Genomic landscape of subspecies defined by phenotypic criteria:Analyses of the mangrove species complex, Avicennia marina

Zhengzhen Wang[1], Zixiao Guo[1], Cairong Zhong[2], Haomin Lyu[1], Xinnian Li[1], Norman Duke[3], and SUHUA SHI[4]

[1]Sun Yat-Sen University
[2]Hainan Dongzhai Harbor National Nature Reserve
[3]James Cook University
[4]Sun Yat-sen University

September 28, 2020

Abstract

Subspecies designation is widely used to describe taxa below species but above geographical populations. What patterns of genomic variation is expected if taxa are designated as subspecies? In this study, we carry out such a survey on the mangrove tree Avicennia marina of the Indo-West Pacific coasts. This species has three subspecies, distinguished by morphological traits and geographical distribution. We collected samples from 16 populations (577 individuals) covering all three subspecies and sequenced 94 nuclear genes. We reveal comprehensive genetic divergence among subspecies, generally higher than among geographical populations within subspecies. The level of genetic diversity differs among the three subspecies, possibly hinting at a degree of separation among their gene pools. We observed that divergence varies from locus to locus across the genome. A small portion of the genome is most informative about subspecies delineation while the rest is undifferentiated or slightly differentiated, hinting at uneven gene flow and incomplete isolation. The three subspecies likely split simultaneously with gene flow among lineages. This reticulate evolution results in some discordance between morphology and genetics in areas of population contact. In short, A. marina subspecies show species-like patterns in some respects and population-like patterns in others. This “ambiguity” is expected at a stage between structured populations and full species, thus the observed patterns strengthen the subspecies designation. We propose that subspecies designation is informative in predicting genomic landscape of divergences and useful in making conservation decisions.

INTRODUCTION

Species is widely considered as the basic entity of biodiversity in nature, although different species concepts have been proposed and used (Zachos, 2016). The most popular biological species concept (BSC) uses reproductive isolation as the gold standard to designate species. In taxonomy, subspecies is used to denote recognizable infraspecific differentiation above populations. Conventionally, subspecies is defined as “a geographically defined aggregate of local populations which differ taxonomically from other subdivisions of the species” by E. Mayr (1940, 1963). In the context of BSC, subspecies can interbreed without a fitness penalty (Patten, 2015), although a test of this ability is usually not practically possible in the wild. The designation of subspecies has been criticized as arbitrary and rejected by some taxonomists (Wilson & Brown, 1953; Hawlitschek, Nagy, & Glaw, 2012; Phillimore & Owens, 2006; Torstrom, Pangle, & Swanson, 2014).) Others insist on the value of the subspecies rank (Durrant, 1955; Mayr, 1982; Phillimore & Owens, 2006), with the emphasis on unique geographic range or habitat, phylogenetically concordant phenotypic characters, and unique natural history (O’Brien & Mayr, 1991).

Given the taxa designated as subspecies, conventionally basing on morphological and geographical evidences, we would expect that the designation itself represents a level of genetic variation at somewhere between geographical populations and full species. It requires some examinations on the pattern of genetic variations, which may strengthen or weaken the subspecies designation. Many studies have tested whether defined subspecies are monophyletic on phylogenetic trees constructed using a handful of genetic markers (Moritz, 1994; Phillimore & Owens, 2006). However, it provides little assessment of the divergence level, because monophyly is also evidence of full species and the alternative is normally interpreted as a deficit of divergence. Instead, we propose that divergence among, given polymorphism within, subspecies should be assessed using population genetic analyses, since speciation proceeds at the population level.

The reduction in cost of sequencing allows us to quantify genetic divergence across the genome in large population samples. With such data in hand we can ask the following questions: What are the patterns of genomic variation among taxa *a priori* designated as subspecies? Do these patterns strengthen or weaken the subspecies designation? We start from investigating the taxa that can be reasonably designated as subspecies by conventional criteria. We perform such a study on the mangrove tree *Avicennia marina* . It is the most wide-ranging mangrove species, reaching the most marginal mangrove patches of the Indo-West Pacific region (Duke, 2006; Tomlinson, 2016). The taxonomy of Indo-West Pacific (IWP) *Avicennia* had been troublesome before Duke's comprehensive revision (Duke, 1991). In that assessment, *A. marina* were divided into three varieties (Duke, 1991). After that division, "varieties" or "subspecies" were used to refer to the three groups by different authors (Duke, 2006; Duke, Benzie, Goodall, & Ballment, 1998; Maguire, Peakall, Saenger, & Maguire, 2002; Maguire, Saenger, Baverstock, & Henry, 2000).

The three subspecies show remarkable differences in morphological traits and geographical distribution (Duke, 1991, 2006). Details of these differences are described in the following section. A previous study used allozyme markers to determine genetic divergence among these subspecies, in which no fixed differentiation among subspecies was identified and the populations were genetically clustered into two groups (Duke et al., 1998). However, the reliability of that study is greatly compromised because they used very few genetic markers. We sought to obtain a comprehensive determination of genetic divergence among the three subspecies as well as reconstructing their evolutionary history through collecting single nucleotide polymorphisms across close to a hundred genomic loci.

The clarification of genetic divergence among subspecies may encourage us to treat these subspecies as different conservation units, particularly in projects such as transplanting and breeding. As the most widely distributed mangrove tree, this significance is valuable for*A. marina* . Mangrove species are all a conservation priority because these species are ecologically important in sheltering coastal regions from hurricanes, supporting the intertidal ecosystem, and sequestering carbon, but they are under great threat of global climate change in combination with more direct human disturbances (Gilman, Ellison, Duke, & Field, 2008; Guo et al., 2018a).

METHODS

Morphological characters, sampling, and DNA extraction

The three subspecies of *Avicennia marina* are *marina, eucalyptifolia* , and *australasica* . The subspecies *marina*is widely distributed from eastern Africa, through the Middle East, South Asia, Southeast Asia, and north to South China (Figure 1). It is also found in western Australia. The subspecies *eucalyptifolia* is mainly distributed in northern Australia and extends to southern Philippines, western Indonesia, and the Southwestern Pacific islands. There is a significant range overlap of the two subspecies in western Australia. *Australasica* is restricted to south-eastern Australia and northern New Zealand (Figure 1). *Australasica* can be morphologically distinguished from the other two by its fully pubescent calyx lobes and bracts (Duke, 1991, 2006). These structures are more glabrous in the other two subspecies. The bark of *australasica* is grey fissured, with short longitudinal fissures or reticulate lines, while the bark of the other two subspecies is smooth green or chalky white with flaky patches. *Eucalyptifolia* is mainly distinguished by its lanceolate leaves (as opposed to ovate to elliptic), as well as the style in open flowers which are positioned level with upper edges of anthers (instead of the lower edges of anthers) (Duke, 1991, 2006).*Marina* may also be distinguished by its larger flowers and thicker leaves. However, these distinctions in morphological characters may be inconclusive where two putative subspecies coexist (Duke, 2006). Typical for mangrove trees, propagules of *A. marina* are bouyant on sea water and disperse over sea to nearby locations with mangorve habitats (Duke, 2006).

We sampled 16 populations, 577 individuals (16 to 100 individuals per population) from East Africa, South China, Southeast Asia, Australia to New Zealand, covering *A. marina* ’s range (Table 1, Figure 1). To avoid sampling offspring from the same tree, sampled individuals were at least five meters apart. At each site, we sampled as many individuals as were available, but no more than 100. Leaves from each individual were dried, labeled, and stored for DNA extraction. DNA was extracted using the modified CTAB method (Doyle & Doyle, 1987). DNA content of each extraction was measured by NanoDrop 2000. For each population, we pooled 300 ng of DNA from each plant to make one DNA pool, ensuring that it contains the same proportion of DNA from each individual. Sixteen DNA pools were used in our experiments.

PCR and Illumina high-throughput sequencing

Based on about 200 DNA sequences from a library of *A. marina*expressed sequence tags (Huang et al., 2014), we developed a new set of primers anchored at exons but spanning at least one intron. The 94 pairs of primers producing amplicons 500 to 1500 bps long were used in this study. We performed polymerase chain reaction (PCR) amplification on DNA pool from each population using our 94 primer pairs. To reduce amplification errors, TaKaRa high-fidelity PrimerStar HS DNA polymerase was used. The 30 µL PCR mixture consists of 3 µL 10x TaqBuffer (Mg2+), 3 µL dNTPs (2mM/µL), 1.5 µL of each primer (10µM/µL), 0.5 µL HS DNA Polymerase, 3 µL DNA template (˜10ng/µL) and 19 µL deionized water. The PCR program was: 4 min at 94°C; 30 cycles of 10 s at 94°C, 30 s of annealing at the corresponding temperature (Table S1 in the online supplementary file), extension at 72°C for 2 min; followed by 8 min final extension at 72°C. Reactions were held at 16°C before PCR products were subjected to electrophoresis on 1.2% agarose gels. Target bands were excised under ultraviolet light and extracted using the Pearl DNA Gel Extraction Kit (Pearl, Guangzhou, China). Extracted DNA was examined by NanoDrop 2000 to ensure that the amount of each gene product was no less than 100ng. PCR products of the 94 loci from the same population were again pooled, using 100 ng of DNA per locus. We thus obtained 16 PCR product pools, each including amplicons from 94 loci.

PCR product pools from each population were delivered for sequencing on the Illumina Genome Analyzer and Illumina HiSeq 2000 platform at BGI (Shenzhen) following the manufacturer’s instructions. 200 bp DNA libraries were constructed for these mixtures and an 8 bp index in the adapter was used to distinguish the populations. Method details used for library construction were the same as those detailed in the supplementary materials of our previous publication (Guo et al., 2016). Raw reads produced from the Illumina Genome Analyzer platform were 90 bps in length (all populations except MC, BB, and DW; abbreviations of population names are defined in Table 1), while those from the Illumina HiSeq 2000 platform were 130 bps in length (MC, BB, and DW).

Read mapping and variant calling

The quality of short reads produced by the Illumina sequencing platforms was first examined by FastQC (Andrews, 2010). Short reads were then mapped to reference sequences using MAQ 0.7.1(Li, Ruan, & Durbin, 2008). Notably, the reference sequences were obtained by sequencing DNA amplicons of all 94 loci from one *A. marina* individual using the Sanger method. We also did this for one *A. alba* individual for use as outgroup. In mapping and pileup, the mutation rate between reference and read was set to 0.002, the threshold of mismatch base quality sum was 200, and the minimum mapping quality of reads was 30. To exclude false-positive mismatches, we counted the mismatch rate for each site across the read and mismatch rate for each base quality. We trimmed the first and last 10 bases of each read and filtered bases with quality score less than 30.

By identifying variant sites using MAQ 0.7.1, we obtained nucleotide polymorphism information within each population. To avoid bias introduced by sequencing errors, we discarded sites with insufficient site coverage

(<100 reads) and those with minor allele frequency less than 0.01 in each population (He et al., 2013). We obtained a list of single nucleotide polymorphisms (SNPs) per population, with allele frequencies. To reduce false SNPs introduced by homopolymers or insertions/deletions, putative variants in those regions were masked. The 16 sets of SNPs were used in the analyses below.

Genetic divergence and diversity estimation

To estimate absolute genetic divergence between populations, we computed pairwise D_{XY} following the formula derived by Nei (Nei & Li, 1979). When calculating D_{XY} , two alleles at each SNP were interpreted as two haplotypes and corresponding allele frequencies as haplotype frequencies. PairwiseD_{XY} values were summed over all SNPs and the sum was normalized by effective sequence length. For each pair of populations, the effective sequence length was defined by sites without missing data in both populations. The obtainedD_{XY} matrix was used in multidimensional scaling using the 'cmdscale' package implemented in R (Figure 2), as well as neighbor-joining tree constructed using MEGA7 (Kumar, Stecher, & Tamura, 2016). We also performed Principal Component Analysis (PCA) on the SNP frequency matrix (summarizing the frequency of each SNP in each population) using the "prcomp" function in R (Venables & Ripley, 2002) to test whether the SNP frequencies differed among populations. Finally, to assess the extent to which genetic polymorphisms were fixed,F_{ST} statistics were computed following a method for many SNPs (Nei & Miller, 1990; Willing, Dreyer, & van Oosterhout, 2012).

The levels of genetic diversity within populations were measured by π and Watterson's ϑ statistics. π summarizes the average number of nucleotide differences between two sequences randomly sampled from a population (Nei, 1987), while Watterson's ϑ estimates nucleotide polymorphism based on the number of observed segregating sites (Watterson, 1977). To correct systematic errors of high-throughput sequencing, we computed ϑ values following a published algorithm (He et al., 2013).

Analyses of molecular variance (AMOVA) basing onD_{XY} and F_{ST} are used to test whether genetic variation was partitioned by subspecies or geographical region. In the test for geographical region, the populations are assigned into three groups with the Malay Peninsula and Wallacea as the boundaries, which are two major discontinuities revealed in mangrove species (Guo et al., 2018b, 2016; J. Li et al., 2016; Yang et al., 2017). The first group includes MC, PN and LS, the second group includes BB, CA, DW, BS and AK, and the last group includes all the other populations.

Mantel tests of D_{XY} andF_{ST} against geographic distance was performed to test the Isolation by Distance model. Geographical distances between sampling sites were approximated either by spheric distance or dispersal pathway along coasts (called coastline distance). The coastline distance is estimated according to the simulation of one-month oceanic dispersal ability using the methods described in (Van der Stocken, Carroll, Menemenlis, Simard, & Koedam, 2019), with approximate ruler of 350 km.

Geographic barriers delineating the largest genetic discontinuities between pairs of populations were identified using BARRIER 2.2 (Manni, Gue, & Heyer, 2004). By randomly selecting half of the 94 genes, we calculated one F_{ST} matrix for the 47 genes. We repeated this process 100 times and obtained 100F_{ST} matrices. Robustness of each inferred barrier was thus assessed by the 100 matrices.

Haplotype inference to illustrate genomic divergence

The portion of the genome unaffected by gene flow increases as speciation proceeds (Feder, Egan, & Nosil, 2012; Feder, Flaxman, Egan, Comeault, & Nosil, 2013; Nadeau et al., 2013; Wu, 2001; Wu & Ting, 2004). As subspecies are somewhere in the speciation continuum, how is differentiation distributed across the genome? The pattern can be visualized by inferring haplotypes of loci and comparing the haplotype networks. The method developed by He et al. (2019) was used to infer haplotypes. This method uses SNP linkage information in each short-read pair to infer haplotypes and frequency of each haplotype in the population, following an expectation-maximization algorithm (Bilmes, 1998; Dempster, Laird, & Rubin, 1977). If two adjacent SNPs were not covered by any read pair, we broke the gene into segments. In this case, the midpoint of the two adjacent SNPs is defined as the breakpoint of two consecutive segments. The accuracy of this method in inferring haplotypes has been validated by sequencing individuals using the Sanger method (He et al., 2019). We selected eight populations representing different subspecies and different regions for inferring haplotypes: two*eucalyptifolia* (CA and DW), two *australasica* (AK and BS), and four *marina* populations (BB, LS, TN, and SY). Genes were split into 454 linked segments and haplotypes were inferred for each segment (Table S2). Before constructing haplotype networks, we filtered out segments with length less than 100 bps or with missing data. For each of the 231 retained segments, we computed a haplotype network using the NETWORK software (Polzin & Daneshmand, 2003). For some segments, the sequences were blasted against the database of National Center for Biotechnology Information for function annotation.

Modelling the pattern of lineage-splitting within A. marina

To infer the lineage-splitting pattern within *A. marina* , we compared our real sequences against simulated sequences under eight models assuming a variety of topologies (Simulation 1). Simulated sequences under these models were produced using the ms software (Hudson, 2002). The models are: (1) panmictic; (2) *eucalyptifolia*by itself and the other two subspecies together; (3) *australasica*by itself and the other two subspecies together; (4) *marina* by itself and the other two subspecies together; (5) three separate lineages with *eucalyptifolia* diverging first; (6) three lineages with *marina* diverging first; (7) three lineages with*australasica* diverging first. (8) three lineages diverging simultaneously. In simulation 1, groups were divided according to subspecies designation in the prior. As a control, we constructed artificial groups by pooling two populations each from one subspecies. Using these groupings, we repeated the simulations and model selection on the eight models described above (Simulation 2).

The effective population sizes of the lineages (N) and coalescent times (T) were common among all models. Notably, to reduce the complexity of parameter setting and to speed up computation, all population size parameters were derived from a single parameter N$_0$randomly chosen from the prior distribution. In models with more than one lineage, N$_0$ was assigned to any one of the lineages (using as baseline). N of other lineages were produced by multiplying N$_0$ by ϑ_x/ϑ_0, where ϑ_x and ϑ_0 are the observed ϑ of the current and baseline lineage respectively.

For each model, we performed 100,000 coalescent simulations using the ms program (Hudson, 2002). Each simulation contained 80 loci of 1000 base pairs. Mutation rate was set at 3.26x10^{-8}/generation/bp, estimated from phylogenomic comparisons to closely related species with whole genomes (He et al., 2020). The sample size of each group was consistent with our real field sampling (Table 1). Demographic parameters were drawn randomly from a uniform prior distribution. Identical prior distributions of corresponding parameters were set for models within each set (Table S3 & S4).

Ten summary statistics were calculated for each simulated data set, including segregating site number (S), Watterson's estimator (ϑ), nucleotide polymorphism (π) and Tajima's D within each group, as well asD_{XY} and F_{ST} for each pair of groups. Summary statistics were calculated for each simulation independently. Euclidean distances were calculated by comparing simulated statistics with corresponding observed summary statistics. The tolerance of retaining simulated data was set to 0.05. Bayesian posterior probabilities of each model were then estimated following the Approximate Bayesian Computation (ABC) schema (Beaumont, Zhang, & Balding, 2002) using the "abc" package in R (Csilléry, François, & Blum, 2012). The "postpr" function together with "neuralnet" option in the "abc" R package was used to perform model selection.

We also built four models (v1, v2, v3, and v4) to test whether the population from Bunbury, Australia (BB, Table1) genetically belongs to*marina* or *eucalyptifolia* (Simulation 3, Table S5). In model v1 and v2, BB (constant effective population size of N$_{bb}$) and *marina* (N$_{ma}$) coalesced at vT$_1$ generations ago and then the common ancestor further coalesced with *eucalyptifolia* (effective population size N$_{eu}$) at vT$_0$ generations ago (vT$_0$>vT$_1$). Model v1 differed from v2 by presence or absence of gene flow (m$_1$and m$_2$) between BB and *eucalyptifolia* . Similarly, in models v3 and v4, BB (N$_{bb}$) coalesced with*eucalyptifolia* (N$_{eu}$) at vT$_1$generations ago. The common ancestor then coalesced with *marina*(effective population size N$_{ma}$) at vT$_0$generations ago (vT$_0$>vT$_1$). Nine summary statistics, Watterson's estimator (ϑ) for each population and pairwise F_{ST} and D_{XY} , were used in the model selection procedure similar to the one previously described.

Detection of gene flow between subspecies

We used the statistical model implemented in TreeMix to infer patterns of splits and mixtures among populations (Pickrell & Pritchard, 2012). As revealed from the F_{ST} statistic above, some populations are genetically similar, e.g. Andaman Sea on the west of Malay Peninsula and the South China Sea (Gulf of Thailand and Hainan Island). Hence, one representative population from each region was used in this analysis. The twelve populations were related to the common ancestor through a graph of ancestral populations, which was inferred by allele frequency and a Gaussian approximation to genetic drift (Pickrell & Pritchard, 2012). Gene flow events were inferred by adding admixtures onto the Maximum Likelihood population splitting topology.

RESULTS

Among-subspecies genetic divergence

We obtained 76 to 87 kb of DNA sequence covering 88 to 94 genes (Table 1). By mapping short reads to reference sequences, we identified 74 to 1657 segregating sites within each population (Table 1). We calculated among-population pairwise D_{XY} values to assess genetic divergence and used the resulting distance matrix to construct a neighbor-joining tree. The D_{XY} matrix shows clear divergence between the three subspecies, with the BB population the sole exception (Figure 2b). The largest D_{XY} values were observed between the *australasica* populations and the other two subspecies, ranging from 7.7 to 9.9/kb (Table S6). Lower divergence was observed between *eucalyptifolia* and *marina*populations, with D_{XY} values between 6.5 and 7.4/kb. By pooling populations within each subspecies, we estimated theD_{XY} to be 8.2/kb between *eucalyptifolia*and *australasica* , 6.7/kb between *marina* and*eucalyptifolia* and 9.1/kb between *marina* and*australasica* .

Genetic divergence was generally lower among populations than among subspecies (Fig. 2d). The two *australasica* populations diverged little from each other (D_{XY} =2.2/kb). The pair of*eucalyptifolia* populations diverged more but still less than among subspecies (D_{XY} = 5.48/kb). Within*marina* , we see two major geographical groups: one containing MC, LS, and PN (west of the Malay Peninsula) and the other TN, BK, SS, SY, WC, SB, CB, and BL (east of the Malay Peninsula, Figure S1).D_{XY} per kb ranges from 1.27 to 3.75 within the first and from 0.94 to 4.69 within the second geographical group. Between the two geographical groups, D_{XY} ranges from 4.32 to 5.69, still lower than between subspecies. The BB population is an outlier and has diverged far from other *marina*populations (D_{XY} = 7.76-8.43/kb), to a level among subspecies. The AMOVA indicates 65.1% of genetic divergences (D_{XY}) is accounted by subspecies division. In contrast, 50.8% of the D_{XY} variance is accounted by geographical division.

D_{XY} provides a measurement of how far the populations diverged from each other. We also measured the extent of divergence by comparing the allele frequencies of polymorphisms within populations (Cruickshank & Hahn, 2014). Plotting principal components of the allele frequency matrix, populations of each subspecies generally cluster together but diverged from other subspecies at PC2, except that the DW population (*eucalyptifolia*) is close to *marina*populations and the BB population (*marina*) is again different from all the other *marina* populations (Figure 2c). In PC1, only population DW diverges largely from all other populations. In addition, the CA population (*eucalyptifolia*) diverges from other populations largely in PC3 and PC4 (Figure S2).

The F_{ST} statistic quantifies these genetic differences. The 120 values of pairwise F_{ST}estimates calculated for the 16 populations are generally high, with the average value of 0.61 (first and third quartiles are 0.50 and 0.76 respectively). Populations from the South China Sea, i.e. TN, BK, SS, SY, and WC (Figure S1), have relatively low pairwise differentiation.F_{ST} between the two populations on the west coast of Malay Peninsula (LS and TN) are also low (Figure S1).

The Mantel tests show a significant relationship (P value <0.01) between genetic differentiation and geographic distance. This is regardless of whether the geographic distance was estimated using the spherical or coastline method (Figure S3, see Methods for details). All four tests have P values less than 0.01and survive a multiple-test correction. This correlation indicates that geographical distance contributes, at least partly, to the high level of genetic differentiation among *A. marina* populations. However, the two geographical groups around the Malay Peninsula show genetic differentiation greater than what we would expect from the distance separating them, indicating that other factors are also important (Figure S3).

The BARRIER analysis reveals that major barriers (with >80% bootstrap support) roughly lie along the Sunda shelf and between Australasia and Southeast Asia. Minor barriers are also identified between Africa and Southeast Asia, as well as between Western Australia and Northern Australia. The major barrier in the historic Sunda Land corresponds to the obvious deviation ofF_{ST} values from the expectation based on distance alone (Figure S3 & S4).

Isolation among subspecies indicated by high divergence and inferred barriers may influence genetic diversity within populations. Both the nucleotide diversity (π) and Watterson's estimator of nucleotide polymorphism (ϑ) show different levels of within-population genetic variation. The two *eucalyptifolia* populations have the highest genetic diversity, on average ϑ (across segments) = 2.82 and 3.94/kb and π = 3.41 and 4.06/kb (Figure 3). In contrast, *marina* populations are low in genetic diversity, with average ϑ ranging from 0.21 to 0.91/kb and π from 0.15 to 1.39/kb (Table1, Figure 3). The BS population (*australasica*) has intermediate diversity, while the AK population (*australasica*) is unusually monomorphic (Table1, Figure 3). The very low diversity of the AK population is likely due to its marginal location, similar to WC and SY.

Haplotype network variation across the genome

We inferred haplotype networks across the 94 loci we sequenced. Using an expectation-maximization method to infer among-SNP linkage disequilibrium, we split these regions into 454 linked segments (Table S2). Segments with missing data and those less than 100bp in length were discarded, retaining 231 segments for haplotype network reconstruction, with *A. alba* as the outgroup (Figure 4).

Among these segments, 134 were not genetically distinguishable among subspecies with only one or a few haplotypes identified and all haplotypes closely related to each other and shared among the three subspecies. The other 66 segments reliably distinguish*australasica* from the other two subspecies. Among these 66 segments, the BB population shares haplotypes with *australasica*instead of *marina* at seven loci. The third type of segments, 14 in total, delimits *marina* from the other two subspecies. Five segments distinguish *eucalyptifolia* , but BB shares haplotypes with *eucalyptifolia* in all cases. Most importantly, in three segments, haplotypes split into three clusters and each subspecies contains haplotypes from a single cluster. These three segments provide the best subspecies delineation. At other eight segments, each subspecies also contains a cluster of haplotypes, except BB shares haplotypes with *eucalyptifolia* . Finally, one segment separates*marina* and *australasica,* but *eucalyptifolia*contains haplotypes from both clusters.

The three segments clearly delineating subspecies are from three genomic loci, Am0259, Amc232, and Amc302. We roughly estimate that about 3% of the *A. marina* genome is highly differentiated among subspecies (three out of the 94 genomic loci surveyed). Am0259 partially covers a protein coding gene, the ortholog of which in *Arabidopsis thalina*is annotated as "shaggy-related protein kinase." Amc232 and Amc302 are noncoding. The eight segments that follow subspecies delineation with the exception of the BB population are from seven genomic loci. Similarly, we estimate that about 7% (7 out of 94) of the *A. marina* genome is highly diverged among subspecies but the divergence is eliminated in populations where subspecies coexist.

A reticulate evolutionary history of the three subspecies

To infer the lineage-splitting pattern within *A. marina* , we fitted several models using approximate Bayesian computation (ABC) to test whether we can distinguish population histories. Our ABC approach shows that simulated sequences under the model with each subspecies diverging simultaneously provides the best fit to the observed data (Figure 5a). This conclusion was validated by three repetitions and high posterior probability of this model (> 0.6, Table 2). This result indicates the three subspecies diverged from each other simultaneously. In contrast, simulations with artificial groups (Simulation 2) allow no robust model selection.

The BB population morphologically diagnosed as *marina* shows lower genetic divergence and differentiation from *eucalyptifolia* than*marina* (Figure 2). Is it an *eucalyptifolia* mis-diagnosed as *marina* or a *marina* exchanging genes with*eucalyptifolia* ? Our ABC simulation (Simulation 3) shows that BB has descended from *marina* but experiences gene flow with*eucalyptifolia* populations (model v2, posterior probability 0.933, Table 2 and Figure 5b). This indicates that subspecies, while significantly differentiated, are genetically permeable. We also used TreeMix to capture potential gene flow events among populations. We identified five such events on the population splitting graph (Table S7). Three such events occurred between subspecies and the other two events occurred between BB and the outgroup species *A. alba*(Figure 5c).

DISCUSSION

Substantial genetic divergence among subspecies

In this study we comprehensively sampled *A. marina* populations across their geographical range, assembled an extensive SNP data set, and used it to quantify the genetic differentiation among the three morphologically recognized subspecies. Our study finds a robust genetic split of *A. marina* into three groups, noting that this divergence was observed both in the genetic distance D_{XY}matrix and in PCA clustering based on a SNP frequency matrix. The genetic grouping pattern is generally consistent with the morphological classification of the three subspecies, *marina* ,*eucalyptifolia* , and *australasica* . The levels of within-subspecies genetic diversity are found to differ among subspecies, implying that gene pools of the three subspecies are separated to some degree.

Genetic differentiation among populations, usually attributed to isolation by distance or isolation by geographic barriers, have been documented in many mangrove species, such as the deep genetic differentiation between populations on the opposite sides of the Malay Peninsula in *Rhizophora* (Guo et al., 2016; Wee et al., 2015)*, Ceriops* (Tan et al., 2005) *, Lumnitzera* (J. Li et al., 2016), and *Xylocarpus* (Guo et al., 2018b). Like previous findings, the differentiation on the two sides of Malay Peninsula is also observed in populations of the subspecies*marina* and is attributed to isolation of the Malay Peninsula currently and the whole Sundaland historically. Although propagules of *A. marina* are buoyant on sea water and disperse over via currents (Steinke & Ward, 2003), they are reported to be relatively weak in dispersal (Clarke, Kerrigan, & Westphal, 2001; Duke et al., 1998). Our estimates of differentiation among *A. marina*subspecies exceed those based on geographical isolation. In addition, there is no geographical barrier inferred between the regions inhabited by *australasica* and *eucalyptifolia.* There must be some other factors causing their substantial genetic divergence. These findings indicate that the subspecies designation in *A. marina*indeed represents a stage beyond structured populations on the speciation continuum.

Despite substantial divergence, these subspecies are not completely isolated. Genetics is not in concordance with morphology in some populations where two subspecies occur in coexistence or adjacently. The individuals from Bunbury, Australia, (BB) are morphologically diagnosed as *marina,* but genetically closer to *eucalyptifolia* as shown by the neighbor-joining tree and MDS clustering ofD_{XY} . A recently published study focusing on the*A. marina* population on the west coast of Australia revealed genetic differentiation across geographical distance but not between subspecies, though the samples likely contain both *marina* and*eucalyptifolia* (Binks et al., 2019). This implies that constraint on gene flow between subspecies appears to be relaxed once geographical isolation (by distance or barrier) is removed. This is compatible with a subspecies designation because full species are less likely to allow gene flow, although accidental introgression via hybridization cannot be completely ruled out.

Genomic landscape of among-subspecies divergence

The genic view of speciation has provided a schema for thinking about how genetic divergence across the genome evolves as speciation proceeds under the antagonistic forces of natural selection and gene flow (Feder et al., 2012; Wu, 2001; Wu & Ting, 2004). Initially gene flow is extensive across the genome, except at a few loci under strong divergent selection. These loci exhibiting excess of divergence are like islands emerging over sea. Genomic islands expand gradually via genetic hitchhiking. As genetic differentiation associated with reproductive isolation accumulates, genetic hitchhiking grades into genomic hitchhiking. Lastly, complete reproductive isolation is established and gene flow is impeded by various forms of behavioral, ecological, or genetic incompatibilities (Abbott, 2017; Abbott et al., 2013; Seehausen et al., 2014). With the establishment of full species, genomic islands with high divergence have expanded to a whole plateau, i.e. high divergence across most or all of the genome (Wu, 2001; Wu & Ting, 2004; Feder, Egan, & Nosil, 2012; Feder, Flaxman, Egan, Comeault, & Nosil, 2013).

We estimate that only about 10% of the genome shows excess genetic divergence among the three subspecies of *A. marina.* The proportion not affected by gene flow is around 3%. This pattern indicates a small portion of the genome belongs to genomic islands of speciation. Some degree of genetic differentiation may exist in the rest of the genome in one subspecies or some populations, or almost no differentiation among subspecies. At this stage, analyses using markers covering 10% of the genome may recognize the three taxa as full species, while those using markers sampled from the rest may indicate no more than structured populations.

Reticulate evolutionary history of the three subspecies

The pattern of genomic divergence provides clues to infer how the three subspecies evolved. A previous phylogenetic analysis indicated that the three subspecies split in a bifurcated manner, with *australasica*diverging first at about 2.7 Mya and *marina* diverging from*eucalyptifolia* at about 1.8 Mya (X. Li et al., 2016). However, given the high variability of genetic divergence across the genome we described above, such phylogenetic analyses using a handful of markers are not reliable in resolving taxa below species. Our Approximate Bayesian Computation modeling supports a simultaneous split of the three subspecies. Such trifurcate split is probably a nascent event of speciation radiation, although the number of diverging lineages is not that large.

Mantel tests indicate that geographical distance might have played a role in isolating populations of *A. marina* . Considering that the three subspecies are distributed along a continuum, we expect to observe a significant positive correlation between genetic divergence and geographical distance in between-subspecies population pairs. However, the divergence cannot be completely explained by isolation by distance. Obvious geographical barriers are inferred between Southeast Asia and Australasia, roughly consistent with the boundary of the *marina*and *eucalyptifolia* ranges. Based on the clines described above, we hypothesize a scenario of *marina* –*eucalyptifolia* split: New Guinea was connected to Australia during glacial ages when sea level was low (Duke et al., 1998) and split *A. marina* populations east and west of the Torres Strait, followed by westward expansion of*eucalyptifolia* during periods of high sea level and opening of the Torres Strait (Gordon, 2005; Hall, 2009). On the other side,*eucalyptifolia* may have differentiated from *australasica*between Rockhampton and Brisbane on the east coast of Australia via the bifurcation of the North Caledonian Jet into the North Queensland and the East Australian Currents (Ganachaud et al., 2007; Schiller et al., 2008) and the latitudinal change in environmental conditions such as temperature. Further studies may clarify these hypotheses.

The discordance between morphology and genetics in some populations (e.g. BB) hints at gene flow among subspecies and is supported by the TreeMix inferences. The BB population on the west coast of Australia is morphologically diagnosed as subspecies *marina* (Duke, 1991) but shows high genetic divergence from other populations and is genetically closer to *eucalyptifolia* than *marina* . Remarkably, all subspecies-informative loci we surveyed show either *marina* or*eucalyptifolia* haplotypes in BB. Hence, BB is highly likely an admixed population. Our simulation confirmed this. In addition, the CA and DW populations show very different patterns of SNP allele frequencies but have not diverged much. This contrast is probably due to both populations harboring composite alleles introduced from either*marina* or *australasica* . CA is likely admixed with*australasica* and DW with *marina,* since they neighbor each other. The sharing of haplotypes with respective subspecies of the two populations appears to support this speculation (Figure 4).

The three subspecies appear to have evolved reticulately, with gene flow among them. This is consistent with their subspecies designation, which indicates they are in the continuum of speciation. Gene flow during speciation has been reported in many taxa, especially at the early stages (Brandvain, Kenney, Flagel, Coop, & Sweigart, 2014; Clarkson et al., 2014; Harr, 2006; Poelstra et al., 2014; Wang, He, Shi, & Wu, 2020). How gene flow recedes as speciation nears completion remains to be addressed. Gene flow is recently proposed to exist even at later stages of speciation (Wang, He, Shi, & Wu, 2020). The role of gene flow in the evolution of populations at the subspecies stage would be interesting to pursue in further studies.

Observed patterns strengthen subspecies designation

In summary, the genetic patterns we revealed strengthen the subspecies designation of *A. marina* . The generally higher level of average genetic divergence among subspecies than structured populations, the distinct levels of genetic diversity within each subspecies, and the clear delineation of subspecies in a small portion of the genome complement the diagnostic morphological differences and indicate substantial divergence among subspecies analogous to full species. The obvious admixture when in contact and low differentiation across the majority of the genome indicate that the subspecies have not yet formed full species. The recognizable trifurcate split of the designated subspecies is coupled with gene flow events, leading to reticulate evolution within *A. marina* . In short, the subspecies show species-like patterns in some respects and population-like patterns in others. These patterns support the idea that these taxa are intermediate between geographical populations and full species. Hence, the subspecies designation is reasonable and informative.

Utility for conservation

The clarification of genetic divergence and evolutionary history of subspecies highlights the predictive value of subspecies designation. The availability of genomic divergence data strengthens the necessity to treat these subspecies as different conservation units, helping to avoid neglect of important genetic resources. The cases of cryptic species going extinct before human recognition should be alarming (Yan et al., 2018). In practice, our assessment provides instructions for selecting source plants for transplanting or breeding in mangrove restoration projects. The conservation of individual species and intraspecific genetic diversity is of great importance in mangrove conservation. The stability of the ecosystem may be cumulatively enhanced by weak effects of individual units of diversity, analogous to gene regulatory networks (Chen et al., 2019). As one of the most widely distributed mangrove species, *A. marina* is important for the ecological health of coastal ecosystems, especially as the global climate continues to change.

ACKNOWLEDGEMENTS

We thank Chung-I Wu for insightful comments that improved the manuscript. This study was supported by the National Natural Science Foundation of China (grant numbers 31830005 and 31900200); the National Key Research and Development Plan (2017FY100705); the China Postdoctoral Science Foundation (grant number 2019M663212); the Science and Technology Program of Guangzhou (201707020035) and the Chang Hungta Science Foundation of Sun Yat-Sen University.

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DATA ACCESSIBILITY

GenBank accession numbers of reference sequences for the genes we sequenced are KC928137-KC928228, KC954697 and KF918414-KF918415 (the detailed information could be found in the supplementary Table S2 of He et al (2019), doi: 10.1093/nsr/nwy078). Short-reads generated in this study are deposited in GSA (Genome Sequence Archive) with accession number CRA003253.

AUTHOR CONTRIBUTIONS

Z. Guo and S. Shi supervised the project. S. Shi, C. Zhong, X. Li, H. Lyu and N. C. Duke collected the samples. Z. Wang, H. Lyu and X. Li produced the data. Z. Wang and Z. Guo analyzed the data. Z. Guo and Z. Wang wrote the manuscript. S. Shi and N. C. Duke helped in improving the manuscript. All the authors read and approved the final manuscript.

Table 1 Sample information and population genetic statistics.

	Location	Longitude & Latitude	Site ID	N[1]	G[2]	Total reads	Depth	Total length	S[3]
1	Meed Creek, Kenya	39°58'6"E, 3deg20'33"S	MC	16	92	6870508	4670	83438	97
2	Laemson, Thailand	98°27'57"E, 9deg36'14"N	LS	35	91	10373578	5966	85999	322
3	Penang, Malaysia	100°22'5"E, 5deg31'34"N	PN	26	93	11894482	6979	88648	287
4	Thongnian, Thailand	99°48'10"E, 9deg18'6"N	TN	35	93	10605220	6100	87742	275
5	Samut Sakon, Thailand	100° 2'6"E, 13deg22'28"N	SS	19	93	12150330	6998	87532	384
6	Ban Kunsha, Thailand	100°26'33"E, 13deg30'1"N	BK	35	93	12291212	6990	87583	382
7	Sanya, China	109°41'16"E, 18deg15'33"N	SY	100	91	15241634	8087	85329	136
8	Wenchang, China	110°50'0"E, 19deg33'35"N	WC	100	93	15431782	7512	86924	118
9	Cebu, Philippines	124° 0'25"E, 10deg21'57"N	CB	26	94	11863938	6938	89399	366
10	Sabah, Malaysia	117°59'27"E, 5deg48'44"N	SB	35	93	11763230	6567	86849	89
11	Bali, Indonesia	115°14'8"E, 8deg42'59"S	BL	35	93	10450180	5837	87181	268
12	Bunbury, Australia	115°39'0"E, 33deg19'33"S	BB	40	93	6834914	3789	82804	358
13	Darwin, Australia	130°54'14"E, 12deg27'44"S	DW	40	92	6746212	4084	84700	165
14	Cairns, Australia	145°47'37"E, 16deg57'22"S	CA	35	88	11609894	6518	77737	104
15	Brisbane, Australia	153° 6'42"E, 27deg21'3"S	BS	40	93	11274220	6062	87426	759
16	Auckland, New Zealand	174°40'44"E, 36deg52'28"S	AK	22	88	11468068	5929	76119	74

Note: [1] N is the sample size, [2] G is the number of genes sequenced, [3] S is the number of segregating sites. Populations 1-12 are the subspecies *marina,*populations 13-14 are *eucalyptifolia* and populations 15-16 are*australasica* .

Table 2 Posterior probabilities of models using Approximate Bayesian Computation

		model 1	model 2	model 3	model 4	model 5	model 6	model 7	model 8
Simulation 1	replicate1	0.0007	0.3002	0.0000	0.0000	0.0001	0.0000	0.0000	0.6990
	replicate2	0.0000	0.0844	0.0000	0.0000	0.0788	0.0000	0.0000	0.8368
	replicate3	0.0927	0.1977	0.0000	0.0006	0.0804	0.0000	0.0000	0.6287
Simulation 2	replicate1	0.4001	0.1128	0.1997	0.0000	0.0000	0.0002	0.0000	0.2873
	replicate2	0.1020	0.0081	0.2922	0.0000	0.2188	0.0355	0.0010	0.3435
	replicate3	0.2007	0.2006	0.0000	0.0555	0.0994	0.0000	0.0446	0.3993
		model v1	model v1	model v2	model v2	model v3	model v3	model v4	model v4
Simulation 3		0.0515	0.0515	0.9333	0.9333	0.0118	0.0118	0.0034	0.0034

Figure 1 *Avicennia marina* distribution range and sampling locations. Ranges of the three subspecies are shown in colors as indicated in the legend. Sampling locations are indicated by circles. Location information and population abbreviations are listed in Table 1. Leaf, flower, and fruit morphological differences are presented on the right and summarized in the imbedded table. Imbedded drawings of morphological traits were adapted from Duke (1991).

Figure 2 Genetic divergence and differentiation among*Avicennia marina* populations. (a-c): colors indicate subspecies. (a) Multi-dimensional scaling analysis of the $F_{ST}$and D_{XY} matrices of 16 *A. marina*populations. (b) The neighbor-joining tree on the right was constructed using the D_{XY} matrix. (c) Clustering of the*A. marina* populations using principal component analysis (PCA). PCA was performed on the SNP frequency matrix. (d) boxplots ofD_{XY} values. "au," "ma," and "eu" indicate*australasica* , *marina,* and *eucalyptifolia*respectively. "maWest" and "maEast" refer to the two recognized geographical groups of *marina* populations west and east of the Malay Peninsula (see the Results section). "BB" refers to the population from Bunbury, Australia.

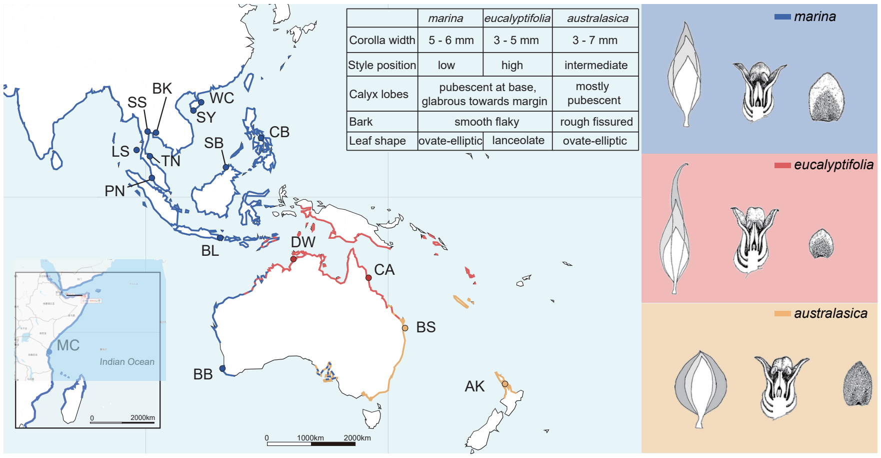
Figure 3 Different levels of genetic diversity among subspecies. Boxplots of ϑ computed for each gene in each population and points with line linked indicate mean ϑ and π values computed by pooling all SNPs in a population.

Figure 4 Networks and geographical distribution of haplotypes inferred in eight *Avicennia marina* populations. Haplotypes are indicated by different colours. Lines linking haplotypes reflect mutations, with mutations exceeding a single step marked. The geographic distribution of haplotypes is also indicated. The presented a to f cases are six typical ones to represent six types of haplotype networks. Among the 231 segments, 134, 66, 14, 11, 5, and 1 segment are classified to each type of a to f respectively.

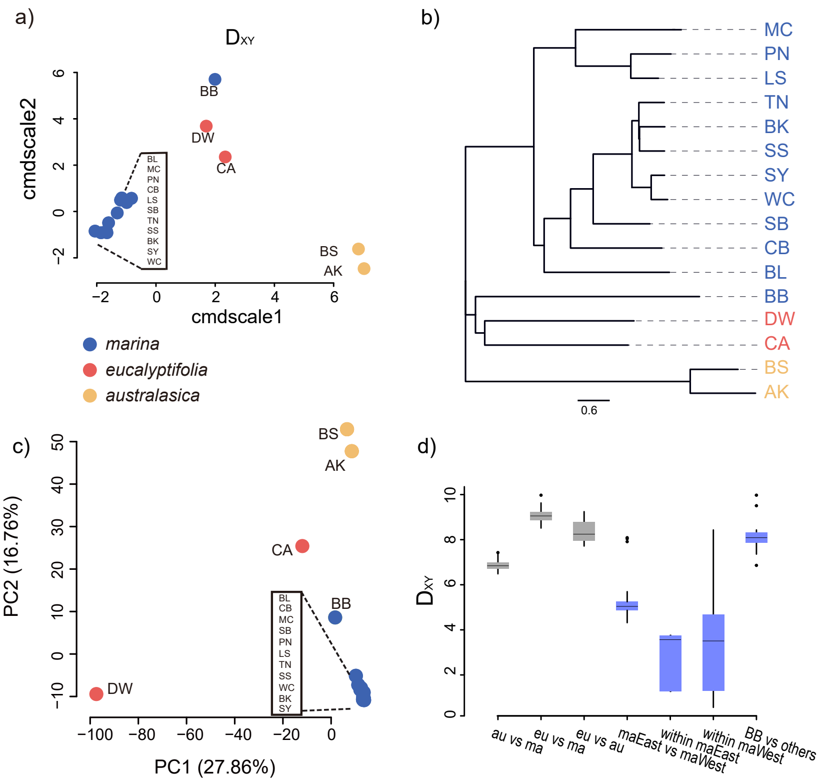
Figure 5 evolutionary history of *Avicennia marina*subspecies. (a) Simulations reconstructing demographic history of*Avicennia marina* populations. Graphical presentation of the eight models of the three subspecies. N stands for effective size and T stands for time of split. (b) Graphical presentation of the four models to investigate the contrast between morphological and genetic characters of the BB population in western Australia. vT_0 and vT_1 indicate divergence time points and N_{eu}, N_{bb}, and N_{ma}indicated effective population size. The constant bi-directional migration rates are denoted by m_{a} and m_{b}. (c) TreeMix to capture gene flow events on a population splitting graph. On the Maximum likelihood tree, each yellow line indicates a gene flow event between branches it links, with color indicating migration weight. Horizontal branch lengths of the tree are proportional to the amount of genetic drift that has occurred on the branch. The triangle matrix on the right indicates residual fit from the maximum likelihood tree. Residuals above zero imply candidate admixture events.

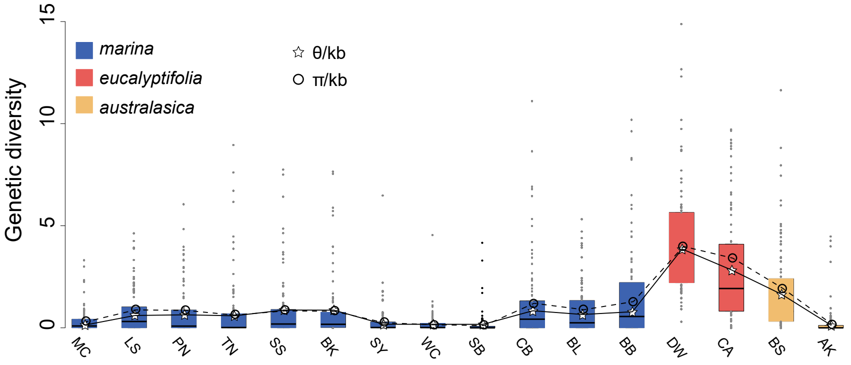
SUPPORTING INFORMATION

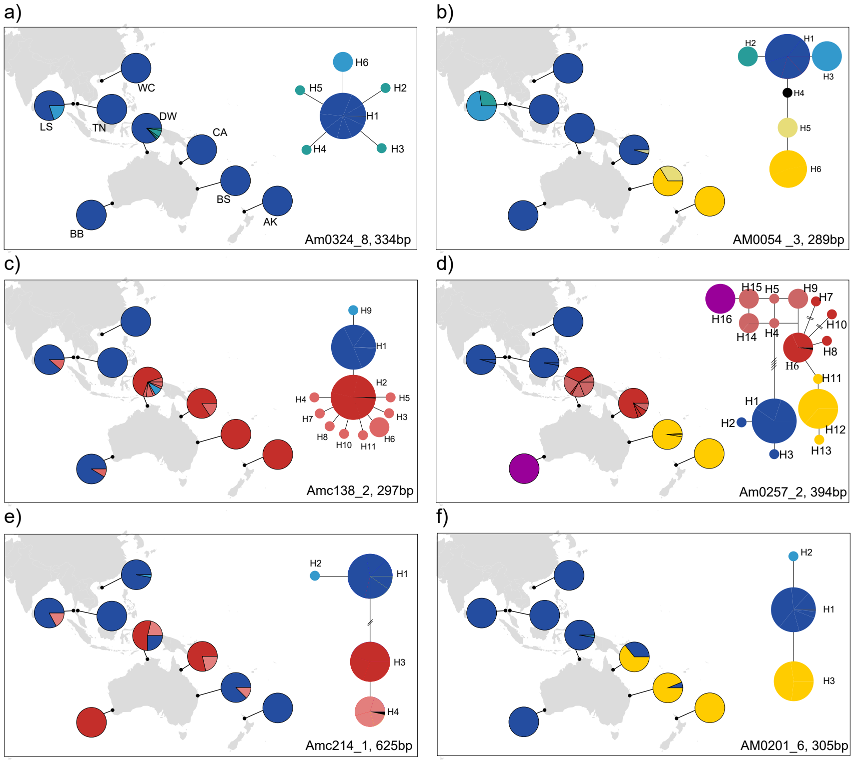
The online supplementary file contains Table S1-S7 and Figure S1-S5.

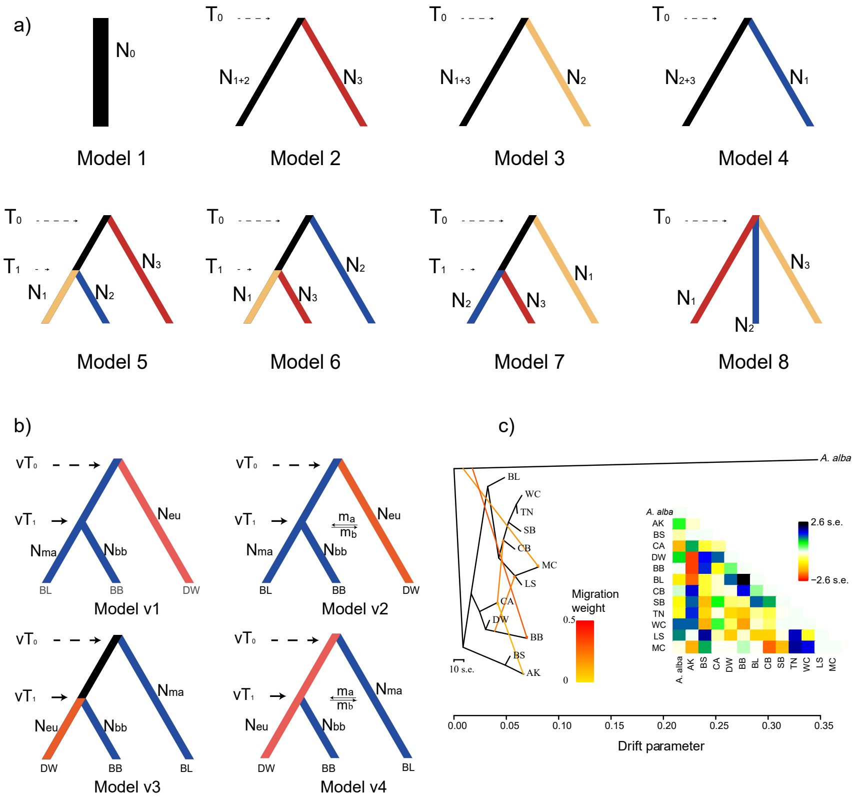


	marina	*eucalyptifolia*	*australasica*
Corolla width	5 - 6 mm	3 - 5 mm	3 - 7 mm
Style position	low	high	intermediate
Calyx lobes	pubescent at base, glabrous towards margin		mostly pubescent
Bark	smooth flaky		rough fissured
Leaf shape	ovate-elliptic	lanceolate	ovate-elliptic




15
10
5
0
Genetic diversity
marina
eucalyptifolia
australasica
θ/kb
π/kb
MC
LS
PN
TN
SS
BK
SY
WC
SB
CB
BL
BB
DW
CA
BS
AK


a)
WC
LS
TN
DW
CA
BS
AK
BB
H6
H5
H2
H1
H4
H3
Am0324_8, 334bp
b)
H2
H1
H3
H4
H5
H6
AM0054 _3, 289bp
c)
H9
H1
H2
H4
H5
H7
H3
H8
H10
H11
H6
Amc138_2, 297bp
d)
H15
H5
H9
H7
H16
H10
H14
H4
H8
H6
H11
H1
H2
H12
H3
H13
Am0257_2, 394bp
e)
H2
H1
H3
H4
Amc214_1, 625bp
f)
H2
H1
H3
AM0201_6, 305bp


a)
N_0
Model 1
T_0
N_{1+2}
N_3
Model 2
T_0
N_{1+3}
N_2
Model 3
T_0
N_{2+3}
N_1
Model 4
T_0
T_1
N_1
N_2
N_3
Model 5
T_0
T_1
N_1
N_3
N_2
Model 6
T_0
T_1
N_2
N_3
N_1
Model 7
T_0
N_1
N_2
N_3
Model 8
b)
vT_0
vT_1
N_{ma}
N_{bb}
N_{eu}
BL
BB
DW
Model v1
vT_0
vT_1
m_a
m_b
N_{ma}
N_{bb}
N_{eu}
BL
BB
DW
Model v2
vT_0
vT_1
N_{eu}
N_{bb}
N_{ma}
DW
BB
BL
Model v3
vT_0
vT_1
m_a
m_b
N_{eu}
N_{bb}
N_{ma}
DW
BB
BL
Model v4
c)
A. alba
BL
WC
TN
SB
CB
MC
LS
CA
DW
BB
BS
AK
10 s.e.
Migration weight
0.5
0
A. alba
AK
BS
CA
DW
BB
BL
CB
SB
TN
WC
LS
MC
2.6 s.e.
−2.6 s.e.
0.00
0.05
0.10
0.15
0.20
0.25
0.30
0.35
Drift parameter