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Analysis of genetic diversity in Pa Co pine (*Pinus kwangtungensis* Chun ex Tsiang) using RAPD and ISSR markers

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Abstract:

***Pinus kwangtungensis* (Pa Co pine) is one of three five-needle pine species in Vietnam, found on the slopes of limestone mountains at altitudes between 1200 and 1500 m. Global warming and long-term deforestation threaten the existence of the species in nature. The genetic diversity of plant populations provides a background for future conservation and improvement programmes. However, the genetic diversity of Pa Co pine is unknown. This study aimed to use inter-simple sequence repeat (ISSR) and random amplified polymorphic DNA (RAPD) genetic markers to evaluate the genetic-diversity parameters of *P. kwangtungensis* to understand the genetic effects of small and fragmented populations, as well as provide the genetic background for its conservation. Total genomic DNA was extracted from fresh needles of 40 trees in four different areas and amplified with 15 RAPD and 16 ISSR markers. Results indicated that the genetic diversity index (h) of *P. kwangtungensis* was 0.2530 with RAPD and 0.223 with ISSR. High genetic variation was found within populations (72% with RAPD and 87% with ISSR). Principal coordinates analysis based on RAPD analysis revealed that the presence of three groups was in accordance, whereas no clear cluster was formed according to ISSR analysis. The results from this study enhance the understanding of the genetic effects of small and fragmented populations of native species that are rare, vulnerable, and require conservation.**

Keywords: **conservation, genetic diversity, ISSR, *Pinus kwangtungensis*, RAPD.**

Classification number: **3.5**

Introduction

Pa Co pine (*Pinus kwangtungensis* Chun ex Tsiang) is one of three five-needle pines in Vietnam; the two others are Da Lat pine (*P. dalatensis* Ferre) and Xuan Nha pine (*P. armandii* subsp. *xuannhaensis* L.K.Phan). It grows naturally in the Northwest region on the slopes of limestone mountains at altitudes between 1200 and 1500 m [1]. This region has a type of tropical climate, but the winters are cold. The mean annual temperature is 14-20°C and the average rainfall exceeds 1200 mm [2]. Pa Co pine is found in northern Vietnam (including Cao Bang, Son La, Hoa Binh, and Thanh Hoa) provinces. Trees can reach 20 m in height and 70 cm in diameter at breast height. The two images in Fig. 1 show a *P. kwangtungensis* tree. It has scaly, brown, and rough bark, with leaves (needles) in bundles of 2-5 per fascicle. The needles are 3-7 cm long and 1-1.5 mm wide. Female cones are cylindrical or ovoid, up to 8 cm long, and 1.5-7 cm wide. The pendant has a short angled peduncle at maturity, either solitary or in pairs. Seed scales are obovate with rhombic apophysis, a thin apex, and umbo depressed. Seeds are ellipsoid- or ovoid-shaped, 0.8-1.2 cm in size, and with wings 2-3 cm long. When on the tree, the opening and release of seeds are not persistent. Furthermore, seed maturation occurs 2 years after pollination. The species has multiple uses. Its timber is useful for constructing houses and furniture as well as developing infrastructure. In addition, local people often use this tree for medicine [3] and to make bonsai trees [2] for ornamental purposes. However, this species is threatened by the rapid global population growth rate along with climate change. In the IUCN Red List of Threatened Species, *P. kwangtungensis* is listed as Near Threatened [4]. In Vietnam, Pa Co pine is listed as a vulnerable species (VU A1acd, B1+2bce). Pa Co

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pine populations are small, fragmented, restricted habitat, and lack of the natural regeneration. These populations are also persistent logging while the number of mature trees is limited [5]. Therefore, the conservation of this vital species is essential to prevent it from going extinct.

Fig. 1. Pa Co pine tree (left) and branch with needles and cone (right). Photo: Trinh Ngoc Bon, Silvicuture Research Institute, Vietnamese Academy of Forest Sciences.

Studying the genetic diversity in a plant population provides basic information for future conservation and improvement programmes. The application of a molecular marker system is a quick and effective tool for studying genetic diversity. Several molecular marker systems, including random amplified polymorphic DNA (RAPD), inter-simple sequence repeat (ISSR), and restriction fragment length polymorphism have been used to study the genetic diversity of conifers [6-9]. Previous studies on the genetic variation of conifers such as *Cunninghamia lanceolata* var. *konishii* [10], *Fokienia hodginsii* [11], *Glyptostrobus pensilis* [12], and *Pinus kremfii* [9] in Vietnam have revealed low levels of genetic differentiation among populations. Genetic diversity studies have also been conducted for other crucial native species such as *Erythrophleum fordii* Oliv. [13], *Hopea cordata* Vidal [14], *Afzelia xylocarpa* Kurz [15], and *Dipterocarpus alatus* Roxb [16]. These studies have been significant for the conservation plans of these species. At present, knowledge on the genetic diversity of *P. kwantungensis* is lacking, which is a barrier to the development of a conservation strategy. Therefore, exploring the genetic diversity in *P. kwangtungensis* is critical for designing a future conservation plan for this species.

The objective of this study was to analyse the existing level of genetic variability in *P. kwangtungensis* populations using RAPD and ISSR markers. Compared with other molecular markers, RAPD and ISSR are easy, cost-effective, and fast tools for studying genetic diversity. Moreover, they do not require prior knowledge of the flanking sequence of the genome of the species concerned [17]. The results will provide the background for the conservation, management, and restoration of this species.

Methodology

Sample collection

Fresh leaves of 40 individual *P. kwangtungensis* trees were collected from four sites, and these are listed in Table 1. The sizes of the populations were 15, 3, 20, and 2 in Moc Chau town - Moc Chau - Son La, Muong Sang - Moc Chau - Son La, Hang Kia - Mai Chau - Hoa Binh, and Pa Co - Mai Chau - Hoa Binh, respectively. The samples were kept in plastic bags with silica gel in the field, transferred to Molecular Biology Laboratory (Institute of Forest Tree Improvement and Biotechnology), and stored at 4°C until DNA extraction.

Table 1. Sample collection locations of *P. kwangtungensis* and the trees' status.

No	Sample ID	Regions	Geographic location		Elevation (m)	Tree status
			Longitude	*Latitude*		
1	MC1	Moc Chau town - Moc Chau - Son La	20°53'18.9"	104°38'7.2"	1189	Old
2	MC2	Moc Chau town - Moc Chau - Son La	20°53'18.7"	104°38'7.2"	1196	Old
3	MC3	Moc Chau town - Moc Chau - Son La	20°53'18.9"	104°38'7.2"	1197	Old
4	MC4	Moc Chau town - Moc Chau - Son La	20°53'18.6"	104°38'7.2"	1204	Old
5	MC5	Moc Chau town - Moc Chau - Son La	20°53'18.5"	104°38'7.2"	1220	Old
6	MC6	Moc Chau town - Moc Chau - Son La	20°53'18.3"	104°38'7.2"	1222	Old
7	MC7	Moc Chau town - Moc Chau - Son La	20°53'18.2"	104°38'7.2"	1224	Old
8	MC8	Moc Chau town - Moc Chau - Son La	20°53'17.4"	104°38'7.2"	1226	Old
9	MC9	Moc Chau town - Moc Chau - Son La	20°53'17.0"	104°38'7.2"	1230	Old

10	MC10	Moc Chau town - Moc Chau - Son La	20°53'16.5"	104°38'7.2"	1225	Old
11	MC11	Moc Chau town - Moc Chau - Son La	20°53'16.6"	104°38'7.2"	1219	Young
12	MC12	Moc Chau town - Moc Chau - Son La	20°53'16.3"	104°38'7.2"	1222	Old
13	MC13	Moc Chau town - Moc Chau - Son La	20°53'16.9"	104°38'7.2"	1221	Young
14	MC14	Moc Chau town - Moc Chau - Son La	20°53'15.5"	104°38'7.2"	1219	Young
15	MC15	Moc Chau town - Moc Chau - Son La	20°53'12.6"	104°38'7.2"	1212	Old
16	MS1	Muong Sang - Moc Chau - Son La	20°53'36.6"	104°37'8.2"	1180	Young
17	MS2	Muong Sang - Moc Chau - Son La	20°53'39.4"	104°37'5.7"	1212	Old
18	MS3	Muong Sang - Moc Chau - Son La	20°53'39.7"	104°37'4.8"	1208	Old
19	HK1	Hang Kia - Mai Chau - Hoa Binh	20°44'0.8"	104°53'23.1"	1409	Young
20	HK2	Hang Kia - Mai Chau - Hoa Binh	20°44'0.9"	104°53'23"	1412	Young
21	HK3	Hang Kia - Mai Chau - Hoa Binh	20°44'1.5"	104°53'23.2"	1412	Old
22	HK4	Hang Kia - Mai Chau - Hoa Binh	20°44'1.9"	104°53'23.6"	1411	Old
23	HK5	Hang Kia - Mai Chau - Hoa Binh	20°44'2.5"	104°53'24.1"	1386	Tree regeneration
24	HK6	Hang Kia - Mai Chau - Hoa Binh	20°44'13.3"	104°53'25.0"	1398	Young
25	HK7	Hang Kia - Mai Chau - Hoa Binh	20°44'4.7"	104°53'25.4"	1398	Old
26	HK8	Hang Kia - Mai Chau - Hoa Binh	20°44'4.9"	104°53'25.3"	1394	Old
27	HK9	Hang Kia - Mai Chau - Hoa Binh	20°44'4.5"	104°53'25.6"	1393	Young
28	HK10	Hang Kia - Mai Chau - Hoa Binh	20°44'33.5"	104°53'46.8"	1411	Old
29	HK11	Hang Kia - Mai Chau - Hoa Binh	20°44'35.5"	104°53'44.6"	1411	Old
30	HK12	Hang Kia - Mai Chau - Hoa Binh	20°44'35.7"	104°53'44.3"	1410	Old
31	HK13	Hang Kia - Mai Chau - Hoa Binh	20°44'36.2"	104°53'43.6"	1407	Old
32	HK14	Hang Kia - Mai Chau - Hoa Binh	20°44'36"	104°53'44.2"	1400	Old
33	HK15	Hang Kia - Mai Chau - Hoa Binh	20°44'36.3"	104°53'43.8"	1406	Old
34	HK16	Hang Kia - Mai Chau - Hoa Binh	20°44'37.8"	104°53'43.2"	1389	Old
35	HK17	Hang Kia - Mai Chau - Hoa Binh	20°44'37.9"	104°53'43"	1384	Old
36	HK18	Hang Kia - Mai Chau - Hoa Binh	20°44'37.9"	104°53'42.9"	1382	Old
37	HK19	Hang Kia - Mai Chau - Hoa Binh	20°44'38.2"	104°53'42.7"	1366	Old
38	HK20	Hang Kia - Mai Chau - Hoa Binh	20°44'38.5"	104°53'42.6"	1357	Old
39	PC1	Pa Co - Mai Chau - Hoa Binh	20°44'38.2"	104°53'42.7"	1306	Young
40	PC2	Pa Co - Mai Chau - Hoa Binh	20°44'38.5"	104°53'42.6"	1326	Young

DNA extraction

Total genomic DNA was extracted from fresh needles. Approximately 100 mg of each sample was used. Leaves were ground into a fine powder in liquid nitrogen and DNA was extracted using the hexadecyltrimethylammonium bromide (CTAB) method [18]. DNA was run on 0.8% agarose gel in 1X TAE buffer through electrophoresis at 90V for 20 mins. DNA concentrations were measured using a the NanoDrop™ ND-1000 UV-Vis spectrophotometer (Thermo Scientific, USA) and then aliquoted to a concentration of 10 ng/µl.

Polymerase chain reaction (PCR) amplification

We used 15 RAPD and 16 ISSR primers (Integrated DNA Technologies, USA) for this study. Table 2 lists the primers and their sequences. PCR amplification was performed in a 20 µl volume containing 50 ng of DNA, 2X PCR MasterMix Buffer (Thermo Scientific, USA), and 1 µM primers. The RAPD-PCR steps were as follows: 3 min at 94°C, followed by 40 cycles of 1-min denaturing at 94°C, 1 min at 37°C, and 1.5 min of elongation at 72°C, before ending with 7 min at 72°C. The ISSR-PCR steps were as follows: 5 min at 94°C, followed by 40 cycles of 45 s at 94°C, 45 s at 56°C, and 1 min 30 s at 72°C, before ending with 7 min at 72°C.

The PCR products were run on 2% agarose gels in 1X TAE buffer to separate the bands; a 1 kb ladder (Thermo Scientific, USA) was used as the DNA standard. The gels were visualised and captured using the DigiDoc-It™ Imagine system (Analytik Jena Company, USA).

Table 2. List of RAPD and ISSR primers used for PCR amplification.

No	RAPD primer	Sequence (5'-3')	No	ISSR primer	Sequence (5'-3')
1	OPA4	AATCGGGCTG	1	UBC807	AGAGAGAGAGAGAGAGT
2	OPA6	GGTCCCTGAC	2	UBC818	CACACACACACA CACAG
3	OPC15	GACGGATCAG	3	UBC824	TCTCTCTCTCTCTCTCG
4	OPC19	GTTGCCAGCC	4	UBC834	AGAGAGAGAGAGAGAGYT
5	OPD12	CACCGTATCC	5	UBC835	AGAGAGAGAGAGAGAGYC
6	OPE3	CCAGATGCAC	6	UBC836	AGAGAGAGAGAGAGAGYA
7	OPE14	TGCGGCTGAG	7	UBC843	CTCTCTCTCTCTCTCTGA
8	OPF1	ACGGATCCTG	8	UBC851	GTGTGTGTGTGTGTGTCTG
9	OPL18	ACCACCCACC	9	UBC855	ACACACACACACACACT
10	OPP9	GTGGTCCGCA	10	UBC856	ACACACACACACACACYA
11	OPR3	ACACAGAGGG	11	UBC881	GGGTGGGGTGGGGTG
12	OPV15	CAGTGCCGGT	12	HB10	GAGAGAGAGAGACC
13	OPAB6	GTGGCTTGGA	13	HB12	CACCACCACGC
14	UBC210	GCACCGAGAG	14	HB15	GTGGTGGTGGC
15	UBC218	CTCAGCCCAG	15	ISCS14	AGTGAGTGAGTGAGTGAGTGA
			16	ISCS34	TGTGTGTGTGTGTGTGRC

Data analysis

The amplification fragments from using RAPD and ISSR were scored according to a binary matrix, where 0 and 1 were coded for the absence and presence of a band, respectively. The genetic diversity index was calculated using the software POPGENE v1.32 [19]. Analysis of molecular variance (AMOVA) and principal coordinate analysis (PCoA) were conducted using GenAlEx v6.502 software [20, 21].

Results

Amplification results of RAPD and ISSR

For the 40 samples from four populations of *P. kwangtungensis*, 15 RAPD primers generated 59 bands ranging in size from 250 to 2000 bp, in which 54 bands were polymorphic loci (91.53%). The 16 ISSR primers produced a total of 142 fragments ranging in size from 250 to 3000 bp, in which 134 bands were polymorphic loci (94.37%). Figs. 2 and 3 are examples of primer amplification results in agarose gel through electrophoresis.

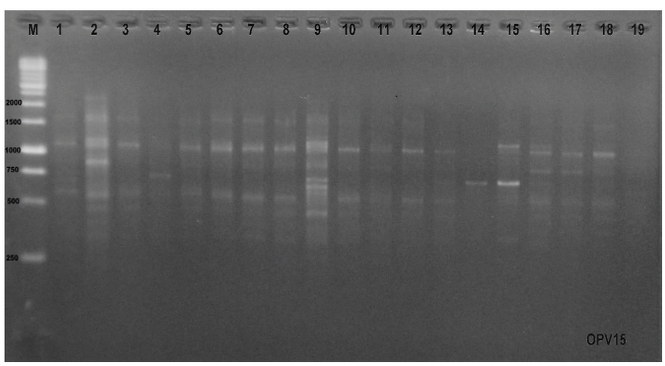

Fig. 2. RAPD amplification results using OPV15 primer. Lane 1-19: representative DNA samples. M: marker 1 kb.

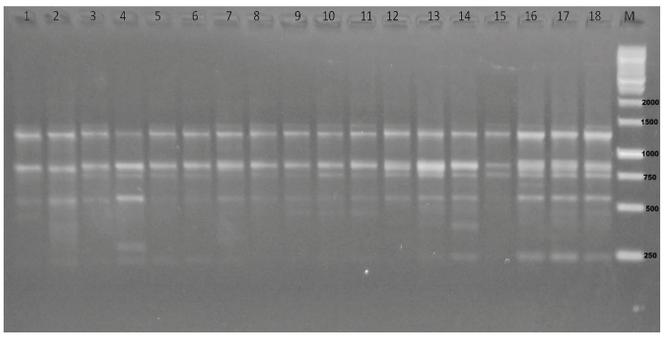

Fig. 3. ISSR amplification results using UBC807 primer. Lane 1-18: representative DNA samples. M: marker 1 kb.

Genetic diversity index

Table 3 shows the genetic diversity of the four populations. Based on RAPD analysis, the range of the mean number of alleles was 1.0508-1.7797, the effective number of alleles (*Ne*) was 1.0360-1.4037, the Shannon index (*I*) was 0.0307-0.3663, and Nei's genetic diversity (heterozygosity, *h*) was 0.0211-0.2404. Based on ISSR, the range of the mean number of alleles was 1.1338-1.7817, *Ne* = 1.0946-1.3363, *I* = 0.0809-0.3279, and *h* = 0.0554-0.2092. The Moc Chau population exhibited the highest genetic diversity based on RAPD, whereas the Hang Kia population exhibited the highest based on ISSR. The Pa Co population had the lowest variation in both analyses.

Table 3. Genetic diversity parameter of the four populations.

Marker	Index		Moc Chau	Muong Sang	Hang Kia	Pa Co	All
RAPD	N_a	Mean	1.7797	1.1356	1.6441	1.0508	1.1953
		SD	0.4180	0.3453	0.4829	0.2216	0.2809
	N_e	Mean	1.4037	1.1114	1.3344	1.0360	1.4216
		SD	0.3622	0.2961	0.3693	0.1567	0.3592
	h	Mean	0.2404	0.0598	0.1974	0.0211	0.2530
		SD	0.1886	0.1552	0.1978	0.0918	0.1783
	I	Mean	0.3663	0.0854	0.2993	0.0307	0.3904
		SD	0.2609	0.2200	0.2801	0.1340	0.2382
	Number of polymorphic loci		6	8	38	3	54
	PPB (%)		77.97	13.56	64.41	5.08	91.53
ISSR	N_a	Mean	1.7535	1.2746	1.7817	1.1338	1.9437
		SD	0.4325	0.4479	0.4146	0.3416	0.2314
	N_e	Mean	1.3190	1.1989	1.3363	1.0946	1.3467
		SD	0.3437	0.3521	0.3321	0.2416	0.3063
	h	Mean	0.1959	0.1115	0.2092	0.0554	0.2223
		SD	0.1802	0.1883	0.1753	0.1415	0.1582
	I	Mean	0.3076	0.1626	0.3279	0.0809	0.3561
		SD	0.2507	0.2705	0.2451	0.2066	0.2128
	Number of polymorphic loci		107	39	111	19	134
	PPB (%)		75.35	27.46	78.17	13.38	94.37

Note: N_a: number of observed alleles; N_e: number of effective alleles; *h*: Nei's (1973) gene diversity; *I*: Shannon index; PPB: percentage of polymorphic bands.

The results of the AMOVA (Table 4) in both the RAPD and ISSR analyses revealed that most of the variation was within populations (72% for RAPD and 87% for ISSR).

Table 4. Analysis of molecular variance of *P. kwangtungensis*.

	RAPD markers	ISSR markers
Among populations	28%	13%
Within populations	72%	87%
PhiPT	0.283, p≥0.001	0.126, p≥0.001

Genetic similarity and cluster analyses of genetic distances

Tables 5 and 6 show the Nei's [22] genetic identity and distance of populations based on RAPD and ISSR, respectively. For both markers, the largest genetic distance was found between Muong Sang and Pa Co (0.3042 with RAPD and 0.2470 with ISSR), and the smallest was found between Moc Chau and Hang Kia (0.0924 with RAPD and 0.0397 with ISSR). The genetic identity showed the same result when the largest identity was between populations of Moc Chau and Hang Kia, and the smallest was between Muong Sang and Pa Co.

Table 5. Nei's genetic identity (above diagonal) and genetic distance (below diagonal) using RAPD markers.

Population	Moc Chau	Muong Sang	Hang Kia	Pa Co
Moc Chau	-	0.8884	0.9117	0.8178
Muong Sang	0.1183	-	0.7928	0.7377
Hang Kia	0.0924	0.2321	-	0.8845
Pa Co	0.2011	0.3042	0.1227	-

Table 6. Nei's genetic identity (above diagonal) and genetic distance (below diagonal) by ISSR markers.

Population	Moc Chau	Muong Sang	Hang Kia	Pa Co
Moc Chau	-	0.9159	0.9611	0.8553
Muong Sang	0.0879	-	0.9066	0.7811
Hang Kia	0.0397	0.0981	-	0.8858
Pa Co	0.1563	0.2470	0.1213	-

A dendrogram-based Nei's genetic distance using UPGMA (Unweighted Pair Group Method with Arithmetic Mean), which was modified from the NEIGHBOR procedure of PHYLIP Version 3.5, is shown in Fig. 4 for RAPD and Fig. 5 for ISSR to reveal the genetic relationship among the four populations. These four populations were divided into three groups: Moc Chau and Hang Kia were in one group with low genetic distance, whereas Pa Co and Muong Sang were separated into two different groups.

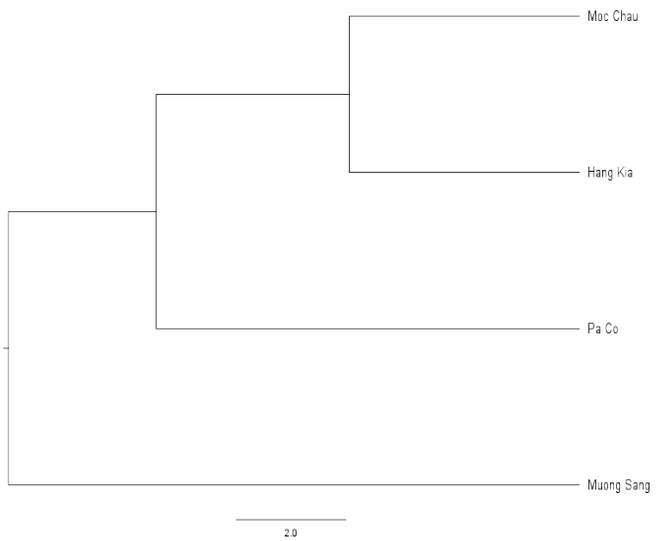

Fig. 4. Genetic distance dendrogram for populations of *P. kwangtungensis* using RAPD markers.

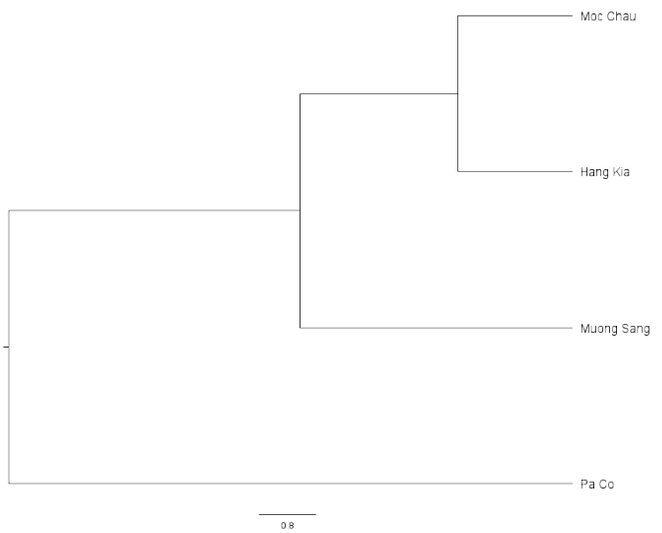

Fig. 5. Genetic distance dendrogram for populations of *P. kwangtungensis* using ISSR markers.

Figures 6 and 7 presents the results of the PCoA using RAPD and ISSR markers, respectively. The first two components of PCoA explained 37.54% of the variation in RAPD and 16.52% in ISSR markers. In the RAPD PCoA (Fig. 6), three clusters were generated. All Muong Sang population samples and most Moc Chau samples formed one group. The second group consisted of most individuals of the Hang Kia population. The Pa Co population formed the third group with some representative accessions of Hang Kia (HK1, HK2, HK4, HK5, HK6, and HK18) and Moc Chau populations (MC1, MC4, and MC14). No distinct cluster was identified in the ISSR PCoA analysis (Fig. 7).

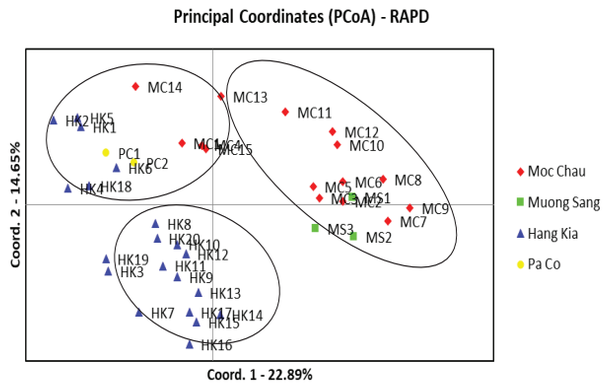

Fig. 6. PCoA revealing the genetic relationships among individuals using RAPD markers.

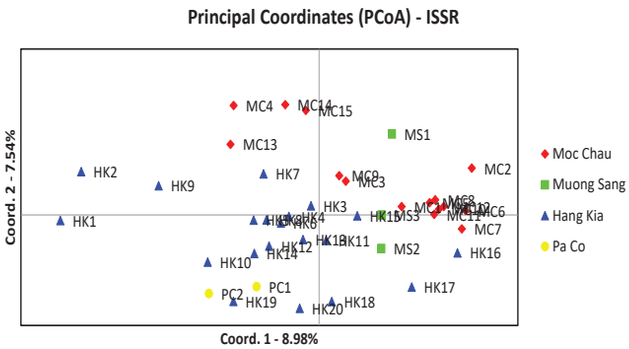

Fig. 7. PCoA revealing the genetic relationships among individuals using ISSR markers.

Discussion and conclusions

This is the first attempt to study the genetic diversity of *P. kwangtungensis* in Vietnam using molecular markers. Based on the RAPD analysis, the genetic parameters revealed the widest genetic diversity in the Moc Chau (Son La) populations and the narrowest in Pa Co (Hoa Binh). By contrast, the ISSR analysis showed the highest variation in the Hang Kia (Hoa Binh) populations and lowest in Pa Co. Moreover, the number of individuals in each population varied considerably. The sizes of the populations were 15 in Moc Chau and and 20 in Hang Kia. By contrast, the Muong Sang (Son La) and Pa Co populations only had three and two samples, respectively. Further analysis with the higher number of samples of these two populations should be conducted in the future to fully examine the genetic resources of *P. kwangtungenesis* in Vietnam.

Using ISSR markers, the mean of genetic diversity for *P. kwangtungensis* in this study (h = 0.2223) was slightly higher than two other fine-needle pines in Vietnam, namely *P. dalatensis* (h = 0.115) [23] and *P. armandii* subsp. *xuannhaensis* (h = 0.114) [24]. Furthermore, these results were consistent with other studies of genetic diversity in threatened conifer species in Vietnam, such as *Taxus chinensis* (I = 0.202) and *Taxus wallichiana* (I = 0.217) [25] as well as *Cunninghamia lanceolata* var. *konishii* (I = 0.2355) [10]. The AMOVA revealed that most of the genetic diversity resided within *P. kwangtungensis* populations (Table 5). These findings were similar to those of studies on other conifer species [12, 23, 25].

The high level of genetic variability within the species might mainly be caused by: (1) the size and fragmented distribution of natural populations; (2) changes in the original vegetation structure and/or the invasion of exotic species in small forest patches of the species; and (3) logging activities or human interference. The natural distributions of *P. kwangtungensis* occurred in Vietnam's Northwest region on the slopes of limestone mountains at altitudes between 1200 and 1500 m [1] and remain in such small patches. These small and fragmented habitats may prevent gene flow among the populations and result in breeding, thereby leading to a decrease in genetic diversity [24]. In addition, human activities such as timber exploitation and agricultural-land expansion contribute to the low number of observed individuals in the natural population.

In conclusion, the genetic diversity of species is crucial for the conservation of genetic resources. In this study, we found a wide range of variation among accessions. The Moc Chau population showed the highest level of genetic diversity and the Pa Co population showed the lowest. This study explored three distinct groups of populations from 40 collected samples of Pa Co pine. Strong genetic similarities were observed between the Moc Chau and Hang Kia populations. The large variability in the number of samples from different populations may have influenced the identification of actual variability within and among the populations. The low number of trees available in the natural habitat emphasised the urgency of developing and implementing a conservation strategy for this species. The long-term conservation of this species should involve in-situ conservation through strict protection from illegal logging, ex-situ conservation through propagating and replanting in new places, and extending genetic diversity by artificial crossing.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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