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Gene flow of *Ceriops tagal* (Rhizophoraceae) across the Kra Isthmus in the Thai Malay Peninsula

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ABSTRACT. The Malay Peninsula (formerly ancient Sundaland) is regarded as a barrier that isolates organisms of the South China Sea from those of the Bay of Bengal. During the interglacial period, approximately 5 Mya, sea levels rose and organisms migrated across the narrowest part of this peninsula, the Kra Isthmus. In the present study, we examine the chloroplast genomes of *Ceriops tagal* along the coasts of both sides of the Kra Isthmus to retrace divergence events and to evaluate the probability of previous long distance dispersal. The haplotype distributions support the hypothesis that the Kra Isthmus was an effective geographic barrier that caused genetically differentiated populations. Based on comparison of the chloroplast genomes, the estimated time of divergence between the two populations is consistent with the emergence time of the Kra Isthmus. However, ancient and recent gene flow obscures the phylogenetic relationships between eastern and western haplotypes. We used nested clade analysis (based on user-defined-distances corresponding to the distances across the peninsula and the sea route around it) and provide evidence of pre-isthmus range expansion and restriction of gene flow that resulted from geographic isolation. Trans-isthmus long distance dispersal probably occurred at the pre-isthmus region ~5 Mya via the southern Malay Peninsula. Our results indicate that the Malay Peninsula has had separate populations on opposite sides of the Kra Isthmus since its formation, but that interglacial migration at the Strait of Malacca may have provided a corridor for gene flow. This is an instance of arrested allopatric speciation due to genetic homogenization via rare long distance dispersal.

Keywords: *Ceriops tagal*; Dispersal; Divergence time; Kra Isthmus; Land barrier; Vicariance.

INTRODUCTION

Geographic isolation is considered to be a major cause of population differentiation (Braillet et al., 2002; Roy et al., 2006; Liao et al., 2007) and speciation (Near and Benard, 2004; Stevens and Hogg, 2004; Hoskin et al., 2005; Hayashi and Kawata, 2006; Starrett and Hedin, 2007; Sheue et al., 2009). The best-known cases are marine organisms that are isolated by isthmuses. For example, the Isthmus of Panama, which emerged approximately 3.5 Mya (Coates et al., 1992), caused substantial differentiation of populations and divergence of species in the Atlantic and Pacific Oceans (Steeves et al., 2005; Smith et al., 2006). Similar barriers have been documented in the Malay Peninsula (Karns et al., 2000; Lessios et al., 2001; Heatwole et al., 2005) and the Baja California Peninsula (Muniz-Salazar et al., 2005). During short periods of geological history, organisms that are isolated by such isthmuses can rapidly accumulate genetic differences and drive population differentiation. However, population differentiation will not occur if there is insufficient time for accumulation of genetic differences (i.e. genetic drift has not completed to fix to one allele) or if occasional dispersal and migration maintains connections between the populations (Trakhtenbrot et al., 2005; Berthier et al., 2006; Wiens et al., 2006).

Most plant seeds cannot disperse over long distances, but long distance dispersal (LDD) and colonization can prevent speciation of geographically isolated populations (Cain et al., 2000). Thus, population divergence generally proceeds slowly, often via short incremental steps over long periods of time. For instance, the average seed dispersal rates of forest understory species generally range from 0.0 to 2.5 m/yr (Cain et al., 2000). However,

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for plants near oceans, a small number of seeds can be dispersed over very long distances (Portnoy and Willson, 1993), especially by sea currents.

Salinity can harm seeds or fruits that have prolonged contact with the sea, as originally discussed by Darwin (1859). Even mangrove seeds, commonly considered to be salt-tolerant, may be harmed by prolonged soaking in saline solutions, e.g. only 8.3±3.3% of the propagules of *Ceriops tagal* developed roots after 23 days of soaking (Clarke et al., 2001). In addition, establishment success and niche availability are important determinants of long-distance colonization (Moore and Elmendorf, 2006; Viard et al., 2006).

The globally discontinuous distribution of mangrove species (Duke et al., 2002) indicates that they can overcome problems associated with LDD and can effectively colonize geographically distant areas (Cain et al., 2000). However, mangrove LDD and colonization is likely to be stochastic and unpredictable (Duke et al., 2002; Minchinton, 2006). Many researchers have examined the disjunct geographic distribution of mangroves (Duke, 1995; Duke et al., 2002) and discontinuities in their of genetic composition (Duke et al., 1998; Chiang et al., 2001; Tan et al., 2005; Su et al., 2006, 2007; Liao et al., 2007). However, little research has been devoted to the underlying processes (e.g. Chiang et al., 2001; Liao et al., 2007).

Our previous study examined chloroplast genetic differentiation in *C. tagal* between populations of the South China Sea (SCS) and the Bay of Bengal (BOB) (Liao et al., 2007). *Ceriops tagal* typically grows in inner mangroves and is geographically widespread from East Africa through India and Malaysia to South China (Tomlinson, 1986). Seeds of *C. tagal* germinate in its viviparous fruit (hypocotyl), which is defined as the propagule. These are slender, long, and sharply angular. We found that ancient Sundaland (which connected the Philippines and the islands of Borneo, Java, and Sumatra with the Thai-Malay Peninsula during the glacial epochs) was a geographic barrier that hindered dispersal of mangrove propagules (Liao et al., 2007). Based on Voris's description of the ancient basin of Sundaland (Voris, 2000), the Sundaland river system provided dispersal routes for mangroves, and this explains their present distribution. Recently, a large-scale phylogeographic study of *Ceriops* (Huang et al., 2007) indicated that the limited ability for LDD in *Ceriops tagal* propagules led to low genetic diversity and substructured populations. This is consistent with the field experiments of McGuinness (1997). Furthermore, based on analysis of inter-simple sequence repeats (ISSR), the phylogenetic split of *C. tagal* populations between SCS and BOB was not completed (Huang et al., 2007). The population substructure indicates these populations have been sorted well. However, the unresolved phylogenetic relationships and incomplete lineage sorting suggest that propagule dispersal across the Malay Peninsula has occurred recently, perhaps during the interglacial period. If recent LDD between the SCS and BOB has occurred, it may have been via the Strait of Malacca (which connects the SCS and BOB) or via the Kra Isthmus (the narrowest and lowest part of the Thai-Malay Peninsula) when the sea level rose during the warmer interglacial climate.

The Thai-Malay Peninsula appears to be a dispersal barrier for mangroves, but it is unclear whether it can stop all mangrove gene flow between the SCS and BOB, which may occur via the Kra Isthmus or around the Malay Peninsula. In this paper, we use *Ceriops tagal* (Perr.) C. B. Rob., a species present on both sides of the Kra Isthmus, to investigate the divergence of high-diversity cpDNA neutral spacers (*atp*B-*rbc*L and *trn*L-*trn*F) among these separate populations, and we consider various mechanisms of trans-isthmus LDD of mangroves. The cpDNA properties of high variability and maternal inheritance are useful in tracing the propagule dispersal of mangroves.

MATERIALS AND METHODS

Sample collection and cpDNA analysis

We sampled leaves from a total of 89 *C. tagal* individuals on the east and west coasts of the Malay Peninsula (Table 1 and Figure 1). There were four populations on the east coast and five populations on the west coast. All sampled individuals were separated by at least 10 m. The collected leaves were dried immediately with silica gel prior to DNA extraction. Total genomic

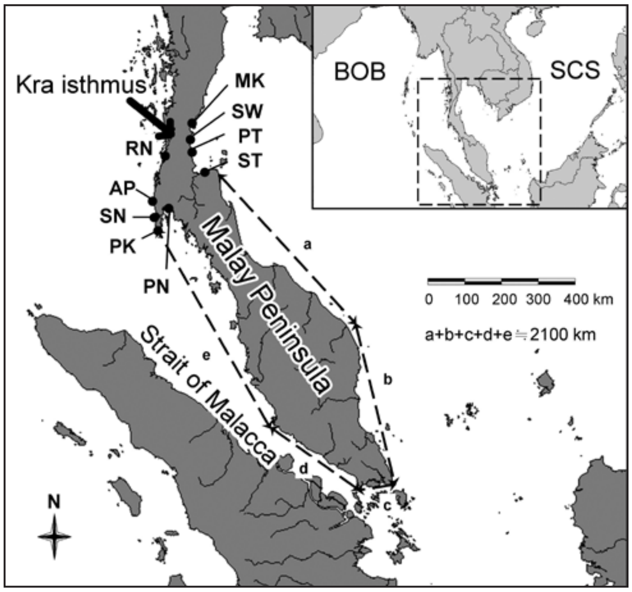

Figure 1. Map of the sampling sites. The sum of the distances of dotted lines is the shortest distance between the most southern populations of the east and west Kra Isthmus around the Malay Peninsula and is roughly equal to 2,100 km (a+b+c+d+e≒2,100 km). This distance was used for the sea-route-distance based NCA. Location abbreviations are given in Table 1.

DNA was isolated from samples using a commercial DNA extraction kit (BIOMAN Co.), dissolved in TE buffer (10 mM Tris, 0.1 mM EDTA, pH 8.0), and stored at -20°C until analysis. Two chloroplast regions (*atp*B-*rbc*L and *trn*L-*trn*F) were selected for amplification and sequencing. The primers, PCR conditions, and sequencing protocols are described in Liao et al. (2007). Sequences obtained in this study have been deposited in the NCBI database under the accession numbers: DQ983274-DQ983276, DQ983280-DQ983281 and DQ983284-DQ983307 (*trn*L-*trn*F); DQ983241, DQ983245, DQ983248, DQ983250-DQ983252, DQ983257-DQ983260 and DQ983262-DQ983273 (*atp*B-*rbc*L).

Population genetic analyses

The local alignment with default settings was performed by ClustalX (Thompson et al., 1997). After sequence alignment, nucleotide diversity (π), haplotype diversity (Hd), and θ (based on the total number of mutations [η] and segregating sites [S]) were estimated for each population using DnaSP 4.0 (Rozas et al., 2003). Pairwise population differentiation was estimated by calculating F_{ST} with 1000 permutations using ARLEQUIN v. 3.0.1 (Excoffier et al., 2005), and the index of gene flow (Nm) was calculated from $Nm=(1-F_{ST})/2F_{ST}$. A neighbor-joining tree, based on the matrix of pairwise F_{ST} values, was drawn to visualize the magnitude of genetic differentiation and relationships among the populations using MEGA 3.1 (Kumar et al., 2004). The overall magnitude of population differentiation (Φ_{ST}) and the variance of the hierarchical structure of populations were measured by Analysis of Molecular Variance (AMOVA) using ARLEQUIN v. 3.0.1 (Excoffier et al., 2005).

Phylogeographic analyses

For phylogeographic analyses, every long-fragment indel was treated as arising from a single evolutionary event and recoded as described by Liao et al. (Liao et al., 2007). The phylogenetic relationships among haplotypes were determined with the unrooted neighbor-joining method performed by TOPALi, version 2.17 (Milne et al., 2004). The model was selected for F84+Gamma with the transition/transversion ratio=0.51, alpha shape parameter=0.10, and Kappa parameter=1.22. The statistical significance of phylogenetic groups in the resulting tree was tested by bootstrap resampling (1000 replicates in each case), using MEGA 3.1.

A haplotype network was constructed by the minimum spanning network method, using ARLEQUIN v. 3.0.1 (Excoffier et al., 2005). The genetic data and geographic information of the populations were entered into GEODIS v. 2.0 (Posada et al., 2000) to assess the significance of associations between genetic and geographic distributions. This was accomplished by examining the clade distance, D_C (average distance of an individual from the geographic center of all individuals within the same nesting clade) and nested clade distance, D_N (relative geographic distribution to other clades in the same higher-level nesting clade), generated from the population data (Templeton et al., 1995; Templeton, 2001). Two nested clade analyses (NCAs) were performed, one using geographic (great circle) distances, and the other using sea-route distances. The great circle distances among populations were determined from latitudinal and longitudinal coordinates (Table 1), and the sea-route distances were determined as described in Figure 1. The approximate sea-route distance from an eastern Kra population (pop E) to a western Kra population (pop W) is:

$$D_{E\text{-}W} = D_{E\text{-}ST} + 2{,}100 \text{ km} + D_{W\text{-}PK},$$

where ST and PK are the most southerly populations of the eastern and western Kra Isthmus, respectively, $D_{E\text{-}ST}$ is the distance from pop E to pop ST, and $D_{W\text{-}PK}$ is the distance from pop W to pop PK. Templeton's (2004) inference key was then applied to the results to determine the NCA outcome. In this analysis, we ignored *C. tagal* populations that were present in the southern Malay Peninsula, so some answers from the inference key may yield the result "Sampling Design Inadequate."

Table 1. Sampling sites.

Population	Abbreviation	Longitude	Latitude	Geographic area
Ao Phangna National Park	AP	08°24' N	98°31' E	West Kra, Bay of Bengal
Phang-Nga	PN	08°24' N	98°30' E	West Kra, Bay of Bengal
Southern Phuket island	PK	07°52' N	98°23' E	West Kra, Bay of Bengal
Ranong	RN	09°55' N	98°37' E	West Kra, Bay of Bengal
Sirinat National Park	SN	08°11' N	98°17' E	West Kra, Bay of Bengal
Mu Ko Chumphon National Park	MK	10°22' N	99°10' E	East Kra, Gulf of Thailand
Surat Thani (Korn Nan)	ST	09°07' N	99°20' E	East Kra, Gulf of Thailand
Thungkra-Swi Amphur Sawi	SW	10°15' N	99°05' E	East Kra, Gulf of Thailand
Tumethong Amphur Patew	PT	10°02' N	99°08' E	East Kra, Gulf of Thailand

RESULTS

Genetic diversity

We used sequence data of the *atp*B-*rbc*L and *trn*L-*trn*F cpDNA spacers for all of the genetic analyses in this study. We obtained 1198 base-pair length fragments after alignment, containing 110 polymorphic sites, which included 10 long-fragment indels and several substitutions and one-base indels. When we recorded each long-fragment indel as a single mutation event (site), there were 78 variable sites, including 20 singletons and 58 informative mutations. Among the 89 samples, we obtained 43 haplotypes and an overall haplotype diversity of 0.974. The diversity within individual populations ranged from 0.400 (AP) to 1.000 (RN). Overall nucleotide diversity (π) was 0.00949 and ranged from 0.00037 (AP) to 0.01861 (RN) within populations. Estimates for θ ranged from 0.00044 (AP) to 0.01589 (MK) within populations and had an overall value of 0.01703.

Table 2. Genetic diversity of *Ceriops tagal* populations, according to estimates of haplotype diversity (*Hd*), nucleotide diversity (π), and genetic diversity index (θ) (estimated by segregating sites using the equation 10 from Tajima, 1996). *N* is the sample size and *H* is the number of haplotypes.

Population	*atp*B-*rbc*L + *trn*L-*trnF*				
	N	*H*	*Hd*	π	θ
MK	9	8	0.972	0.01104	0.01589
SW	10	8	0.956	0.00217	0.00232
PT	8	7	0.964	0.00191	0.00181
ST	9	7	0.917	0.00410	0.00410
RN	10	10	1.000	0.01861	0.01546
AP	5	2	0.400	0.00037	0.00044
SN	21	13	0.957	0.00940	0.00940
PK	8	3	0.607	0.00215	0.00250
PN	9	5	0.806	0.00268	0.00239
E-Kra	36	22	0.967	0.00584	0.01228
W-Kra	53	26	0.966	0.01160	0.01381
Total	89	43	0.974	0.00949	0.01703

The average number of nucleotide differences between populations on the east and west sides of the isthmus was 9.479, and nucleotide substitutions averaged 0.00918 per site (K) between them. Eastern and western populations shared 28 mutations, excluding gaps. Thirty-seven mutations were polymorphic in western populations but monomorphic in eastern populations; 24 mutations were polymorphic in eastern populations but monomorphic in western populations. Thus, western populations had slightly higher genetic diversity than eastern populations. This result is supported by the π and θ estimates (Table 2).

Population differentiation and gene flow

All of the pairwise F_{ST} values were significant at the $P<0.05$ level, except for those in the following four pairs of populations: RN-PN, MK-ST, SW-PT, and PT-ST. Both members of each of these pairs were located on the same side of the Kra Isthmus (Table 3). The pairwise *Nm* values, estimated from the reciprocal F_{ST} values between eastern populations, were mostly higher than 1.0. This indicates that gene flow via propagule dispersal of *C. tagal* has occurred frequently among eastern populations. We also obtained high *Nm* values in several pairwise comparisons of western populations (AP-RN, PN-RN, AP-SN, PN-SN, and RN-SN). However, these *Nm* values were greater than

Table 3. Matrix of F_{ST} (below) and *Nm* (above) between nine populations of *Ceriops tagal* based on chloroplast *atp*B-*rbc*L and *trn*L-*trn*F spacers. Bold values indicate comparisons between populations on the two coasts of the Kra Isthmus.

	East Kra populations						West Kra populations				
	TR	Kh	MK	SW	PT	ST	PN	PK	SN	AP	RN
TR		0.756	1.279	0.426	0.462	0.744	**0.227**	**0.253**	**0.614**	**0.246**	**0.475**
Kh	0.398		24.500	0.184	0.173	2.064	**0.069**	**0.064**	**0.546**	**0.031**	**0.975**
MK	0.281	0.020		2.293	3.021	28.912	**0.592**	**0.559**	**0.870**	**0.779**	**1.273**
SW	0.540	0.731	0.179		9.704	8.272	**0.097**	**0.052**	**0.352**	**0.052**	**0.772**
PT	0.520	0.743	0.142	0.049*		28.912	**0.095**	**0.050**	**0.377**	**0.043**	**0.901**
ST	0.402	0.195	0.017*	0.057	0.017*		**0.520**	**0.306**	**0.573**	**0.498**	**1.332**
PN	**0.688**	**0.878**	**0.458**	**0.838**	**0.840**	**0.490**		0.127	0.601	0.230	2.104
PK	**0.664**	**0.886**	**0.472**	**0.906**	**0.909**	**0.620**	0.797		0.759	0.081	1.006
SN	**0.449**	**0.478**	**0.365**	**0.587**	**0.570**	**0.466**	0.454	0.397		0.614	0.789
AP	**0.670**	**0.942**	**0.391**	**0.905**	**0.921**	**0.501**	0.685	0.860	0.449		2.606
RN	**0.513**	**0.339**	**0.282**	**0.393**	**0.357**	**0.273**	0.192*	0.332	0.388	0.161	

*Denotes insignificant statistical support from 1023 permutations ($P>0.05$).

1.0 for just two of the pairwise comparisons of eastern and western populations (MK-RN and RN-ST). These results indicate that the cpDNA gene flow (propagule dispersal) was higher within eastern and western populations than between these populations. Thus, the Kra Isthmus appears to reduce dispersal of mangroves between the east and west coasts, but it is not an absolute barrier that prevents all dispersal.

Population genetic structure

The results of the hierarchical AMOVA indicate that within-population variation accounts for 52.0% of the observed genetic variation and variation between populations on the east and west sides of the isthmus accounts for 31.6% of the variation. Variation among populations within these groups accounted for 16.5% of the total variation. The hierarchical Φ statistics revealed the same pattern. It showed that most of the genetic variation is within populations ($\Phi_{ST} = 0.480$), followed by variation among groups ($\Phi_{CT} = 0.316$), and variation among populations within groups ($\Phi_{SC} = 0.241$). All of the Φ statistics and differences in percentages of variation are statistically significant ($p<0.005$, Table 4). The grouping of populations in the NJ tree derived from the pairwise F_{ST} values is consistent with their geographical distribution although the short branch length between the east and west populations indicates minor divergence between these groups (Figure 2). Taken together, the AMOVA results and NJ population tree indicate that the Kra Isthmus has a hand in structuring the *C. tagal* populations although the genetic distance between populations on the east and west sides is short.

Phylogeographic inferences from NCA

The apparent discrepancies between the groupings in the haplotype phylogenetic tree and actual geographic distribution result in unresolved, ambiguous, and statistically weak relationships of gene-tree topology. This tree indicates recent gene flow (Bernardi et al., 2003) but provides no evidence of population history. By contrast, the minimum spanning network separates the east and west clades although only by one or a few steps (Figure 4). Roughly speaking, eastern populations were located in the interior clades and western populations in the terminal clades. The central clades, with presumably the most ancient evolutionary information, included

Table 4. Summary of results of molecular variance (AMOVA) for East and West Kra areas of *Ceriops tagal*. The significance (*P*) of the variance was based on 1000 permutations.

Source of Variation	d.f.	% total variance	*Φ*	*P*
Among groups	1	31.56	0.31560	<0.005
Among populations within groups	7	16.48	0.24079	<0.0001
Within populations	80	51.96	0.48040	<0.0001

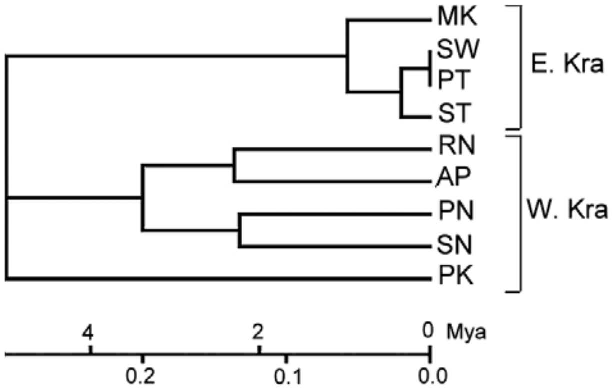

Figure 2. Neighbor-Joining tree of *Ceriops tagal* constructed by pairwise F_{ST}. The scale bar indicates F_{ST} differences and divergence time. The time scale, based on the estimated time of divergence of the Gulf of Thailand and the Bay of Bengal populations, is approximately 5 Mya.

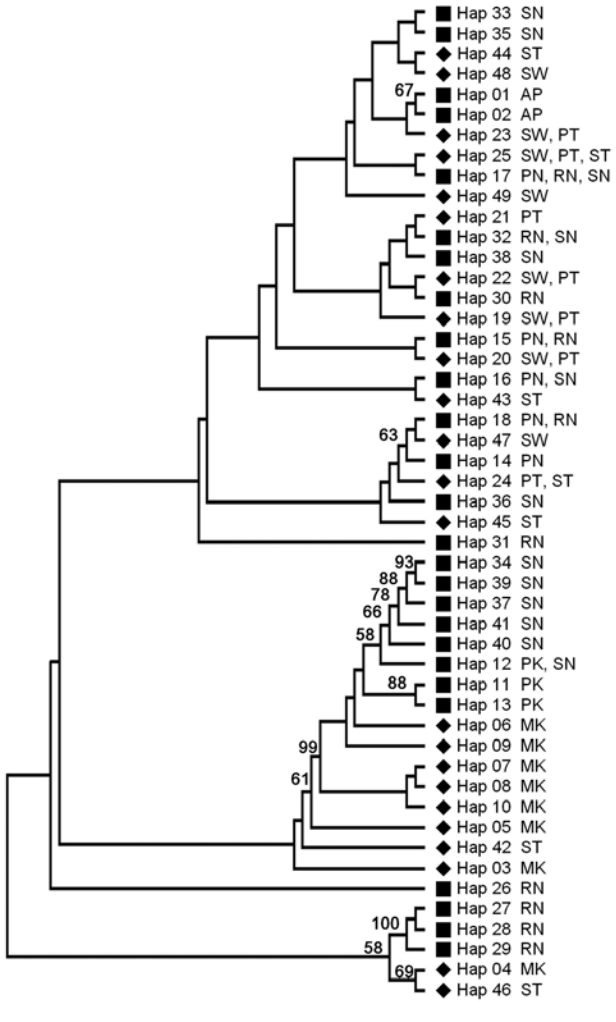

Figure 3. Unrooted Neighbor-Joining tree of *Ceriops tagal* haplotypes. Numbers on branches are bootstrap values of 1,000 replicates. Diamonds indicate haplotypes on the east side of the isthmus, and squares indicate haplotypes on the west side of the isthmus. The scale bar indicates the inferred frequency of substitutions per nucleotide site.

eastern and western haplotypes (subclades I-7 and I-16). Furthermore, several haplotypes located in the terminal clades were loosely connected with the long-missing links (Figure 3), suggesting a longer evolutionary separation of these haplotypes than merely an accumulation of a few step mutations. Great-circle and sea-route distance-based analyses detected insignificant nested-clade distance associations among lower level clades. This indicates that the null hypothesis of no association between haplotype and geographical distribution should not be rejected for these clades.

However, great-circle-distance and sea route distance-based nested clade analyses generated four clades that showed significant associations (permutational chi-squared probabilities for geographic structure less than 0.05) with D_C, D_N or I-T values (Table 5). Both analyses yielded similar inferences for three of these four clades. We infer a restricted gene flow with isolation by distance (IBD) evolutionary scenario for Clade II-10. This indicates that within this centrally located clade of the western Kra Isthmus, short-distance dispersal may have occurred, but only rarely. In contrast, we infer that Clade II-5 (an eastern clade connected to two western haplotypes by single missing links), arose from long distance dispersal (LDD), according to the analyses based on both types of geographic-distance. Clade III-3 is a clade of western haplotypes, with many long missing links (inferred to be consequences of "contiguous range expansion") and one eastern haplotype.

In contrast, the inferences from the total cladograms generated using the great circle and sea route distances are distinctly different. The great circle distance-based results indicate there was "inadequate sampling" to discriminate IBD from LDD. The sea route distance-based results indicate a restricted gene flow with an IBD scenario. The highest-clade inferences indicate a better use of the sea route distance mode on tracking the evolutionary events of mangrove dispersal (Table 5).

DISCUSSION

The Thai-Malay Peninsula as a barrier

Previously, we showed a significant genetic difference between the SCS and the BOB populations of *C. tagal* and speculated that the Sundaland served as a geographic barrier (Liao et al., 2007). Research on mangrove population genetics by Shi and colleagues (Tan et al., 2005; Huang et al., 2007; Su et al., 2006,

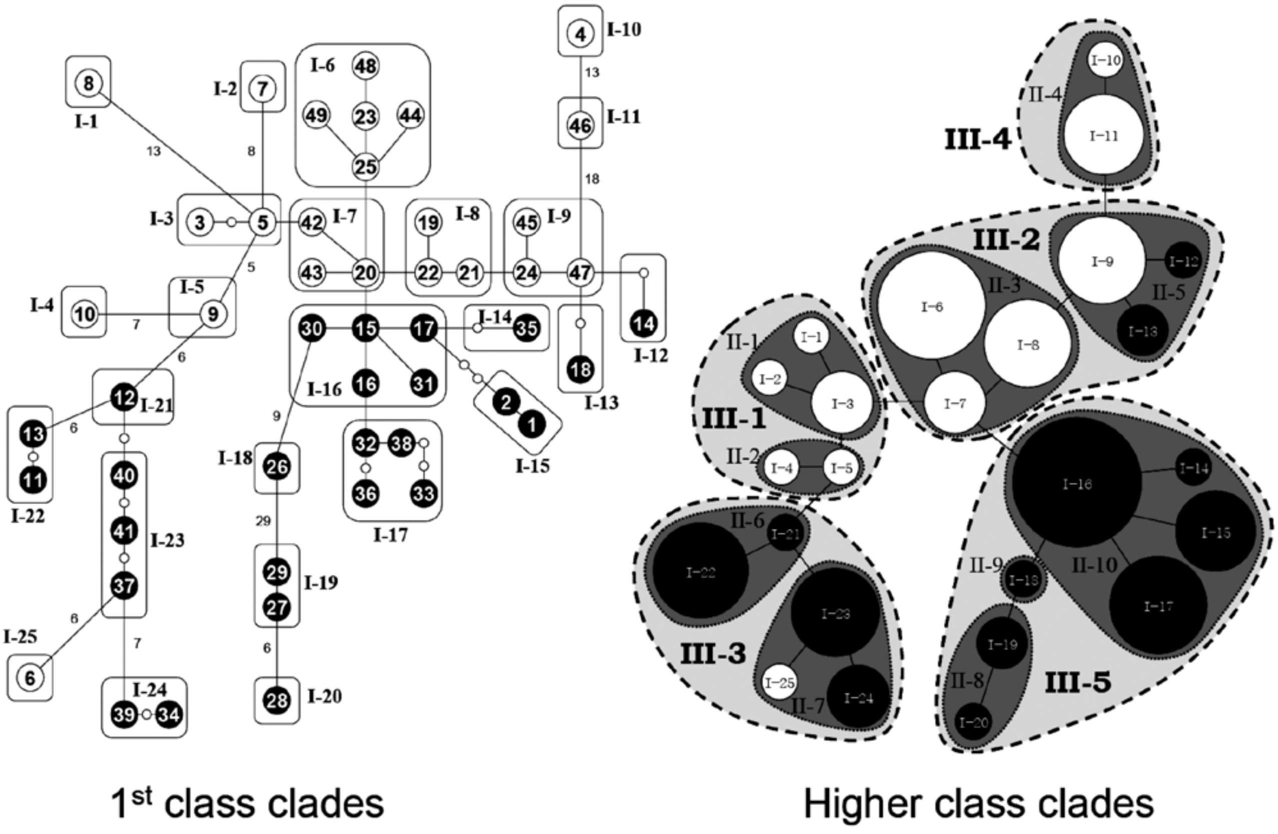

Figure 4. Nesting scheme for one-step (1st class) and higher (2nd and 3rd class) clades. Small open circles in the 1st class clades are inferred missing haplotypes we did not observe in the dataset. The haplotypes or clades observed in the east Kra Isthmus are presented in white and those observed in west Kra Isthmus are black. In the higher class clades, the size of circles represents sample size. The Arabic numbers within the 1st-class-clades circles are haplotype numbers; the Roman numbers followed by Arabic numbers are assigned numbers for hierarchical clades.

Table 5. Summary of nested clade phylogeographic analyses for clades showing statistically significant associations between haplotypes and geographical distances.

Clade nesting	Great circle distance		Sea-route distance	
	Chain of inference	Inference	Chain of inference	Inference
Clade II-5	1→2→11→12→13→21Yes	Long-distance movement	1→2→11→12→13→21Yes	Long-distance movement
Clade II-10	1→2→3→4No	Restricted gene flow with isolation by distance	1→2→3→4No	Restricted gene flow with isolation by distance
Clade III-3	1→2→11→12No	Contiguous range expansion	1→2→11→12No	Contiguous range expansion
Total cladogram	1→2→3→5→6→7→8No	Sampling design inadequate to discriminate between isolation by distance (short distance movements) versus long distance dispersal	1→2→3→4No	Restricted gene flow with isolation by distance

2007) also concluded that a biogeographic event caused differentiation of SCS and BOB populations. Population genetic studies of starfish (Benzie, 1999) and vetigastropods (Imron et al., 2007) also revealed an isolation of populations in the western Pacific and the eastern Indian Oceans. It thus seems that the Thai-Malay Peninsula (formerly Sundaland) is an effective geographic barrier to the migration of species.

However, oceanographic data (Woodruff, 2003) and a biogeographic study of populations of the giant freshwater prawn (*Macrobrachium rosenbergii*) between northern and southern sites of the Kra Isthmus (de Bruyn et al., 2005) suggest that species have migrated between the SCS and BOB in the past. In the case of *C. tagal*, the similar genetic composition of eastern and western Kra Isthmus populations implies that gene flow may have occurred across the peninsula, before and/or after the formation of the Kra Isthmus. According to Wolfe et al.'s (1987) estimation of the evolutionary rate of cpDNA ($1.0\text{-}3.0\times 10^{-9}$ per site per year), the divergence time between east and west Kra Isthmus populations was about 4.59-1.53 Mya. This is similar to the estimated time of formation of the Thai-Malay Peninsula land barrier after the last marine transgressions during the Pliocene era, 5.5-4.5 Mya (Woodruff, 2003). Although dating the variation of cpDNA markers includes a large margin of error, the approximate agreement between the dates of the genetic and paleoclimatic data suggests that geographical and climatic changes caused a divergence of eastern and western populations of *C. tagal*.

The geographic isolation imposed by the Kra Isthmus contributes to about one-third of the total genetic variation present in the *C. tagal* populations that we studied (Table 4). This divergence apparently began in the Pliocene, when a land barrier emerged that separated the eastern and western populations. In addition, 30% of the pairwise-compared *Nm* values between the eastern and the western populations are higher than 0.8, and 56.7% are higher than 0.5 (Table 3). This indicates that the Thai-Malay Peninsula might allow some gene flow across the Kra Isthmus (Figure 3).

The haplotypes located at the center of a network are usually considered the most ancestral. Thus, clades II-3 and II-10 (Figure 4) probably include the most ancient haplotypes of eastern and western populations and presumably carry the maximum amount of genetic information regarding the ancestral populations of *C. tagal* ~5 Mya. In addition, the star-like topology that we observed, with short interior branches that lead to long terminal clades with missing links, is indicative of exponential growth events (Anderson et al., 2003). Populations of other organisms in the Indo-Malay region, such as the mollusc *Haliotis asinina* (Imron et al., 2007), also seem to have rapidly expanded ~5 Mya. The rapid expansion of *C. tagal* populations is supported by the unresolved splitting of phylogenetic relationships among haplotypes (Figure 3). The unresolved haplotype relationships of the eastern and western populations may be due to ancient gene flow and incomplete lineage sorting, caused by climatic changes that led to fluctuations of sea level.

Comparison of geographic distance-based NCAs

The two geographic distance-based NCAs yielded the same inferences for the lower-class network, but different inferences for the total cladograms. It may be that in our network the lower class clades are almost exclusively composed of populations on the same side of the isthmus, between which great-circle and sea-route distances are similar. For the highest clade, a different interpretation is necessary due to the substantial differences between the great-circle and sea-route distances that separate the eastern and western populations. The great-circle distance-based analysis failed to discriminate between IBD and LDD. This may be due to the short great-circle distances between populations on the two sides of isthmus and between populations on the same sides of the isthmus. Thus, cross-isthmus dispersal of propagules cannot be distinguished from around-peninsula dispersal using great-circle distances. However, using the more realistic sea route-based geographic distances, we infer a scenario with restricted gene flow and IBD. This is supported by the absence of tight connections between haplotypes representing eastern and western populations within all but two clades (Clades II-5 and II-7).

This pattern excludes the possibility of propagule exchange across the Kra Isthmus mediated by changes in stream drainage patterns or rising sea levels. Indications of migration around the peninsula in the 5 million years since the Malay Peninsula emerged are also provided by the high pairwise-*Nm* values derived from the F_{ST} data (Table 4). Since different inferences can be drawn from analyses using the two types of distances, Fetzner and Crandall (2003) suggested utilization of both types of distances to provide insight to historical and contemporary processes. However, the sea-route-distance mode provides more precise inferences of mangrove dispersal that reflect the difficulties of LDD, and are consistent with the field experiment results of McGuinness (1997). We cannot completely exclude the possibility of LDD. In particular, when verifying other analytic results (i.e. *Nm*) and geological evidence (i.e. glacial/interglacial cycles), it is realistic to consider rare instances of LDD. We conclude that (a) dispersal events have occurred between populations on the same side of the Kra Isthmus and between populations on different sides of the isthmus; (b) cross-side dispersal has been far less frequent than within-side dispersal; and (c) since its emergence, the Kra Isthmus has played an important role in differentiating and structuring the populations of *C. tagal*.

Potential means of dispersal across the Kra Isthmus

Our network analysis (Figure 4) revealed unexpected associations of eastern and western populations in subclades II-5 and II-7. Dates based on the estimated evolutionary rate of cpDNA indicate that the contiguous range expansion of subclade II-7 (III-3) occurred ca. 7.04-2.35 Mya, and the LDD of subclade II-5 occurred 0.49-0.17 Mya. Thus, contiguous range expansion of III-3 occurred at about the same time as the Kra Isthmus formed. This indicates that haplotype exchanges may have occurred before the isthmus formed. In other words, the NCA concluded that rapid range expansion of *C. tagal* occurred ~5 Mya, at which time rising sea levels promoted frequent gene flow between mangrove populations situated on the SCS and BOB coasts. However, LDD events also seem to have occurred less than one million years ago, indicating that propagules have dispersed across the Kra Isthmus via a sea route or land bridge.

There are two possible mechanisms by which *C. tagal* may have dispersed from one side of the Kra Isthmus to the other. The first is directly across the isthmus, perhaps through openings in the Kra Isthmus during interglacial periods or through human-assisted introductions. There is no evidence that sea levels have risen by more than 20 meters above present levels in the past 0.5 million years (Rohling et al., 1998). This is much less than the 100 meters required for such openings. Human-mediated dispersal also seems unlikely because ancient "Heidelberg Man" (*Homo heidelbergensis*) and/or their relatives living in the area roughly 0.2 to 0.6 Mya had no apparent uses for mangrove seedlings or reasons to aid dispersal. Although we cannot exclude the possibility of animal-mediated dispersal, there is no evidence that mangroves are dispersed this way. Thus, human- or animal-mediated LDD is unlikely to account for the apparent cross-isthmus gene flow that we detected.

The second mechanism of dispersal across the isthmus is LDD around the Malay Peninsula, presumably via the Malacca Strait. Straits are generally considered to provide corridors for species migration in the waters they connect. A classic example is the Strait of Gibraltar, in which organisms passed between the Atlantic and Mediterranean (Seidenkrantz et al., 2000; Betzler et al., 2006; Gonzalez-Wanguemert et al., 2006). Similarly, the Malacca Strait plays an important role in the passage of marine organisms (and merchant vessels) between the Indian Ocean and the SCS. In addition, the eastern and western populations of *C. tagal* may have been isolated during glacial periods (when the Sunda Shelf emerged above sea level) and reconnected during interglacial periods. The likely dates for the LDD interglacial events are apparently associated with subclade II-5 of *C. tagal* and range from 0.49-0.17 Mya (including the last part of the Günz-Mindel, ~ 0.62-0.45 Mya and all of the Mindel-Riss ~0.3-0.2 Mya). Thus, despite the uncertainty of molecular dating, it is consistent with the proposal that interglacial sea-level rises facilitated trans-isthmus gene flow of mangroves.

On the other hand, irrespective of the routes involved, we provided evidence of a few rare LDD events between eastern and western populations, and these appear to have hindered allopatric speciation. The unresolved nature of our haplotype tree, which make determining the time of divergence between haplotypes of the two coasts difficult, also indicates recent contact between eastern and western populations (Figure 3). A similar phenomenon has been reported for Baja California's marine fishes (*Halichoeres semicinctus*, *Semicossyphus pulcher*, *Hermosilla azurea*, and *Sebastes macdonaldi*). Bernardi et al. (2003) suggested that the lack of tree topology resolution for these species was probably due to recent or ongoing dispersal, with high levels of gene flow (although they could not exclude the possibility of insufficient time for lineage sorting due to the use of slowly evolving markers). Thus young isthmuses, such as Baja California and the Kra Isthmus, appear to inhibit migration and dispersal but do not entirely prevent low-frequency LDD.

In summary, our results suggest that populations of *C. tagal* diverged by a vicariance mechanism during formation of the Kra Isthmus. The formation of this land barrier is considered to have prevented gene exchange between SCS and BOB populations of other organisms, including sea snakes (Karns et al., 2000; Heatwole et al., 2005), sea urchins (Lessios et al., 2001), and other mangrove species (Tan et al., 2005; Su et al., 2006). However, we detected gene flow between the eastern and western sides of the Kra Isthmus after increasing sample sizes. We consider two types of gene flow: (a) ancient gene flow by direct exchange across a leak in the Kra area approximately 5 Mya, and (b) infrequent but repeated LDD around the Malay Peninsula and through the Strait of Malacca during interglacial periods. These ancient and recent gene flows caused a mixed haplotype distribution and unresolved phylogenetic relationships among the eastern and western populations of *C. tagal*. In other words, the Malay Peninsula does not completely prevent gene flow of mangroves between the SCS and BOB. Thus, in agreement with previous studies (Tan et al., 2005; Su et al., 2006; e.g. Su et al., 2007; Liao et al., 2007) our results challenge the common belief that the Thai-Malay Peninsula has stopped the LDD of mangroves between SCS and BOB. Most researchers have ignored the significance of rare LDD and have only focused on "major events," such as the vicariance caused by Sundaland. However, some minor dispersal events can have large impacts of population structure and even retard or impede the process of allopatric speciation. Our study of *C. tagal* provides important insight into the significant role of rare LDD in the prevention of allopatric speciation.

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分布於馬來半島克拉地峽的細蕊紅樹 (紅樹科) 之基因交流研究

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馬來半島在冰河時期時，連接婆羅州形成巨大的古巽他大陸，長久以來被認為阻隔了南中國海 (西太平洋) 和孟加拉灣 (印度洋) 間的交流。然而，在古地質事件約五百萬年前的間冰期，因海平面上升，使得馬來半島最狹窄的克拉地峽 (Kra Isthmus) 被海水淹沒，原本分布在兩個隔離海域的生物得以互相交流。在此研究中，我們利用了母系遺傳的葉綠體 DNA 作為分子標幟，試圖追溯分布在克拉地峽兩岸的紅樹林之細蕊紅樹是否受到馬來半島的隔離影響，導致遺傳上的分歧，進而評估橫跨海域的長距離擴散的可能性。由其單套型分佈的情形顯示，克拉地峽的確形成有效的地理屏障，造成兩岸族群的遺傳分化。經由單套型在親緣分析結果顯示，在地峽東、西兩岸的細蕊紅樹族群無法呈現良好的解析力，顯示古地質事件造成的族群擴張和近代海流造成的長距離基因交流導致此結果。分別利用直線距離和海路距離等兩種地理距離估算，進行巢狀支系分析 (nested clade analysis, NCA)，結果顯示現今地峽東、西兩岸的細蕊紅樹族群分布原因為地峽形成前的族群區域性擴散和隔離後所致侷限的基因交流所造成。此外，經由嚴格分子時鐘估算結果，呈現之時間尺度吻合五百萬年前橫跨克拉地峽的基因交流發生時間。本研究結論指出在馬來半島形成後，有效阻隔克拉地峽兩側的族群，但在間冰期導致海平面上升，馬來半島南端麻六甲海峽的通道提供兩海域生物長距離遷徙的路徑。此研究提供了經由少數個體長距離擴散事件，導致族群遺傳均質化的結果，遏止了長時間隔離造成的異域種化的案例。

關鍵詞：細蕊紅樹；克拉地峽；地理屏障；擴散；隔離；分歧時間。