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Full Length Research Paper

Genetic diversity on the tropical rare wood species of *Dalbergia* in Vietnam revealed by inter-simple sequence repeat (ISSR) markers

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Genetic diversities of three rare hardwood species of *Dalbergia* (*D. assamica*, *D. nigrescens* and *D. tonkinensis*) were evaluated for conservation based on inter-simple sequence repeat (ISSR) markers. A total of 47 ISSR primers were used for the analysis, but only 31 ISSR primers were successfully amplified for 25 samples from each species. There were 166 fragments across the 75 samples produced, in which 153 were polymorphic with an average of 4.94 polymorphic fragments per primer. The number of amplified fragments ranged from 1 (ISSR13, ISSR54 and ISSR59) to 11 (ISSR14) and their size varied from 200 to 1700 bp. The similarity coefficient ranged from 67.0 to 98.9% in *D. assamica*; from 71.2 to 98.5% in *D. nigrescens* and from 68.5 to 95.2% in *D. tonkinensis*. The estimated value of molecular diversity parameters within species such as the effective number of alleles, Shannon's information index, intralocus gene diversity and Nei's gene diversity were low among the individuals of the different *Dalbergia* species (1.227, 0.195, 0.662 and 0.146, respectively in *D. assamica*; 1.135, 0.111, 0.425 and 0.109, respectively in *D. nigrescens*; 1.198, 0.166, 0.526 and 0.123, respectively in *D. tonkinensis*). The analysis of molecular variance (AMOVA) of ISSR data indicated that the greater proportion of total genetic variation existed among species rather than within species. The correlation between genetic and geographic distance was also found in the three *Dalbergia* species.

Key words: *Dalbergia*, endemic species, genetic similarity, ISSR markers.

INTRODUCTION

Dalbergia, a large genus of small to medium-sized trees, shrubs and woody climbers, belongs to the family Fabaceae, subfamily Faboideae and distributed mainly in tropical and sub-tropical regions (Rout et al., 2003). Several species of *Dalbergia* are important timber trees and valuable for construction and ornamentation such as *D. nigrescens*, *D. tonkinensis* and *D. assamica*. The genus has 27 species distributed from the North to the

South of Vietnam. Of those species, *D. tonkinensis* is on Vietnam's Red List (Dang and Nguyen, 2007) prohibiting exploitation, shipping and storage. The other species are also in danger of extinction. Therefore, it is necessary to understand patterns of genetic variation of these species in order to establish conservation strategies and protect them from further genetic erosion.

Over the years, the genetic diversity of forest trees has been detected and assessed extensively using both morphological and molecular methods. Several molecular marker techniques are now used in diversity studies. The most commonly used systems are restriction fragment length polymorphism (RFLP) (Garcia-Mas et al., 2000),

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random amplification of polymorphic DNA (RAPD) (Williams et al., 1990), amplified fragment length polymorphism (AFLP) (Vos et al., 1995) and inter simple sequence repeat (ISSR) (Lalhruaitluanga and Prasad, 2009). In higher plants or animals, ISSR markers are in more demand, because they are known to be abundant, very reproducible, highly polymorphic, highly informative and quick to use (Zietkiewicz et al., 1994, Borner et al., 2002). ISSRs were proposed for genetic diversity by Lalhruaitluanga and Prasad (2009) and commonly used in population genetics, taxonomy and phylogeny of many plant species (Wolf and Randle, 2001). ISSR primers can also confirm specific amplified DNA polymorphic fragments within varieties (Li and Ge, 2001). Studies on biodiversity of valuable hardwood trees in *Dalbergia* using RAPD, ISSR and AFLP markers have also been implemented in several countries. For instance, Olivarimbola et al. (2004) established genetic relationships of 122 individuals of *Dalbergia monticola* in Madagascar using 60 RAPD and 3 cpSSR markers. Results indicate that the population in the Central North originated from a region in the South. Similarly, French, Indian and Brazilian researchers have applied RAPD and ISSR markers, and specific genetic sequences to investigate genetic relationships among species and populations of *Dalbergia* (Rout et al., 2003; Subhash and Manojkumar 2004; Juchum et al., 2007; Andrianoelina et al. 2006).

This report is however, not on the phylogeny in the genus *Dalbergia*. In this study, we aimed to report the genetic relationships and genetic diversity among the individuals of three *Dalbergia* species revealed by ISSR markers.

MATERIALS AND METHODS

Leaf samples or wood pieces of *D. assamica*, *D. tonkinensis* and *D. nigrescens* were collected from a natural stand grown in the Yordon National Park (Dak Lak province), Cuc Phuong National Park (Ninh Binh province), Phong Nha - Ke Bang National Park (Quang Binh province), Copia Nature Reserve (Thuan Chau district, Son La province) and some streets of Ha Noi city in Vietnam.

Yordon National Park is the largest of Vietnam's nature preserves and one of seven internationally important Centers of Plant Diversity in Vietnam. This park encompasses over 1,000 km² and extends from Eastern Cambodia into Northern Dak Lak and Southern Gia Lai Provinces in Vietnam. The topography of most of this park is flat, with an elevation of approximately 200 m. Cuc Phuong is the oldest National Park in Vietnam, located only 120 km southwest of Hanoi and nestled between the provinces of Ninh Binh, Hoa Binh and Thanh Hoa. Cuc Phuong is situated in the foothills of the northern Annamite Mountains. The park consists of verdant karst mountains and lush valleys. Elevation varies from 150 to 656 m at the summit of May Bac Mountain or Silver Cloud Mountain. The area of this park is about 220,00 km². Phong Nha - Ke Bang National Park is located in the middle of the Annamite Mountain Range, 40 km from Dong Hoi, 500 km from Vietnam's capital of Hanoi and close to the Vietnam - Laos border, just several kilometers to the west. Phong Nha-Ke Bang National Park is one of the world's two largest limestone regions. The park covers a total area of 857.54 km², which are divided into three zones, a "strictly protected zone" (648.94 km²), an "ecological recovery zone"

(174.49 km²) and an "administrative service zone" (34.11 km²). Copia proposed nature reserve is located in Thuan Chau district, Son La province. The proposed nature reserve is centered on Mount Copia, a 1,800 m peak. The area of this park is about 19,253 ha.

There were 25 individuals of each species used in this study (Figure 1 and Table 1). The amount of population of each species was limited in Vietnam. The samples were stored at -20°C until DNA was extracted.

DNA isolation

Total genomic DNA was extracted from leaves and wood specimens using the cetyl trimethylammonium bromide (CTAB) method described by Doyle and Doyle (1990). The concentration of DNA was determined with a UV-visible light spectrophotometer (UVS 2700, Labomed, USA) and the DNA samples were diluted to 10 to 20 ng μL⁻¹ and used as templates for polymerase chain reaction (PCR) amplification.

ISSR marker amplification

ISSR primers were obtained from Integrated DNA Technologies, USA (Table 2). The ISSR sequences were collected based on the publications of Arif et al. (2009); Borner et al. (2002); Bhattacharya et al. (2010); Gupta et al. (2008); Lalhruaitluanga and Prasad (2009); Djè et al. (2006); Hou et al. (2005) and Goswami and Tripathi (2010). The reaction mixtures contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 to 20 ng genomic DNA, 10 pmol of primer, 2 to 4 mM MgCl₂, 300 to 400 μM of each dNTP, and 0.8 to 1.2 U of Taq DNA polymerase (Amersham). The temperature profile consisted of an initial denaturation step at 94°C for 4 min, followed by 35 cycles: 94°C for 1 min, 38 to 55°C for 1 min and 72°C for 1 min. After the final cycle, samples were incubated for 10 min to ensure complete extension. The product was stored at 4°C. The PCR products were separated on 1.5% agarose gel in 0.5 x Tris-borate-ethylenediaminetetraacetic acid (TBE) buffer. The size of amplified DNA fragments was estimated with 1 kb ladders (Fermentas, USA). The gels were visualized under UV using gel documentation (CSL-MiCRODOC, Cleaver, England).

Data analysis

DNA fingerprints were scored for the presence (1) or absence (0) of bands of various molecular weight sizes in the form of binary matrix. The Simqual program was used to calculate Jaccard's coefficients (Jaccard, 1908); these were calculated as follows: $S_{ij} = a / (a + b + c)$, in which S_{ij} is the coefficient of similarity between two individuals i and j ; a is the number of fragments shared by samples; b represents amplified fragments in sample i ; and c represents fragments in sample j . The UPGMA-based dendrogram was constructed using NTSYS 2.0 software, version 2.0 (Rohlf, 1992). Win-Boot software (Yap and Nelson, 1996) was used to compute bootstrap-based P-values to assess the strength of evidence for clustering; this data was bootstrapped with 1,000 replications, a long Jaccard's coefficient. The polymorphism information content (PIC) of each locus was determined as described by Weir (1990): $PIC = 1 - \sum P_i^2$, where P_i is the frequency of the i th allele in the genotypes. Nei's gene diversity was calculated using the formula: $H_i = h_1 + h_2 / \text{total number of loci}$ where h_1 and h_2 [i.e., $h_j = (1 - p^2 - q^2)$] represent intralocus gene diversity. The analysis of AMOVA was calculated using GenAlEx 6.3 software (Peakall and Smouse, 2006) whereas, diversity in the frequency of fragment size of ISSR patterns was apportioned within and among species of *Dalbergia* using Shannon's information index (i) and gene diversity



Figure 1. Location of the three *Dalbergia* species in the Vietnam.

Table 1. Details of the three *Dalbergia* species employed for the study of genetic diversity from different location of Vietnam

| Number | Scientific name | Code used in this study | Number of sample | Location | Conservation status* |
|--------|------------------------------|-------------------------|------------------|--|------------------------------|
| 1 | <i>Dalbergia assamica</i> | Da | 25 | Ha Noi and Cuc Phuong National Park (Ninh Binh) | Endemic to Vietnam |
| 2 | <i>Dalbergia tonkinensis</i> | Dt | 25 | Ha Noi and Phong Nha - Ke Bang National Park (Quang Binh) | VU (A1acd) and IUCN Red List |
| 3 | <i>Dalbergia nigrescens</i> | Dn | 25 | Yordon National Park (Dak Lak) and Copia Nature Reserve, Thuan Chau (Son La) | Endemic to Vietnam |

*Vietnam Red List; (Dang and Nguyen, 2007); (IUCN, 1998)

Table 2. Code and sequences of the 31 ISSR primers used for the study of genetic diversity of three *Dalbergia* species

| Number | Primers code | Primer sequence | Amplified product range (bp) | Number | Primers code | Primer sequence | Amplified product range (bp) |
|--------|--------------|-----------------|------------------------------|--------|--------------|-----------------|------------------------------|
| 1 | ISSR1 | (CAG)5 | 450-1100 | 17 | ISSR46 | (AG)8T | 300-1200 |
| 2 | ISSR2 | (CAA)5 | 500-1300 | 18 | ISSR49 | (GA)8T | 200-1100 |
| 3 | ISSR3 | (GACA)4 | 300-1400 | 19 | ISSR51 | (GA)8A | 300-950 |
| 4 | ISSR5 | (CCG)6 | 500-1700 | 20 | ISSR52 | (CT)8G | 400-1400 |
| 5 | ISSR6 | (CTC)6 | 600-1200 | 21 | ISSR54 | (TC)8G | 1400 |
| 6 | ISSR7 | (GGC)6 | 400-1200 | 22 | ISSR55 | (AC)8T | 400-1200 |
| 7 | ISSR8 | (GAA)6 | 400-800 | 23 | ISSR56 | (AC)8G | 450-1000 |
| 8 | ISSR9 | (TG)8GA | 750-800 | 24 | ISSR59 | (GA)8CT | 500 |
| 9 | ISSR10 | (CTC)8 | 450-1200 | 25 | ISSR61 | (AC)8TG | 400-850 |
| 10 | ISSR11 | (CCA)5 | 300-900 | 26 | ISSR62 | CTC(AG)7 | 300-850 |
| 11 | ISSR12 | (CCCT)4 | 600-800 | 27 | ISSR63 | CTC(GA)7 | 250-800 |
| 12 | ISSR13 | (GT)8C | 700 | 28 | ISSR64 | ACA(GT)7 | 300-950 |
| 13 | ISSR14 | (CTCT)4GTC | 300-1400 | 29 | ISSR65 | CAC(TG)7 | 400-1200 |
| 14 | ISSR15 | (CA)8A | 400-1400 | 30 | ISSR67 | (ATG)6 | 600-1200 |
| 15 | ISSR17 | (CT)8T | 400-600 | 31 | ISSR69 | (GGGTG)3 | 450-1300 |
| 16 | ISSR18 | (CT)8A | 500-1000 | | | | |

index (H_i) following Nei (1973) with the help of PopGen 32 software.

RESULTS

A total of 47 ISSR markers were used for this study, but only 31 ISSR primers were successfully amplified for 25 samples of each *Dalbergia* species. 29 primers showed polymorphism, 2 primers (ISSR7 and ISSR13) could not show distinguish within the species, while 6 primers showed monomorphic bands within the species. Most of the amplification reactions were duplicated and only bands that were consistently reproduced across amplifications were considered for the analysis. Bands with the same mobility were considered as identical fragments, receiving equal values, regardless of their staining intensity. When multiple bands in a region were difficult to resolve, data for that region of the gel was not included in the analysis.

PCR amplification of DNA using 31 ISSR primers produced 166 DNA fragments that were scored in all genotypes. The number of fragments varied from 1 (ISSR13, ISSR54 and ISSR59) to 11 (ISSR14) and their sizes varied from 200 to 1700 bp. Of the 166 amplified fragments, 153 were polymorphic with the average number of bands per primer and polymorphic bands per primer as 5.36 and 4.94, respectively (Table 7). The total

number of polymorphic band, average number of band/primer and average numbers of polymorphic band/primer were 59, 3.74 and 2.19, respectively in *D. assamica*; 33, 3.08 and 1.32, respectively in *D. nigrescens* and 47, 3.58 and 1.81, respectively in *D. tonkinensis* (Table 7).

PIC value varied from 0 (ISSR 7 and ISSR13) to 0.564 (ISSR52), with an average of 0.352 (Table 3). The patterns of ISSR fragments produced by primers ISSR55 are shown in Figure 2. Further analysis of these ISSR profiles for band similarity indices could clearly differentiate all the species of *Dalbergia* (for instance at the size about 750 bp, all individuals of *D. tonkinensis* have amplified DNA fragment) (Figure 2C). More also, the results of the genetic similarity matrix obtained after multivariate analysis using Nei and Li's coefficient (Nei and Li, 1979) among the individuals of different *Dalbergia* species ranged from about 67.0 (Da10 and Da21) to 98.9% (Da2 and Da4) in *D. assamica*; from 71.2 (Dn2 and Dn25) to 98.5% (Dn10 and Dn12) in *D. nigrescens* and from 68.5 (Dt5 and Dt20) to 95.2% (Dt2 and Dt3) in *D. tonkinensis* (Tables 4, 5 and 6).

The dendrogram based on UPGMA analysis grouped 75 samples into three main clusters (I, II, and III) of which each main cluster contained one species. All the species shared more than 28% similarity among themselves. Genetic similarity within clusters ranged from about 28 to 72% with a bootstrap value of 70.5 (Figure 3). Genetic

Table 3. The statistical data for 31 ISSR primers used for the study of genetic diversity of three *Dalbergia* species

| Number | Primers code | PIC | Poly band | Mono band | Total band | Nei's gene diversity among species | Nei's gene diversity | | |
|--------|--------------|--------|-----------|-----------|------------|------------------------------------|----------------------|-------|-------|
| | | | | | | | Da | Dn | Dt |
| 1 | ISSR1 | 0.305 | 4 | 1 | 5 | 0.201 | 0.271 | 0.000 | 0.000 |
| 2 | ISSR2 | 0.318 | 6 | 0 | 6 | 0.431 | 0.134 | 0.000 | 0.000 |
| 3 | ISSR3 | 0.431 | 10 | 0 | 10 | 0.369 | 0.128 | 0.196 | 0.370 |
| 4 | ISSR5 | 0.243 | 2 | 3 | 5 | 0.106 | 0.000 | 0.067 | 0.080 |
| 5 | ISSR6 | 0.320 | 5 | 0 | 5 | 0.379 | 0.026 | 0.115 | 0.330 |
| 6 | ISSR7 | 0.000 | 0 | 5 | 5 | 0.000 | 0.000 | 0.000 | 0.000 |
| 7 | ISSR8 | 0.211 | 1 | 1 | 2 | 0.222 | 0.000 | 0.000 | 0.000 |
| 8 | ISSR9 | 0.428 | 2 | 0 | 2 | 0.440 | 0.077 | 0.000 | 0.000 |
| 9 | ISSR10 | 0.227 | 5 | 1 | 6 | 0.340 | 0.064 | 0.142 | 0.000 |
| 10 | ISSR11 | 0.485 | 6 | 0 | 6 | 0.343 | 0.246 | 0.134 | 0.000 |
| 11 | ISSR12 | 0.283 | 2 | 0 | 2 | 0.418 | 0.160 | 0.000 | 0.000 |
| 12 | ISSR13 | 0.000 | 0 | 1 | 1 | 0.000 | 0.000 | 0.000 | 0.000 |
| 13 | ISSR14 | 0.419 | 11 | 0 | 11 | 0.383 | 0.342 | 0.106 | 0.235 |
| 14 | ISSR15 | 0.471 | 10 | 0 | 10 | 0.363 | 0.305 | 0.408 | 0.280 |
| 15 | ISSR17 | 0.347 | 2 | 0 | 2 | 0.405 | 0.202 | 0.000 | 0.000 |
| 16 | ISSR18 | 0.495 | 2 | 0 | 2 | 0.374 | 0.000 | 0.000 | 0.246 |
| 17 | ISSR46 | 0.334 | 6 | 0 | 6 | 0.432 | 0.154 | 0.000 | 0.093 |
| 18 | ISSR49 | 0.505 | 7 | 0 | 7 | 0.365 | 0.266 | 0.000 | 0.000 |
| 19 | ISSR51 | 0.401 | 8 | 0 | 8 | 0.340 | 0.134 | 0.341 | 0.206 |
| 20 | ISSR52 | 0.564 | 4 | 0 | 4 | 0.288 | 0.421 | 0.000 | 0.038 |
| 21 | ISSR54 | 0.423 | 1 | 0 | 1 | 0.444 | 0.000 | 0.000 | 0.000 |
| 22 | ISSR55 | 0.344 | 9 | 0 | 9 | 0.432 | 0.096 | 0.090 | 0.117 |
| 23 | ISSR56 | 0.330 | 5 | 0 | 5 | 0.236 | 0.243 | 0.125 | 0.203 |
| 24 | ISSR59 | 0.423 | 1 | 0 | 1 | 0.444 | 0.000 | 0.000 | 0.000 |
| 25 | ISSR61 | 0.315 | 4 | 1 | 5 | 0.342 | 0.093 | 0.157 | 0.000 |
| 26 | ISSR62 | 0.470 | 6 | 0 | 6 | 0.318 | 0.000 | 0.145 | 0.229 |
| 27 | ISSR63 | 0.286 | 7 | 0 | 7 | 0.385 | 0.000 | 0.109 | 0.215 |
| 28 | ISSR64 | 0.386 | 9 | 0 | 9 | 0.355 | 0.254 | 0.218 | 0.308 |
| 29 | ISSR65 | 0.483 | 8 | 0 | 8 | 0.388 | 0.000 | 0.263 | 0.000 |
| 30 | ISSR67 | 0.324 | 4 | 0 | 4 | 0.461 | 0.245 | 0.000 | 0.182 |
| 31 | ISSR69 | 0.346 | 6 | 0 | 6 | 0.265 | 0.074 | 0.098 | 0.054 |
| | Total | 10.917 | 153 | 13 | 166 | 10.269 | 3.935 | 2.714 | 3.186 |
| | Average | 0.352 | 4.935 | 0.419 | 5.354 | 0.331 | 0.146 | 0.109 | 0.123 |

similarity among individuals of *D. tonkinensis* in cluster I ranged from 77 to 96% with a bootstrap value of 100. Clusters II of *D. nigrescens* had the genetic similarity about 80 to 98% with a bootstrap value of 100, while clusters III, that included *D. assamica*, had the genetic similarity of about 76 to 99% with a bootstrap value of 100.

Values of effective number of alleles, Shannon's information index, intralocus gene diversity (H_j) and Nei's gene diversity (H_i) among the individuals of different *Dalbergia* species using 31 ISSR markers were 1.227, 0.195, 0.662 and 0.146, respectively in *D. assamica*; 1.135, 0.111, 0.425 and 0.109, respectively in *D. nigrescens*; and 1.198, 0.166, 0.526 and 0.123 in *D.*

Table 4. Similarity matrix for Nei and Li's coefficient among the 25 individuals of *D. assamica*.

| | Da1 | Da2 | Da3 | Da4 | Da5 | Da6 | Da7 | Da8 | Da9 | Da10 | Da11 | Da12 | Da13 | Da14 | Da15 | Da16 | Da17 | Da18 | Da19 | Da20 | Da21 | Da22 | Da23 | Da24 | Da25 | |
|-------------|------|------|------|------|------|------|------|------|------|------|------|------|------|-------|------|------|------|------|------|------|------|------|------|------|------|--|
| Da1 | 100 | | | | | | | | | | | | | | | | | | | | | | | | | |
| Da2 | 90.0 | 100 | | | | | | | | | | | | | | | | | | | | | | | | |
| Da3 | 89.8 | 95.5 | 100 | | | | | | | | | | | | | | | | | | | | | | | |
| Da4 | 91.1 | 98.9 | 94.4 | 100 | | | | | | | | | | | | | | | | | | | | | | |
| Da5 | 85.2 | 91.0 | 86.5 | 90.0 | 100 | | | | | | | | | | | | | | | | | | | | | |
| Da6 | 78.3 | 80.0 | 79.6 | 79.2 | 81.1 | 100 | | | | | | | | | | | | | | | | | | | | |
| Da7 | 79.3 | 81.1 | 80.6 | 82.1 | 80.2 | 89.7 | 100 | | | | | | | | | | | | | | | | | | | |
| Da8 | 77.2 | 80.9 | 80.4 | 80.0 | 80.0 | 91.8 | 95.2 | 100 | | | | | | | | | | | | | | | | | | |
| Da9 | 77.8 | 81.5 | 81.1 | 80.6 | 80.7 | 79.8 | 83.0 | 82.8 | 100 | | | | | | | | | | | | | | | | | |
| Da10 | 70.0 | 68.4 | 69.6 | 69.5 | 72.7 | 80.0 | 85.5 | 83.1 | 71.3 | 100 | | | | | | | | | | | | | | | | |
| Da11 | 82.2 | 85.9 | 83.5 | 84.9 | 85.2 | 82.2 | 81.3 | 83.1 | 81.8 | 75.9 | 100 | | | | | | | | | | | | | | | |
| Da12 | 73.6 | 75.5 | 75.0 | 74.7 | 80.5 | 85.9 | 87.1 | 89.2 | 81.2 | 83.8 | 81.6 | 100 | | | | | | | | | | | | | | |
| Da13 | 78.0 | 81.7 | 81.3 | 80.9 | 83.0 | 84.1 | 83.1 | 85.1 | 88.1 | 75.6 | 86.2 | 85.7 | 100 | | | | | | | | | | | | | |
| Da14 | 78.0 | 83.7 | 81.3 | 82.8 | 87.2 | 78.0 | 77.2 | 76.9 | 83.7 | 71.6 | 84.1 | 79.3 | 83.9 | 100.0 | | | | | | | | | | | | |
| Da15 | 71.1 | 73.1 | 72.5 | 72.3 | 75.9 | 77.0 | 78.2 | 77.9 | 74.4 | 78.8 | 81.2 | 82.7 | 74.7 | 78.8 | 100 | | | | | | | | | | | |
| Da16 | 73.1 | 76.8 | 78.3 | 76.0 | 77.8 | 80.9 | 80.0 | 77.8 | 76.4 | 70.5 | 78.9 | 82.4 | 80.7 | 76.7 | 75.6 | 100 | | | | | | | | | | |
| Da17 | 74.5 | 80.0 | 77.7 | 79.2 | 81.1 | 82.2 | 81.3 | 79.1 | 77.8 | 73.9 | 82.2 | 83.7 | 82.0 | 82.0 | 81.2 | 87.2 | 100 | | | | | | | | | |
| Da18 | 72.6 | 80.0 | 77.7 | 79.2 | 77.2 | 80.2 | 81.3 | 79.1 | 79.8 | 71.9 | 78.3 | 79.5 | 80.0 | 82.0 | 75.0 | 85.1 | 86.4 | 100 | | | | | | | | |
| Da19 | 72.5 | 76.3 | 79.8 | 75.5 | 77.3 | 76.4 | 77.5 | 75.3 | 75.9 | 69.8 | 80.5 | 75.6 | 80.2 | 82.4 | 77.1 | 85.5 | 84.7 | 84.7 | 100 | | | | | | | |
| Da20 | 71.0 | 80.4 | 78.0 | 79.6 | 79.5 | 78.7 | 81.8 | 81.6 | 78.2 | 74.1 | 78.7 | 84.3 | 78.4 | 80.5 | 79.5 | 83.5 | 82.8 | 84.9 | 81.0 | 100 | | | | | | |
| Da21 | 71.7 | 75.5 | 75.0 | 74.7 | 76.4 | 73.6 | 74.7 | 72.5 | 73.0 | 67.0 | 77.5 | 74.7 | 77.3 | 79.3 | 76.2 | 86.7 | 90.4 | 81.6 | 84.1 | 75.9 | 100 | | | | | |
| Da22 | 74.2 | 79.8 | 79.3 | 78.9 | 75.0 | 78.0 | 79.1 | 78.9 | 79.5 | 73.6 | 80.0 | 75.3 | 77.8 | 75.8 | 78.8 | 80.7 | 82.0 | 82.0 | 78.2 | 82.6 | 79.3 | 100 | | | | |
| Da23 | 70.8 | 72.8 | 74.2 | 72.0 | 73.6 | 70.8 | 73.9 | 73.6 | 72.1 | 69.9 | 74.7 | 73.8 | 76.5 | 76.5 | 73.2 | 84.0 | 81.0 | 78.8 | 79.0 | 72.9 | 87.2 | 76.5 | 100 | | | |
| Da24 | 70.7 | 74.5 | 73.9 | 73.7 | 71.4 | 72.5 | 73.6 | 75.3 | 71.9 | 67.8 | 76.4 | 71.6 | 76.1 | 70.3 | 70.9 | 81.2 | 76.4 | 76.4 | 80.7 | 74.7 | 75.6 | 86.7 | 76.8 | 100 | | |
| Da25 | 73.4 | 80.9 | 80.4 | 80.0 | 78.0 | 77.2 | 80.2 | 80.0 | 78.7 | 72.7 | 79.1 | 80.5 | 83.0 | 76.9 | 73.9 | 83.9 | 85.2 | 81.1 | 79.3 | 81.6 | 80.5 | 87.2 | 79.8 | 85.7 | 100 | |

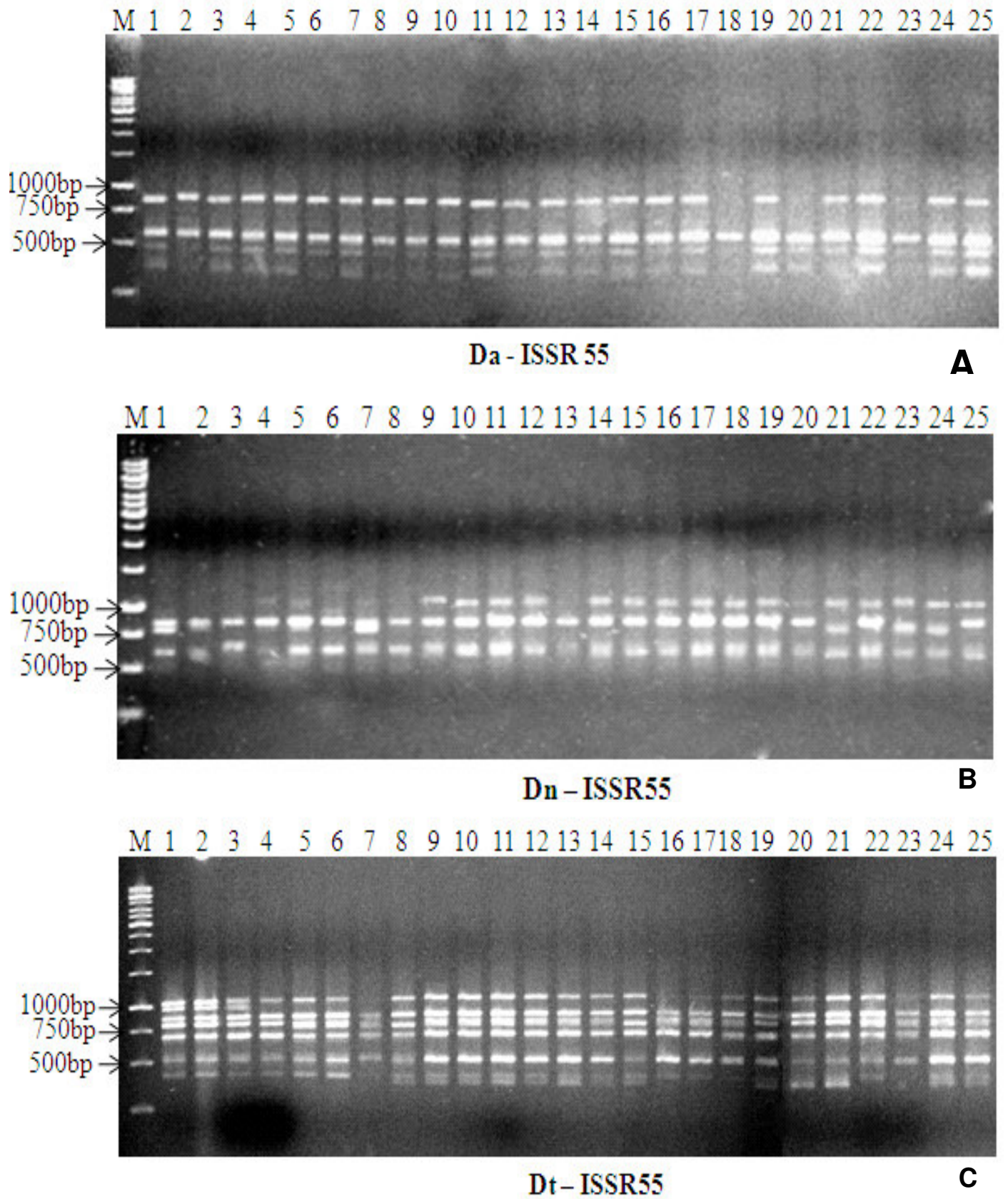


Figure 2. ISSR patterns of the three species of *Dalbergia* generated by primer ISSR55 (A, *D. assamica*; B, *D. nigrescens*; C, *D. tonkinensis*).

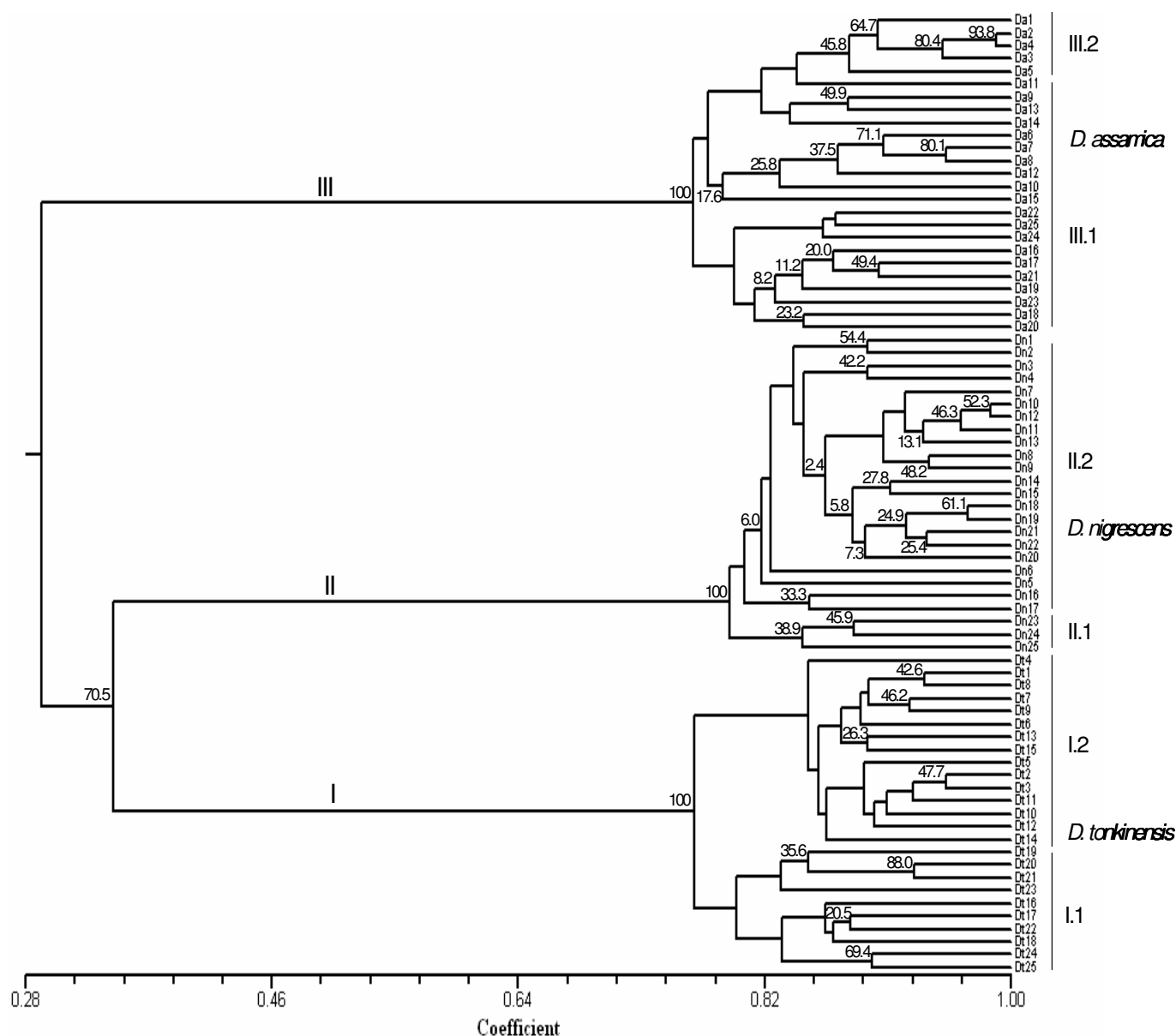


Figure 3. Dendrogram of cluster analysis of ISSR markers, illustrating the genetic among three species of *Dalbergia*.

tonkinensis (Tables 8). AMOVA of ISSR data revealed that a greater proportion of total genetic variation existed among species (80%) rather than within species (20%) (Table 9).

DISCUSSION

In our studies, ISSR markers were effective tools for understanding the genetic diversity of *Dalbergia*. This result was not surprising because these techniques have been used successfully in population genetic studies and in detecting genetic diversity in many species (Arif et al., 2009; Li and Ge, 2001; Djè et al., 2006; Blair et al.,

1999). The polymorphism of the three tropical hardwood species of *Dalbergia* genus was compared (Table 7). The total number of polymorphic bands (59) and average number of polymorphic bands/primer (2.19) detected by ISSR primers were much higher in *D. assamica*. This method is obviously advantageous in differentiating closely related cultivars and has been used for cultivar identification in numerous plant species, including rice (Joshi et al., 2000), apple (Goulaõ and Oliveira, 2001), mulberry (Zhao et al., 2006) and strawberry (Arnau et al., 2003). The results indicate that the mean levels of genetic variation were low among the individuals of different *Dalbergia* species. Molecular markers have become common for assessing diversity within plant

Table 5. Similarity matrix for Nei and Li's coefficient among the 25 individuals of *D. nigrescens*.

| | Dn1 | Dn2 | Dn3 | Dn4 | Dn5 | Dn6 | Dn7 | Dn8 | Dn9 | Dn10 | Dn11 | Dn12 | Dn13 | Dn14 | Dn15 | Dn16 | Dn17 | Dn18 | Dn19 | Dn20 | Dn21 | Dn22 | Dn23 | Dn24 | Dn25 |
|-------------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| Dn1 | 100 | | | | | | | | | | | | | | | | | | | | | | | | |
| Dn2 | 89.6 | 100 | | | | | | | | | | | | | | | | | | | | | | | |
| Dn3 | 82.4 | 84.5 | 100 | | | | | | | | | | | | | | | | | | | | | | |
| Dn4 | 86.4 | 85.7 | 89.6 | 100 | | | | | | | | | | | | | | | | | | | | | |
| Dn5 | 80.3 | 77.5 | 80.9 | 84.8 | 100 | | | | | | | | | | | | | | | | | | | | |
| Dn6 | 80.6 | 80.3 | 78.6 | 79.7 | 79.1 | 100 | | | | | | | | | | | | | | | | | | | |
| Dn7 | 79.7 | 81.9 | 82.9 | 89.6 | 83.6 | 83.8 | 100 | | | | | | | | | | | | | | | | | | |
| Dn8 | 82.6 | 84.7 | 83.1 | 84.3 | 81.2 | 81.4 | 91.2 | 100 | | | | | | | | | | | | | | | | | |
| Dn9 | 82.4 | 84.5 | 80.3 | 78.9 | 75.7 | 81.2 | 85.5 | 94.0 | 100 | | | | | | | | | | | | | | | | |
| Dn10 | 85.3 | 84.7 | 83.1 | 87.0 | 83.8 | 86.8 | 94.0 | 94.1 | 91.2 | 100 | | | | | | | | | | | | | | | |
| Dn11 | 84.3 | 88.9 | 84.7 | 85.9 | 82.9 | 83.1 | 90.0 | 92.9 | 90.0 | 95.7 | 100 | | | | | | | | | | | | | | |
| Dn12 | 84.1 | 86.1 | 84.5 | 85.7 | 85.3 | 85.5 | 92.6 | 92.8 | 89.9 | 98.5 | 97.1 | 100 | | | | | | | | | | | | | |
| Dn13 | 86.4 | 83.1 | 81.4 | 88.1 | 84.8 | 87.9 | 92.4 | 92.5 | 86.8 | 95.5 | 91.3 | 94.0 | 100 | | | | | | | | | | | | |
| Dn14 | 83.8 | 83.3 | 84.3 | 82.9 | 79.7 | 85.3 | 89.7 | 84.5 | 81.7 | 89.9 | 86.1 | 88.6 | 88.2 | 100 | | | | | | | | | | | |
| Dn15 | 83.8 | 85.9 | 84.3 | 85.5 | 77.1 | 85.3 | 87.0 | 84.5 | 81.7 | 89.9 | 88.7 | 88.6 | 88.2 | 91.2 | 100 | | | | | | | | | | |
| Dn16 | 83.3 | 80.3 | 81.2 | 85.1 | 79.1 | 82.1 | 83.8 | 81.4 | 81.2 | 81.4 | 80.6 | 80.3 | 79.7 | 82.6 | 80.0 | 100 | | | | | | | | | |
| Dn17 | 81.2 | 80.8 | 79.2 | 80.3 | 72.2 | 75.0 | 81.7 | 79.5 | 84.3 | 79.5 | 78.7 | 78.4 | 75.3 | 83.1 | 80.6 | 85.3 | 100 | | | | | | | | |
| Dn18 | 86.2 | 80.3 | 83.8 | 87.9 | 84.6 | 82.1 | 86.6 | 84.1 | 78.6 | 86.8 | 83.1 | 85.5 | 90.8 | 88.1 | 85.3 | 82.1 | 80.0 | 100 | | | | | | | |
| Dn19 | 86.4 | 83.1 | 84.1 | 88.1 | 84.8 | 82.4 | 89.6 | 87.0 | 81.4 | 89.7 | 85.9 | 88.4 | 90.9 | 91.0 | 88.2 | 85.1 | 82.9 | 96.8 | 100 | | | | | | |
| Dn20 | 82.1 | 84.3 | 85.3 | 83.8 | 80.6 | 80.9 | 85.3 | 85.5 | 82.6 | 88.2 | 87.1 | 89.7 | 89.4 | 86.8 | 89.6 | 78.3 | 76.4 | 86.4 | 89.4 | 100 | | | | | |
| Dn21 | 83.6 | 85.7 | 86.8 | 88.1 | 84.8 | 79.7 | 89.6 | 84.3 | 78.9 | 87.0 | 85.9 | 88.4 | 88.1 | 91.0 | 88.2 | 82.4 | 80.3 | 90.8 | 93.8 | 89.4 | 100 | | | | |
| Dn22 | 80.9 | 83.1 | 86.8 | 85.3 | 82.1 | 79.7 | 86.8 | 84.3 | 81.4 | 87.0 | 85.9 | 88.4 | 88.1 | 88.2 | 88.2 | 82.4 | 80.3 | 90.8 | 93.8 | 92.3 | 93.8 | 100 | | | |
| Dn23 | 75.8 | 73.2 | 79.1 | 77.6 | 74.2 | 74.6 | 81.8 | 82.1 | 81.8 | 82.1 | 78.6 | 80.9 | 80.3 | 77.9 | 77.9 | 80.0 | 75.4 | 80.0 | 80.3 | 78.8 | 77.6 | 83.1 | 100 | | |
| Dn24 | 80.3 | 75.0 | 78.3 | 79.4 | 78.8 | 76.5 | 83.6 | 83.8 | 83.6 | 83.8 | 80.3 | 82.6 | 82.1 | 79.7 | 79.7 | 84.6 | 82.4 | 87.5 | 87.7 | 80.6 | 82.1 | 84.8 | 88.5 | 100 | |
| Dn25 | 73.5 | 71.2 | 74.3 | 77.9 | 80.0 | 83.1 | 79.4 | 79.7 | 82.1 | 82.4 | 78.9 | 81.2 | 83.3 | 75.7 | 75.7 | 75.0 | 73.2 | 80.3 | 77.9 | 79.1 | 72.9 | 77.9 | 83.9 | 85.7 | 100 |

populations (Smith and Wayne, 1996; Lalhruaitluanga and Prasad, 2009).

The dendrogram analysis of all the three clusters considering them as three populations generated an over-view of population distribution (Figure 3). It is interesting to note that three populations comprising of three distinct *Dalbergia* species are seen as genetically distinct groups. Analysis of molecular variance of ISSR markers data (Table 9) revealed that the genetic variation

within species (20%) was lower than those among species (80%). And the analysis of intralocus gene diversity (H_i) and Nei's gen diversity (H_i) (0.662 and 0.146, respectively) in *D. assamica* also were higher than those of two species; *D. nigrescens* (0.425 and 0.109, respectively) and *D. tonkinensis* (0.526 and 0.123, respectively) (Table 8). Compared to the other species in *Dalbergia* at the population level of genetic diversity, Shannon's diversity index and Nei's genetic diver-

sity were 0.239 and 0.358 for *D. sissoo* (Wang et al., 2011), 0.223 and 0.150 for *D. monticola* (Andrianoelina et al., 2006) and 0.205 and 0.135 for *D. odorifera* (Yang et al., 2007). While in our present study, they were 0.195 and 0.146 for *D. assamica*, 0.111 and 0.109 for *D. nigrescens* and 0.166 and 0.123 for *D. tonkinensis* (Table 8). The analysis of Shannon's information index and Nei's gene diversity values within species showed the existence of higher genetic diversity in *D.*

Table 6. Similarity matrix for Nei and Li's coefficient among the 25 individuals of *D. tonkinensis*.

| | Dt1 | Dt2 | Dt3 | Dt4 | Dt5 | Dt6 | Dt7 | Dt8 | Dt9 | Dt10 | Dt11 | Dt12 | Dt13 | Dt14 | Dt15 | Dt16 | Dt17 | Dt18 | Dt19 | Dt20 | Dt21 | Dt22 | Dt23 | Dt24 | Dt25 |
|-------------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| Dt1 | 100 | | | | | | | | | | | | | | | | | | | | | | | | |
| Dt2 | 90.5 | 100 | | | | | | | | | | | | | | | | | | | | | | | |
| Dt3 | 92.8 | 95.2 | 100 | | | | | | | | | | | | | | | | | | | | | | |
| Dt4 | 87.8 | 88.1 | 85.9 | 100 | | | | | | | | | | | | | | | | | | | | | |
| Dt5 | 89.4 | 89.7 | 89.7 | 84.9 | 100 | | | | | | | | | | | | | | | | | | | | |
| Dt6 | 91.1 | 84.5 | 84.5 | 86.3 | 85.7 | 100 | | | | | | | | | | | | | | | | | | | |
| Dt7 | 89.0 | 84.9 | 84.9 | 84.3 | 86.0 | 89.9 | 100 | | | | | | | | | | | | | | | | | | |
| Dt8 | 93.7 | 84.7 | 86.9 | 84.1 | 85.9 | 87.3 | 90.0 | 100 | | | | | | | | | | | | | | | | | |
| Dt9 | 89.2 | 87.2 | 87.2 | 84.5 | 90.6 | 87.7 | 92.6 | 90.1 | 100 | | | | | | | | | | | | | | | | |
| Dt10 | 90.4 | 92.9 | 90.6 | 90.2 | 89.5 | 84.3 | 82.6 | 84.5 | 84.9 | 100 | | | | | | | | | | | | | | | |
| Dt11 | 91.5 | 91.7 | 94.0 | 82.4 | 90.6 | 85.4 | 83.5 | 85.5 | 88.1 | 89.3 | 100 | | | | | | | | | | | | | | |
| Dt12 | 87.8 | 90.4 | 88.1 | 83.1 | 87.1 | 86.3 | 84.3 | 86.4 | 86.7 | 90.2 | 91.4 | 100 | | | | | | | | | | | | | |
| Dt13 | 87.3 | 85.4 | 85.4 | 80.2 | 82.1 | 83.3 | 88.5 | 85.9 | 86.3 | 82.9 | 86.3 | 84.8 | 100 | | | | | | | | | | | | |
| Dt14 | 85.5 | 90.4 | 92.7 | 83.1 | 82.8 | 79.5 | 82.1 | 81.9 | 84.5 | 83.5 | 86.7 | 83.1 | 84.8 | 100 | | | | | | | | | | | |
| Dt15 | 90.0 | 85.7 | 88.0 | 87.5 | 89.2 | 88.5 | 88.8 | 88.6 | 88.9 | 85.5 | 88.9 | 85.2 | 89.5 | 85.2 | 100 | | | | | | | | | | |
| Dt16 | 82.2 | 82.6 | 84.6 | 82.0 | 81.7 | 80.7 | 81.1 | 76.9 | 81.3 | 80.4 | 81.3 | 78.0 | 75.3 | 82.0 | 79.8 | 100 | | | | | | | | | |
| Dt17 | 85.9 | 82.0 | 82.0 | 83.5 | 81.1 | 84.3 | 82.6 | 80.2 | 80.7 | 83.9 | 80.7 | 79.3 | 80.7 | 77.3 | 83.3 | 86.5 | 100 | | | | | | | | |
| Dt18 | 79.3 | 79.8 | 79.8 | 81.2 | 75.0 | 77.6 | 76.1 | 73.9 | 74.4 | 81.6 | 74.4 | 75.0 | 74.1 | 77.0 | 76.7 | 86.4 | 85.9 | 100 | | | | | | | |
| Dt19 | 77.9 | 78.4 | 78.4 | 77.6 | 75.6 | 78.3 | 78.8 | 76.5 | 79.1 | 78.2 | 77.0 | 75.6 | 72.6 | 77.6 | 77.4 | 78.9 | 82.4 | 82.1 | 100 | | | | | | |
| Dt20 | 74.7 | 75.3 | 73.3 | 78.8 | 68.5 | 75.0 | 69.4 | 71.1 | 69.8 | 77.1 | 71.8 | 70.2 | 69.1 | 74.4 | 72.0 | 71.9 | 75.0 | 83.5 | 84.4 | 100 | | | | | |
| Dt21 | 76.2 | 74.7 | 74.7 | 78.0 | 70.0 | 74.4 | 72.9 | 72.6 | 71.3 | 78.6 | 71.3 | 69.8 | 68.7 | 73.8 | 73.5 | 75.3 | 78.6 | 85.0 | 85.9 | 93.0 | 100 | | | | |
| Dt22 | 79.5 | 78.0 | 80.0 | 79.3 | 77.2 | 77.9 | 78.4 | 76.1 | 76.7 | 79.8 | 74.7 | 71.4 | 72.4 | 75.3 | 77.0 | 86.5 | 88.2 | 88.1 | 86.7 | 79.3 | 85.2 | 100 | | | |
| Dt23 | 75.3 | 75.9 | 75.9 | 77.1 | 73.0 | 77.8 | 78.3 | 75.9 | 74.4 | 77.6 | 72.4 | 75.0 | 76.3 | 75.0 | 76.8 | 76.4 | 81.9 | 86.3 | 84.8 | 81.6 | 83.1 | 84.1 | 100 | | |
| Dt24 | 77.0 | 75.6 | 77.5 | 74.7 | 76.7 | 77.4 | 77.9 | 73.6 | 76.1 | 77.3 | 74.2 | 72.7 | 75.9 | 74.7 | 76.5 | 82.0 | 85.7 | 81.2 | 79.8 | 74.4 | 78.0 | 88.0 | 81.5 | 100 | |
| Dt25 | 78.8 | 79.3 | 79.3 | 76.5 | 78.4 | 77.1 | 75.6 | 75.3 | 75.9 | 77.0 | 75.9 | 72.4 | 77.8 | 78.6 | 78.3 | 79.8 | 85.5 | 81.0 | 79.5 | 76.3 | 75.6 | 83.3 | 81.3 | 89.9 | 100 |

assamica population compared to *D. nigrescens* and *D. tonkinensis*.

In our study, the relative genetic distances within species showed the separation of samples from the geographical distances. For instance, for the species *D. tonkinensis*, 15 genotypes; Dt1-Dt15 (collected from Ha Noi province) were grouped into subgroup I.2 and 15 genotypes; Dt16-Dt25 (collected from Quang Binh province) were grouped into subgroup I.1 with a bootstrap value of 100; similar three genotypes, Dn23, Dn24

and Dn25 of *D. nigrescens* species population (collected from Son La province) were grouped into subgroup II.1 to a clade of 22 rest genotypes (Dn1-Dn22) with the bootstrap value of 100 and five genotypes (Da1, Da2, Da3, Da4 and Da5) of *D. assamica* species population (collected from Hanoi province) were grouped into a minor subgroup III.2 to a clade of 20 rest genotypes (Da6-Da25) with the bootstrap value of 45.8 (Figure 3).

The genetic structure of any species are

normally affected by a number of evolutionary factors including mating system, gene flow, seed dispersal, mode of reproduction, as well as natural selection (Hamrick et al., 1992). Infact, little occurred within and between the individual populations of *Dalbergia* species, which is more concordant with the hypothesis of a highly clonal population resulting from reproduction by seeds. Mohana et al. (2001) reported that the pairs of seeds developing within a pod of *D. sisso* are genetically more similar than any random pairs of

Table 7. A comparative list of genetic diversity of three *Dalbergia* species ISSR primers.

| Primer | Among three species | Da | Dn | Dt |
|--|---------------------|------|------|------|
| Total number of polymorphic bands | 153 | 59 | 33 | 47 |
| Total number of monomorphic bands | 13 | 42 | 44 | 46 |
| Total number of bands | 166 | 101 | 77 | 93 |
| Average number of bands/primer | 5.36 | 3.74 | 3.08 | 3.58 |
| Average number of polymorphic bands/primer | 4.94 | 2.19 | 1.32 | 1.81 |

Table 8. Genetic diversity parameters characterizing of three *Dalbergia* species using ISSR primers.

| Primer | Among <i>Dalbergia</i> species | Da | Dn | Dt |
|---|--------------------------------|-------|-------|-------|
| Sum of effective number of alleles (SENA) | 1.187 | 1.227 | 1.135 | 1.198 |
| Shannon's Information index (I) | 0.157 | 0.195 | 0.111 | 0.166 |
| Polymorphic information content (PIC) | 0.352 | 0.103 | 0.091 | 0.095 |
| Intralocus gene diversity (H _j) | 1.806 | 0.662 | 0.425 | 0.526 |
| Nei's gene diversity (H _i) | 0.331 | 0.146 | 0.109 | 0.123 |

Table 9. Summary of AMOVA analysis based ISSR primers of three *Dalbergia* species.

| Source | df | SS | MS | Est. Var. | Percentage |
|----------------|----|----------|---------|-----------|------------|
| Among species | 2 | 1566.720 | 783.360 | 31.030 | 80 |
| Within species | 72 | 547.520 | 7.604 | 7.604 | 20 |
| Total | 74 | 2114.240 | | 38.635 | 100 |

SS, Sums of squares; MS, mean sums of squares; Est. Var, estimated variance.

seeds in a tree. Thus, formation of two-seeded pods appears to be associated with increased genetic relatedness among the developing seeds. The lowest levels of genetic similarity among the populations of *D. sissoo*, *D. assamica*, *D. latifolia*, *D. paniculata* and *D. spinosa* were 82.8, 87.3, 84.0, 87.5 and 84.5, respectively. It could be the reason for the observed genetic differentiation between the individuals of the *D. assamica*, *D. nigrescens* and *D. tonkinensis* populations growing from 120 to 1600 km apart.

This is the first study on the DNA diversity of some species of *Dalbergia* genus in Vietnam. Besides the high level of genetic diversity between species, a significant variation within a species was also found. This may be the result of a long-term of adaptation in diversity climatic and geographical conditions of Vietnam. Nevertheless, a further assignment should be established in more natural conditions to study the relationship between them and provide precise information for the protection of diversity.

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