J. Viet. Env. **2014**, Vol. 6, No. 1, pp. 69-76
DOI: 10.13141/jve.vol6.no1.pp69-76

JOURNAL of VIETNAMESE ENVIRONMENT

Genetic population of threatened *Hopea odorata* Roxb. in the protected areas of Vietnam

Đa dạng di truyền quần thể loài Sao Đen đang bị đe dọa ở các khu vực bảo tồn ở Việt Nam

Research article

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Hopea odorata Roxb. is widely distributed in lowland forests in Vietnam and native to South-East Asia. Due to over-exploitation and habitat destruction, this species are now threatened and mainly restricted to protected areas for survival. In total, 70 adult individuals of three populations include BGM, TP and BE of *Hopea odorata* were investigated base on nine microsatellite loci. Analysis of molecular variance showed most genetic variation (73%) was within individuals. The mean values of genetic differentiation among populations was high with F_{ST} = 0.251. Allelic richness ranged from 2.444 - 3.293, pair wise differentiation was significant. Bayesian cluster analysis and F_{ST} values suggest three populations of *Hopea odorata* could be divided into two groups. Individual trees from the BGM and TP populations were more closely related than those of the BE population. Inbreeding within population was not significant, no null allele evidence was found but Evidence for recent bottleneck events were found for the BE population at TPM = 0.01 suggesting that A reduction in the number of individuals could be the result of overharvesting in the past. This research provides additional useful information for conservation, management, and restoration of populations to the Protection Forestry Department, Vietnam.

Sao Đen là loài cây gỗ phân bố rộng rãi trong các khu rừng đất thấp ở Việt Nam và các nước Đông Nam Á. Do khai thác quá mức và môi trường sống bị phá hủy mà loài này đang đứng trước nguy cơ bị đe dọa và hiện chỉ còn phân bố giới hạn trong các khu bảo tồn. Chúng tôi đã tiến hành phân tích đa dạng di truyền của ba quần thể loài Sao đen gồm BGM, TP và BE từ 70 mẫu cây trưởng thành dựa trên chín locus microsatellite. Kết quả phân tích cho thấy sự đa dạng di truyền xảy ra chủ yếu trong các cá thể (73%). Giá trị sai khác di truyền giữa các quần thể là khá cao (F_{ST} = 0.251), hệ số đa dạng alen dao động từ 2.444 đến 3.293, sự sai khác di truyền giữa các quần thể là có ý nghĩa. Kết quả phân tích cấu trúc di truyền và sự sai khác di truyền giữa ba quần thể đều khá cao. Hiện tượng tự thụ phấn xảy ra trong ba quần thể nghiên cứu chưa có ý nghĩa, không có các bằng chứng về sự xuất hiện của các alen vô nghĩa (điều mà thường xảy ra khi sử dụng chung locus microsatellite cho các loài hoặc các chi có mối quan hệ gần gũi). Nguyên lý thắt cổ chai trong di truyền (bottleneck) có khả năng xảy ra ở quần thể BE cho thấy khả năng trước đây nơi đây đã xảy ra sự khai thác quá mức làm số lượng cá thể giảm đột ngột. Nghiên cứu này cung cấp những thông tin về di truyền quan trọng cho cục Kiểm Lâm trong việc bảo tồn, phục hồi và quản lý bền vững quần thể loài Sao Đen ở Việt Nam.

Keywords: Dipterocarp, *Hopea odorata,* conservation, population genetics, microsatellite

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http://dx.doi.org/10.13141/JVE
ISSN: 2193-6471

1. Introduction

Many species of Dipterocarpaceae have predominated in the international tropical timber market, and therefore play an important role in the economy of many Southeast Asian countries (Poore, 1989). The dipterocarps also constitute important timber for domestic needs in the seasonal evergreen forests of Asia. The largest genera in Dipterocarpaceae are Shorea (about 250 species), followed by Hopea (105 species), Dipterocarpus (70 species) and Vatical (65 species). In Vietnam, there are more than 40 known species from six genera (Anisoptera, Hopea, Shorea, Parashorea, Vatica, Dipterocarpus), all native and often endemic. Hopea odorata Roxb. has a scatted distribution in Vietnam, Laos, Cambodia, Thailand, Malaysia, Myanmar and India. In Vietnam, Hopea odorata was found in central highland, Southeast and Thanh Hoa province (Nghia NH, 2005) but like most dipterocarps, a long period of selective logging has reduced the size of stands to small groups of trees or isolated individuals, it is threatened by deforestation, changes in land use systems and exploitation for timber. It has been listed in the critically endangered category (Vulnerable A1cd+2cd) by the International Union for Conservation of Nature (IUCN 2013). The sapwood is pale yellow or greyish yellow turning pale brown on exposure, heartwood yellowish-brown to brownish red sometimes with dark streaks, turning purplish on exposure, with lustrous white resin canals at irregular intervals, becoming dull with age. It is chiefly used for boat-building, dug-out canoes and for construction purposes, where durability and strength are of primary importance. It is also used for carts, presses flooring, roofing, piles, fence-posts, ploughs, furniture, furniture, veneer and a number of other uses. It is a first class sleeper wood.

Best growth of Hopea odorata is obtained in areas with annual rainfall more than 1200 mm and mean annual temperature of 25°–27°C. It is suitable for rehabilitation of degraded lands and is also widely planted as an ornamental and shade tree. The timber of Hopea odorata is a heavy, strong, light colored hardwood. The bark is rich in tannin suitable for tanning leather; it produces resin, though of inferior quality (rock dammar). Evergreen tree up to 45 m tall with diameter of 120 cm and prominent buttress. Leaves are simple and alternate, 10–20 cm long with slightly unequal base. Inflorescence is a branched panicle, terminal or axillary. Flowers are small, unisexual, with 5 pinkish petals with hairs on both sides. As with any other dipterocarp species, mass flowering and fruiting of Hopea odorata is irregular and may occur once in 2 to 3 years. Trees reach reproductive maturity at the age of 8-10 years. The fruits are dispersed by wind and seeds germinate readily on falling to the ground. Some Hopea odorata fruits are polyembryonic, one fruit may produce up to seven platets. Apomixis in Hopea odorata has been inferred from embryological studies. Isozyme and DNA profiles of Hopea odorata seedlings revealed genetic variation between multiple seedlings from single seeds indicating sexual and asexual reproduction in this species. Pollen can be dispersed as far as 700 m by small insects (Mahani, 2002).

Due to the exploitation of these species for their valuable timber and resin by local people and forestry enterprises, their habitats are heavily affected by deforestation, forest fragmentation, and unsustainable management such as selective logging. Logging results in intense fragmented habitats and low density populations. These threaten the long-term survival of the genetic resource of species Hopea odorata.

Conservation and management of a species requires information on the ecological and genetic diversity within and among populations. In order to obtain such information, especially a better understanding of genetic processes, powerful biological techniques are required. Understanding the amount of genetic diversity provides information for the development of conservation strategies and sustainable utilization of a species. Microsatellite markers have been used for dipterocarp studies on gene flow, genetic structure, and mating systems (Ujino et al., 1998; Iwata et al., 2000; Takeuchi et al., 2004; Pandey and Geburek, 2009). Several species showed an overall high level of gene diversity but low overall differentiation such as Dryobalanops aromatica Gaertn. (G_{ST} = 0.067, Lim et al., 2001), Shorea leprosula Miq. (G_{ST} = 0.117, Lee et al., 2000) and Shorea lumutensis Sym. (G_{ST} = 0.048, Lee et al., 2004).

The objective of this study was to investigate the level of genetic variability within and between remnant populations of Hopea odorata in protected areas of Vietnam and to test for potential distance-related effects for local inbreeding using microsatellite markers in adult and aged trees.

2. Materials and methods

2.1 Plant materials

This research was carried out in three sites in Vietnam including Bu Gia Map National Park (Binh Phuoc province), Tan Phu Protective forests (Dong Nai province), and Ben En National Park (Thanh Hoa province) (Table 1).

Table 1. Collection locations *H. odorata* populations

Population	Sample size	Symbols sample	Collection locality	Altitude	Latitude	Longitude
Bu Gia Map	23	SDBGM 1 -> SDBGM23	Bu Gia Map, Binh Phuoc	130m	10º56'N	106º59'E

Tan Phu	29	SDTP1 -> SDTP29	Tan Phu, Dong Nai	100m	11º12'N	107º09'E
Ben En	18	SDBE1 -> SDBE18	Ben En, Thanh Hoa	100m	19º35'N	105º30'E

Parts of the native vegetation at Bu Gia Map (Binh Phuoc), Ben En (Thanh Hoa), and Tan Phu (Dong Nai) have been destroyed because of agricultural expansion. This has led to an alteration of the spatial distribution and age class structure of trees in these sites. However, vegetation structures were still characterized by three strata.

The inner bark was sampled from 18 to 29 mature trees (>20cm at dbh) that were accessible in each of the three populations, within the natural range of *Hopea odorata*. The samples were immediately placed into paper envelopes and plastic bags with silica gel, then transferred to Laboratory of Molecular Systematics and Conservation Genetics, Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology; and stored at -86^{o}C until DNA extraction. The samples were identified on the basis of previous taxonomic treatments of collected specimens from these populations and verified with *rbc*L sequences The GenBank accession number KM 267144 with sequence rbcL *Hopea odorata* voucher specimens were stored at Botany department, Institute of Ecology and Biological Resources.

2.2 DNA extraction

Total DNA was extracted from the samples by using the modified CTAB method proposed by Doyle and Doyle (1987). Liquid nitrogen was added to about 100 mg of each sample, which was then ground by hand. Total DNA yield and purity were assessed by spectrophotometer with OD 260 and OD 280, visualization on 1% agarose electrophoresis. Stock DNA was diluted to a concentration of 10 ng/µl.

2.3 SSR amplification

Fifteen SSR primers of the related species **Shorea curtisii *(Ujino et al. 1998)*** and *Neobalanocarpus* (Iwata et al., 2000) were initially tested for cross amplification in five samples. Based on their amplification, nine primers that gave polymorphic PCR products were selected for the examination of the whole sample set (Table 2).

Table 2. Microsatellite sequences of primer pairs for *Hopea odorata* with SSR loci, primer sequences, repeat motif and number of alleles per locus

SSR locus	Repeat motif	Primers sequences	Size (bp)	Number of alleles
Shc9	$(CT)_{12}$	F:TTTCTGTATCCGTGTGTTG R:GCGATTAAGCGGACCTCAG	235-335	5
Shc2	$(CT)_2CA(CT)_5$	F: CACGCTTTCCCAATCTG R: TCAAGAGCAGAATCCAG	135-145	2
Shc3	$(CT)_8$	F:TTGAAGGGAAGGCTATG R:CTTCTCAACTACCTTACC	115-125	2
Shc17	$(CT)_5AT(CT)_4$	F:CTAGAATCCGCCATTTCC R:CACAAATACGTCTCCATATC	100-111	1
Shc1	$(CT)_8(CA)_{10}CT$ $(CA)_4CTA$	F:GCTATTGGCAAGGATGTTCA R:CTTATGAGATCAATTTGACAG	130-144	3
Nhe5	$(CT)_{14}$	F:GGAGGTGTAAACAAACTCAGTG R:CTACATAATTGTGCAAACTAGGC	113-129	3
Nhe19	$(GA)_{14}$	F:ATCAGAGTAGCCATGTTGCTTG R:GGAGAGACTGGGCTTGCTC	198-350	9
Nhe11	$(GA)_{19}$	F:CCATCTGAGGGTGTTGAAAG R:GAGTAGAAGAAGGCAGGTGATTA	144-272	7
Nhe4	$(GA)_{19}$	F:ACGCAAGCCAACACATCC R:TTTGCCATTTCACAATCATCAC	214-370	9

Polymerase Chain Reaction was performed in a 25 µl reaction mixture containing 5 µl of total DNA (equivalent 50ng of DNA), 2,5 µl of 10x PCR buffer, 200 nM of each primer, 1U of taq DNA polymerase (Omega), 2.5

mM $MgCl_2$ and 0,2 mM of each dNTP. PCR reactions were performed in a thermal cycler (Bio-Rad Mycycler) using following conditions: 1 cycle at 95°C for 5 mins, followed by 35 cycles at 95°C for 1 min, 45°C for 1 min, 72°C for 1 min and a final extension at 72°C for 5 mins. PCR products were separated by capillary electrophoresis on a Qiaxcel system (Qiagen).

2.4 DNA analysis

A suite of genetic parameters were calculated using GenAlex (Peakall and Smouse, 2006) and FSTAT (Goudet, 1995), including the number of alleles (A) per locus, allelic richness (Ar) per locus and population, observed (Ho) and expected (He) heterozygosities, the coefficient of excesses of homozygotes or heterozygotes compared with panmictic expectations within populations (F_{IS}, 1000 permutations), and the genetic differentiation ($F_{ST,}$ 1000 permutations) between populations. F-statistics were determined after Weir and Cockerham (1984) as used in FSTAT software with Jackknifing procedure applied over loci in deriving significance level. These parameters of population structure are defined as the correlations between pairs of gene within individual (Capf), between individuals in the same population (theta) and within individuals within population (smallf) and are analogous to Wright's F_{IT}, F_{ST} and F_{IS}, respectively. Each locus was checked for evidence of null alleles, scoring errors and allele drop out using Micro-checker (Van Oosterhout et al. 2004). We tested for recent bottlenecks in each population under the two-phase model (TPM) with 95% single-step mutations and 5% multiple-step mutations (Wilcoxon's test 1-tailed) using BOTTLENECK 1.2.02 (Piry et al., 1999).

Exact tests of deviation from the Hardy-Weinberg equilibrium for all loci and among populations were performed at the significance level (P = 0.05). Significance testing for variance components in the analysis of molecular variance (AMOVA) was implemented on the basis of 1000 permutations. A Neighbor Joining Tree for populations of each species was generated to determine the genetic association among populations by using 1000 permutations in Poptree 2 (Takezaki, 2010). Pairwise F_{ST}-values based on θ were calculated between all pairs of populations and tested for significant differentiation using 999 permutations. Isolation-by-distance between pairs of populations and their geographical distances was tested with θ /(1-θ) considering straight flight distances, log transformed (Rousset, 1997) between populations in a Mantel test using 1000 randomizations (Mantel, 1967). A Bayesian clustering method (Pritchard et al., 2000) was carried out using STRUCTURE version 2.3.4. We tested K in ten independent runs from 1 to 16 (10,000 burn-in and 50,000 Markov chain Monte Carlo replicates in each run), without using sampling location as a prior to assess convergence of ln (PD). Runs were carried out assuming admixture and an independent model of allele frequencies. The results were uploaded into Structure Harvester (Earl, 2011), which estimates the most likely K value. The number of clusters was determined from the K with the highest posterior probability and using the second-order rate of change of the likelihood function ΔK, as suggested by Evanno et al. (2005).

UPGMA cluster analysis of genetic distances was generated to determine the genetic association among populations by using TFPGA (Miller, 1997).

3. Results

3.1 Genetic variation

The nine SSR markers produced a total of 41 different alleles ranging in size from 100 bp to 370 bp, across all 70 trees of nine populations of *Hopea odorata.* The proportion of polymorphic loci was high in all populations averaged 76,67%. Allelic richness (Ar) ranged from 2.444 (at BE) to 3.293 (BGM), the frequency of observed heterozygotes (Ho) ranged from 0.297 (at TP) to 0.472 (at BE). All three populations showed moderate levels of genetic diversities (Ho and He < 0.5). The mean of observed heterozygosity (Ho) was 0.366 and higher than the expected (He = 0.356) (Table 3). The F_{IS} value in BE populations was -0.198 showed an excess of heterozygotes (F_{IS} < 0). In contrast, F_{IS} values in TP was high positive (0.152) indicating a lack of heterozygotes suggesting the presence of null alleles, which are commonly found when microsatellite loci are cross-amplified among far-related species or genera. Microchecker results indicated no evidence for scoring error due to stuttering, no evidence for large allele dropout and no null alleles might be present. To determine the population "genetic reduction signatures", characteristic of recent reductions in effective population size, the Wilcoxon's heterozygosity excess test (Piry et al. 1999), standard differential test, sign test and the allel frequancey distribution mode shift analysis (Luikart et al. 1998) were performed using Bottleneck software. According to the data for the heterozygosity excess examined under the two-phase model (TPM), we found evidence of recent population reduction or bottleneck for BE population of *H. odorata* (TPM = 0.00976).

Table 3. Genetic variation within populations at nine microsatellite loci

Population	N	A	Ar	P	Ho	He	F_{IS}
BGM	23	3.2	3.293	80	0.33	0.348	0.09
TP	29	2.7	2.943	70	0.297	0.338	0.152
BE	18	2.2	2.444	80	0.472	0.382	-0.198
Mean		2.7	2.893	76.67	0.366	0.356	0.015

Notes: N, population size; A, mean number of alleles per locus; Ar, mean number of alleles richness; P, the percentage of polymorphic loci; Ho and He, mean observed and expected heterozygosity, respectively; F_{IS}, Wright's inbreeding coefficient with P < 0.05.

3.2 Genetic structure

Table 4. Analysis of molecular variance of *Hopea odorata* from nine populations

Source of variation	d.f.	Sum of squares	Est.variance component	Total variation (%)
Among populations	2	59.192	0.606	25%
Among individuals	67	123.530	0.040	2%
Within individuals	70	123.500	1.764	73%
Total	**139**	**306.221**	**2.410**	**100%**

Table 5. Amova-F_{ST} results (F_{IS}, F_{ST} with P-value after 1000 permutations)

	F_{ST}	F_{IS}	F_{IT}	G_{ST}
Hopea odorata	0.251*	0.022^{NS}	0.268*	0.193

Notes: F_{IS}, Wright's inbreeding coefficient, with *P < 0.001; NS: none significant
**: significant at P<0.05

The AMOVA revealed that most of the variation remains within the individual (73%). The proportion among populations and within population was 25% in *Hopea odorata* (Table 4). The genetic differentiation (F_{ST}) was 0.251, the total fixation (F_{IT}) was 0.268 (Table 5).

Among population variation

The large differentiation (0.31) was found between populations of Ben En (BE) and Tan Phu (TP) for *Hopea odorata*, the differentiation between BGM and TP was 0.17 (Table 6).

Table 6. Population pairwise (FST) and significant values.

	BGM	TP	BE
BGM	-	**	**
TP	0.17	-	**
BE	0.30	0.31	-

Bayesian assignment of individuals with structure showed most individuals of *Hopea odorata* were not mixed (Fig 2). We found two genetic cluster in all populations of *Hopea odorata* and *Hopea hainanensis* species.

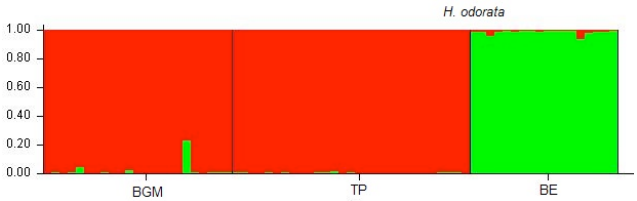

Figure 2. Bayesian assignment

With the Ln(PD) values were high for K=2. Delta K values for *H. odorata* were 577.11

A Mantel test within each species gave no significant IBD although a positive trend was obtained for *Hopea odorata.*

Un-weighted Pair Group Method (UPGMA) analysis based genotype matrix showed the relationship between individuals within species *Hopea odorata*. All individual studies were separated into two different groups, close relationships between individuals within each group. Group 1 includes individuals of populations Bu Gia Map and Tan Phu, demonstrating a close relationship between the two populations, while the second group consists of individuals of the population Ben En. Methods of distribution are the group into two perfectly matched by coordinates sample collection.

4. Discussion

Outcrossing species with a great potential for genetic movement will maintain high levels of genetic diversity within populations and species (Hamrick and Godt, 1989). Information on the high values of genetic diversity from microsatellite loci in dipterocarp species such as *Shorea lumutensis* (He = 0.700, Lee et al., 2004; He = 0.648, Boshier, 2011), *Shorea leprosula* (He = 0.709, Rimbawanto and Isoda, 2001; He = 0.686, Keiya et al., 2001) and at their population levels was reported previously. Our results showed *Hopea odorata* species had moderate levels of genetic diversity within populations, mean He = 0.356. This can be explained from their life strategy because these *Hopea odorata* species are regionally or narrowly distributed, have a long-life, high fecundity, are predominantly outcrosser, pollinated by insects (Appanah and Chan, 1981) and late successional. Seeds are dispersed over short distances. The TP populations showed lower levels of genetic diversity. This might be due to the smaller available sample size in populations of adults and aged trees, resulting in part from human activities (e.g., logging). Small populations might be subject to within population inbreeding, which can reduce gene diversity. However,

for three studied populations, the inbreeding levels were none significant. An excess of the heterozygotes appeared in populations of *H. odorata* (Ben En, F_{IS} = -0.198) which can be explained from secondary forest and forestry practices.

The differentiation between populations (pairwise F_{IS} and overall G_{ST}) can be explained on basic of geographic distance. *Hopea odorata* has fairly high G_{ST} = 0.193 when compared to other dipterocarp species, such as *Dryobalanops aromatica* (G_{ST} = 0.067, Lim et al., 2001), *Shorea leprosula* (G_{ST} = 0.117, Lee et al., 2000) and *Shorea lumutensis* (G_{ST} = 0.048, Lee et al., 2004). These results for *Hopea odorata* is in contradiction with the expectation of low variability among populations of long-lived and outcrossing species (Hamrick and Godt, 1989). The limited gene flow via either pollen or seed dispersal thus could play an important role in *Hopea*. Dipterocarp species are insect-pollinated (Appanah and Chan, 1981; Dayanandan et al., 1990) which occurs over only short distances, typically not further than a few kilometers. The high differentiation values at populations have high geographic distance suggest that historical gene exchanges among populations were limited in relation to larger distributional ranges of *Hopea odorata* even populations from Northern and Southern Vietnam (≈ 1000 km distance). This might indicate genetic depauperation in this species.

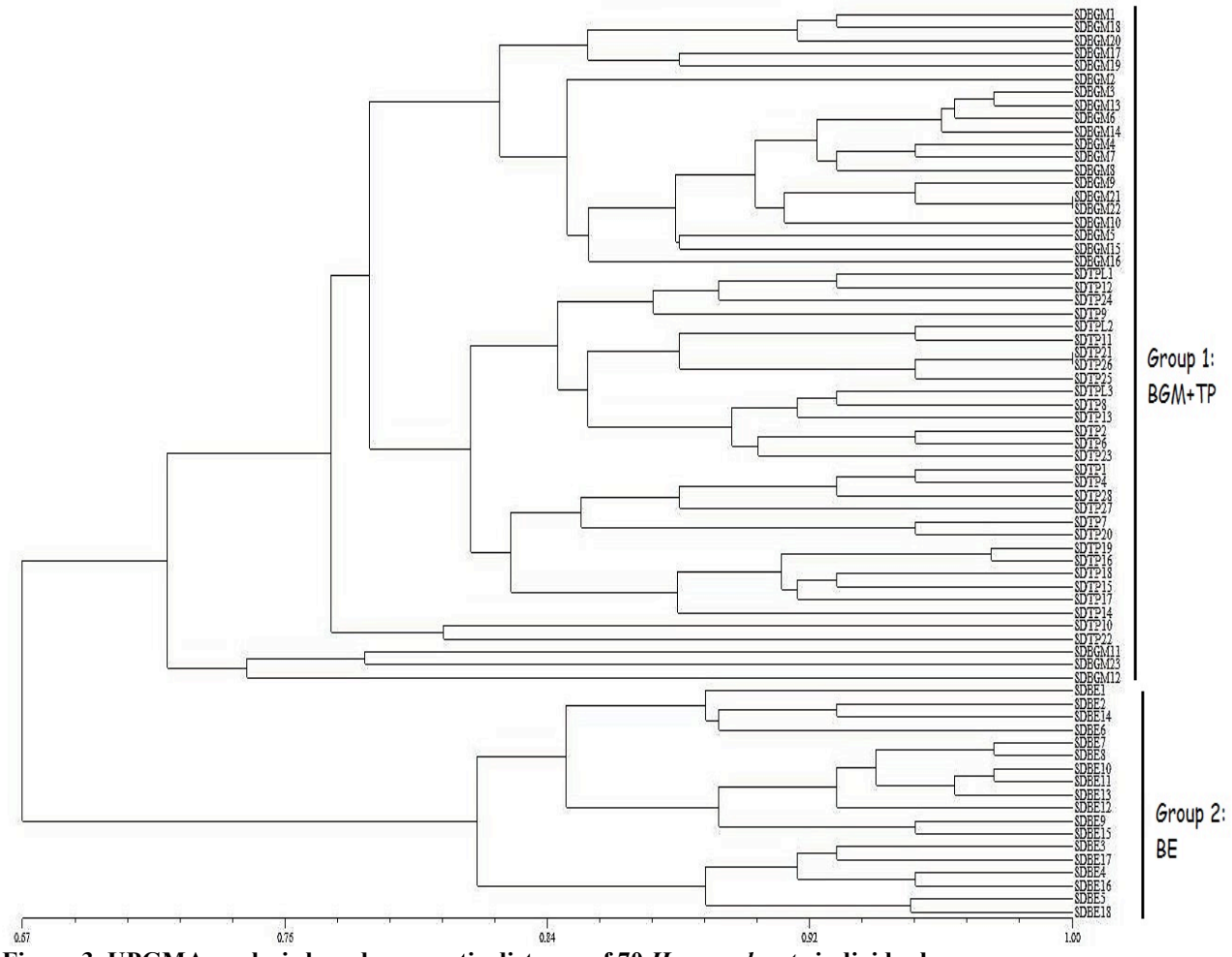

Figure 3. UPGMA analysis based on genetic distance of 70 *Hopea odorata* individuals

Evidence for bottleneck were found at BE population which mean that in the recent past, the BE population of *Hopea odorata* was subjected to a reduction in the number of individuals resulting by over harvested in the pass from human activities. Comparing the genetic structure of each species, Bayesian cluster analysis and F_{ST} values suggested three populations of *Hopea odorata* could be dived into two groups because of high genetic differentiation. The UPGMA result also confirmed Bayesian analysis when it also divided three populations of *Hopea odorata* into two clades. The strong genetic differentiation among regions showed that there is a lack of connectivity between populations in each area. But even have strong differentiation, each population of *Hopea odorata* still showed high heterozygotes. Our result indicated even these population have small size but they showed high genetic diversity suggesting the forestry protection department should protect and replicate more population of *Hopea odorata*.

In conclusion, our result give a better understand the genetic implications towards the conservation of threatened *Hopea odorata* species in Vietnam. There was no evidence for inbreeding and high level of population differentiation. From a conservation point of view, effective management strategies should be worked out for both in situ and ex situ activities. Protection of *Hopea odorata* species is necessitates survival of populations in

different protected areas because of the small population sizes of adult trees. This provides additional useful information for conservation, management, and restoration of populations to the Protection Forestry Department, Vietnam.

Our research only used adult individuals because using mature individuals can show the genetic signal of past demographic change. Genetic studies of seedling will provide information about current conditions affecting the genetic variation of the species. Future work can track change in demography and genetic structure with genetic survey performed in next generation.

5. Acknowledgments

This research was funded by IEBR.CBT.TS01/14 and BAS42 (Vrije Universiteit Brussel).

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