RESEARCH ARTICLE

Genetic diversity and population structure of endangered *Aquilaria malaccensis* revealed potential for future conservation

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Abstract

The endangered *Aquilaria malaccensis*, is an important plant with high economic values. Characterization of genetic diversity and population structure is receiving tremendous attention for effective conservation of genetic resources. Considering important repositories of biological diversity, the genetic relationships of 127 *A. malaccensis* accessions from 10 home gardens of three states of northeast India were assessed using amplified fragment length polymorphism (AFLP). Of the 1153 fragments amplified with four AFLP primer combinations, 916 (79.4%) were found to be polymorphic. Polymorphic information content (PIC) and marker index (MI) of each primer combination correlate significantly with the number of genotypes resolved. Overall, a high genetic diversity (avg. 71.85%) was recorded. Further, high gene flow (N_m: 3.37), low genetic differentiation (F_{ST}: 0.069) and high within population genetic variation (93%) suggests that most of the genetic diversity is restricted within population. Neighbour joining (NJ), principal coordinate analysis (PCoA) and Bayesian-based STRUCTURE grouped all the accessions in two clusters with significant intermixing between populations, therefore, revealed that two genetically distinct gene pools are operating in the *A. malaccensis* populations cultivated in home gardens. Based on the various diversity inferences, five diverse populations (JOH, FN, HLF, DHM and ITN) were identified, which can be potentially exploited to develop conservation strategies for *A. malaccensis*.

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Introduction

The primary goal of plant conservation is to maintain genetic diversity of endangered species. Among the different genetic recourses, home garden plantations are proved to be very efficient for *ex situ* conservation of rare/endangered species (Kabir and Webb 2008). Although, very little is known about the genetic diversity status of the home garden populations and their potential use to conserve species genetic variations (Li *et al.* 2005).

A. malaccensis Lam. (Thymelaeaceae) is an evergreen, hermaphroditic and an obligate outcross species having entomophilous mode of pollination with most of the insects in the immediate vicinity of the tree (Borah *et al.* 2012; Shankar 2012). Commonly known as 'sasi', is the main source of agarwood in India. Globally, it is distributed in Bangladesh, Bhutan, India, Indonesia, Iran, Malaysia, Myanmar, Philippines, Singapore and Thailand (IUCN 2013). In India, natural distribution and cultivation of A. malaccensis is confined to Arunachal Pradesh, Assam, Manipur, Meghalaya, Mizoram, Nagaland, Sikkim, Tripura and West Bengal (Barden *et al*. 2000).

Indiscriminate cutting has led to the depletion of its natural population, leading to it being listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) since 1995 (http://www.cites.org), and The Red List of Threatened Plants (IUCN 2013). It is assumed that species is almost 'extinct in wild' in Assam (Anonymous 2003) and mainly survives in plantations and home gardens in India.

Among DNA-based marker systems, the amplified fragment length polymorphism (AFLP) have been proven powerful marker for accurate estimation of genetic diversity and population structure to devise suitable conservation strategies for endangered plant species (Meudt and Clarke 2007;

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Mohapatra *et al.* 2008; Nag *et al.* 2015). In the present study, AFLP markers were used to characterize genetic diversity, differentiation and population structure of 127 *A. malaccensis* accessions from 10 different home gardens of northeast India. To our knowledge, this is the first report on genetic characterization of this endangered species.

Materials and methods

In the present study, a total of 127 *A. malaccensis* accessions collected from 10 different home gardens of northeast India were used for genetic diversity and population structure analysis (figure 1; table 1). Among these accessions, five were from Tripura, seven from Itanagar (Arunachal Pradesh) and remaining 115 were from different home gardens of Assam. Both experimental field (EFN) and farm at NEIST (FN) contain very old populations of *Aquilaria* and are maintained as permanent fields. These populations have not been raised as a result of any breeding programme or experimental crossings, hence they are also considered as home garden populations. Young leaves of the *A. malaccensis* were collected and dried in silica gel by changing it periodically, until the sample was completely dried. Total genomic DNA was isolated according to CTAB method of Doyle and Doyle (1990) with minor modifications. DNA concentration was determined on 0.8% agarose gel using uncut λ DNA as a standard and by Nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA).

AFLP protocol was followed as described by Vos *et al.* (1995) with minor modifications. Genomic DNA (250 ng) was restricted with *Eco*RI/*Mse*I enzyme mix and ligated to standard adapters using T4 DNA ligase. The adapter-ligated DNA served as a template for preamplification, with PCR parameters of 20 cycles at 94°C for 30 s, 56°C for 1 min and 72°C for 1 min. After initial screening of 24 primer pairs in 10 individuals from 10 populations, four primer pairs that revealed clear and reproducible profiles were extrapolated for molecular profiling of all genotypes. The AFLP analysis was done with the help of ABI 3730xl automated DNA Analyzer (Applied Biosystems, Hitachi, Japan). Selective amplification was carried out with 2.5 μL of these diluted products using *Eco*RI primers (fluorescently labelled with NED, FAM and JOE) and *Mse*I primers, *Taq* polymerase, PCR buffer, $MgCl_2$, each dNTPs and deionized water in a final volume of 10 μL. The first selective amplification cycle consisted of 94°C for 30 s, 65°C for 30 s and 72°C for 1 min. The annealing temperature was lowered by 0.7°C per cycle during the next 12 cycles, followed by 23 cycles at 94°C for 30 s, 56°C for 30 s and 72°C for 1 min. All PCR reactions were performed on i-cycler PCR system (Biorad, USA). 0.5 μL of each selective PCR product was mixed with 0.3 μL Gene

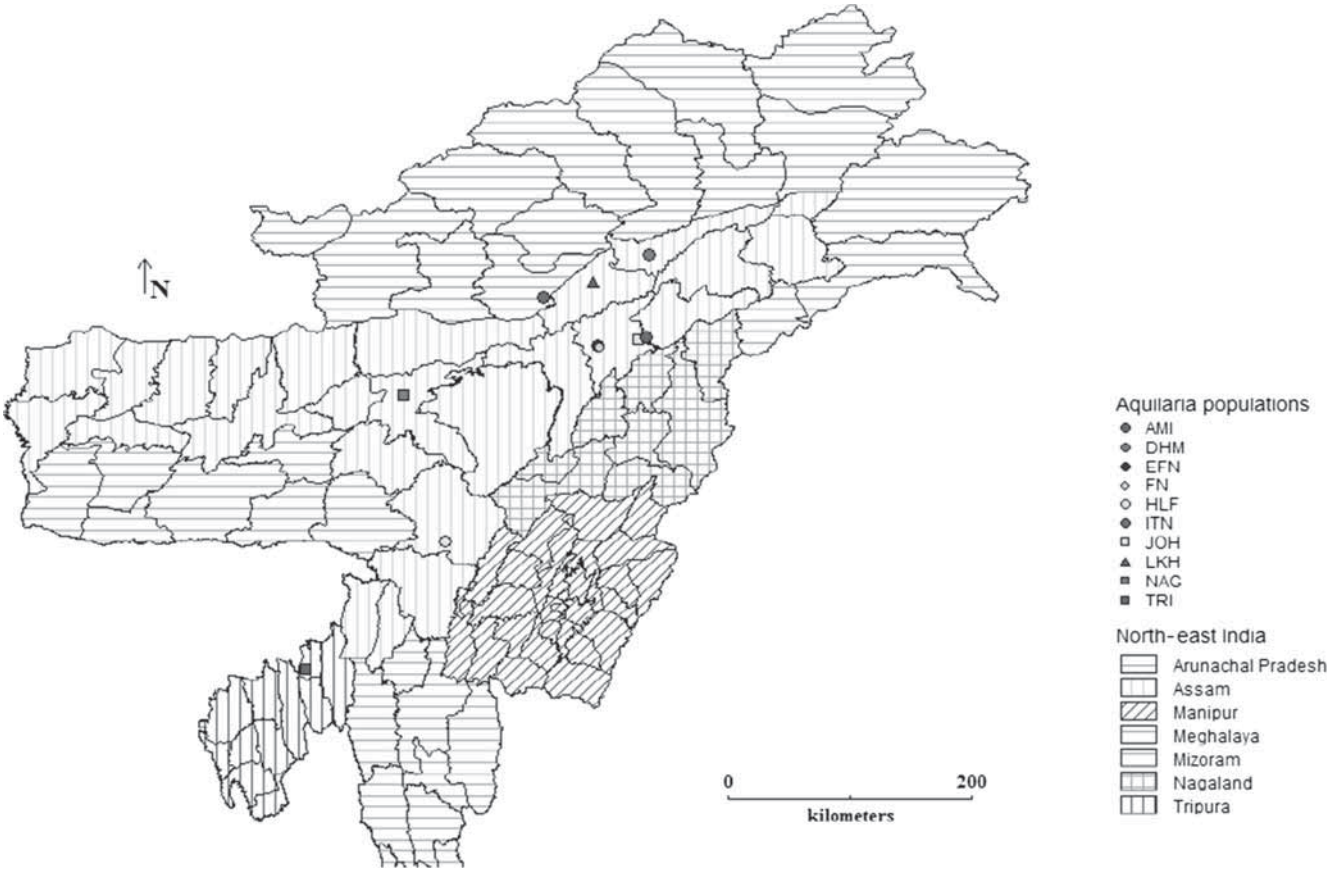

Figure 1. Map displaying the location of *A. malaccensis* collected from different home gardens of northeast India. The experimental samples of collection sites were marked as solid symbols.

Table 1. Details of geographical location of ten plantations of *A. malaccensis* from the northeast India.

Population	Code	Sample size	Latitude	Longitude
Dhemaji	DHM	8	94°32′51.65″E	27°28′24.44″N
Experimental field, NEIST	EFN	15	94°09′25.17″E	26°44′20.55″N
Farm NEIST	FN	31	94°09′49.24″E	26°44′59.89″N
Halflong	HLF	10	93°00′41.52″E	25°10′34.58″N
Itanagar	ITN	7	93°44′55.46″E	27°07′50.50″N
Lakhimpur	LKH	7	94°07′27.84″E	27°15′02.73″N
Jorhat	JOH	33	94°27′56.68″E	26°47′20.83″N
Tripura	TRI	5	91°57′35.92″E	24°08′56.72″N
Amiguri	AMI	6	94°31′15.80″E	26°48′17.69″N
Nagaon	NAG	5	92°42′03.11″E	26°20′58.69″N

Scan-500 ROX size standard (Applied Biosystems) and 9.2 μL highly deionized formamide. This mixture was denatured at 94°C for 5 min, followed by immediate chilling on ice and these denatured products were loaded on an ABI 3730xl automated DNA Analyzer (Applied Biosystems) to detect the amplified fragments.

Software GeneMapper ver. 4.0 (Applied Biosystems) was used to analyse electropherograms generated in automated genotyping through ABI 3730xl automated DNA Analyzer (Applied Biosystems). A large amount of data generated by automated DNA analyzer was checked manually to exclude unreliable detection and to increase the quality of data. The size range of amplified fragments, peak height threshold in terms of relative fluorescence units (rfu) and band width were considered as the most important scoring parameters, and the amplified fragments of 50–500 bp having present (1) and absent (0) peaks were extracted using software GeneMapper ver. 4.0 (Applied Biosystems). The resulted binary matrix was exported in the form of comma separated text for data analysis.

Polymorphism information content (PIC) of each marker was calculated according to Anderson *et al.* (1993). Marker index (MI) was calculated by using the formula given by Varshney *et al.* (2007). The diversity measures for each population were calculated using GenAlEx 6.5 (Peakall and Smouse 2006, 2012) which computes percentage polymorphism (%P), observed number of alleles (n_a), effective number of alleles (n_e), gene diversity (h), Shannon's information index (I) and number of private bands. To compensate the impact of varying sample size, rarefaction was used to measure the mean allelic richness (A_r) and mean private allelic richness (P_r) per locus by using HP-RARE 1.0 (Kalinowski 2005). Overall genetic diversity was calculated based on Jaccard dissimilarity coefficient using DARwin5 ver. 5.0.158 (Perrier and Jacquemoud-Collet 2006).

F_{ST} was calculated using Arlequin ver. 3.5.1.2 (Excoffier and Lischer 2010). The input file for ARLEQUIN was prepared by using a program CONVERT ver. 1.31 (Glaubitz 2004). Gene flow (N_m) was calculated using Wright's (1951) equation,

$$F_{ST} = 1/(4N_e m + 1),$$

where N_e is the effective population size, m is the proportion of individuals that are immigrants and $N_e m$ is the number of migrants. Bayesian model based clustering method of STRUCTURE ver. 2.3.4 (Pritchard *et al.* 2000; Falush *et al.* 2007) was employed to estimate the genetic structure. Five independent runs with K value ranging from 1 to 14 and three iterations for each value of K was set. Length of burn-in period and number of Markov chain Monte Carlo (MCMC) repeats after burn-in were set at 100,000 each. Results of STRUCTURE were visualized using STRUCTURE HARVESTER (Evanno *et al.* 2005; Earl 2012) to get the best-fit value of K for the data. Analysis of molecular variance (AMOVA) and principal coordinate analysis (PCoA) was carried out using GenAlEx 6.5. The dendrogram was computed on the basis of Jaccard coefficient using unweighed pair group method with arithmetic mean (UPGMA) for drawing a neighbour joining (NJ) tree with DARwin5 ver. 5.0.158 (Perrier and Jacquemoud-Collet 2006).

Results

Four AFLP primer combinations resulted in the amplification of 1153 fragments, of which 916 remained polymorphic across 127 accessions from 10 populations of *A. malaccensis* (figure 1; table 1). The number of fragments scored for each primer combination ranged from 215 (E-ACA+M-CAT) to 335 (E-ACC+M-CAT) with an average of 288.2, while polymorphic fragments varied from 126 (E-ACA+M-CAT) to 299 (E-ACC+M-CAT). Polymorphism rate was

Table 2. Details of AFLP analysis of *A. malaccensis.*

Primer combination	NB	PB	PIC	MI
E-ACA+M-CAT	215	126	0.215	27.09
E-AGG+M-CTC	282	204	0.240	48.96
E-ACC+M-CAG	321	287	0.272	78.06
E-ACC+M-CAT	335	299	0.273	81.63
Total	1153	916	–	–
Average	288.25	229	0.250	58.94

NB, number of bands; PB, number of polymorphic bands; PIC, polymorphism information content; MI, marker index.

Table 3. Estimates of genetic diversity in 10 plantations of *A. malaccensis.*

Population	No. of polymorphic loci	% P	n_a	n_e	h	I	Private bands	A_r	P_r
DHM	421	45.52	1.455	1.241	0.149	0.229	17	1.427	0.027
EFN	469	48.25	1.483	1.209	0.132	0.209	13	1.385	0.014
FN	627	68.45	1.685	1.244	0.155	0.25	52	1.448	0.026
HLF	499	52.73	1.527	1.245	0.155	0.244	22	1.462	0.051
ITN	408	43.01	1.430	1.241	0.147	0.224	14	1.416	0.024
LKH	324	31.88	1.319	1.172	0.106	0.163	2	1.307	0.008
JOH	646	70.52	1.705	1.25	0.16	0.258	55	1.464	0.026
TRI	330	33.84	1.338	1.214	0.128	0.19	3	1.338	0.010
AMI	359	31.00	1.310	1.193	0.115	0.171	5	1.308	0.012
NAG	325	32.75	1.328	1.208	0.124	0.184	7	1.328	0.018

%P, percentage of polymorphic loci; n_a, no. of different alleles; n_e, no. of effective alleles; h, gene diversity; I, Shannon's information index; A_r, mean no. of allelic richness per locus after rarefaction; P_r, mean no. of private alleles per locus after rarefaction.

highest for primer combination E-ACC+M-CAG (89.41%) and E-ACC+M-CAT (89.25%), followed by E-AGG+M-CTC (72.34%). Primer combination E-ACA+M-CAT had the lowest polymorphism at 58.6% (table 2). To display discriminatory power of AFLP primer combinations, different indices such as average PIC and MI were also estimated. The average PIC and MI were found to be 0.250 and 58.93, respectively, where, PIC ranged from 0.215 (E-ACA+M-CAT) to 0.273 (E-ACC+M-CAT), while, MI for each primer combination ranged from 27.09 to 81.63 (table 2). There was a very strong correlation between PIC and MI ($r^2 = 0.998$). The overall genetic diversity found to be high which varied from 39.36 to 100% with an average of 71.85%.

In an individual population, the percentage of polymorphic loci ranged from 31.0 to 70.52% with an average of 45.8%. The observed number of alleles (n_a) varied from 1.310 to 1.705, while the effective number of alleles (n_e) ranged from 1.172 to 1.250. Gene diversity (h), and Shannon's information index ranged from 0.106 to 0.160 (avg. 0.137), 0.163 to 0.258 (avg. 0.212), respectively. The number of private bands ranged from 2 to 55 with an average of 21. The highest number of private bands was observed in population JOH (55) followed by FN (52). The allelic richness (A_r) ranged from 1.307 to 1.464, while the private allelic richness varied from 0.008 to 0.051 per locus (table 3).

The population pairwise F_{ST} ranged from 0.040 to 0.261 (table 4). The highest value of population pairwise F_{ST} was observed between AMI and HLF populations (0.261) and the lowest was observed between JOH and EFN (0.040). The overall F_{ST} (0.069) suggests low genetic differentiation among the tested populations. The gene flow (N_m) was calculated from F_{ST} using Wright's equations (1951) was 3.37. The partitioning of molecular variance (AMOVA) revealed that most of the variance is partitioned within the population (93%) and very less (7%) molecular variance was partitioned between the populations, with Φ_{PT} value 0.069 which indicates low genetic differentiation (table 5).

The UPGMA-based dendrogram (figure 2) and PCoA (figure 3) grouped all the 127 individuals in two distinct clusters with no clear differentiation between populations from geographically distinct home gardens. Further, Bayesian model based STRUCTURE analysis (figure 4) revealed a similar pattern and all the 10 populations could be classified into two clusters (K = 2), best fit the dataset (figure 4a), indicating that two distinct genetic pools existed in *A. malaccensis* populations cultivated in home gardens (figure 4, b and c). None of the home garden populations constituted a distinct group but remained intermixed in two clusters (figure 4c).

Table 4. Population pairwise F_{ST} in *A. malaccensis* ten plantations.

	DHM	EFN	FN	HLF	ITN	LKH	JOH	TRI	AMI	NAG
DHM	0									
EFN	0.17041	0								
FN	0.05315	0.1354	0							
HLF	0.16946	0.24953	0.12527	0						
ITN	0.09007	0.13205	0.07914	0.13893	0					
LKH	0.13316	0.13554	0.11918	0.22909	0.08629	0				
JOH	0.0863	0.03981	0.05816	0.14518	0.05658	0.06978	0			
TRI	0.10501	0.1718	0.08624	0.16429	0.106	0.13513	0.08533	0		
AMI	0.24986	0.08426	0.20852	0.26097	0.19263	0.23106	0.09047	0.23538	0	
NAG	0.1179	0.13285	0.0792	0.17556	0.09178	0.1213	0.05941	0.13057	0.20921	0

Table 5. Total genetic variance analysis (AMOVA) among and within populations of *A. malaccensis* based on binary data obtained from AFLP results.

Source of variation	DF	SQ	Variance components	% of total variance	Φ_{PT}	*P* value
Among population	9	1225.750	5.389	7	0.069	<0.0001
Within population	117	8484.596	72.518	93	–	–

DF, degrees of freedom; SQ, sum of squares; *P*, level of significance for estimation of genetic variation based on 9999 permutations.

Discussion

AFLP inferences derived in this study comprehensively provided insights on genetic diversity and population structure among the home garden populations of *A. malaccensis* existing in the northeast India. Sufficient polymorphic bands (916; 79.4%) obtained were useful for estimation of population genetic parameters. This is in agreement with the fact that an ideal molecular marker should uncover hundreds of polymorphic loci representing the entire genome in a simple and reliable experiment (Staub *et al.* 2000; Mariette *et al.* 2002; Luikart *et al.* 2003), and AFLP markers used in this study fulfilled these criteria. Discrimination power and marker attributes, such as polymorphic rate, PIC and MI are yet another criteria displayed in previous genetic diversity studies (Laurentin and Karlovsky 2007; Tatikonda *et al.* 2009). The primer combinations E-ACC+M-CAT and E-ACC+M-CAG showed highest number of polymorphic alleles, PIC and MI values suggest that these markers will be suitable for future population biology studies in this species. Strong correlation among PIC and MI ($r^2 \geq 0.9$) suggests that genetic diversity inferences are reliable. Overall high genetic diversity suggests the potential of home garden populations for implementation of conservation plan.

High within population genetic variance (93%) in AMOVA suggests that these populations are panmictic and not genetic divergent. Further, gene diversity (*h*) and F_{ST} also revealed similar trends that majority of genetic diversity was restricted to within population. Between populations, the genetic diversity as reflected by F_{ST} and Φ_{PT} value (0.069), was low although statistically significant, indicating a weak genetic differentiation among home garden

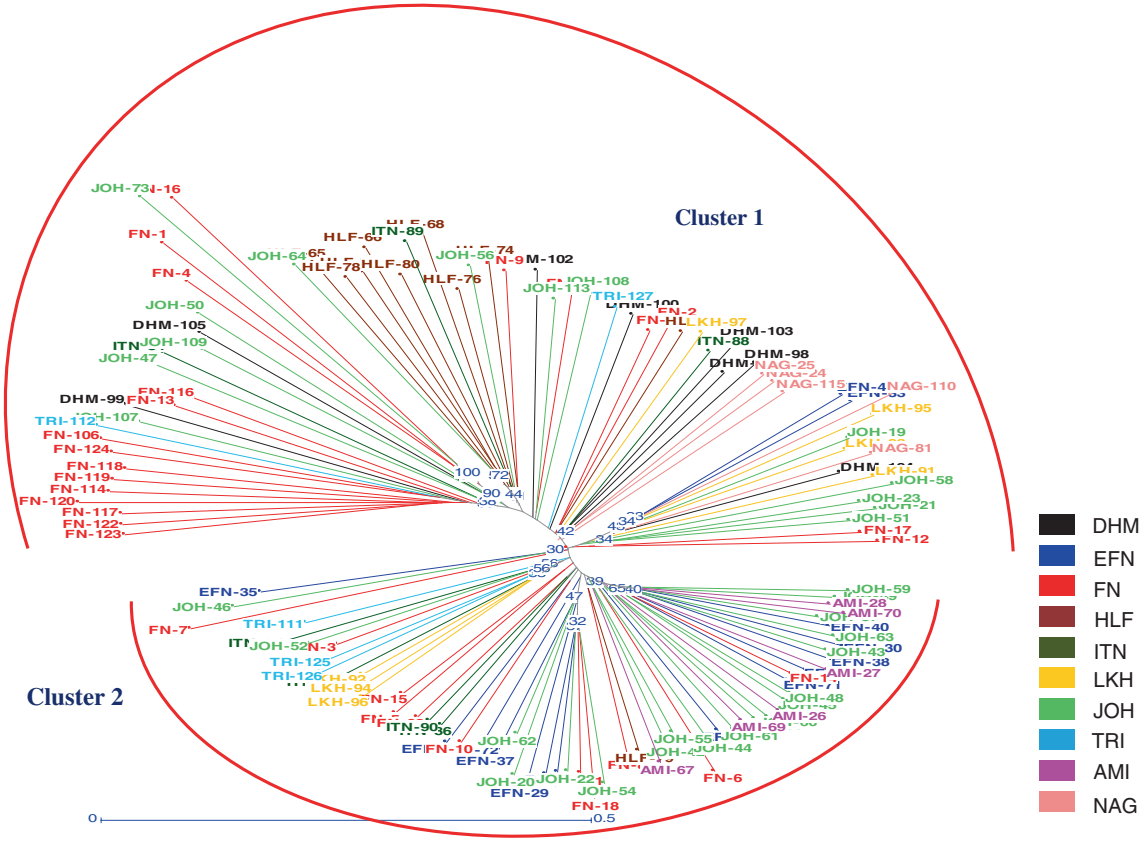

Figure 2. Bootstrapped neighbour–joining (NJ) tree of 127 *A. malaccensis* accessions using 916 polymorphic AFLP fragments.

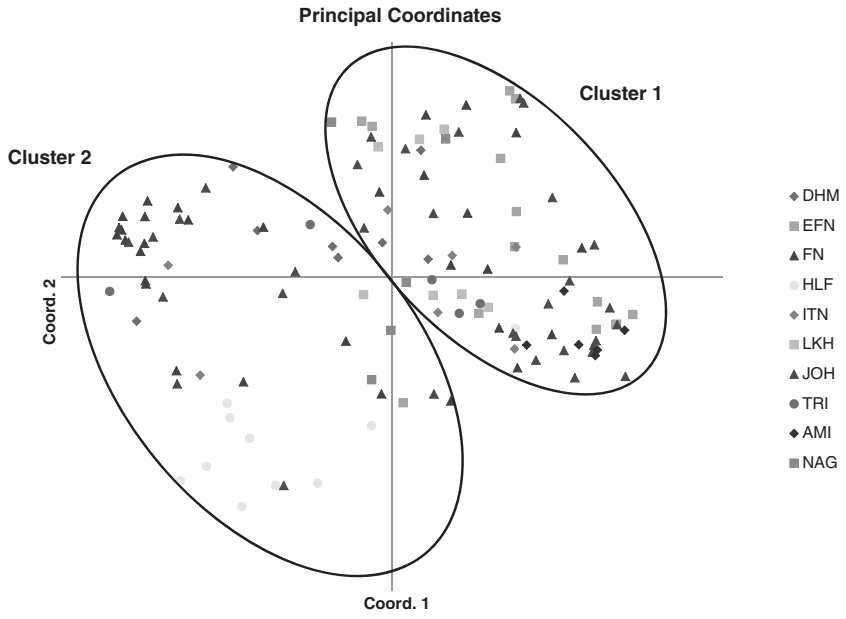

Figure 3. Principal coordinate analysis revealing clustering pattern of 127 *A. malaccensis* accessions.

populations, which is predominantly observed in other tropical outcrossing tree species (Souza *et al.* 2013). According to Bawa (1990), the understory plants may have restricted pollen flow and seed dispersal, generating low genetic variation and inbreeding. *A. malaccensis* in wild have short distance pollen flow and limited seed dispersal (Shankar 2012), but in home gardens the seed dispersal mechanism is controlled by anthropogenic activity and this counteracts the short distance pollen dispersal observed in the wild. This phenomenon leads to a high gene flow as observed in current study. Further, high gene flow within population diversity resulted from the number of other factors such

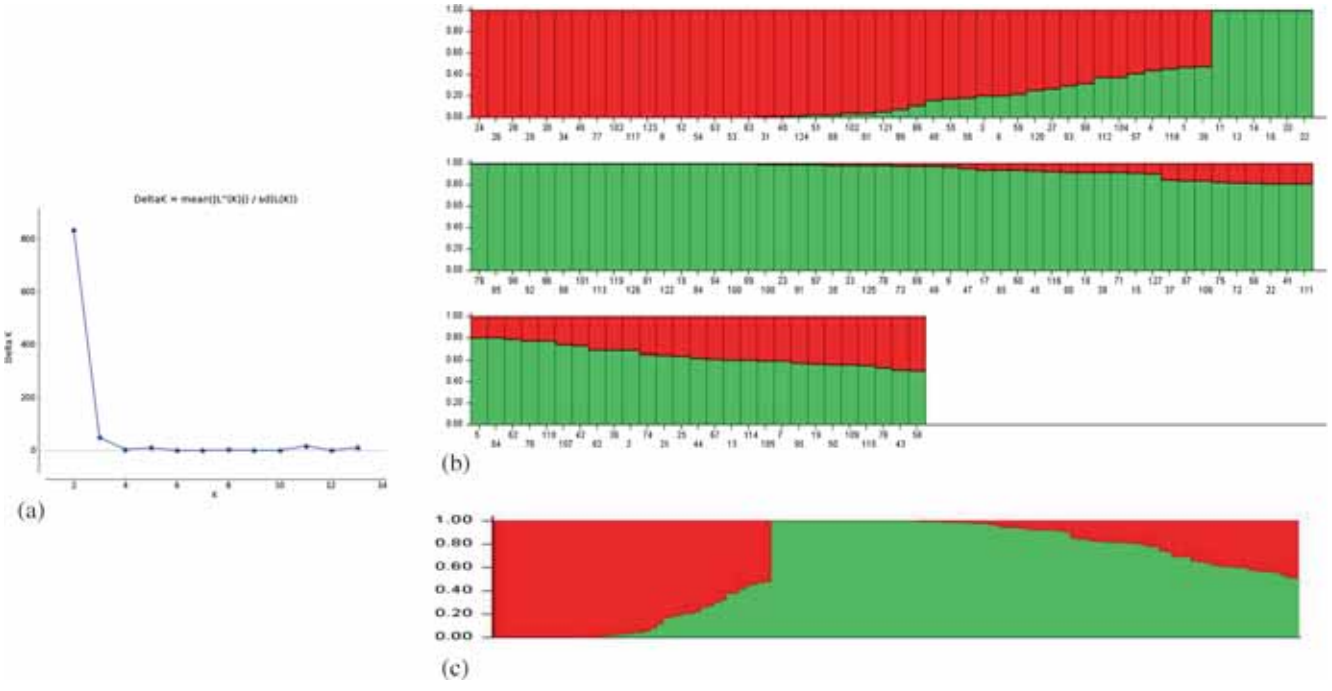

Figure 4. STRUCTURE analysis based clustering of 127 individuals of *A. malaccensis.* (a) Plot showing population size and corresponding value of delta K, (b) histogram showing two genetic pools in population-wise clustering and (c) histogram showing individual-wise clustering pattern.

as successional status, life form, ecological and life history traits. Late successional taxa reveal higher within population variation than the early or mid successional taxa (Hamrick and Godt 1996; Gitzendanner and Soltis 2000; Nybom and Bartish 2000). High variance partitioned within population revealed by AMOVA suggest that *A. malaccensis* is a late successional taxon. Nonetheless, five populations, namely, DHM, FN, HLF, ITN and JOH revealed higher values of gene diversity, Shannon's information index and private alleles suggests that these populations have relatively high genetic diversity.

As observed in many outcrossing species (Nybom and Bartish 2000), isolation by distance may be one of the important factors that can influence the structure of *A. malaccensis* natural populations, considering their larger distribution from West Bengal to the northeastern states. Species having such a distribution pattern is likely to be genetically differentiated due to spatial isolation (González-Astorga and Núñez-Farfán 2001). However, two major groups with significant intermixing of the populations in cluster analysis in NJ, PCoA and Bayesian-based STRUCTURE analysis was expected due to high level of anthropogenic activities in home garden populations. Additionally, wide spread gene flow has taken place in the region sampled in the home garden cultivations, therefore, suggests no population groups separately.

Current inferences on genetic diversity and population structure will allow developing appropriate sampling strategies for optimization and implementation of *ex situ* conservation in *A. malaccensis* genetic resources. AFLP-based high within population genetic diversity in *A. malaccensis* home garden populations revealed that the current study can be potentially utilized for implementation of conservation strategies in the northeast India.

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