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GENETIC STRUCTURE OF *ANISOPTERA COSTATA* IN SOUTHEAST VIETNAM

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SUMMARY

Anisoptera costata is an endangered species in Vietnam, from habitat loss and over-exploitation. To protect this species, we analyzed 56 adult trees using eight microsatellites to investigate the genetic diversity within and among three *A. costata* populations, representing the range of distribution of this species in southeastern Vietnam. Our results showed low genetic diversity (N_A = 2.5, H_O = 0.244, H_E = 0.268). Genetic population differentiation among populations was low (F_{ST} = 0.139), indicating high gene flow (N_m = 2.838). Genetic variation among individuals was 79.77%. These suggest a lack of genetic diversity in *A. costata*. The clustering analysis also showed two genetic clusters. These results will provide necessary information to improve the efficiency of conservation and management of this species.

Keywords: Dipterocarp, genetic diversity, species conservation, bottleneck events, microsatellites

INTRODUCTION

The family Dipterocarpaceae includes 17 genera with about 580-680 species in the world. Dipterocarps are mainly distributed throughout the tropics, and play an important role for many countries, especially in Southeast Asia (Ashton, 1998). They not only provide valuable wood, but also many different products for human life such as aromatic oils (*Shorea guiso, Dipterocarpus costatus* and *Sh. robusta*). More than 45 species in six different genera of *Anisoptera, Hopea, Shorea, Parashorea, Vatica* and *Dipterocarpus* have been found in Vietnam (Nguyen Hoang Nghia, 2005). In recent decades, due to different causes, such as over-exploitation of wood for commercial purposes and local people needs, as well as deforestation for agricultural land expansion, many dipterocarps were lost and degraded their habitats. Furthermore, they occur only in small and isolated remnants of secondary forests. *Anisoptera costata* is distributed in southern Vietnam (Nguyen Hoang Nghia, 2005), threatened, and is listed as endangered based on the IUCN Red List Categories (Nguyen *et al.*, 2017) and Vietnam Red Book (MOST, VAST, 2007). Understanding the genetic diversity within and among *A. costata* populations is necessary for its conservation and management. To obtain these information, microsatellites (SSRs) were used as powerful tools to analyze genetic variation in plant species (Ng *et al.*,

2004; Abasolo *et al.*, 2009; Muhammad *et al.*, 2016; Harada *et al.*, 2018; Tam *et al.*, 2019). In the present study, we assessed the genetic diversity within and among *A. costata* populations, using SSR markers to provide a platform for the conservation, management and restoration of this species in Vietnam.

MATERIALS AND METHODS

The materials were derived from three sites presenting the range of *A. costata* habitats in Southeastern Vietnam, one site each in Binh Phuoc, Dong Nai and Tay Ninh (Table 1).

A total of 56 adult trees were randomly sampled from three *A. costata* populations. Inner barks were collected and preserved in markered plastic bags with silica gel in the field, and then transferred to the Laboratory of Molecular Biology, Institute of Ecology and Biological Resources and stored at -70ºC until used.

Genetic DNA was extracted using the modified CTAB protocol described by Doyle J and Doyle L (1990). Liquid nitrogen was added to about 100 mg of sample, which was then ground using Mixer mill MM 400 (Retsch). The concentration of total DNA was determined by electrophoresis on 1% agarose gel, as well as by spectrophotometry using the NanoDrop 2000C (Thermo Sci. USA). Total DNA was diluted to a concentration of 10ng/µL.

The polymerase chain reaction (PCR) was performed in a 25 µL solution volume containing 1.5 µL of 10 ng/ µL of *A. costata* genomic DNA, 12 µL of 2x Taq Master Mix, 9.5 µL deionized water, and 1 µL of each primer. The eight microsatellites (Table 2) were chosen based on testing six microsatellites for *Dipterocarpus tempehes* (Isagi *et al*. 2002), five for *Dryobalanops lanceolata* (Terauchi, 1994) and *Shorea curtisii* (Ujino *et al*., 1998). The amplification conditions were performed as follows an initial denaturation step at 94ºC for 2 min, followed by 40 cycles of denaturation at 94ºC for 1 min, annealing for each primer pair at 52-56ºC for 1 min (Table 2), extension at 72ºC for 1 min, with a final extension at 72ºC for 10 min to complete the extension of any remaining product using Amp PCR System 9700 (Applied Biosystem, USA) before storing the DNA samples at 4ºC. Products of amplification were separated by electrophoresis on 7% polyacrylamide gels in 1 x Tris-acetate-EDTA (TAE) buffer using a Sequi-Gen®GT DNA electrophoresis system, and then stained by GelRedRed™ Nucleic Acid Gel Stain. Allele sizes were detected by Gel-Analyzer software of GenoSens1850 (Clinx Sci. Ins. Co., Ltd) with a 25 bp DNA ladder (Invitrogen).

Table 1. Sampling locaties for *A. costata* in southeastern Vietnam.

Populations	No of samples	Locality	Altitude	Latitude	Longitude
Bu Gia Map	18	Bu Gia Map, Binh Phuoc	23-323 m	12º14'N	107º11'E
Cat Tien	17	Cat Tien, Tan Phu, Dong Nai	120-129 m	11º26'N	107º17'E
Lo Go-Xa Mat	21	Tan Bien, Tay Ninh	18-35 m	11º21'N	107º36'E

Based on microsatellite genotyping and allele frequencies, genetic variation measures of the three *A. costata* populations were determined. Genetic diversity within and over all populations was detected as the number of alleles per locus (N_A), private alllele, percentage of polymorphic loci, effective alleles per locus (A_E), observed (H_O) and expected (H_E) heterozygosities; fixation index (F_{IS}), inbreeding coefficient of an individual relative to the total populations (F_{IT}) and the effect of subpopulations compared with the total

populations (F_{ST}) using GenAlEx 6.5 (Peakall, Smouse, 2012) and FSTAT (Goudet, 2001). Hardy-Weinberg equilibrium (HW) test was determined using Cervus 3.0 (Kalinowski *et al.,* 2007). The Wright's F-statistics over all population for each locus and locus-by-locus analysis of molecular variance (AMOVA) were determined using ARLEQUIN 3.5 (Excoffer *et al.,* 2005). Testing recent bottleneck events for each population through the stepwise mutation model (SMM) and two-phase model (TPM) was implemented using BOTTLENECK 1.2 (Piry *et al.*, 1999). The F_{IS} values were corrected for null allele frequency base on the individual inbreeding model (IIM) using INEst (Chybicki, Burczyk, 2009). The gene flow between populations was calculated using F_{ST} value N_m = (1/F_{ST}-1)/4. Principal coordinate analysis (PCoA) was performed at the population level on the basis of the pairwise G'_{ST} matrix using GenAlEx. The Neighbor-joining (NJ) tree of genetic distances was generated to determine the genetic association among populations using POPTREE2 (Takezaki *et al.,* 2010).

Table 2. Nucleotide sequences of SSRs and allelic size range for *A. costata.*

Primer	Primer sequences (5'-3')	Repeat motif	Allele size (bp)	T_m°C	Sources
dipt1	F: ATGCTTACCACCAATGTGAATG R: CTCGCAGCAGAACAACTTTCTA	$(GA)_6$	117-133	54	Terauchi (1994)
dipt2	F: TAGGGCATATTGCTTTCTCATC R: CTTATTGCAGTCATCAAGGGAA	$(AG)_{15}$	117-205	54	Isagi *et al.,* 2002
dipt3	F: TGGCAAACAAGCTACTGTTCAT R: CATGGGTTTAGCAACCTACACA	$(TA)_8$	198-216	52	Isagi *et al.,* 2002
dipt4	F: CTTCCCTAAATTCCCCAATGTT R: TAATGGTGTGTGTACCAGGCAT	$(AG)_{15}$	212-226	55	Isagi *et al.,* 2002
dipt5	F: ACAATGAAACTTGACCACCCAT R: CAAAAGGACATACCAGCCTAGC	$(GA)_{24}$	227-249	55	Isagi *et al.,* 2002
dipt6	F: GCTATTGGCAAGGATGTTCA R: CTTATGAGATCAATTTGACAC	$(CT)_8(CA)_{10}$ $CT(CA)_4CTCA$	148-172	56	Ujino *et al.,* 1998
dipt7	F: ATGTCCATGTTTGAGTG R: CATGGACATAAGTGGAC	$(CT)_8CA(CT)_5CACC$ $C(CTCA)_3CT(CA)_{10}$	168-188	54	Ujino *et al.,* 1998
dipt8	F: ATCTGTTCTTCTACAAGCC R: TTAGAACTTGAGTCAGATAC	$(CT)_4TT(CT)_5$	156-184	54	Ujino *et al.,* 1998

RESULTS AND DISCUSSIONS

The eight microsatellite loci produced 20 different alleles with an average of 2.5 alleles for each locus across all 56 tree samples in three *A. costata* natural populations. Fifty-four out of 132 tests were significant at the 5% level. All loci were polymorphic (Table 3). At the species level, two alleles were detected at 5 loci (dipt3, dipt4, dipt5, dipt7 and dipt8), while three alleles were detected at two loci (dipt1 and dipt6). Four alleles were found at only locus (dipt2). Homozygotes were found at two loci of dipt3 and dipt4 at two populations Bu Gia Map and Lo Go-Xa Mat. The most common alleles (allelic frequencies >0.8) were found at dipt1 (Lo Go-Xa Mat), dipt3 (Cat Tien), dipt5 (all three populations), dipt6 (Cat Tien), dipt7 (Bu Gia Map and Lo Go-Xa Mat) and dipt8 (Cat Tien and Lo Go-Xa Mat). The observed (H_O) and expected (H_E) heterozygosity varied from 0.078 at dipt3 to 0.399 at dipt2 and from 0.109 at dipt4 to 0.424 at dipt2, respectively. The mean fixation index (F_{IS}) overall populations for each locus were 0.06.

Table 3. Genetic parameters in eight loci for *A. costata.*

Locus	N_A	A_E	H_O	H_E	F_{IS}	F_{IT}	F_{ST}	HW	N_m
dipt1	3	1.6	0.213	0.356	0.402	0.599	0.329	*	0.51
dipt2	4	1.8	0.399	0.424	0.06	0.119	0.062	nd	3.773
dipt3	2	1.1	0.078	0.097	0.19	0.292	0.125	ns	1.75
dipt4	2	1.2	0.137	0.109	-0.259	-0.074	0.147	**	1.446
dipt5	2	1.2	0.183	0.188	0.027	0.065	0.039	nd	6.142
dipt6	3	1.6	0.345	0.351	0.017	0.064	0.048	nd	4.939
dipt7	2	1.5	0.314	0.281	-0.119	0.212	0.295	***	0.596
dipt8	2	1.6	0.283	0.337	0.161	0.216	0.066	ns	3.55
Mean		1.4	0.244 (0.034)	0.268 (0.033)	0.06 (0.071)	0.187 (0.071)	0.139 (0.04)		2.838 (0.735)

Notes*:* N_A: number of alleles; A_E: effective alleles; H_O and H_E: observed and expected heterozygosity; F_{IS}: fixation index; F_{IT}: coefficient of total inbreeding; F_{ST}: genetic differentiation index of Weir and Cockerham (1984); *HW*: Hardy-Weinberg equilibrium; N_m: number of migrants; SE: standard error, *p<0.01, **p<0.001, ***p<0.0001

Table 4. Genetic diversity values and results of bottleneck tests for three *A. costata* populations

Populations	*N*	*P* (%)	N_A	A_E	H_O (SE)	H_E (SE)	F_{IS} (SE)	F_{IS} IIM	*P* value of bottleneck		
									A	B	C
Bu Gia Map	18	75	1.9	1.5	0.264	0.287	0.11*	0.068	ns	ns	nd
Cat Tien	17	100	2.1	1.5	0.301	0.335	0.129	0.077	0.006	0.01	ns
Lo Go-Xa Mat	21	75	2	1.3	0.167	0.181	0.105**	0.065	ns	ns	ns
Mean		83.3	2	1.4	0.244 (0.034)	0.268 (0.033)	0.115	0.07			

Notes*: N*: sample size; *P*: percentage of polymorphic loci; N_A: alleles per locus; A_E: effective alleles; H_O and H_E: observed and expected heterozygosities; F_{IS}: fixation index; F_{IS}IIM: corrected inbreeding coefficient for null alleles; A: heterozygosity deficit, one-tailed test; B: heterozygosity excess, one-tailed test; C: heterozygosity excess or deficit, two tailed test; SE: standard error, *p<0.05, **p<0.01.

Table 5. Nei's genetic distance (below diagonal) and genetic identify (above diagonal) for *A. costata* population pairs.

	Bu Gia Map	Cat Tien	Lo Go-Xa Mat
Bu Gia Map		0.911	0.939
Cat Tien	0.093		0.848
Lo Go-Xa Mat	0.063	0.165	

Six of the eight loci had a positive fixation index, indicating an excess of homozygotes and inbreeding. The two loci had negative values. At the population level, the values for genetic diversity were presented in Table 4. The percentage of polymorphic loci was high in the Cat Tien population (100%) and lower in two remaining populations (75%). Private alleles were found at two loci dipt1 and dipt2 for Lo Go-Xa Mat, dipt3 and dipt4 for the Cat Tien population and dipt9 for two populations Cat Tien and Lo Go-Xa Mat, whereas the remaining loci did not have any private alleles in all populations. The mean alleles per locus (N_A) averaged 2, ranging from 1.9 alleles in the Bu Gia Map population to 2.1 alleles in Cat Tien. The mean number of effective alleles (A_E) was 1.4, ranging from 1.3 in Lo Go-Xa Mat to 1.5 in two populations of Bu Gia Map and Cat Tien. The observed heterozygosity (H_O) averaged 0.244, ranging from 0.167 in Lo Go-Xa Mat to 0.301 in Cat Tien. The expected heterozygosity (H_E) averaged 0. 268, ranging from 0.181 in Lo Go-Xa Mat to 0.335 in Cat Tien.

Our results were lower than compared to other dipterocarp species, such as *Shorea leprosula* (H_O=0.63-0.66, H_E=0.69-0.71, Ng *et al.*, 2004), *Hopea odorata* (H_O=0.366, H_E=0.356, Trang *et al*., 2014), *Sh. leprosula* (H_E=0.709, Rimbawanto, Isoda, 2001), *Dipterocarpus dyeri* (H_O=0.527, H_E=0.601, Tam *et al*. 2020). However, the mean number of alleles in our results was low compared to *Sh. leprosula* (N_A=11.0-11.4, Ng *et al*., 2004), *Dryobalamops aromatic* (N_A=5.1, Lim *et al.,* 2001). Thus, the present study showed the deforestation, habitat degradation and over-exploitation of the studied species are main factors for the low heterozygosity and affect the number of alleles in all the studied populations. The fixation index F_{IS} (inbreeding coefficient) varied from 0.105 in Lo Go-Xa Mat to 0.129 in Cat Tien, with an average of 0.115. All populations had excess in homozygosity (F_{IS}>0.1) and significant inbreeding, except Cat Tien. The inbreeding corrected for null alleles based on the individual inbreeding model (F_{IS}IIM) averaged 0.07, ranging from 0.065 in Lo Go-Xa Mat to 0.077 in Cat Tien, also indicating homozygote excess. However, this value was slightly low compared to the fixation index F_{IS}. The inbreeding coefficient calculated for the total populations (F_{IT}) varied from -0.074 at dipt4 to 0.599 at dipt1, with an average of 0.187, suggesting an excess of homozygosity in the populations. Genetic differentiation among populations was 0.139 (F_{ST}) indicating moderate genetic differentiation. The BOTTLENECK analysis showed that a significant heterozygosity deficit was found in one population of Cat Tien (p<0.05). This suggests the Cat Tien population appeared signs of a recent bottleneck.

The mean value of Nei's genetic distance and genetic identify for *A. costata* population pairs were presented in Table 5. The Nei's genetic distance varied from 0.063 for the population pair of Lo Go-Xa Mat and Bu Gia Map to 0.165 between two populations of Lo Go-Xa Mat and Cat Tien. Similarly, the genetic identify averaged 0.899 (0.848 - 0.939).

The genetic differentiation ranged from 0.039 at dipt5 to 0.329 at dipt1, and the gene flow was 2.838 (Table 3). The population pairwise differentiations (F_{ST}) were significant for *A. costata* (p<0.001).

Table 6. Pairwise genetic differentiation (F_{ST}) between the three *A. costata* populations.

Population	Bu Gia Map	Cat Tien	Lo Go-Xa Mat
Bu Gia Map		+++	+++
Cat Tien	0.090		+++
Lo Go-Xa Mat	0.093	0.184	

Note: $^{+++}$p<0.001

The F_{ST} value varied from 0.09 between two populations of Cat Tien and Bu Gia Map to 0.184 between Lo Go-Xa Mat and Cat Tien with an average of 0.139 (Table 6). Low differentiation was found between Cat Tien and Bu Gia Map, and high differentiation was between Cat Tien and Lo Go-Xa Mat. These results were higher compared to another dipterocarp such as *Sh. javasnia* (F_{ST}=0.076, Rachmat *et al.*, 2010) and lower compared to *H. odorata* (F_{ST}=0.26, Trang *et al.*, 2014).

The analysis of molecular variance (AMOVA) revealed genetic variation within and between populations (Table 7). Most of the total variance was attributed to variation within individuals (79.77%), while the lower variation among population was 2.23%. The variation between populations was significant (p<0.001). This showed a gene migration between *A. costata* populations.

PCoA analysis showed that two populations of Bu Gia Map and Lo Go-Xa Mat were clustered together to firm one group (Figure 1). The first and second principal coordinate explained 77.63% and 22.37% of the variation, respectively. Similarly, neighbor-Joining tree showed two populations Bu Gia Map and Lo Go-Xa Mat clustered to form one group (Figure 1). These populations have the lowest genetic distance (Table 5).

Table 7. Analysis of molecular variance from three *A. costata* populations.

	Sum of squares	Variance components	Total variation (%)	P value
Among populations	22.463	0.273	20.23	<0.001
Within populations	117.394	1.077	79.77	
Total	621.7	1.350		

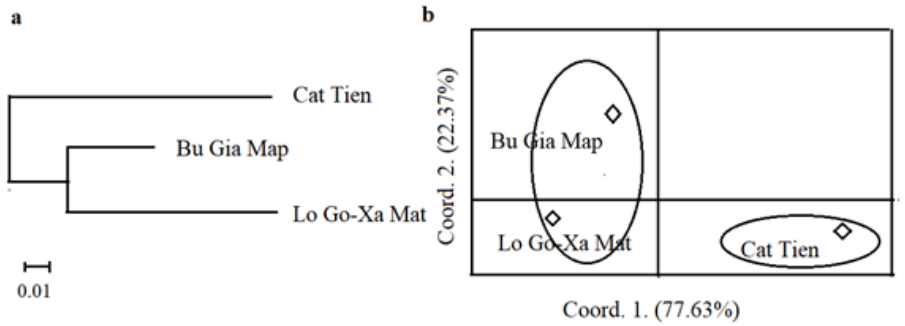

Figure 1. a) Neighbor-joining tree based on the Nei's unbiased genetic distance produced from POPTREE. b) Principal coordinates (PCoA) based on the F_{ST} values from GenAlEx

CONCLUSION

Low genetic diversity and genetic population differentiation were maintained in *A. costata*. Clustering analysis showed two groups. The most genetic variation was found within populations. The results also showed that human activities reduced the genetic diversity of this species. Therefore, conservation activities could be a focus on maintaining all individuals for each population. These activities could be implemented with *ex situ* conservation to avoid inbreeding.

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