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Genetic diversity and differentiation of an endangered tree species, *Afzelia xylocarpa* (Kurz) craib in Thailand revealed by nuclear microsatellite markers

Greuk Pakkad[1,3]*, Seiichii Kanetani[2,3#] and Stephen Elliott[1]

[1]Forest Restoration Research Unit, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand 50200.
[2]Forest Ecosystem Research Group, Kyushu Research Center Forestry and Forest Products Research Institute, 4-11-16 Kurokami, Chuo-ku, Kumamoto 860-0862, Japan.

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***Afzelia xylocarpa* is listed as an endangered species on the IUCN *World list of Threatened Trees*, due to over exploitation for its valuable timber and habitat loss, have resulted in a rapid decline of populations size and local population extinction. Understanding of the processes that determine population genetic structure, gene flow and mating systems is important to conserve and manage the existing populations for this endangered tree species. This study describes the level of genetic diversity and differentiation of fifteen populations of *A. xylocarpa* in Thailand. Genetic variations at seven nuclear microsatellite loci were examined. The seven nuclear microsatellite loci employed detected a total of 53 alleles (n=432). The nSSRs data indicate that a high level of genetic diversity (H_S = 0.575) and low level of genetic differentiation among the 15 examined *A. xylocarpa* populations. The observed level of genetic differentiation among the 15 populations was low, as revealed by F_{ST} = 0.074 and G_{ST} = 0.078. The results for the nSSRs suggested that all of the populations in North Eastern, Central Thailand and the Klong Lan populations harbored the high genetic diversity and less divergent from the other populations. Therefore, these populations should be assigned the highest priority for conservation of this species.**

Key words: *Afzelia xylocarpa*, genetic diversity, endangered species, tropical tree, conservation.

INTRODUCTION

Deforestation is one of the most serious threats to biodiversity. The causes of deforestation are varied, including population pressure, shifting cultivation, agricultural development, transmigration, forest fire and unsupervised, poor logging practices (World Resources Institute, 1991). Between 1980 and 1990 tropical forests were destroyed at a global average rate of more than 0.8% per annum, implying that the area of tropical forests has diminished by a 10th during the last 12 years (FAO, 1997). In Thailand for example, natural forest cover has fallen to less than 20% of the country's land area, despite 15% of the country being designated conservation areas (Forest Restoration Research Unit, 1998). It causes climatic change, recurrent floods, soil erosion, and loss of fertility,

*Corresponding author: E-mail: greuk@hotmail.com. Tel: +66-53-943358. Fax: +66-53-892259.

Abbreviations: SSRs, Simple sequence repeats; **STRs,** short tandem repeats; **CTAB,** cetyl trimethyl ammonium bromide; **PCR,** polymerase chain reaction. #These authors contributed equally to this work.

degradation of watersheds, deterioration in the quality of life and loss of wildlife habitats. This result in loss of species populations, reductions in remnant population sizes, changes in densities of reproductive individuals, reduced reproductive success, increased isolation of remnant populations and reduced genetic variability (Prober and Brown, 1994) through genetic bottlenecks and mating systems.

Genetic diversity is directly related to a specie's ability to survive environmental change. It is the basis of all biodiversity and is widely recognized as a key requirement for the long-term survival of species on an evolutionary time-scale. It provides the template for adaptation, evolution and survival of populations and species, especially in environments that are subject to climate changes or the introduction of new pests, pathogens or competitors (Rajora and Mosseler, 2001). The loss of a single species can set off a chain reaction affecting many other species distributing each forest ecosystems. Thus, conserving genetic diversity is one of the most profound challenges facing forest managers relying on either natural or artificial.

Genetic analysis of populations requires suitable markers that can elucidate fine-scale details of spatial structure (Streiff et al., 1998) and reconstruct gene flow patterns (Streiff et al., 1999). Microsatellites have become the preferred marker in many studies because of their high allelic diversity, reliability of scoring and co-dominant inheritance. Microsatellites, also known as simple sequence repeats (SSRs) (Tautz, 1989) and short tandem repeats (STRs) (Edwards et al., 1991), have strong discriminatory power and are becoming a popular tool for the studying of genetic diversity, gene flow and conservation of natural plant populations (Chase et al*.,* 1996; Dow et al*.,* 1995; Dayanandan et al*.,* 1997; Streiff et al*.,* 1998; Ueno et al*.,* 2000). Microsatellites are short stretches of tandem repeated, simple DNA sequences such as $(GT)_n$ or $(CAC)_n$ (each generally less than 5 base pairs in length) (Zhao and Kochert, 1993). However, application of microsatellite markers (or very large numbers of markers) must be used with some caution in conservation genetics (Hedrick, 2001), because statistically significant differences might not reflect biological important differences, or might give a different signal than do other markers (Balloux et al., 2000)

The microsatellite markers for *Afzelia xylocarpa* have been developed using dual suppression polymerase chain reaction (PCR) technique (Pakkad et al., 2009). These loci provide microsatellite markers with high polymorphism. The markers are available for more detailed investigation of population genetic structure and gene flow among *A. xylocarpa* populations. The objectives of the present study were to assess genetic variation among and within populations of *A. xylocarpa* in Thailand, using nuclear microsatellite markers. Specifically, the present study aimed to determine levels and the distribution of genetic variability within and among populations as well as develop guidelines for conserving the species using the acquired genetic information.

MATERIALS AND METHODS

Study species

A. xylocarpa (Kurz) Craib (Caesalpinioideae) is a deciduous tree, reaching heights up to 30 m and diameters at breast height of up to 150 cm. It is native to Cambodia, Laos, Myanmar, Thailand and Vietnam. In the natural distribution, it occurs in scattered locations in mixed deciduous, dry evergreen or dry dipterocarp forest, at elevation of 100 - 650 m in areas with uniform rainfall regime, 1000 – 1500 mm/year, a dry season of 5-6 months, mean annual temperature of 20 - 32°C and an absolute minimum temperature of 10°C.

The attractive reddish timber is very valuable in the region and is often used for construction, furniture, plywood, veneer, musical instruments, interior decoration and wood carving. The wood from the large branches is also used and the lumped wood near the base of the trunk is highly priced. The young green seed are edible and the seed pulp can be used to make cigarettes. The bark is used for tanning leather and herbal medicine. It is a nitrogen fixing tree and suitable for agroforestry and for soil improvement, but planted only on a small scale. Currently, it is listed as an endangered species on the IUCN World list of Threatened Trees, due to over exploitation for its valuable timber, habitat fragmentation and a low rate of nature regeneration. Its population has declined rapidly with several local population extinctions.

Sample collection and DNA extraction

Young leaves were collected in November to December 2009 from *A. xylocarpa* trees in populations at the following 4 locations in Northern Thailand, 3 locations in North Eastern Thailand, 4 locations in Central Thailand and 4 locations in Western Thailand. The number of trees sampled in each population varied from 12 in Sam Roi Yod National Park (12) to 30 in the other populations (Table 1). Total genomic DNA was extracted from the leaves of each sampled tree using a modified cetyl trimethyl ammonium bromide (CTAB) method described by Murray and Thompson (1980).

Microsatellite markers and genotyping

Seven nuclear microsatellite markers were selected for genotyping *A. xylocarpa*: Axy015, Axy087, Axy2-49, Axy2-58, Axy2-58(2) (Pakkad et al., 2009), Axy061 (Accession: AB441865.1) and Axy 2-27 (Accession: AB441868.1) developed for *A. xylocarpa* (Pakkad, unpublished data). The sequences of forward primers for both locus Axy061 and Axy2-27 were ACACACACACACAGAGAGAGAG and the reverse primers for the locus Axy061 AND Axy2-27 were GAAAGAGAAAGATAGGTCAGGGA and CCAAATCTCAATTTAACCATCAA, respectively.

PCR amplification was performed in 10 μl reaction mixtures containing 10 ng of template DNA, 1X PCR buffer (20 mM Tris-HCl pH 8.3, 50 mM KCl), 200 μM of each dNTP, 1.5 mM $MgCl_2$ 0.2 μM of each primer and 0.25 units of *Taq* polymerase. The PCR conditions were an initial denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, annealing temperature for 30 s, 72°C for 30 s, then a final extension at 72°C for 7 min. PCR products were separated using a 3100 ABI prism Sequencer with GeneScan software (Applied biosystems)

Statistical analysis

The 15 populations were divided into four groups (Northern, North Eastern, Central and Western Thailand), based on their geographical

Table 1. Sites and sample size for 15 populations of *Afzelia xylocarpa*.

Group	Population	Sample size	Latitude (N)	Longitude (E)
Northern Thailand	Mae Ping National Park (1)	**30**	17.693512°	98.882245°
Northern Thailand	Pha Glong National Park (2)	30	18.156166°	100.011497°
Northern Thailand	Mae Yom National Park (3)	30	18.639029°	100.187089°
Northern Thailand	Doi Khun Tan National Park (15)	30	18.489503°	99.254301°
North Eastern Thailand	Phu Pha Man National Park (4)	30	16.771604	102.03111°
North Eastern Thailand	Phu Pha Yol National Park (5)	30	17.006613°	104.080230
North Eastern Thailand	Phu Sa Dok Bua National Park (6)	30	16.254499°	104.850428°
Central Thailand	Western part of Kao Yai National Park (7)	30	14.459303°	101.439303°
Central Thailand	Sa Kae Raj Environmental Research Center (Biosphere Reserve) (8)	30	14.516048°	101.983373°
Central Thailand	Eastern part of Kao Yai National Park (9)	30	14.228265°	101.951273°
Central Thailand	Phud Ta Chay National Park (10)	30	14.442896°	101.001058°
Western Thailand	Klong Lan National Park (11)	30	16.10°	99.12°
Western Thailand	Sam Roi Yod National Park (12)	12	12.193134°	99.957202°
Western Thailand	Kaeng Kra Chan National Park (13)	30	12.739616°	99.669255°
Western Thailand	Salak Pra Wildlife Sanctuary (14)	30	14.147858°	99.376278°

locations, for genetic analysis (Figure 1). The Northern and western Thailand are characterised by high mountains and steep river valleys, lies on the fringe of the Himalayan foothills, which give way to the plains of the North Eastern. The North Eastern or Korat Plateau is an arid region characterized by a rolling surface and undulating hills. The Chao Phraya River and its four main tributaries have formed the alluvial floodplain of the Central Thailand. The genetic diversity at the nSSR loci in each population and each group was quantified in terms of the number of alleles per locus (*A*), allelic richness (*A*r) (El-Mousadik and Petit, 1996), the mean observed heterozygosity (*Ho*), and gene diversity (*H*) (Nei, 1987) using FSTAT 2.9.3 software (Goudet, 1995). The numbers of ''private alleles'' (Slatkin, 1985), that is, the numbers of alleles not present in other populations or groups (*K*p) were counted. Deviations from Hardy–Weinberg expectations were determined by χ^2 analysis and calculating inbreeding coefficients (F_{IS}, Weir and Cockerham, 1984) using Genepop web Version 3.4 (Raymond and Rousset, 1995; http://genepop.curtin.edu.au/genepop) and their significance ($F_{IS} \neq 0$) was tested by 1000 permutations.

Bayesian clustering approach implemented in STRUCTURE Version 2.1 (Pritchard et al*.,* 2000) was used to detect clusters structure and estimate the number of populations (*K*) in a sample and to assign individuals to one or more of these populations (*K*). This approach assumes Hardy–Weinberg equilibrium and linkage equilibrium between loci within populations. The analyses were conducted under the admixture model and the option of correlated allele frequencies between populations. The number of genetically distinct clusters (*K*) was set to vary from 1 to 15 (total number of populations). The model was run five independent simulations for each *K*, used a burn-in length of 30,000 and a run length of 100,000 iterations. Other parameters were set to default values as suggested by Pritchard and Wen (2003). The ΔK statistic, based on the rate of change of log likelihood of data [*L* (*K*)] between successive K values was used to select the optimal *K* fallowed Evanno et al*.* (2005).

Contributions of each population to the total diversity (*CT*) and the total allelic richness (*CTR*) were calculated following the study of Petit et al. (1998). Two components of the total contribution of each population to diversity – the diversity within it (*CS* and *CSR*) and its differentiation (*CD* and *CDR*) – were also estimated for total diversity and allelic richness, respectively. These statistics satisfy the following relationships: *CT* = *CS* + *CD* and *CTR* = *CSR* + *CDR* (Petit et al*.,* 1998). The gene diversity in the total population (H_T), the average gene diversity within populations (H_S) (Nei, 1987), the proportion of the total genetic diversity that occurs among population (G_{ST}) (Nei, 1973), and the coefficient of genetic differentiation among populations under an infinite allele model (IAM) (F_{ST}) (Wier and Cockerham, 1984) were estimated using FSTAT. The significance of differentiation at each locus was tested by the loglikelihood (G)-based exact test (Goudet et al., 1996), using a Markov chain method in Genepop web Version 3.4.

Analysis of molecular variance (AMOVA; Excoffier et al., 1992) was performed to examine the hierarchical genetic structure, using the program ARLEQUIN Version 2.000 (Schneider et al., 2000) with three levels of population structure. As shown in Figure 1 and Table 2, four geographic groups were defined a priori. Genetic variation was partitioned into three levels: among groups, among populations within groups and within populations. The significance of the variance components and the differentiation statistic was tested with 10,000 permutations. Mantel tests (Mantel, 1967) were carried out using ARLEQUIN to test the significance of isolation by distance patterns, regressing pairwise population $F_{ST}/(1 - F_{ST})$ values against the log-transformed geographic distances between the respective pairs of populations (Rousset, 1997). The geographic distances used were the great-circle distances between populations calculated from their longitudes and latitudes, using Excel software. In addition, the gene flow estimates (*N*m) were calculated as $Nm = (1 - G_{ST})/4G_{ST}$ (Slatkin and Barton, 1989).

For nSSRs, Nei's standard genetic distances (Nei, 1972) were used to construct UPGMA and neighbor-joining (NJ) phylograms and confidence levels for the topologies were estimated by resampling over loci with 1000 bootstrap replicates. The genetic distance estimates, bootstrapping, unweighted pair group method using arithmetic averages (UPGMA) and neighbor-joining (NJ) phylograms, and consensus tree construction procedures were carried out using ''Populations 1.2.28'' software (Langella, 1999).

RESULTS

The seven nSSRs loci were found to be moderately polymorphic, with numbers of detected alleles for each locus ranging from 2 (Axy015) to 15 (Axy2-58(2)) (Table 3). A total of 53 alleles were identified among the 432

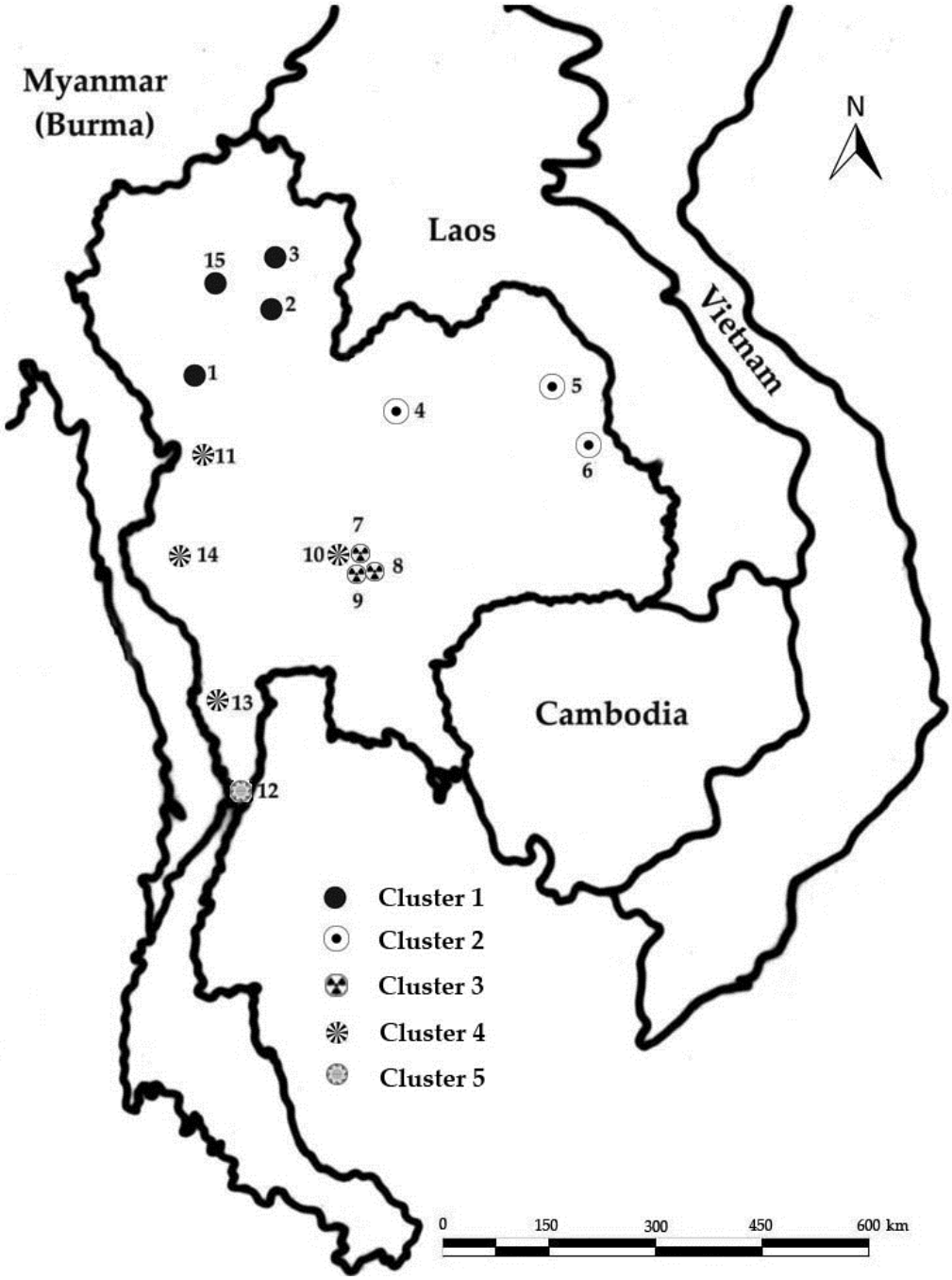

Figure 1. Map of Thailand showing the locations of sampling sites. Population numbering is as follows: 1, Mae Ping; 2, Pha Glong; 3, Mae Yom; 4, Phu Pha Man; 5, Phu Pha Yol; 6, Phu Sa Dok Bua; 7, Western part of Kao Yai; 8, Sa Kae Raj; 9, Eastern part of Kao Yai; 10, Phud Ta Chay; 11, Klong Lan; 12, Sam Roi Yod; 13, Kaeng Kra Chan; 14, Salak Pra; 15, Doi Khun Tan.

individuals with a mean of 7.57 per locus. The average expected heterozygosity per locus over 15 populations ranged from 0.358 (Axy015) to 0.776 (Ay2-49) (Table 3), with a mean of 0.620 over all loci. The heterozygosity at locus Axy015 was low since only two alleles were detected.

Bayesian clustering of the information from the seven nSSRs loci demonstrated that the model with K = 5 explained the data satisfactorily. This finding suggests that the most probable number of clusters was 5 based on our data for 432 individual from 15 populations. The most individuals of Mae Ping (1), Pha Glong (2), Mae

Table 2. Genetic diversity of the four designated groups among the 15 *A. xylocarpa* populations investigated in this study.

Population	*N*	*A*	*Ar*	K_p	*H*	H_O	F_{IS}
Northern Thailand	118.7	5.3	5.27	0	0.519	0.463	0.108^{NS}
Mae Ping (1)	29.9	3.9	3.33	0	0.474	0.407	0.143*
Pha Glong (2)	29.9	4.3	3.72	0	0.509	0.431	0.157*
Mae Yom (3)	29.9	4.7	4.02	0	0.542	0.531	0.023^{NS}
Doi Khun Tan (15)	29.1	4.1	3.55	0	0.492	0.490	0.007 ^{NS}
Average	29.7	4.3	3.66	0	0.504	0.465	0.051^{a}
North Eastern Thailand	89.4	6.0	6.00	0	0.654	0.587	0.103^{NS}
Phu Pha Man (4)	29.6	5.6	4.65	0	0.627	0.585	0.066 ^{NS}
Phu Pha Yol (5)	29.9	5.4	4.73	0	0.658	0.550	0.164*
Phu Sa Dok Bua (6)	30.0	5.0	4.35	0	0.651	0.624	0.041 ^{NS}
Average	29.8	5.3	4.58	0	0.645	0.586	0.090^{a}
Central Thailand	119.7	7.1	7.14	6	0.640	0.563	0.120^{NS}
Western part of Kao Yai (7)	29.9	5.4	4.52	2	0.638	0.589	0.078
Sa Kae Raj (8)	30.0	5.1	4.26	0	0.649	0.529	0.186*
Eastern part of Kao Yai (9)	30.0	5.0	4.19	2	0.589	0.533	0.095 ^{NS}
Phud Ta Chay (10)	29.9	5.4	4.41	0	0.644	0.603	0.066 ^{NS}
Average	30.0	5.2	4.35	1	0.630	0.564	0.080^{a}
Western Thailand	101.3	5.1	5.11	0	0.574	0.509	0.113^{NS}
Klong Lan (11)	29.9	4.1	3.80	0	0.600	0.574	0.043 ^{NS}
Sam Roi Yod (12)	11.9	3.3	3.24	0	0.514	0.518	-0.003 ^{NS}
Kaeng Kra Chan (13)	29.9	4.0	3.19	0	0.517	0.479	0.077 ^{NS}
Salak Pra WS (14)	29.7	4.0	3.36	0	0.517	0.476	0.084 ^{NS}
Average	25.4	3.9	3.40	0	0.537	0.512	0.050^{a}

N, Number of individuals analyzed; *A*, total number of alleles or haplotypes detected; *Ar*, allelic richness for 12 diploid individuals in nSSRs ; K_p, number of population- or group-specific alleles; *H*, gene diversity; H_O, observed heterozygosity; F_{IS}, fixation index; *P<0.01; ^{NS}, non significant; ^{a} Not available.

Table 3. Characteristics of the nuclear microsatellite markers for *A. xylocarpa* used in this study.

Locus	T_a (^oC)	Size range (bp)	*N*	*A*	H_o	*H*	F_{IS}
Axy2-49	55	96-116	431	11	0.776	0.815	0.0473
Axy2-58	55	211-227	431	10	0.466	0.538	0.0988
Axy2-27	55	247-261	431	4	0.525	0.506	-0.045
Axy087	55	110-124	431	7	0.599	0.548	-0.092
Axy061	55	141-147	419	4	0.327	0.537	0.3856
Axy015	55	223-225	431	2	0.358	0.360	0.0062
Axy2-58(2)	55	361-380	430	15	0.638	0.722	0.0909

T_a, PCR annealing temperature; *N*, number of individuals analyzed; *A*, total number of alleles detected; H_O, observed heterozygosity; *H*, gene diversity; F_{IS}, fixation index.

Yom (3) and Khun Tan (15) populations were assigned to cluster I; the most individuals of Phu Pha Man (4), Phu Pha Yol (5) and Phu Sa Dok Bua (6) populations were assigned to cluster II; the most individuals of Western part of Kao Yai (7), Sa Kae Raj (8) and Eastern part of Kao Yai (9) populations were assigned to cluster III; the most individuals of Phud Ta Chay (10), Klong Lan (11), Kaeng Kra Chan (13) and Salak Pra (14) were assigned to cluster IV, while the most individuals of Sam Roi Yod population were assigned to cluster V (Figure 2).

Within the 15 populations, the average number of alleles over the seven loci ranged from 3.3 (Sam Roi Yod (12)) to 5.6 (Phu Pha Man (4)) at the population level and 3.9 (Western Thailand) to 5.3 (North Eastern Thailand) at the group level (Table 2). Allelic richness (A_R) was calculated for 24 gene copies; there was a maximum value of

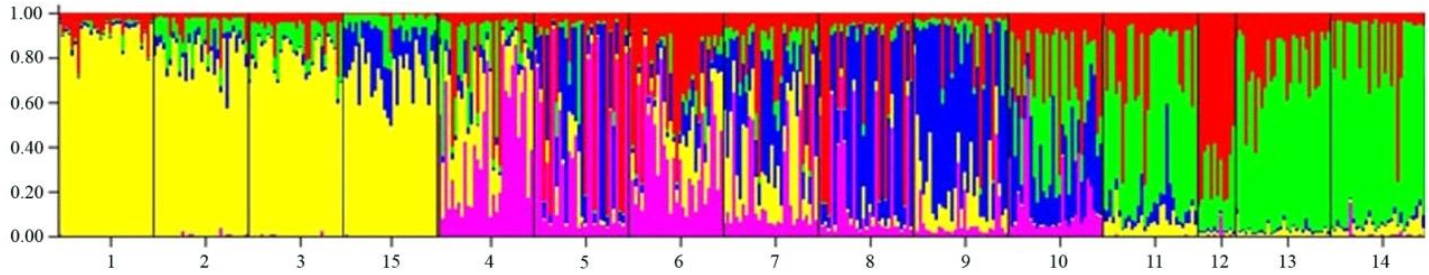

Figure 2. Genetic relationships among the 15 populations, estimated using STRUCTURE (Pritchard et al., 2000) and data on seven nSSRs loci. Population numbering is as follows: 1, Mae Ping; 2, Pha Glong; 3, Mae Yom; 4, Phu Pha Man; 5, Phu Pha Yol; 6, Phu Sa Dok Bua; 7, Western part of Kao Yai; 8, Sa Kae Raj; 9, Eastern part of Kao Yai; 10, Phud Ta Chay; 11, Klong Lan; 12, Sam Roi Yod; 13, Kaeng Kra Chan; 14, Salak Pra; 15, Doi Khun Tan.

4.73 in the Phu Pha Yol (5) population and a minimum value of 3.19 in the Kaeng Kra Chan (13) population. The private alleles were found only two populations of the Western part (7) and Eastern part (9) of Kao Yai. The mean observed heterozygosity (H_O) across loci per population ranged from 0.407 in Mae Ping (1) to 0.624 in Phu Sa Dok Bua (6). The expected heterozygosity across loci per population ranged from 0.474 in Mae Ping (1) to 0.658 in Phu Pha Yol (5). Fixation indices (Weir and Cockerham), which are measures of heterozygote deficits, were positive for all of the populations, except the Sam Roi Yod (12) population. F_{IS} deviated significantly from zero for the Mae Ping (1), Pha Glong (2), Phu Pha Yol (5) and Sa Kae Raj (8) populations due to an excess of homozygosity.

The Phu Pha Yol (5) population contributed most to the total diversity (CT), via its large intrinsic diversity within it and the Phu Sa Dok Bua (6) population contributed nearly as much (Figure 3). The contributions to total diversity of the Phu Pha Man (4), Western part of Kao Yai (7) Sa Kae Raj (8) and Phud Ta Chay (10) populations were positive, due their own diversity. The contribution to total diversity of the Mae Ping (1), and Sam Roi Yod (12) populations were also positive, however due to divergence from the other populations. The contributions to total diversity of the other populations were negative, due to a lack of inherent diversity in the Pha Glong (2) and Mae Yom (3) populations, and due to a lack of divergence from the other populations in the Eastern part of Kao Yai (9), Klong Lan (11), Kaeng Kra Chan (13), Salak Pra (14) and Doi Khun Tan (15) populations.

The results based on the contributions to the total allelic richness (CTR) were similar to those based on total diversity (CT), except the Mae Ping (1), Phud Ta Chay (10) and Sam Roi Yod (12), which were negative result (Figure 3). The Phu Pha Yol (5) population also showed the highest value, due to its own diversity. The positive contributions to the total allelic richness made by the Phu Pha Man (4), Phu Sa Dok Bua (6), Western part of Kao Yai (7) and Sa Kae Raj (8) were mainly due to their own allelic richness (Figure 3).

An AMOVA of hierarchical gene diversity revealed that genetic variation among individuals within populations accounted for 91.57% of the total molecular variance (Table 4). A further 3.66% of the total variance was distributed among populations within groups, whereas 4.76% of the variance occurred among groups. All differentiation statistics were highly significant ($P < 0.001$). The coefficient of genetic differentiation among populations was estimated with different statistics. The F_{ST} and G_{ST} values were estimated to be 0.074 and 0.078. The UPGMA and NJ trees based on Nei's genetic distances for nSSRs are shown in Figure 4. The relationship between genetic differentiation and geographical among pairwise comparisons of populations was investigated using the Mantel test. Mantel test showed a significant correlation between populations differentiation measured as $F_{ST}/(1- F_{ST})$ and the natural logarithm of the geographical distance between populations ($r = 0.5518$; $P < 0.001$ with 1,000 permutations) (Figure 5).

DISCUSSION

Forest fragmentation reduces the size and increases the spatial isolation of populations; therefore, it is theoretically expected to reduce the heterozygosity, to cause the loss of intermediate-frequency alleles, to increase the inbreeding coefficient, consequently, to reduce the effective population size and to increase the genetic differentiation among populations due to restricted gene flow and genetic drift (Hamrick et al*.,* 1992; Young et al*.,* 1996; Young and Boyle, 2000).

In the present study, the nSSRs data indicate that a high level of genetic diversity ($H_S = 0.575$) and low level of genetic differentiation among the 15 examined *A. xylocarpa* populations. The observed level of genetic differentiation among the 15 populations was low, as reveled by $F_{ST} = 0.074$ and $G_{ST} = 0.078$. This result was further confirmed by the AMOVA analysis, with 91.57% of the genetic variation existed within populations (Table 4). *Afzelia* (Caesalpinioideae) species are pollinated by large *Xylocopa* bee (Kato et al*.,* 2008) which have been reported

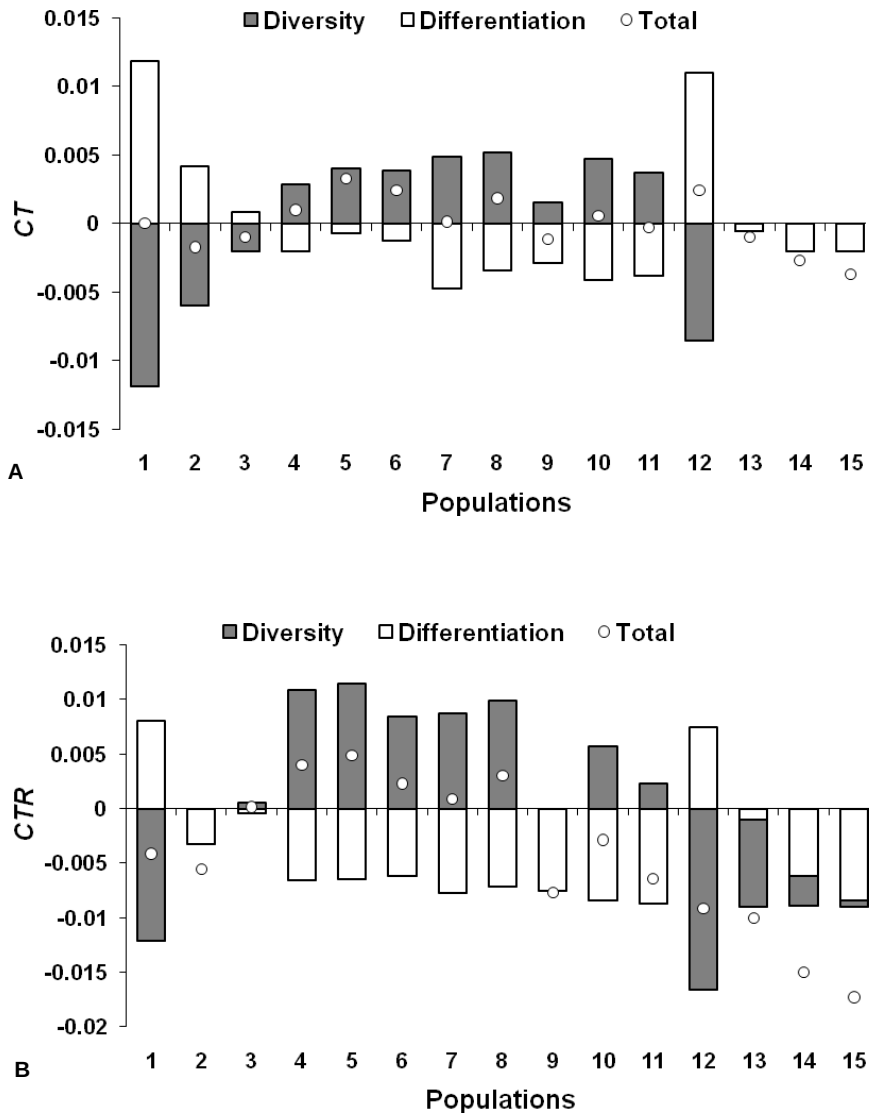

Figure 3. Contribution to the total diversity (*CT*) and allelic richness (*CTR*) of each population of *A. xylocarpa*. Population numbering is as follows: 1, Mae Ping; 2, Pha Glong; 3, Mae Yom; 4, Phu Pha Man; 5, Phu Pha Yol; 6, Phu Sa Dok Bua; 7, Western part of Kao Yai; 8, Sa Kae Raj; 9, Eastern part of Kao Yai; 10, Phud Ta Chay; 11, Klong Lan; 12, Sam Roi Yod; 13, Kaeng Kra Chan; 14, Salak Pra; 15, Doi Khun Tan.

to be capable of flying distances of several kilometers thus affecting pollen dispersal between plants at great distances. The gene flow estimates (*N*m) was estimated at 3.13, indicating a very high migration rate among 15 populations. According to the study of Wright (1931), migration rates of greater than one migrant per generation may be sufficient to prevent the differentiation among populations. The migration rates estimated from this study was above that level and appeared to be sufficiently high to counteract of the fragmented distribution on population structure. Field observation also showed that this species also can re-establish from stump sprouts which the potential importance of stump sprouting in the logged populations as a mechanism maintaining genetic diversity. Four populations exhibited a significantly positive inbreeding coefficient (Table 2), possibly due to the presence of null alleles and/or the non random mating of individuals within these populations. For the other populations, low F_{IS} values and did not significantly different from zero, indicate that the populations are largely out breeding with minor degrees of inbreeding.

High genetic diversity and low genetic differentiation among populations in rare plants is attributable to a number of factors including: insufficient length of time for isolation (Coates, 1988), adaptation of genetic system to small population conditions (Coates, 1988; Rossetto et al., 1995; James, 2000), recent fragmentation of a once continuous genetic system (Rossetto et al., 1995), or

Table 4. Results of AMOVA for nuclear microsatellites. *P*-values are based on permutation tests.

Sources of variation	d.f.	Sum of squares	Variance components	Percentage of variation	ϕ-statistics	*P*-value
Among groups	3	86.9	0.10	4.76	ϕ_{CT}=0.047	<0.0001
Among populations within groups	11	72.0	0.08	3.66	ϕ_{SC}=0.038	<0.0001
Within populations	849	1691.2	1.99	91.57	ϕ_{ST}=0.084	<0.0001

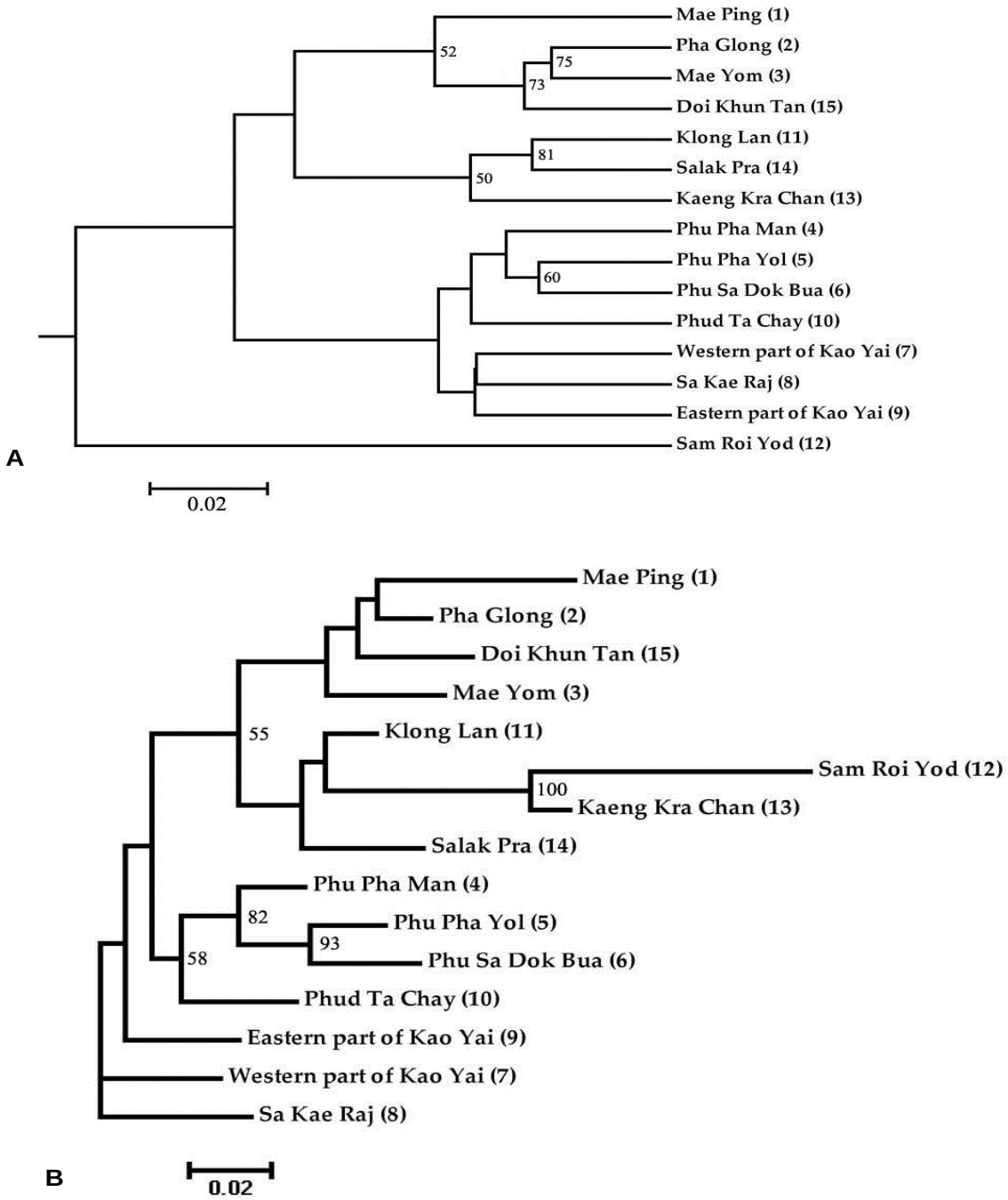

Figure 4. Dendrograms for the 15 *A. xylocarpa* populations based on Nei's genetic distances obtained using (a) the UPGMA and (b) the neighbor-joining method. The numbers are percentage values over 1000 bootstrap replicates. Only bootstrap values over 50% are shown.

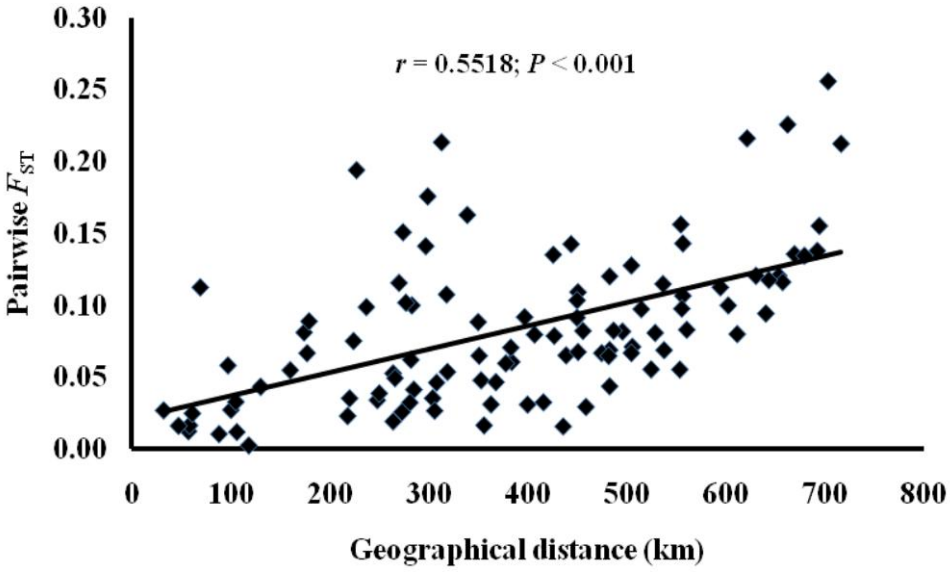

Figure 5. Correlation between pairwise F_{ST} of *Afzelia xylocarpa* and geographical distance.

extensive, recurrent gene flow between populations due to the combination of bird pollination and high outcrossing rates (Maguire and Sedgley, 1997; Chiang et al*.,* 2006). Such results were achieved previously in a number of studies of rare and endangered species, including *Acacia anomala* (Coates, 1988), *Swietenia humilis* (White et al., 1999), *Swietenia macrophylla* (Lemes et al., 2003), *Magnolia stellata* (Setsuko et al., 2007), *Prunus africana* (Farwig et al., 2008), *Dalbergia monticola* (Andrianoelina et al., 2009) and *Shorea robusta* (Pandey and Geburek, 2010). On the other hand, low genetic diversity and high genetic differentiation among populations had been detected in *Eugenia dysenterica* (Zucchi et al., 2003), *Caesalpinia echinata* (Cardoso et al., 1998), *Glyptostrobus pensilis* (Li et al., 2005), *Vitellaria paradoxa* (Sanou et al., 2005).

The Phu Pha Man (4), Phu Pha Yol, Phu Sa Dok Bua (6), Western part of Kao Yai (7), Sa Kae Raj (8), Eastern part of Kao Yai (9) and Phud Ta Chay (10) populations harbored high genetic diversity (Table 2). These possibilities due to all of these populations are located in North Eastern and Central part of Thailand. In the past, these areas were a large and continuous mixed deciduous, dry evergreen and dry dipterocarp forest, which is the habitat of this species. Therefore, the North Eastern and Central part of Thailand seem to be a center of species' distribution and old source of this species. An older source population is expected to harbor more genetic variation than a population founded more recently from it, because of a longer time to accumulate mutations (Jorde et al*.,* 1998). In addition, the connectivity between the populations in these groups as a result of gene flow between them is likely to be important in maintaining their genetic diversity and minimizing genetic drift. The contribution profile (Figure 3) shows that all of the populations in the North Eastern and Central Thailand contribute strongly to the total diversity through their inherent diversity and were less divergent from the other populations. Millar and Libby (1991) suggest that it may not be the genetically distinct outlier populations that should rank highest but the core population from the center of the species range, and that within-stand diversity may be qualitatively more important than the uniqueness of a population.

In contrast, the populations in Northern Thailand had the lowest genetic diversity (Table 2). These populations are located in a small plain area and surround by the high mountain. The explanation may account for the low level of genetic diversity occurred in these populations possible due to mountain barriers to gene flow. Limited gene flow tends to generate marked genetic structures, that is*,* a spatially structured distribution of genotypes and alleles within and among populations. Because plants are sessile and pollen and seed dispersals are limited, marked aggregations of close relatives tend to form. Thus, mating among neighboring individuals often results in inbreeding depression (Waser and Price, 1979; 1991; Campbell and Waser, 1989; Fenster, 1991; Campbell and Dooley, 1992; Moran-Palma and Snow, 1997; Nuortila et al., 2002). The limited gene flow showed some of these effects in Mae Ping (1) and Pha Glong (2) populations, resulted in less genetic diversity than the other populations and positive and significant different from zero for F_{IS} value. The contribution to total diversity (*CT*) of the Mae Ping (1), Pha Glong (2), Mae Yom (3) populations were also very divergent from the other populations (Figure 3). Clearly gene flow with other populations must be absent or extremely low, and actually the coefficient of differentiation between those populations and the rest was the largest of all.

Population structure

Two phylogenetic dendrograms based on Nei's genetic distances were constructed for nSSRs, an UPGMA tree and an NJ tree (Figure 4), both of which agree with the geographic grouping in Table 2. This is consistent with the results of the STRUCTURE analyses, which clearly identified the five clusters of populations. The most individuals of populations in the Northern and Northern Eastern Thailand were assigned to cluster I and II, respectively. The most individuals of populations in the Central Thailand, except for the Phud Ta Chay (10) populations were assigned to cluster III, The most individuals of populations in the Western Thailand (except for the Sam Roi Yod (12)) and Phud Ta Chay (10) populations were assigned to cluster IV (Figure 2). These observations suggest that extensive gene flow has occurred among populations within the large, continuous forested areas. The Sam Roi Yod (12) population of the Western Thailand were assigned to cluster IV, and the contribution to total diversity (*CT*) of this population were also very divergent from the other populations (Figure 3), indicated this population was slightly isolated. A strong correlation between genetic and geographical distances (Mantel test: $r = 0.5518$; $P<0.0001$) revealed a pattern of isolation-by-distance across the distribution range of *A. Xylocarpa* in Thailand. This pattern suggested that the dispersal of this species might be constrained by distance such that gene flow is most likely to occur between neighboring populations (Hutchison and Templeton, 1999; Slatkin, 1993).

Implications for conservation

The maintenance of genetic variation is one of the major objectives for conserving endangered and threatened species (Avise and Hamrick, 1996). *A. xylocarpa* is threatened throughout its range in Southeast Asia as a result of over-exploitation and habitat destruction between 1945 to 1975, which have clearly reduced local population sizes and led many populations to local extinction. Habitat degradation caused by selective logging and, most importantly, through conversion of forest into agriculture land likely to reduce the colonization of new sites, despite the ability of this species to regenerate in disturbed habitats. Understanding genetic variation within and among populations provides essential information in the formation of appropriate management strategies directed towards their conservation (Milligan et al., 1994).

In this study, the genetic diversity and differentiation of 15 populations of *A. xylocarpa* in Thailand were analyzed using nSSRs. The results for the nSSRs suggested that all of the populations in the Northern Eastern, Central Thailand and the Klong Lan (11) populations harbored the high genetic diversity and less divergent from the other populations (Table 2 and Figure 3). Therefore, these populations should be assigned the highest priority for conservation of this species in Thailand. In addition, the majority genetic variation observed in this study resides within rather than among populations suggesting that a subset of populations need only be included in *ex situ* conservation program in order to capture most of the genetic variation within species. This is the first report concerning the genetic diversity and differentiation of this species, and provides basic genetic information that should facilitate attempts to conserve it. In further study, there is a necessity of the evaluating genetic diversity and differentiation in other countries, and comparing with Thailand's populations using results from this study for the establishment conservation scheme of *A. xylocarpa* in Southeast Asia.

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REFERENCES

Andrianoelina O, Eavreau B, Rammanjisoa L, Bouvet JM (2009). Small effect of fragmentation on the genetic diversity of *Dalbergia monticola*, an endangered tree species of the eastern forest of Madagascar, detected by chloroplast and nuclear microsatellites. Ann. Bot. 104: 1231-1242.

Avise JC, Hamrick JL (1996). Conservation genetics: case histories from nature. New York: Chapman and Hall.

Balloux F, Brünner H, Lugon-Moulin N, Hausser J, Goudet J (2000). Microsatellites can be misleading: an empirical and simulation study. Evolution 54: 1414–1422.

Campbell DR, Dooley JL (1992). The spatial scale of genetic differentiation in a hummingbird-pollinated plant: comparison with models of isolation by distance. Am. Nat. 139: 735–748.

Campbell DR, Waser NM (1989). Variation in pollen flow within and among populations of *Ipomopsis aggregata*. Evolution 43: 1444–1455.

Cardoso MA, Provan J, Powell W, Ferreira PCG, de Oliveira DE (1998). High genetic differentiation among remnant populations of the endangered *Caesalpinia echinata* Lam (Leguminosae Caesalpinioideae). Mol. Ecol. 7: 601–608

Chase M, Kesseli R, Bawa, KS (1996). Microsatellite Markers for Population and Conservation Genetics of Tropical Trees. Am. J. Bot. 83: 51-57.

Chiang YC, Hung KH, Schaal BA, Ge XJ, Hsu TW, Chaing TY (2006). Contrasting phylogeographical patterns between mainland and island taxa of the *Pinus luchuensis* complex. Mol. Ecol. 15: 765–779.

Coates DJ (1988). Genetic diversity and population genetic structure in the rare Chittering Grass Wattle, *Acacia anomala* Court. Aus. J. Bot. 36: 273-286.

Dayanandan S, Bawa KS, Kesseli R (1997). Conservation of Microsatellites among tropical trees (Legumninosae). Am. J. Bot. 84:1658-1663.

Dow BD, Ashley MV, Howe HF (1995). Characterization of highly variably $(GA/CT)_n$ microsatellites in the bur oak, *Quercus macrocarpa.* Theor. Appl. Genet. 91: 137-141.

Edwards A, Civitello A, Hammond HA, Caskey CT (1991). DNA typing and genetics mapping with trimeric and tetrameric tandem repeats. Am. J. Hum. Genet. 49: 746-756.
El-Mousadik A, Petit RJ (1996). Chloroplast DNA phylogeography of the argan tree of Morocco. Mol. Ecol. 5: 547–557.
Excoffier L, Smouse PE, Quattro JM (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131: 479–491.
Evanno G, Regnaut S, Goudet J (2005). Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. Mol Ecol 14: 2611–2620.
FAO (1997). State of the World's Forests 1997.
Farwig N, Braun C, Böhning-Gaese K (2008). Human disturbance reduces genetic diversity of an endangered tropical tree, Prunus africana (Rosaceae). Conserv. Genet. 9: 317-326.
Fenster CB (1991). Gene flow in *Chamaecrista fasciculata* (Leguminosae) II. Gene establishment. Evolution 45: 398–409.
FORRU (1998). Forest for the Future: Growing and Planting Native Trees for Restoring Forest Ecosystems. Biology Department, Science Faculty, Chiang Mai University, Thailand. 152 p.
Goudet J (1995). FSTAT (Version 12): a computer program to calculate Fstatistics. J. Hered. 86: 485–486.
Goudet J, Raymond M, DeMeeus T, Rousset F (1996). Testing differentiation in diploid populations. Genetics 144: 1933–1940.
Hamrick JL, Godt MJW, Sherman-Broyles SL (1992). Factors influencing genetic diversity in woody plant species. New Forest 6: 95–124.
Hedrick PW (2001). Conservation genetics: where are we now? Trends Ecol. Evol. 16: 629–636.
Hutchison DW, Templeton AR (1999). Correlation of pairwise genetic and geographic distance measures: inferring the relative influences of gene flow and drift on the distribution of genetic variability. Evolution 53: 1898–1914.
James SH (2000). Genetic systems in the south-west flora: implications for conservation strategies for Australian plant species. Aus. J. Bot. 48: 341-347.
Jorde LB, Bamshad M, Rogers AR (1998). Using mitochondrial and nuclear DNA markers to reconstruct human evolution. Bioessays 20: 126–136.
Kato M, Kosaka Y, Kawakita A, Okuyama Y, Kobayashi C, Phimminith T, Thongphan D (2008). Plant – pollinatorinteractions in tropical monsoon forests in Southeast Asia. Am. J. Bot. 95: 1375–1394.
Langella O, (1999). Populations 1.2.28. Available at http://www.pge.cnrs-gif.fr/bioinfo/populations/index.php.
Lemes MR, Gribel R, Proctor J, Grattapaglia D (2003). Population genetic structure of mahogany (*Swietenia macrophylla* King, Meliaceae) across the Brazilian Amazon, based on variation at microsatellite loci: implications for conservation. Mol. Ecol. 12: 2875–2883.
Li F, Xia N (2005). Population structure and genetic diversity of an endangered species, *Glyptostrobus pensilis* (Cupressaceae). Bot. Bull. Acad. Sin. 46: 155-162
Maguire TL, Sedgley M. 1997. Genetic diversity in *Banksia* and *Dryandra* (Proteaceae) with emphasis on *Banksia cuneata*, a rare and endangered species. Heredity 79: 394-401.
Mantel N A (1967). The detection of disease clustering and a generalized regression approach. Cancer Res. 27: 209 - 220.
Millar CI, Libby WJ (1991). Strategies for conserving clinal, ecotypic, and disjunct population diversity in widespread species. Pages 149–170 in Falk DA, Holsinger KE (editors). Genetics and conservation of rare plants. Oxford University Press, New York.
Milligan BG, Leebens-Mack J, Strand AE (1994). Conservation genetics: beyond the maintenance of marker diversity. Mol. Ecol. 12: 844–855.
Moran-Palma P, Snow AA (1997). The effect of interplant distance on mating success in federally threatened, self-incompatible *Hymenoxys herbacea=Hacaulis* var. *glabra* (Asteraceae). Am. J. Bot. 84: 233–238.
Murray MG, Thompson WF (1980). Rapid isolation of high molecular weight plant DNA. Nucleic Acid Res. 8: 4321–4325.
Nei M (1972). Genetic distance between populations. Am. Nat. 106:283–292.
Nei M (1973). Analysis of gene diversity in subdivided populations. Proc. Nat. Acad. Sci. U.S.A. 70: 3321–3323.
Nei M (1987). Molecular Evolutionary Genetics. Columbia University Press, New York.
Nuortila C, Tuomi J, Laine K (2002). Inter-parent distance affects reproductive success in two clonal dwarf shrubs, *Vaccinium myrtillus* and *Vaccinium vitis-idaea* (Ericaceae). Can. J. Bot. 80: 875–884.
Pakkad G, Ueno S, Yoshimaru H (2009). Isolation and characterization of microsatellite loci in an endangered tree species, *Afzelia xylocarpa* (Kurz) Craib (Caesalpinioideae). Mol. Ecol. Res. 9: 880-882.
Pandey M, Geburek T (2010). Genetic differences between continuous and disjunct populations: some insights from sal (*Shorea robusta* Roxb.) in Nepal. Conserv. Genet. 11: 977-984.
Petit RJ, El Mousadik A, Pons O (1998). Identifying populations for conservation on the basis of genetic markers. Conserv. Biol. 12: 844–855.
Prober S M, Brown AHD (1994). Conservation of the grassy white box woodlands: of *Eucalyptus albens*. Conserv. Biol. 8: 1003-1013.
Pritchard JK, Stephens M, Donnelly P (2000). Inference of population structure using multilocus genotype data. Genetics 155: 945–959.
Pritchard JK, Wen W (2003). Documentation for STRUCTURE software:Version 2. Available from http://pritchard.bsd.uchicago.edu.
Rajora OP, Mosseler A (2001). Challenges and opportunities for conservation of forest genetic resources. Euphytica 118: 197-212.
Raymond M, Rousset F, (1995). GenepopWeb Version 3.4. Available at http://genepop.curtin.edu.au/genepop.
Rossetto M, Weaver PK, Dixon KW (1995). Use of RAPD analysis in devising conservation strategies for the rare and endangered *Grevillea scapigera* (Proteaceae). Mol. Ecol. 4: 357-364.
Rousset F (1997). Genetic differentiation and estimation of gene flow from Fstatistics under isolation by distance. Genetics 145: 1219–1228.
Sanou H, Lovett PN., Bouvet JM (2005). Comparison of quantitative and molecular variation in agroforestry populations of the shea tree (Vitellaria paradoxa C.F. Gaertn) in Mali. Mol. Ecol. 14: 2601-2610.
Schneider S, Roessli D, Excoffier L (2000). Arlequin: A Software for Population Genetics Data Analysis, Ver. 2.000. Genetics and Biometry Lab, Department of Anthropology, University of Geneva.
Slatkin M (1985). Rare alleles as indicators of gene flow. Evolution 39: 53–63.
Slatkin M (1993). Isolation by distance in equilibrium and non-equilibrium populations. Evolution 47: 264–279.
Slatkin M, Barton, HH (1989). Acomparison of three indirect methods for estimating average levels of gene flow. Evolution 43: 1349–1368.
Streiff R, Labbe T, Bacilieri R (1998). Within-population genetic structure in *Quercus robur* L. and *Quercus petraer* (Matt.) Liebl. assessed with isozymes and microsatellites. Mol. Ecol. 7: 317-328.
Streiff R, Ducousso A, Lexer C, Steinkellner H, Gloessl J, Kremer A (1999). Pollen dispersal inferred from paternity analysis in a mixed stand of *Quercus robur* L. and *Quercus petraea* (Matt.) Liebl. Mol. Ecol. 8: 831– 841.
Tautz D (1989). Hypervariability of simple sequences as a general source for polymorphic DNA markers. Nucleic Acids Res. 17: 6463-6471.
Setsuko S, Ishida K, Ueno S, Tsumura Y, Tomaru N (2007). Population differentiation and gene flow within a metapopulation of a threatened tree, *Magnolia stellata* (Magnoliaceae). Am. J. Bot. 94: 128-136.
Ueno S, Tomaru N., Yoshimaru H, Manabe T, Yamamoto S (2000). Genetic structure of *Camellia japonica* L. in an old-growth evergreen forest, Tsushima, Japan. Mol. Ecol. 9: 647-656.
Waser NM, Price MV (1979). Pollen dispersal and optimal outcrossing in *Delphinium nelsoni*. Nature 277: 294–297.
Waser NM, Price MV (1991). Outcrossing distance effects in *Delphinium nelsonii*: pollen loads, pollen tubes, and seed set. Ecology 72: 171–179.
White G, Boshier D, Powell W (1999). Genetic variation within a fragmented population of *Swietenia humilis* Zucc. Mol. Ecol. 8: 1899-1909.
Wier BS, Cockerham CC (1984). Estimating F-statistics for the analysis of population structure. Evolution 38: 1358–1370.
World Resources Institute (1991). A Guide to the global environment. Oxford University Press, New York, p. 383.
Wright, S., 1931. Evolution in Mendelian populations. Genetics 16: 97-159.

Young AG, Boyle T, Brown ADH (1996). The population genetic consequences of habitat fragmentation for plants. Tren. Ecol. Evol. 11: 413–418.

Young AG, Boyle TJ (2000). Forest fragmentation. In: Young, A.G., Boshier, D., Boyle, T.J., (eds) Forest conservation genetics: principles and practice. CSIRO, Melbourne, pp 123–134.

Zhao X, Kochert G (1993). Phylogenetic distribution and genetic mapping of a (GGC)n microsatellite from rice (*Oryza sativa* L.). Plant Mol. Biol. 21: 607-614.

Zucchi M, Brondani RP, Pinheiro JB, Chaves LJ, Coelho AS, Vencovsky R (2003). Genetic structure and gene flow in *Eugenia dysenterica* DC in the Brazilian Cerrado utilizing SSR markers. Genet. Mol. Biol. 26: 449-457.