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Genetic Diversity and Population Structure of *Canarium tramdenum* Dai and Yakovl. in Northern Vietnam

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ABSTRACT

Canarium tramdenum **occurs naturally in subtropical and tropical regions of Indochina and China. The wood is used for making high quality furniture and the fruit and leaves are used in traditional medicine. However, a lack of information on genetic diversity and population structure has handicapped the genetic conservation and domestication of this high-value species. This study evaluated genetic variation within and among four *C. tramdenum* populations. Sixty individuals were collected from four natural populations in Vietnam in the provinces of Ninhbinh, Bacgiang, Nghean, and Backan. Genetic diversity and genetic structure were determined using 20 ISSR markers. A total of 192 DNA fragments with sizes ranging from 110 bp to 3,000 bp were detected, of which 154 segments (80.2%) were polymorphic and 38 segments (19.8%) were monomorphic. The ISSR data indicated a moderate degree of genetic diversity for the species (h = 0.252). The four populations were separated into three genetic clusters with low levels of genetic distance between them. AMOVA result showed that most (78%) of the genetic variation was within the populations. The moderate to high genetic diversity of *C. tramdenum* and the low genetic differentiation among populations suggested that all existing natural populations in the particular regions needed to be preserved to protect the genetic diversity of this species.**

1. Introduction

Canarium tramdenum Dai and Yakovl. is a forest tree species belonging to Burseraceae family, first described as *Pimela nigra* Lour by Loureiro in 1790, and subsequently renamed *Canarium nigrum* (Lour) Engl. and *Canarium pimela* Leench in the 1900s. In the year 1985, it was re-assessed and is now described as Canarium tramdenum Dai and Yakovl. (Nguyen *et al.* 2009). It occurs naturally in the evergreen tropical and subtropical forests of China, Vietnam, Laos, Cambodia, and Thailand.

In Vietnam, the ripe fruits of *C. tramdenum* are generally used as a food and flavouring? ingredient. The leaves are traditionally used for medical purposes, such as treating diarrhoea and rheumatism (Hoang *et al.* 2004, 2008), and more generally as an antibiotic and detoxifying substance in traditional medicine. The fruits and leaves of *C. tramdenum* have been reported as vasorelaxant and antioxidant agents (Thang *et al.* 2014). The bark also contains a high concentration of phenolics and terpenoids and is considered as a traditional cure for reducing risks from diabetes. The high quantites of α-amyrin and β-amyrin that are extracted from *C. tramdenum* bark also suggest the potential for inhibiting the development of inflammation, ulcers and tumours acting as a hyperlipidemic (Nguyen *et al.* 2019). The light and soft timber, grey white in colour without a strong heartwood-sapwood boundary is used for house construction, furniture, veneer, pencils, matches and pulp. Given this range of uses, *C. tramdenum* is planted as a multi-purpose species in agroforestry in Vietnam (Hoang 2004).

Through commercial exploitation, the number of *C. tramdenum* trees in natural habitats is decreasing. Some conservation and utilization programs for this

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species have been implemented (Loc *et al.* 2020). However, there have been no genetic studies on C. tramdenum to date. Genetic diversity information is critically important to guide genetic resource conservation, exploitation and domestication of this species. In recent years, molecular markers have been widely used for the examination of genetic diversity and population structure of forest tree species. Information gained by molecular markers is used to study of genetic differences between natural populations (Agarwal and Shrivastava 2008). In comparison with other types of molecular markers, inter-simple sequence repeat (ISSR) markers are considered an efficient, cost-effective method for examining genetic relationships among the natural populations. Based on its unique characteristics, the ISSR technique can detect more genetic loci than isozymes and has higher reproductiviy than other classes of markers such as RAPD (Fernández *et al.* 2002).

This study was conducted to assess the genetic diversity level and population structure of four *C. tramdenum* populations from different regions in Vietnam usinf ISSR markers. We aimed to evaluate the genetic diversity degree of this species in order to enhance the effectiveness of genetic conservation and development of *C. tramdenum* in Vietnam.

2. Materials and Methods

2.1. Plant Sample Collection

A total of sixty samples of *Canarium tramdenum* were collected from populations in four provinces in northern Vietnam (Ninhbinh, Bacgiang, Nghean, and Backan) (Figure 1). These populations are the main centres of the natural distribution of the species in Vietnam. Fresh leaves were sampled from each of 15 dispersal individuals (>100 m apart) from remnant forests per population, dried in silica gel (Rawat *et al.* 2016), kept separately in zip lock plastic bags and brought to the laboratory in Hanoi for DNA extraction. It was found that the dried leaves could be kept at room temperature (25°C) for 30 days without DNA degradation.

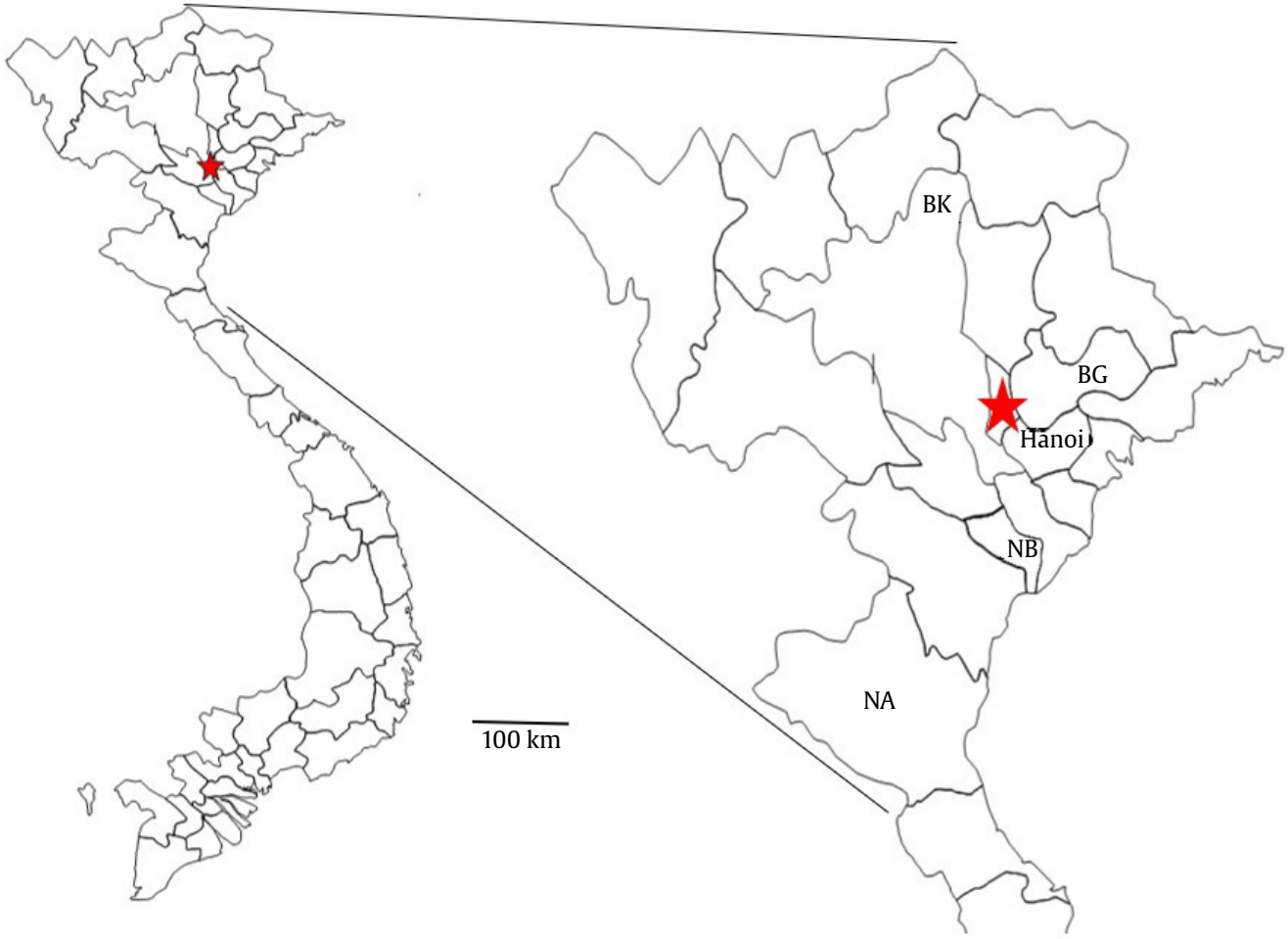

Figure 1. Geographical localities of four *C. tramdenum* populations analysed in this study: BK (Backan), BG (Bacgiang), NB (Ninhbinh) and NA (Nghean).

2.2. DNA Extraction

The DNA extraction process was based on CTAB method by Doyle and Doyle (1987) with some modifications. About 200 mg of leaf tissue from each sample was used. Leaves were grounded into fine powder in liquid nitrogen then put into Eppendorf tube 2 ml, added with 700 µl extraction buffer (EDTA 0.5M pH8, Tris HCl 100 mM pH8, 500 mM NaCl, 2% CTAB, 2% PVP and 0.1% β-mercapto ethanol). The mixture was incubated at 65°C for 60 minutes. In the next stage, the mixture was centrifuged for 20,000 rpm for 10 minutes then the fluid layer was taken out and put into a new Eppendorf tube 2 ml. Then 30 µl RNase was added, and the mixture was kept at 37°C for 30 minutes. In the next step, 700 µl Chloroform: Isoamyl alcohol (ratio 24:1) was added, shaken and centrifuged at 20,000 rpm for 10 minutes. The upper layer of solution was transfered to a new Eppendorf tube 1.5 ml. 750 µl cold Isopropanol was added and the sample kept at -20°C for 30 minutes, then centrifuged at 20,000 rpm for 10 minutes to collect DNA precipitation. DNA precipitation was washed twice in cold Ethanol 70%. DNA was allowed to dry and dissolved with 100µL of 1xTE buffer and stored at -20°C.

After the extraction process, total genomic DNA was run on 0.9% agarose gels and observed by gel scanner using UV rays. The DNA concentration was measured using a spectrophotometer (Nanodrop). The purification of DNA was determined by OD260/280 ratio. Samples with a ratio of OD260/280 more than 1.8 with a concentration ≥20 µg/µl were selected for the subsequent analysis.

2.3. Polymerase Chain Reaction (PCR) Amplification

DNA samples were used as the template for ISSR-PCR. PCR amplification was performed in a 20µl volume containing 3 µl DNA (20 ng/µl), 10µl 2X PCR MasterMix buffer (Thermo Scientific), 1 µM ISSR primers and 6 µl deionized water. The steps of the PCR were as follows: initial denaturation at 94°C for 4 min, 45s for denaturing at 94°C, attached primers in 45s at 56°C, and 1 min of elongation at 72°C, followed by 40 cycles of stages 2-4, final extension at 72°C for 10 min.

In total, 20 ISSR primers (UBC Primer Set No. 9, Biotechnology Laboratory, University of British Columbia, Vancouver, British Columbia, Canada) were used for PCR amplification, the details of these primers are shown in Table 1. The PCR reaction was repeated at least three times for each sample.

Table 1. List of ISSR primers used for PCR amplification

ISSR primers	Sequences (5'-3')
UBC807	AGAGAGAGAGAGAGAGT
UBC808	AGAGAGAGAGAGAGAGC
UBC809	AGAGAGAGAGAGAGAGG
UBC810	GAGAGAGAGAGAGAGAT
UBC811	GAGAGAGAGAGA GAGAC
UBC814	CTC TCT CTC TCT CTC TA
UBC818	CACACACACACA CACAG
UBC823	TGTGTGTGTGTGTGTGGA
UBC824	TCTCTCTCTCTCTCTCG
UBC825	ACA CAC ACA CAC ACA CT
UBC827	ACACACACACACACACG
UBC828	TGTGTGTGTGTGTGTGA
UBC834	AGA GAG AGA GAG AGA GYT
UBC836	AGA GAG AGA GAG AGA GYA
UBC841	GAG AGA GAG AGA GAG AYC
UBC855	ACA CAC ACA CAC ACA CYT
UBC876	GAT AGA TAG ACA GAC A
UBC886	VDV CTC TCT CTC TCT CT
UBC889	DBD ACA CAC ACA CAC AC
UBC898	CCC TCC CTC CCT CCC T

PCR products were resolved on a 2% agarose gel electrophoresis in 1x TAE buffer at 90V

In 70 minutes. Redsafe staining (www.intronbio.com) with a concentration of 5 µl/100 ml was used for visualizing. 1 kb ladder (Thermo Scientific) was used as DNA standard. The gels were visualized under UV using GelDoc system. Bands that were reproducible in successive amplifications over three PCRs replicated and unambiguous (clearest and lightest) on the electrophoresed gel were selected for further analysis.

2.4. Data Analysis

The genotype data samples were scored according to a binary matrix where 0 and 1 code for the absence and presence of a band on the electrophoresed gel, respectively. The polymorphic information content (PIC) of each ISSR indicator is determined using the following formula (Saal and Wricke 1999):

$$PIC_i = 1 - \sum P_{ij}^2$$

Where: P_{ij} is the j^{th} allele frequency of genotype *i*

Genetic diversity parameters were calculated using POPGENE version 1.32 (Yeh *et al.* 1997). Analysis of molecular variance (AMOVA) and Principal Coordinate Analysis (PCoA) were conducted using GenAlEx v6 software (Peakall and Smouse 2006).

The UPGMA dendrogram based on Nei and Li method (1979) was constructed by MEGA X software (Kumar *et al.* 2018).

3. Results

A total of 192 bands, with sizes ranging from 110 bp to 3,000 bp, were obtained from 20 ISSR markers. Among these, 154 bands were revealed as polymorphic (80.2%) and 38 bands were accounted as homomorphic (19.8%). The average number of amplification bands was 9.6 per primer, indicating high genetic diversity across these four natural populations of *C. tramdenum* in Vietnam. The polymorphic information content (PIC) values varied between 0.18 (UBC810) and 0.41 (UBC828), with an average of 0.35, indicating that the loci were moderately informative and could, therefore, detect and quantify the genetic diversity parameters among *C. tramdenum* populations.

Genetic diversity statistics for the populations of *C. tramdenum* are summarized in Table 2. The highest values of Shannon's Information index (I) and genetic diversity (h) were observed in the Backan population (0.440 and 0.293, respectively), while the Nghean population showed the lowest values for all indices (0.320 and 0.210 for Shannon's Information Index and Genetic diversity index, respectively).

At the population level, a relatively moderate to high genetic diversity of *C. trandenum* was detected. The effective numbers of alleles in the Bacgiang, Nghean, Ninhbinh, and Backan populations were 1.447, 1.359, 1.422, and 1.469 respectively (Table 2). Among the studied populations, the genetic diversity index varied from 0.210 to 0.293.

Analysis of molecular variance (AMOVA) results showed that the most of the genetic variation were accounted between individuals within population (78%), whereas among population genetic variation was 22% (Figure 2).

Nei's genetic distance between populations ranged from 0.116 (between Ninhbinh and Nghean populations) to 0.266 (between Backan and Nghean populations), with an average of 0.228 (Table 3).

Table 2. Genetic diversity parameters of four *C. tramdenum* populations

Population	Genetic diversity index			
	Na	Ne	I	h
Bacgiang	1.542	1.447	0.380	0.257
Nghean	1.497	1.359	0.320	0.210
Ninhbinh	1.625	1.422	0.387	0.249
Backan	1.854	1.469	0.449	0.293
Total	1.625	1.424	0.382	0.252
S.E.	0.050	0.026	0.019	0.013

Na: observed number of alleles, Ne: effective number of alleles, I: shannon's information index, h: genetic diversity index

Principal Components Analysis (PCoA) divided the 60 individuals into three different clusters across respective groups mostly reflecting the geographic origins (Figure 3). Genetic information accounted for 38.51% of the observed variance, the first two components explained 15.7 % and 12.4 % of the total variance, respectively. We also observed distinct groups of Backan and Bacgiang natural populations in a biplot of the first two components (Figure 3). These results also revealed that there was an association between genetic relationships of *C. tramdenum* populations and their geographic locations (where Nghean is closer to Ninhbinh than other locations) (Figure 1).

A dendrogram based on Nei's genetic distance using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) also divided the four populations into three groups with a low degree of genetic differentiation (Figure 4).

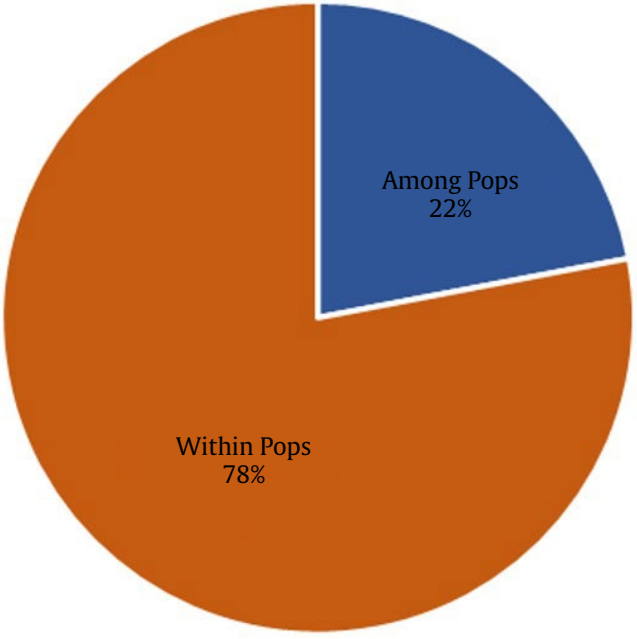

Figure 2. A pie chart of molecular variance estimated by AMOVA

Table 3. Nei's Genetic identity level of four populations

Population	Backan	Nghean	Ninhbinh	Bacgiang
Backan	0.000			
Nghean	0.266	0.000		
Ninhbinh	0.258	0.166	0.000	
Bacgiang	0.237	0.189	0.200	0.000

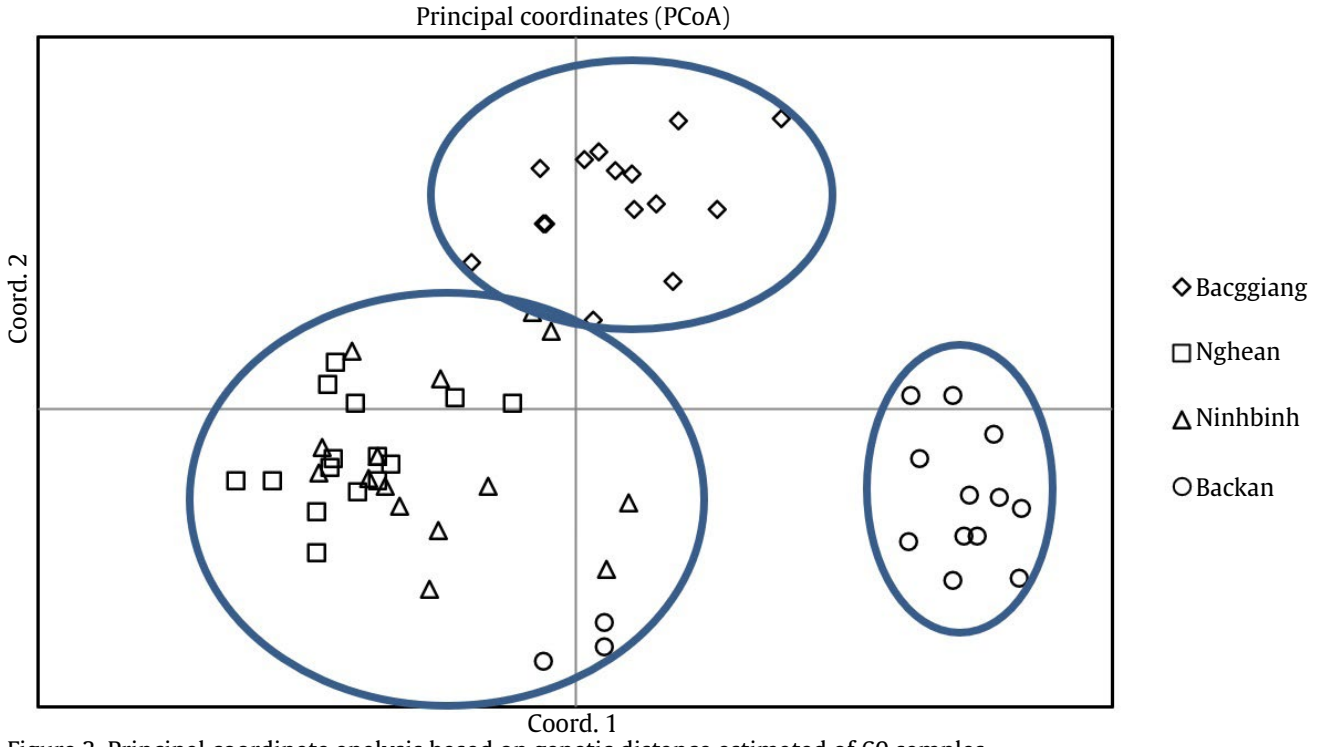

Figure 3. Principal coordinate analysis based on genetic distance estimated of 60 samples

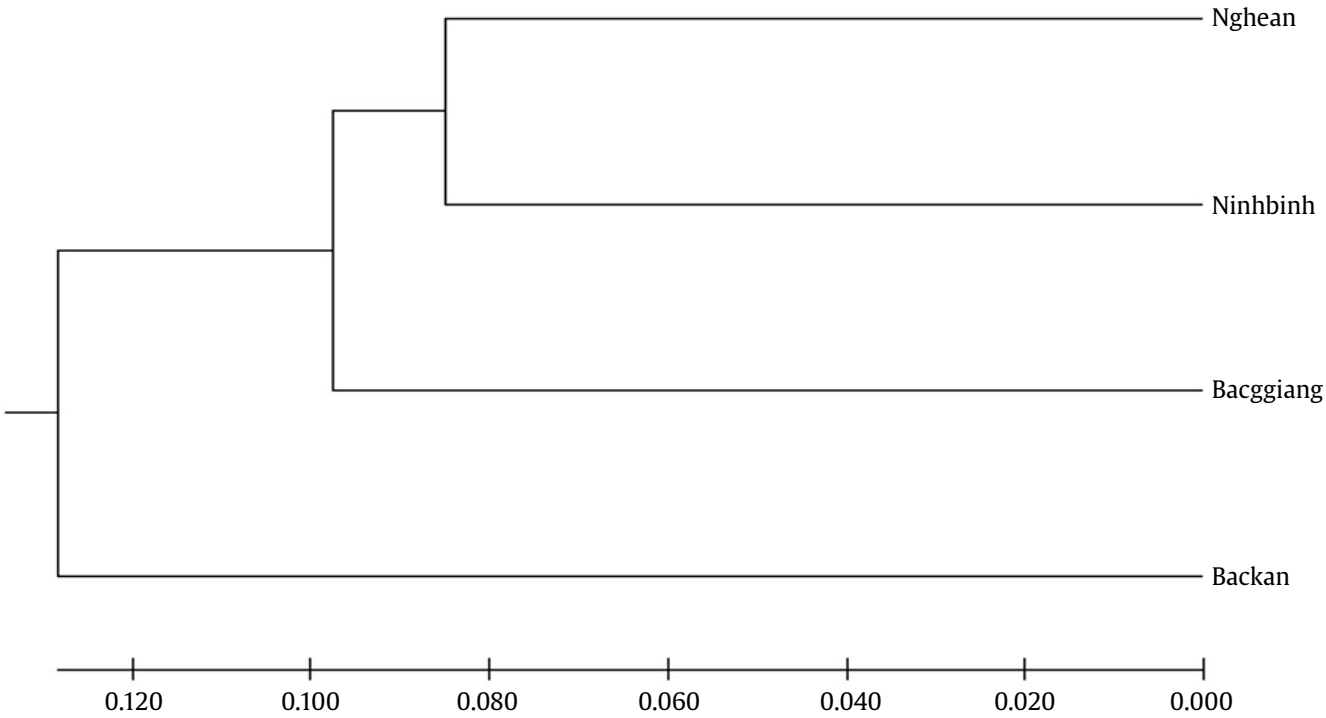

Figure 4. UPGMA dendrogram based on Nei's genetic distance matrix among four *C. tramdenum* natural populations. The bar on the bottom indicates the differentiation index based on the coefficient

4. Discussion

Information on the extent and distribution of genetic diversity is very important for the conservation and development of forest tree genetic resources in their natural habitat (Allendorf and Luikart 2007). This present study is the first application of ISSR markers to characterize and evaluate the genetic diversity within and among *C. tramdenum* populations in Vietnam.

4.1. The Power of ISSR Marker in the Eexamination of Genetic Diversity of *C. tramdenum*

Our study demonstrated the usefulness and utility of ISSR marker system as a molecular diagnostic tool to evaluate the genetic diversity and determine the pattern of genetic structure within and among different *C. tramdenum* populations in Vietnam. The average of amplification bands 9.6 per primer was obtained using 20 ISSR markers. This number was higher than that for the sister species *C. album* in China (average amplification 7.35 bands per marker) (Mei *et al.* 2017). In addition, two private ISSR bands were observed in the Backan population and one in the Bacgiang population. More extensive sampling should be conducted in the next stages to confirm that some natural populations contain private alleles. Private and unique bands could be transformed as distinct fingerprints into STS (Sequence-Tagged Site) and SCAR (Sequence Characterized Amplified Regions) markers (Agarwal and Shrivastava 2008) and therefore, private alleles could be developed as specific markers for the best management and accurate identification of the plant genetic materials of *C. tramdenum.*

The results also indicated the high level of genetic diversity of *C. tramdenum*. The average genetic diversity index (h) of *C. tramdenum* populations was 0.252 which is higher than that in other native species in Vietnam, for example, endemic pines (h = 0.114 and 0.115 for *P. armandii subsp. xuannhaensis* (Dinh *et al.* 2015), *P. dalatensis* (Nguyen *et al.* 2015), respectively), but remarkably lower than that in 19 *Canarium ovatum* accession in Philippines (Mendioro *et al.* 2008). Sandovan *et al.* (2017) also determined the high genetic diversity (0.62) of 79 *C. ovatum* accession and *C. luzonicum* by using microsatellite marker, despite the limitation of sample sizes. The cross-pollination during the cultivation is considered as one of main reason for the high genetic diversity in *C. ovatum* (Mendioro *et al.* 2008; Sandovan *et al.* 2017).

Genetic variation is the result of numerous factors including long-term evolutionary history, mutation, natural selection, mating system, genetic drift, gene flow and human impacts. Outbreeding species are in general likely to have a higher proportion of genetic variation within populations, whereas inbreeding species and those where vegetative propagation predominates are more likely to have higher proportions of between-population genetic variation (Rao and Hodgkin 2002). The AMOVA result, with 78% of the genetic variation within populations, suggests that outcrossing predominates in the breeding system of *C. tramdenum*. The mating system is expected to play a key role in determining the reproduction of the species, therefore, the information of the matting system is a key priority, to inform genetic conservation and breeding programs for *C. tramdenum* in Vietnam.

4.2. The Efficiency of ISSR Markers in Genetic Conservation Management of *C. tradenum*

Understanding the population structure and genetic relationships among natural populations provides valuable information for conservation, germplasm management and planning intraspecific crosses in *C. tramdenun* breeding programs. The AMOVA result showed that genetic variation within populations accounted for 78% of the total variation. The 22% of the variation between populations is a relatively high percentage compared to other studies, which are typically about 15-20% between populations for outcrossing species. The majority of genetic variation was within populations suggesting that genetic conservation efforts should sample more than one population, and furthermore that efforts should be made to sample additional populations in case there is further variation not present in these four populations. However, when the resources are insufficient, the conservation program should preserve at least three different populations: Backan, Bacgiang, and either Ninhbinh or Nghean population (Figure 3). Besides the protection of natural populations in different regions, the arboretum that contains a sufficient number of individuals of at least three populations as above seems to be the most efficient and suitable for long-term conservation of this species.

The genetic distance among populations provides a piece of basic information to support the conservation strategies in selecting genetic materials (natural populations, germplasm, seed) to ensure that the natural resources are well managed and utilized (Rao and Hodgkin 2002). Off spring of crosses between unrelated parents belong to genetically

diverse populations may display more heterosis (Meena *et al.* 2017). In this study, the Nei's genetic distance between populations ranged from 0.116 (between Ninhbinh and Nghean populations) to 0.266 (between Backan and Nghean populations), with an average of 0.228 (Table 3). Individuals belonging to populations with sufficient genetic distance could be potential parents for cross-pollination in *C. tramdenum* breeding programs to explore more heterosis in the future. Backan population with the high level of genetic diversity can be considered as a valuable genetic resource for parental selection in *C. tramdenum* breeding programs.

The results from this study are useful in the future for defining strategies for germplasm conservation, however, a successful management program of *C. tramdenum* cannot be based only on genetic diversity assessment. The combination of molecular, quantitative and chemical analyses is required for efficient genetic development and conservation strategies of this high values species.

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