0.202 (0.155)	1.725 (0.301)	1.117 (> 0.10)
WPO	0.170 (0.089)	0.650 (0.019)	− 0.512 (0.181)	− 0.226 (> 0.10)
NA	0.197 (0.062)	0.719 (0.015)	3.135 (0.115)	1.516 (> 0.10)
Total	0.117 (0.031)	0.385 (0.000)	− 1.618 (0.077)	− 0.546 (> 0.10)

SSD sum-of-squared deviations, H_{Rag} Harpending's raggedness index, *CI* confidence interval

regions or the total sampling locations was obtained in this current study.

Discussion

Genetic diversity

This is the first report of chloroplast DNA sequence variation within and among natural populations of *E. agallocha* across most of its distribution range. At the species level, analysis of cpDNA sequences revealed a high degree of genetic variation in *E. agallocha* with a total genetic diversity (H_T) of 0.740, higher than the average value ($H_T = 0.68$) of 169 plant species which were also obtained from cpDNA-based studies (Petit et al., 2005). Compared with other mangrove species, the genetic variation found in *E. agallocha* cpDNA is higher than that of *Ceriops decandra* ($H_T = 0.270$ using ISSR markers, Huang et al., 2008), *Aegiceras*

corniculatum ($H_T = 0.039$ using ISSR markers, Ge & Sun, 1999), and *Kandelia candel* ($H_T = 0.036$ using allozyme markers, Sun et al., 1998) but lower than that of *Avicennia germinans* ($H_T = 0.87$ using cpDNA markers, Nettel & Dodd, 2007). Undeniably, comparing diversity between marker types should be treated with caution given that they fit different mutational models. The current comparisons, however, could still provide some references for diversity levels between *E. agallocha* and other mangrove species since related studies that used cpDNA markers and simultaneously provided H_T values are limited. These findings are also consistent with the earlier report on the same species using ISSR analysis, which revealed a higher species-level expected heterozygosity ($H_E = 0.277$, Zhang et al., 2008) than that of the mangrove *C. decandra* ($H_E = 0.253$, Tan et al., 2005) and mangrove associate *Heritiera littoralis* ($H_E = 0.236$, Jian et al., 2004) using ISSR analysis. The high level of genetic diversity of *E. agallocha* populations could be attributed to its wide distribution and cross-pollinating nature given that predominately out-crossing woody plants with wide-range distribution usually show high genetic diversity (Hamrick et al., 1992).

At the population level, genetic variation was relatively low ($H_S = 0.150$) compared with that at the species level, implying that the high level of genetic diversity of *E. agallocha* populations may be due to genetic divergence of the populations. In most populations, the levels of genetic variation within population were low. Ten out of 13 populations from EIO and 20 out of 32 populations from WPO, for example, showed no nucleotide variation for all sequences studied. Since *E. agallocha* is a cross-pollinated species, this reduces inbreeding as a cause of low genetic variation. Hence, a plausible explanation is that these local populations may have suffered from bottleneck(s) caused by the climatic oscillations during the Pleistocene. Glaciations, for example, especially the last glaciation and the resulting sea-level fluctuations during this time, are considered to have strongly impacted the coastal mangrove species (Chen et al., 2015). During the last glacial maximum, sea levels fell from + 6 m 120 Kya to approximately—120 m, resulting in the emergence of shelves and the disappearance of relevant coastal areas, e.g., the coastal areas of Hainan disappeared with the emergence of the Sunda Shelf in Southeast Asia, and thus the mangrove forests were mainly restricted to a narrow area on the outer margins of the shelf (Cannon et al., 2009; Woodruff, 2010; Chen et al., 2015). A general pattern of relatively high species-level diversity and relatively low and heterogeneous within-population variation in the cpDNA of *E. agallocha* seem to reflect the geological changes that the species has gone through in the past.

Population genetic structure

Permutation test (with 1,000 permutations) on the total samples showed that N_{ST} was not significantly greater than G_{ST}, indicating no significant phylogeographic structuring based on cpDNA variation in *E. agallocha* of IWP. However, genetic variation among populations was strong ($G_{ST} = 0.798$), indicating that the total genetic diversity was primarily distributed among populations. Obvious genetic structure was also detected in *E. agallocha* by both AMOVA and haplotype network analysis. Like G_{ST}, AMOVA performed on all sampling locations revealed that more genetic variation existed among population (75.03%) than within populations (24.97%) when regional clustering is being disregarded. This pattern is consistent with those observed in other mangrove species like *C. decandra* (Tan et al., 2005) and *L. racemosa* (Su et al., 2006), but in contrast to that observed by Zhang et al. (2008) for the same species using ISSR marker data. Given that cpDNA is haploid, maternally inherited, and evolve relatively slowly (Ye et al., 2014), while nuclear DNA is diploid and biparentally inherited, such inconsistent conclusions, which has been reported in other species (e.g., Feng et al., 2016), can be explained by the different inheritance patterns and evolutionary rates of the markers used.

AMOVA also revealed that a large proportion of variation (56.88%) was due to differences among regions (EIO, WPO, and NA) with significant genetic differentiation coefficient, $F_{CT} = 0.569$, indicating long-term impediments to gene flow (i.e., seed dispersal) among regional populations. This agreed with previous observation that there was little seed dispersal of *E. agallocha* between regions (Zhang et al., 2008). Like other mangrove species with water-dispersed propagules, the gene flow (through propagule dispersal) of *E. agallocha* is largely determined by ocean currents and the species' life history strategies (Duke, 1992; Dodd et al., 2002; Wee et al., 2014). *E.*

agallocha has small seeds (about 3 mM in diameter) with an air space in the seed coat; these characteristics help the seeds to float and disperse via sea surface currents. Although the longevity of *E. agallocha* seeds in transit remains unclear, the absence of suitable currents seems more likely to be the main reason for the limited dispersal ability of seeds observed for this species, and the impacts of geographical distance and the presence of major landscape barriers should also be partially responsible.

An indication of isolation-by-distance (IBD) was observed in *E. agallocha* when taking into account all populations studied, but no such indication was observed when each region was tested separately. Previous findings suggested that genetic structure in mangroves is often more complex than predicted by a simple stepping-stone model (Maguire et al., 2000; Dodd et al., 2002; Ceron-Souza et al., 2015). In combination with the view that tests of IBD may be strongly biased by hierarchical population structure (Meirmans, 2012; Ngeve et al., 2016), the IBD signals observed for *E. agallocha* here is likely caused by mixed factors from both distance and resistance posed by an effective barrier to gene flow.

Phylogeographic subdivision and demographic history

The network analysis provided a well-resolved phylogenetic relationship of the haplotypes, which suggested three star-like evolutionary units separately dominating three lineages in *E. agallocha* of the IWP region. While EIO was found to be fixed for a single lineage (group I haplotypes), WPO comprised two lineages: group II haplotypes distributed in east Malay Peninsula, Borneo, and Bali, and group III distributed in Southeast China, Japan, the Philippines, and Indonesia. Populations in NA also had group III haplotypes. This clustering pattern was further supported by the PCoA analysis.

The split between group I and group II haplotypes, or simply the genealogical break across the Malay Peninsula, has been demonstrated in numerous other mangrove species, such as *Rhizophora apiculata* (Inomata et al., 2009), *Bruguiera gymnorrhiza* (Urashi et al., 2013), *Ceriops decandra* (Huang et al., 2008), and *Lumnitzera racemosa* (Su et al., 2006). During glacial periods in the Pleistocene, the Sundaland was largely exposed due to the lowering of sea levels, forming massive lowland connections between present-day islands in this region (i.e., the Malay Peninsula, Sumatra, Java, and Borneo) and the adjacent continents, impeding genetic exchange between the EIO and WPO regions (Voris, 2000). Our investigation based on Monmonier's algorithm identified a significant barrier between EIO and WPO, indicating that the Malay Peninsula still acts as one of the most important present-day land barriers separating the Pacific and Indian oceans. Similarly, another barrier identified between WPO and NA could be attributed to the connection of New Guinea and Australia at the Torres Straits during the glacial periods in the Pleistocene (Jennings, 1972).

The closer relationship of group III haplotypes to group II instead of group I haplotypes echoed previous interpretation that mangrove populations in the EIO and WPO were derived from populations in two major refugia—one in the Andaman Sea and another in the South China Sea, during the Last Glacial Maximum (Flenley, 1998; Cannon et al., 2009). Since very different results may be observed when using different markers (Feng et al., 2016), the historical range expansion of *E. agallocha* in this region cannot be ruled out although no evidence of demographic expansion was observed using cpDNA data in this study. In fact, our additional work (data not shown) on the past range of *E. agallocha* by ecological niche modeling showed that *E. agallocha* populations in the region experienced frequent demographic fluctuations until the Last Glacial Maximum period and its range has been slowly expanding after that. Similar scenarios of population contraction and range expansion from a refugium after glaciations have also been frequently proposed or detected in other mangrove and non-mangrove species (Saenger & Bellan, 1995; Maguire et al., 2000; Hewitt, 2000; Nettel & Dodd, 2007; Kennedy et al., 2016).

Aside from the two land barriers mentioned above, three other barriers where no geographical obstacles exist were identified (i.e., the barrier between the Bay of Bengal and west Malay Peninsula in EIO, the barrier separating Southeast China from other populations of the South China Sea in WPO, and the barrier between populations ADR and VT in NA). One of the key forces involved in the dispersal of mangrove propagules is water surface currents. Thus, the lack of suitable ocean currents could be responsible for the genetic discontinuity observed in these regions. In

WPO for example, when the seeds of *E. agallocha* ripen in summer, the surface current of the tropical Indian Ocean flows northward into the South China Sea and then through the Bashi Strait into the Pacific Ocean. The seeds of *E. agallocha* growing in Malaysia subsequently would have limited chances to disperse into Southeast China but can possibly reach the Philippines and Taiwan. On the other hand, the existence of two opposing currents, a northeastward current that originates from the southeast of the Hainan Island, flows along Guangdong and extends to the Taiwan Strait (Su & Wang, 1987; Hu & Liu, 1992), as well as a westward current which originates from the Kuroshio and intrudes the northern South China Sea through the Luzon Strait (Chan, 1970; Williamson, 1970; Nitani, 1972), provides opportunity for gene flow from Southeast China to Taiwan and from Iriomote Island to the Philippines. The pattern of ocean currents described above provides reasonable explanation for the coexistence of haplotypes C3 and C6 in the Philippines and Taiwan.

This study represents the most complete sampling of *E. agallocha* throughout their distribution range. In summary, our data based on four distinct cpDNA fragments suggested that *E. agallocha* displayed high genetic diversity at species level and conspicuous differentiation among the regions EIO, WPO, and NA. Genetic data and AMOVA analysis revealed significant genetic structure among *E. agallocha* populations. In contrast to findings by Zhang et al. (2008) using nuclear markers, the high cpDNA diversity in *E. agallocha* was likely due to genetic divergence among populations rather than variation within populations. Studies of genealogy, haplotype distribution, and PCoA suggested the existence of three haplotype groups distributed in distinct geographical locations. Although no compelling evidence for demographic expansion was obtained in the present study, the historical range expansion of *E. agallocha* cannot be fully ruled out. The genetic discontinuities found between distinct distributional areas of *E. agallocha* suggested that both land barrier and oceanic currents may have shaped the geographical distribution, genetic structure, and population demography of this species in the IWP region. These findings shall help to better understand the genetic diversity and evolution of mangroves, which would further contribute to the conservation and management of this important group of plants.

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