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Genetic structure of the threatened *Dipterocarpus costatus* populations in lowland tropical rainforests of southern Vietnam

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ABSTRACT. *Dipterocarpus costatus* is an endangered species restricted to the lowland forests of southern Vietnam. Habitat loss and over-exploitation of *D. costatus* wood are the major threats to this species. We investigated the level of genetic variability within and among populations of *D. costatus* in order to provide guidelines for the conservation, management, and restoration of this species to the Forest Protection Department, Vietnam. Nine microsatellite markers were used to analyze 114 samples from four populations representing the natural range of *D. costatus* in southeast Vietnam. We indicated the low allelic diversity (N_A = 2.3) and low genetic diversities with an average observed and expected heterozygosity of 0.130 and 0.151,

respectively, in the lowland forests of southeast Vietnam. The low genetic diversity might be a consequence of inbreeding within the small and isolated populations of *D. costatus* owing to its habitat loss and over-exploitation. All populations deviated from Hardy-Weinberg equilibrium showing reduced heterozygosity. Alleles were lost from the populations by genetic drift. Genetic differentiation among populations was high (average pairwise F_{ST} = 0.405), indicating low gene flow (<1) and isolated populations due to its destructed habitat and large geographical distances ($P < 0.05$) among populations. Heterozygosity excess tests (except of Bu Gia Map only under infinite allele model) were negative. The high genetic variation (62.7%) was found within populations. The STRUCTURE and neighbor joining tree results suggest strong differentiation among *D. costatus* populations, with the three genetic clusters, Phu Quoc, Tan Phu and Bu Gia Map, and Lo Go-Xa Mat due to habitat fragmentation and isolation. The threatened status of *D. costatus* was related to a lack of genetic diversity, with all its populations isolated in small forest patches. We recommend the establishment of an *ex situ* conservation site for *D. costatus* with a new big population comprising all genetic groups in order to enhance its survival under different environmental stresses.

Key words: Dipterocarps; *Dipterocarpus costatus*; Genetic conservation; Genetic variability; Simple sequence repeats

INTRODUCTION

Dipterocarpus costatus is a dipterocarp restrictedly distributed in the lowland tropical forests in some provinces of southeastern Vietnam. Being an important component of semi-evergreen forests, it forms canopy tree communities, occasionally mixed with scattered trees of other dipterocarps, such as *D. dyeri*, *Shorea roxburghii*, *S. siamensis*, and *Anisoptera costata* on alluvial, granite, and basalt rocks in low relief areas, where water levels rise rapidly during both dry and rainy seasons. The region receives a mean annual rainfall of 2200-2500 mm, and has annual daytime temperatures of 25°-28°C and humidity of 78-83%. *D. costatus* wood is used for general construction and ship building purposes, and its resin is used in caulking boats and preparing torches, paints, varnishes, and lacquers.

Owing to the pressure of a rapidly developing economy, *D. costatus* was over-exploited by local people and forest enterprises in the 1980s and 1990s, leading to its habitat destruction and fragmentation. Thus, this dipterocarp is restricted to the small and isolated patches of secondary forests in protected areas such as Tan Phu rainforests (Dong Nai), Bu Gia Map National Park (Binh Phuoc), Lo Go-Xa Mat National Park (Tay Ninh), and Phu Quoc islands (Kiên Giang). *D. costatus* has been classified as an endangered species on the basis of the IUCN Red List Categories and Criteria version 2014.3 (Ashton, 1998). Two main threats to this dipterocarp are its limited distribution and the absence of natural regeneration. In order to conserve and manage a threatened species, it is important to understand the ecological and genetic diversity within and among populations. Various molecular markers have been identified as efficient tools for studying genetic structure, gene flow, and mating systems, and

estimating genetic variation within a species. Especially, microsatellite markers are useful in analyzing the effective pollen flow and seed dispersal among populations and within a species (Ujino et al., 1998; Iwata et al., 2000; Takeuchi et al., 2004; Pandey and Geburek, 2009; Tam et al., 2014; Trang et al., 2014). The objective of this study was to use simple sequence repeats (SSRs) as genetic markers to investigate the level of genetic variability within and among the populations of *D. costatus* and to provide guidelines for the conservation, management, and restoration of this species to the Forest Protection Department, Vietnam.

MATERIAL AND METHODS

Plant materials

The research was carried out at four sites (Table 1). The original vegetation at these sites had been greatly affected by human activities, including agricultural expansion and over-exploitation of trees for commerce, firewood collection, and construction purposes in 1980s and 1990s. However, the three-storey structure of vegetation was also found in the four studied areas. In degraded habitats, the spatial distribution and age-class structure of forest trees was altered.

Table 1. Locality information for populations of *Dipterocarpus costatus*.

Population	Sample size	Locality	Altitude	Latitude	Longitude
Tan Phu	30	Dinh Quan, Dong Nai Province, Vietnam	80-120 m	11°05'N	107°24'E
Bu Gia Map	27	Bu Gia Map, Binh Phuoc Province, Vietnam	400-450 m	11°13'N	107°10'E
Lo Go-Xa Mat	28	Tan Bien, Tay Ninh Province, Vietnam	10-25 m	11°21'N	106°02'E
Phu Quoc	29	Phu Quoc Islands, Kien Giang Province, Vietnam	255 m	10°24'N	103°50'E

In this study, the inner barks from 114 mature trees were sampled for four populations, representing the natural range of *D. costatus* in southeastern Vietnam. The average geographical distance between the four populations was 130 km, ranging from 55 km (Bu Gia Map/Lo Go-Xa Mat) to 209 km (Phu Quoc/Tan Phu). The samples were placed in labeled plastic bags containing silica gel desiccant and transported to the laboratory for further analysis. The samples were stored at -30°C until DNA extraction.

DNA extraction

DNA was extracted from the samples by using the modified cetyltrimethylammonium bromide (CTAB) method proposed by Doyle and Doyle (1987). Liquid nitrogen was added to approximately 100 mg of each sample, which was then ground using a mixer mill MM 400 (Retsch). The total DNA concentration in each sample was determined through fluorimetry. Each DNA sample was then diluted to a concentration of 10 ng/μL.

DNA amplification for SSRs

Nine microsatellite markers were cross-amplified for *D. costatus* to analyze the genetic diversity within and among its populations (Table 2). Six of them were isolated from *D. tempehes* (Isagi et al., 2002), two from *Shorea curtisii* (Ujino et al., 1998), and one from *Dryobalonops lanceolata* (Terauchi, 1994). All PCR amplifications were performed in 25-μL

reaction volumes containing 10 ng DNA template, 2 mM $MgCl_2$, 0.2 mM each dNTP, 1X PCR buffer containing Tris-HCl (10 mM Tris-HCl, pH 8.3, and 50 mM KCl), 10 pmol each forward and reverse primers, and 1.25 U Taq DNA polymerase (Invitrogen). PCRs were conducted in the GeneAmp PCR System 9700 (Applied Biosystems), under the following conditions: an initial denaturation step at 94°C for 2 min, 40 cycles of 94°C for 1 min, 54° or 56°C for 30 s, and 72°C for 1 min, followed by a final extension step at 72°C for 10 min to complete the extension of any remaining products before holding the samples at 4°C until further analysis.

Table 2. Nucleotide sequences of the simple sequence repeat primers and number of alleles for *Dipterocarpus costatus*.

Primer	Nucleotide sequences (5'-3')	Simple sequence repeats	Number of alleles	References
Dipt1	F: CTTCCCTAAATTCCCCAATGTT R: TAATGGTGTGTGTACCAGGCAT	$(AG)_{15}$	3	Isagi et al., 2002
Dipt3	F: ACAATGAAACTTGACCACCCAT R: CAAAAGGACATACCAGCCTAGC	$(GA)_{24}$	4	Isagi et al., 2002
Dipt4	F: TAGGGCATATTGCTTTCTCATC R: CTTATTGCAGTCATCAAGGGAA	$(AG)_{15}$	4	Isagi et al., 2002
Dipt5	F: TCTCAAAATCTGCAAAGACAGC R: CCATAGTCATCACCTCTAATGGTC	$(GA)_{25}$	2	Isagi et al., 2002
Dipt6	F: TGGCAAACAAGCTACTGTTCAT R: CATGGGTTTAGCAACCTACACA	$(TA)_{8}$	2	Isagi et al., 2002
Dipt7	F: CAGGAGGGGAATATGGAAAA R: AAGTCGTCATCTTTGGATTGC	$(AC)_{9}$	4	Isagi et al., 2002
Dipt8	F: ATGCTTACCACCAATGTGAATG R: CTCGCAGCAGAACAACTTTCTA	$(GA)_{6}$	3	Terauchi, 1994
Shc7	F: ATGTCCATGTTTGAGTG R: CATGGACATAAGTGGAG	$(CT)_8CA(CT)_5CACCC(CTCA)_3CT(CA)_{10}$	3	Ujino et al., 1998
Shc11	F: ATCTGTTCTTCTACAAGCC R: TTAGAACTTGAGTCAGATC	$(CT)_4TT(CT)_5$	2	Ujino et al., 1998

The amplification products were separated by vertical electrophoresis on 6% polyacrylamide gels in 1X TAE buffer (0.04 M Tris-acetate and 0.001 M EDTA) and subsequently stained with ethidium bromide for 10 min using Sequi-Gen®GT (BioRad). The banding patterns were visualized under UV light and photographed using BioDocAnalyze (Biometra, Analytik Jena Company). The sizes of the PCR fragments were determined using a 25-bp DNA ladder (Invitrogen).

DNA analysis

Null alleles and allelic dropouts were determined using Micro-Checker version 2.0 (Van Oosterhout et al., 2004), at the P value equal to 0.05. GenAlex (Peakall and Smouse, 2006) was used to perform population genetic analysis and FSTAT 2.9.3 (Goudet, 2001) was used to determine the number of alleles (N_A), the number of effective alleles (N_E), and number of private alleles (N_P) per locus, observed (H_O) and expected (H_E) heterozygosities, and inbreeding coefficient (F_{IS}). The tests for deviations from Hardy-Weinberg equilibrium for each locus within each population were carried out by estimating F_{IS} values within 720 randomizations and linkage disequilibrium between the pairs of loci in each population was estimated using FSTAT 2.9.3. Hierarchical genetic variation, including intra-population inbreeding coefficient (F_{IS}) and inter-population genetic differentiation coefficient (F_{ST}) among populations, was also calculated using FSTAT. The correction between the matrix of genetic distances expressed as $F_{ST}/ (1 - F_{ST})$ and the matrix of geographical distances among populations was performed using a Mantel test with 1000 randomizations utilizing the FSTAT

software. Arlequin was used to calculate the multilocus pairwise F_{ST} between different populations (Excoffier et al., 2007). The significance of variance components in analysis of molecular variance (AMOVA) was tested using 1000 permutations. The neighbor-joining cluster analysis of Nei's chord distance (Nei et al., 1983) was used to determine the genetic association among populations by using POPTREE2 (Takezaki et al., 2010). A Bayesian clustering approach was implemented to investigate the population structure using STRUCTURE ver. 2.3.4 (Pritchard et al., 2000). Setting the admixture model with correlated allele frequencies, five separate runs of the number of groups in the dataset (K = 1-15) were performed with a burn-in period of 50,000 iterations, followed by 100,000 Markov chain Monte Carlo repetitions. In order to determine the optimal value of K, the number of groups that best fits the dataset (ΔK) was determined as described by Evanno et al. (2005) using the Structure Harvester (Earl and von-Holdt, 2012). The bottleneck events for each population were tested via the infinite allele model (IAM) and two-phase model (95% stepwise mutation model with 5% multi-step mutations and a variance among multiple steps of 12) using BOTTLENECK ver. 1.2 (Piry et al., 1999). The Wilcoxon signed-rank test (Luikart et al., 1998) with default execution settings was used to evaluate any deviation from 50:50 heterozygosity deficiency/excess.

RESULTS

Genetic diversity

No evidence of null alleles and allelic dropouts was found using the Micro-Checker software. Four alleles were revealed at three loci (Dipt3, Dipt4, and Dipt7); three alleles at three loci (Dipt1, Dipt8, and Shc7); and two alleles at three loci (Dipt5, Dipt6, and Shc11). A total of 27 alleles were detected in *D. costatus* in the lowland tropical rainforests of southeast Vietnam. The largest number of alleles (4 alleles) was found at Dipt3 in the Tan Phu population (Dong Nai), Dipt4 in Lo Go-Xa Mat (Tay Ninh), and Dipt7 in Phu Quoc (Kien Giang). One allele was found at three loci (Dipt1, Dipt5, and Dipt6) in Bu Gia Map (Binh Phuoc) and two loci (Dipt5 and Dipt6) in Phu Quoc. The number of polymorphisms at all nine loci was higher in Tan Phu and Lo Go-Xa Mat (100%) than in Phu Quoc (77.8%) and Bu Gia Map (66.7%) populations. The values of genetic diversity for *D. costatus* populations are presented in Table 3. The N_A in different populations ranged from 1.8 to 2.6, with an average of 2.3. The N_E ranged from 1.1 to 1.2, with an average of 1.2. The N_P was found at Dipt3 (allelic frequencies = 0.017) in Tan Phu, Dipt7 (0.052) in Phu Quoc, Dipt1 (0.071), and Dipt4 (0.036) in Lo Go-Xa Mat. The average N_P was 0.11 in Tan Phu and Phu Quoc, and 0.22 in Lo Go-Xa Mat. The highest values for H_O = 0.152 and H_E = 0.174 were detected in Tan Phu. In all the studied populations, there were positive fixation index values, indicating an excess of homozygotes and inbreeding. The inbreeding coefficient was the lowest (F_{IS} = 0.071, P = 0.053) in Bu Gia Map and the highest (F_{IS} = 0.135, P = 0.0.34) in Lo Go-Xa Mat. AMOVA results showed that the genetic variation within populations (62.7%; P < 0.001) was high compared to that among the four populations (37.3%). The heterozygosity excess was detected in all four populations using the three models (P < 0.05), except for Bu Gia Map using IAM (P = 0.109). The mode shift did not detect any evidence of a bottleneck.

Table 3. Genetic variation within *Dipterocarpus costatus* populations at nine SSR loci.

Population	N	N_A	N_E	N_P	H_O	H_E	F_{IS}
Tan Phu	30	2.56	1.21	0.11	0.152	0.174	0.104
Bu Gia Map	27	1.78	1.14	-	0.099	0.110	0.071
Lo Go-Xa Mat	28	2.56	1.20	0.22	0.143	0.166	0.135*
Phu Quoc	29	2.22	1.20	0.11	0.126	0.152	0.106*
Mean		2.27	1.19		0.130	0.151	0.107

N, number of collected samples; N_A, mean number of alleles per locus; N_E, number of effective alleles; N_P, number of private alleles; H_O and H_E, mean observed and expected heterozygosities, respectively; F_{IS}, Wright's inbreeding coefficient. *P < 0.05.

Population structure

Analyses of genetic divergence among populations indicated that all four populations were significantly differentiated (P < 0.05; Table 4). F_{ST} values ranged from 0.315 to 0.481, with an average of 0.405 and the value of gene flow (N_m) was 0.37. The largest genetic distance (0.303) was found between the populations of Lo Go-Xa Mat and Phu Quoc, and the smallest (0.082) between the populations of Bu Gia Map and Lo Go-Xa Mat, with an average of 0.180. Similar results were found for genetic identity; the largest identity (0.921) between Lo Go-Xa Mat and Bu Gia Map and the smallest (0.739) between Phu Quoc and Lo Go-Xa Mat, with an average of 0.837. The obtained value of genetic distance indicated high level of differentiation between Phu Quoc and the remaining populations (Tan Phu, Lo Go-Xa Mat, and Bu Gia Map). According to Mantel, the significant correlation was found between the pairwise F_{ST} values (F_{ST} / (1 - F_{ST}) and the geographic distance between the populations (r = 0.653, P < 0.05).

Table 4. Pairwise F_{ST} values and significant P values (P = 0.05) in *Dipterocarpus costatus* populations.

Population	Tan Phu	Bu Gia Map	Lo Go-Xa Mat	Phu Quoc
Tan Phu		+	+	+
Bu Gia Map	0.402		+	+
Lo Go-Xa Mat	0.321	0.315		+
Phu Quoc	0.481	0.438	0.477	

The population genetic relationship based on Nei's chord distance and the Bayesian analysis, implemented in STRUCTURE, indicated through a bar plot that the most likely number of genetic clusters was 3 (Δk = 150). These results were consistent with the evidence that individuals with similar multilocus genotypes were assigned distinct populations (Figures 1 and 2). Most of the individuals at Tan Phu and Phu Quoc were detected with two distinct clusters. However, there was a little signal of admixture in these populations. The third cluster was composed of two populations - Bu Gia Map (Binh Phuoc) and Lo Go-Xa Mat (Tay Ninh). Some individuals of the Tan Phu population belong to Bu Gia Map and Lo Go-Xa Mat, indicating admixture of populations due to gene flow in the past. In the 1800s, the large habitat of *D. costatus* in the southeastern region of Vietnam included all three Provinces of Dong Nai, Binh Phuoc, and Tay Ninh. The STRUCTURE results suggest distinct differentiation among *D. costatus* populations due to habitat fragmentation and isolation.

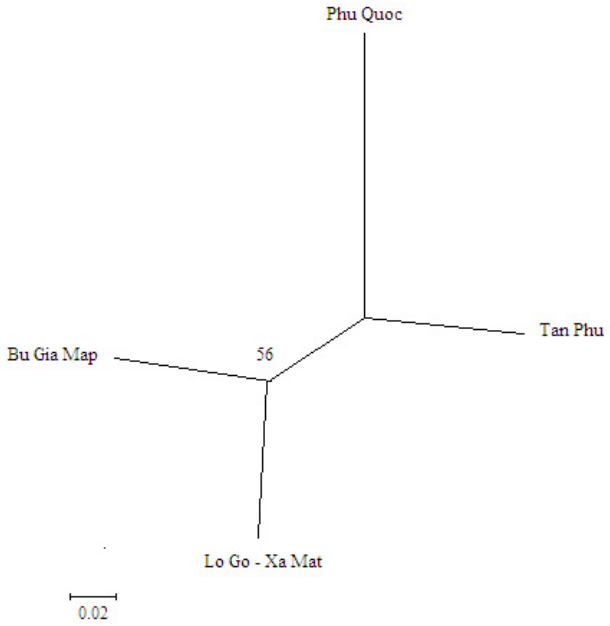

Figure 1. Neighbor joining tree of 4 populations based on Nei's chord distance.

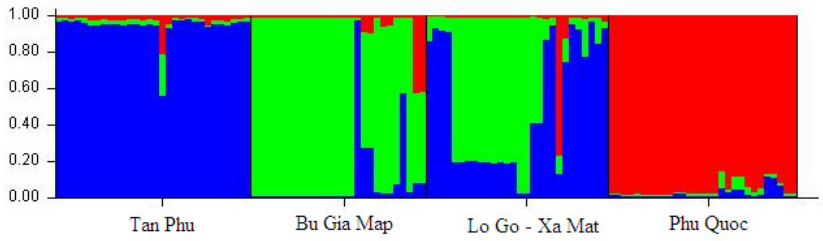

Figure 2. Bar plot of admixture assignment for four populations to the clusters (K = 3) based on Bayesian analysis.

DISCUSSION

The results obtained in the present study indicated that the studied populations of *D. costatus* in the lowland forests of southeastern Vietnam retained the low allelic diversity (N_A = 2.3, N_E = 1.2) and low genetic diversities with H_O and H_E of 0.130 (0.099-0.152) and 0.151 (0.110-0.174), respectively. The observed allelic diversity was low compared to *Shorea leprosula* (N_A = 11.0-11.4; Ng et al., 2004) and *Dryobalanops aromatica* (N_A = 5.1; Lim et al., 2001). Besides, these were compared to the H_O and H_E values that had been reported from other dipterocarp species such as *S. leprosula* (H_O = 0.63-0.66, H_E = 0.69-0.71; Ng et al., 2004), *Parashorea malaanonan* (H_O = 0.26, H_E = 0.46; Abasolo et al., 2009), *Hopea odorata* (H_O = 0.366, H_E = 0.356; Trang et al., 2014), and *D. alatus* (H_O = 0.209, H_E = 0.239; Tam et al., 2014). Therefore, the low genetic diversities might be a consequence of allelic loss within the populations of *D. costatus*. Moreover, the STRUCTURE analysis showed that the

two populations, Tan Phu and Phu Quoc, were genetically separated, with a little signal of admixture. However, the admixture of the Tan Phu population with the Bu Gia Map and Lo Go-Xa Mat populations was 10 and 51%, respectively. The Phu Quoc population was isolated from the remaining populations, suggesting a restricted dispersal among them owing to the large geographical distances of 180 km (126-209 km) between them. The Tan Phu, Bu Gia Map, and Lo Go-Xa Mat populations were distributed in southeast Vietnam. Historically, this region was covered by a large natural forest, where *D. costatus* occurred. The destruction of this forest for agricultural purposes and urbanization led to its fragmentation. Moreover, in 1980s and 1990s, the over-exploitation of forests still continued. Thus, the deforestation, habitat degradation, and over-exploitation of *D. costatus* for commerce (wood and aromatic oily resin) are the major factors that might explain the low heterozygosity values that were found in all the studied populations. The fixation of alleles at the three loci (Dipt1, Dipt5, and Dipt7) for Bu Gia Map and two loci (Dipt5 and Dipt6) for Phu Quoc might explain the low heterozygosity values for this dipterocarp species. This might result from the significantly decreased size of the *D. costatus* populations confined to the small patches in the secondary forest remnants. Clearly, the small population sizes might affect the number of alleles in the studied populations. The occurrence of small and isolated populations for many generations can lead to inbreeding and loss of alleles within the populations by genetic drift. The mean deficit of heterozygote ($F_{IS} = 0.107$) estimated for the four populations indicated inbreeding due to selfing or biparental mating. The significantly positive F_{IS} values of 0.135 and 0.106 were found in Lo Go-Xa Mat and Phu Quoc, respectively ($P < 0.05$). Consequently, the inbreeding coefficient and homozygosity decreased the number of alleles in the *D. costatus* populations.

The results of AMOVA showed 62.7% genetic variance within the populations, whereas 37.3% variance was found among the populations. This level of genetic variation was considered high when compared to *Shorea* species in Indonesia with a 43% genetic variation within populations (Cao et al., 2009), and low when compared to *D. alatus* in Vietnam with a genetic variation of 74.9% within populations and the genetic differentiation of 0.266 (Tam et al., 2014). The high values of pairwise F_{ST} (0.405) showed strong differentiation among the *D. costatus* populations. Due to large geographical distances between the populations (>50 km), the limited gene flow via pollen (pollination by insects within a distance of 200 m; Appanah and Chan, 1981; Fukue et al., 2007) and seed dispersal by wind or gravity (the dispersal distances of up to 500 m; Chan, 1980; Takeuchi et al., 2004). The analysis also revealed the low levels of migration among the studied populations ($N_m = 0.37$). Thus, the level of genetic diversity as well as the mixture of ancestral alleles of the genetic groups were observed in the bar plot. However, these groups were formed with individuals from the genetic clusters comprising the four populations. These results showed low levels of gene flow ($N_m < 1$) among populations. A barrier to gene flow was reflected by significant differentiation ($P < 0.001$) in *D. costatus*.

In conclusion, the low levels of genetic variability and the high levels of genetic differentiation observed within the *D. costatus* populations are the consequence of human interferences and large geographical distances among them. In the recent decades, the over-exploitation of *D. costatus* wood and aromatic oily resin for commercial purposes has greatly destroyed its habitat. All studied populations were confined to small forest patches that were separated by the large geographical distances. The habitat degradation and narrow distribution of *D. costatus* have led to its endangerment. Therefore, it is considered as a priority species for conservation in Vietnam. The results obtained in this study suggest that the threatened status of *D. costatus* was related to the lack of genetic diversity. Thus, the best conservation strategy for

this species is to establish an *ex situ* conservation site using seeds collected from individuals of all populations of *D. costatus*, thereby avoiding the random loss of genetic variability due to the loss of individual ecotypes and enhancing the survival of new self-sustaining populations. The establishment of a new big population of *D. costatus* should provide new alleles, which might improve its fitness under different environmental stresses.

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