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FULL-LENGTH RESEARCH ARTICLE

Genetic Diversity and Population Structure in Chestnut (*Castanea* spp.) Varieties Revealed by RAPD and SRAP Markers

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Abstract Chestnut (*Castanea* spp.) species give delicious fruits containing balanced nutriments and their varieties have been mainly distinguished by nuts and leaves. Because these morphological traits are influenced by environmental factors, it may be impracticable to distinguish various varieties exactly based solely on morphological traits. We used RAPD and SRAP markers to assess genetic relationship among exotic varieties and native ones including 'SongchonBam' and 'KumyaWangBam'. UPGMA phylogenetic tree and PcoA analysis divided 35 chestnut varieties into 2 major clusters at the genetic distance of 0.26–0.30. According to STRUCTURE analysis, all samples were divided into two groups corresponding to two species (*C. mollissima*, *C. crenata*) in case $K = 2$. The markers selected in this study would be useful to provide detailed information about genetic diversity of chestnut germplasms to assist in breeding and conservation strategies.

Keywords Chestnut · RAPD · SRAP · Genetic diversity · Population structure

Introduction

Chestnut (*Castanea* spp.) species belonging to the genus *Castanea,* Fagaceae give delicious fruits containing balanced nutriments. Among 13 chestnut species distributed in Northern Hemisphere, four species such as Korean chestnut (*Castanea crenata* Sieb. and Zucc.), Chinese chestnut (*C. mollissima* Blume), European chestnut (*Castanea sativa* Mill.) and American chestnut [*C. dentata* (Marsh.) Borkh.] are cultivated for fruit production. In DPR Korea, Korean chestnut and Chinese chestnut are cultivated.

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Chestnut trees represent one of the important industrial crops in the Democratic People's Republic of Korea. Chestnut trees give many benefits including delicious fruits, good timbers, flowers and peels as medicinal stuff. In addition, chestnut honey made from flowers is very appreciated and tea made from chestnut leaves and flowers is used for mucolytic, antispasmodic and anti-dysenteric treatments [17]. It has been reported that the leaves have an antioxidant activity [4] and flowers of chestnut trees are useful to treat candidiasis [1].

C. mollissima (Chinese chestnut) is the main native chestnut species of China and '*mollissima*' in its name stems from the presence of soft pubescence on the back of the fresh branches and leaves. Chestnut varieties such as 'SongchonBam', 'HamjongBam', 'Hwagwang', 'Honggwang' and 'Jungil No1.' belong to Chinese chestnut species. *C. mollissima* grows in subtropical, temperate continental and temperate oceanic regions characterized by hot summers and mild winters. *C. mollissima* has been introduced into many countries due to its flexibility and adaptability to different soil and climates. Although *C. mollissima* exhibits the highest resistance to chestnut blight

(*Cryphonectria parasitica*.), ink disease and *Phytophthora* spp. so that it has been introduced to North America and has been used widely as starting material for breeding, it is susceptible to Asian Chestnut Gall Wasp, *Dryocosmus kuriphilus* Yasmatsu.

C. crenata (Korean chestnut) is cultivated in Asia, including DPR Korea and the north-eastern region of China, Japan, and prefers temperate summer and winter climate with sufficient rainfall of 1200–1400 mm/year in summer. Korean chestnut includes majority of chestnut varieties distributed in DPR Korea such as 'Kumya-WangBam' and 'KosongWangBam'. It is considered as one of the most important sources of resistance to *Phytophthora* spp., and its germplasm has been widely used as breeding material to confer *Phytophthora*-resistance. Although many varieties belonging to Korean chestnut exhibit good nut qualities, they would be infected by gall wasp (*Dryocosmus kuriphilus*).

Traits associated with nuts and leaves are important to characterize chestnut varieties. Major characteristics of the nuts include size of fruit, size and shape of hilum, peeling of endothelium, length of style and sugar content in fruits.

Varieties belonging to *C. mollissima* have smaller fruit than those to *C. crenata*. In addition, hilum is close to a square or a rectangle in *C. mollissima* while in *C. crenata,* hilum is round. The endothelium of fruits in *C. mollissima* cultivars peel well, but it is difficult to peel endothelium of fruits in *C. crenata*. In addition, the length of style in *C. mollissima* varieties is a little longer than in *C. crenata*.

Specifically, fruits of *C. mollissima* taste sweeter than those of *C. crenata*. The leaves of *C. mollissima* cultivars are wider and longer than those of *C. crenata* cultivars. In general, chestnut trees have a laciniate at the edge of the leaf, and varieties belonging to *C. mollissima* have wider-spaced, longer and wider laciniate than those of varieties belonging to *C. crenata.* The colour of leaves in *C. mollissima* cultivars is deeper than that of *C. crenata* cultivars. The stalk colour of *C. mollissima* cultivars is greyer than that of *C. crenata* cultivars. However, these morphological observations described are unlikely to be considered effective sufficiently and tend to be influenced by environmental and developmental factors [2]. For example, in *C. sativa* Mill. var. 'Judia', trees growing at different latitudes accompanied by different temperature-exposed areas exhibit significant differences in leaf traits [7, 11]. For these reasons, although traits associated with nuts and leaves are important phenotypic traits that differentiate chestnut cultivars, several studies have been reported to use molecular tools with phenotypic traits in chestnut cultivar classification because morphological traits are likely to be influenced by environmental factors.

Genetic distinction among six cultivars of *Castanea sativa* Mill. has been revealed using RAPD and ISSR markers [13]. Microsatellite markers have been developed and characterized for *Castanea sativa* Mill [6]. Genetic diversity and genetic structure in 10 populations of Chinese chestnut (*Castanea mollissima* BIume) from Shandong Province have been revealed using ISSR markers [5]. Interspecific hybrids between European chestnut and Chinese chestnut with putative resistance to *Phytophthora cinnamomi* have been identified by using SSR markers [12]. Genetic distinction between three European chestnut populations (Huelva, Malaga, Sevilla) has been clarified by using SSR and EST-SSR markers [16]. Although molecular studies on chestnut cultivars have been carried out using RAPD, ISSR, SSR, and EST-SSR markers in European species, molecular study on Chinese and Korean chestnut cultivars grown in DPR Korea has not been reported.

This study aims to use RAPD and SRAP markers to reveal genetic relationship of 35 chestnut cultivars distributed and cultivated in DPR Korea to assist in conservation and breeding strategies.

Materials and Methods

Thirty-five chestnut cultivars (Table 1) cultivated in DPR Korea were used as material. As shown in Table 1, the cultivars such as 'Taesong No.1', 'TaesongOlBam', 'Ryongbong', 'Pukun', 'Paekbam', 'SongchonKunBam', 'OunBam', 'Changgwang' belong to *C. crenata*, and their leaf shape is similar to Chinese chestnut (*C. mollissima*), while 'Hwagwang', 'Honggwang' 'Jungil No.1' belong to *C. mollissima* and the leaf shape is similar to *C. crenata*.

Genomic DNA Isolation

Fresh young leaf samples were collected from one plant of each cultivars. Genomic DNA was isolated from 100 mg of fresh leaf material using CTAB method [8].

Qualitative and Quantitative Estimation of DNA

DNA quality was assessed by using Nanodrop Spectrophotometer (Thermoscientific Nanodrop 1000, USA). DNA samples exhibiting 1.8–2.0 value of absorbance ratio of 260 and 280 nm were considered to be pure DNA. Quantity of DNA was assessed by 0.8% agarose gel run in 1X TAE buffer at 50 V for 45 min.

RAPD PCR Amplification

RAPD PCR amplification was performed to amplify randomly unknown target sequences by using random primers according to the protocol [21]. PCR was performed in a total volume of 20 µl composed of 10X Taq buffer 2 µl,

Table 1 General characteristics of 35 chestnut cultivars used in this study

No	Name	Biotic stress resistance	Abiotic stress tolerance	Flavour	Seed size[a]	Specific name
1	KosongWangBam	High	Low	Ordinary	Big	*C. crenata*
2	PaechonOlBam	Low	Low	Ordinary	Ordinary	*C. crenata*
3	Taesong no.1	High	High	Good	Big	–
4	WangBam no.1	High	Low	Ordinary	Big	*C. crenata*
5	Manjok	Low	Low	Ordinary	Ordinary	*C. crenata*
6	Taesong no.2	Low	Low	Ordinary	Ordinary	*C. crenata*
7	Unjon no.1	Low	Low	Ordinary	Big	*C. crenata*
8	Taesong no.3	High	High	Good	Ordinary	*C. crenata*
9	PukchangOlBam	Low	Low	Ordinary	Ordinary	*C. crenata*
10	Togenashi	Low	Low	Ordinary	Big	*C. crenata*
11	Taesong OlBam	Low	Low	Ordinary	Ordinary	–
12	WangBam no.2	Low	Low	Ordinary	Big	*C. crenata*
13	TosanBam no.13	High	High	Ordinary	Big	*C. crenata*
14	Samjoseng	Low	Low	Ordinary	Ordinary	*C. crenata*
15	KaesongOlBam	High	Low	Ordinary	Ordinary	*C. crenata*
16	RyongbongBam	Low	Low	Good	Big	–
17	Pukun	Low	Low	Ordinary	Big	–
18	PaekBam	Low	Low	Good	Big	–
19	KumyaWangBam	High	High	Ordinary	Big	*C. crenata*
20	WonsanBam no.3	Low	Low	Ordinary	Big	*C. crenata*
21	Okgwang	Low	Low	Ordinary	Ordinary	*C. crenata*
22	UnjonBam	Low	Low	Ordinary	Ordinary	*C. crenata*
23	Pungsan	Low	Low	Ordinary	Ordinary	*C. crenata*
24	WonsanKunOlBam	Low	Low	Ordinary	Ordinary	*C. crenata*
25	SongchonKunYaBam	Low	Low	Ordinary	Ordinary	*C. crenata*
26	OunBam	High	Low	Ordinary	Big	–
27	RyongnamBam	Low	Low	Ordinary	Big	*C. crenata*
28	Hwagwang	Low	Low	Ordinary	Ordinary	–
29	Hongguang	Low	Low	Ordinary	Ordinary	–
30	Jungil no.1	Low	Low	Ordinary	Small	–
31	ChanggwangBam	Low	High	Good	Ordinary	*C. crenata*
32	WonsanBam no.2	Low	Low	Ordinary	Small	*C. crenata*
33	Taedan no.6	High	Low	Ordinary	Big	*C. crenata*
34	SongchonBam	Low	High	Good	Small	*C. mollissima*
35	HamjongBam	Low	High	Good	Small	*C. mollissima*

[a]Seed size: big: 20–30 g, ordinary: 10–20 g, small: < 10 g

2 µl of dNTPs, 2 µL of $MgCl_2$, 1 µl of primer, 0.1 µL 0.1 U/µL Taq polymerase, 1 µl of temple DNA (100 ng/µl) and 12.9 µl of ddH_2O for each sample in a Mastercycler (nexus gradient).

Thirty primers from RAPD primer set (Opéron, sets D, H, I, N and P) were tested on two cultivars and five primers (OPD3, OPI14, OPN4, OPU6 and OPU130) exhibiting high reproductive, and polymorphic rates were selected. The programme used for amplification was as follows: initial denaturation at 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 40 °C for 60 s, and 72 °C for 90 s, final extension at 72 °C for 5 min. Amplification products were separated on 1.5% agarose gels in 1 × TAE buffer at 90 V for 1 h. Gels were stained with ethidium bromide and photographed under UV light by using Geldoc-It™ (USA).

SRAP Analysis

SRAP primer combinations of Me1-Me10 and Em1-Em10 were tested on DNA samples from two cultivars belonging

Table 2 Primers used for RAPD and SRAP analysis, number of bands and their size range

Primer	Sequence (5′ → 3′)	Fragment size range (bp)	Number of bands
OPD3	GTCGCCGTCA	150-3 000	6
OPI14	TGACGGCGGT	100-3 000	11
OPN4	GACCGACCCA	100-3 000	8
OPU6	ACCTTTGCGG	200-2 500	14
OPU13	GGCTGGTTCC	200-2 500	6
Me2-Em1	Me2: TGAGTCCAAACCGGCTT	300-3 000	7
	Em1: GACTGCGTACGAATTATC		
Me5-Em2	Me5: TGAGTCCAAACCGGGTA	100-3 000	15
	Em2: GACTGCGTACGAATTTAT		

to *C. crenata* and *C. mollissima*. Ten SRAP primers exhibiting high reproductive and polymorphic rates were selected and used to analyse 35 accessions. PCR amplification was performed according to the protocol [3]. The SRAP markers were amplified using the following programme: initial denaturation at 94 °C for 5 min, 5 cycles of 94 °C for 1 min, 35 °C for 1 min, and 72 °C for 1 min, 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min, final extension at 72 °C for 5 min. Amplification products were separated on 1.5% agarose gels in 1X TAE buffer at 80 V for 90 min. Gels were stained with ethidium bromide and photographed under UV light by using Gel-doc-It^{TM} (USA).

Data Analysis

Only clear and polymorphic DNA bands were used for data analysis. The bands were scored as present (1) or absent (0) and a binary data matrix was constructed. DNA fragments of identical size amplified with the same primer were considered to be the same DNA marker. A dendrogram was constructed using the unweighted paired group method of cluster analysis using arithmetic averages (UPGMA). Principal coordinate analysis (PCoA) was performed with NTSYSpc version 2.20 [19] to examine the genetic relationship among populations. Based on RAPD and SRAP data, the POPGENE ver. 1.32 [22] was used to estimate values meaning genetic diversity: number of polymorphic bands, observed number of alleles, effective number of alleles Nei (1973) gene diversity (h), Shannon's information index (I) and genetic distances among populations. G_{ST} representing population differentiation and gene flow (Nm) among populations were estimated. Analysis of molecular variance (AMOVA) was performed to evaluate the distribution of genetic variation within and among species using the Arlequin ver. 3.5.2.2 [10]. Fixation index (F_{st}) was estimated. The patterns of the population structure were investigated using the Markov chain Monte Carlo (MCMC) simulation method in STRUCTURE HARVESTER V.2.3.4 [18] with a set of K values ranging from 2 to 5. Ten independent runs for each K value were performed with a burn-in period of 10 000 MCMC repeats in three iterations [9].

Results

RAPD primers, OPD3, OPI14, OPN4, OPU6, OPU13 and SRAP Em1-Me2, Em2-Me5 primer sets were, selected among 30 RAPD and 20 SRAP primers for further molecular analysis due to their high polymorphic indices (> 0.6) (Table 2).

On the basis of RAPD and SRAP profiles (Fig. 1), the genetic relationship was assessed among 35 chestnut cultivars using NTSYSpc2.11 software. Data matrix based on Nei (1973) genetic distance was obtained, and a dendrogram was constructed using UPGMA method. As shown in Fig. 2, at the genetic distance of 0.3, all samples were divided into two groups corresponding to two species (*C. mollissima*, *C. crenata*): group I included varieties belonging to the Korean chestnut species (*C. crenata*) and exotic varieties such as 'Taesong No. 1', 'TaesongOlBam', 'PaekBam', while group II included varieties belonging to the Chinese chestnut species (*C. mollissima*), exotic varieties such as 'RyongbongBam', 'Pukun', 'OunBam', 'Hwagwang', 'Honggwang', and 'Jungil No. 1' (Table 3).

The results of principle component analysis (PCoA) performed using NTSYSpc2.11 software are as follows (Fig. 3) which is coincident with above dendrogram.

Population structure among 35 chestnut varieties using STRUCTURE HARVESTER V.2.3.4 is shown in Fig. 4. All varieties were divided into two subpopulations, red one (group I in Fig. 2 corresponding to *C. crenata*) and green one (group II in Fig. 2 corresponding to *C. mollissima*) in

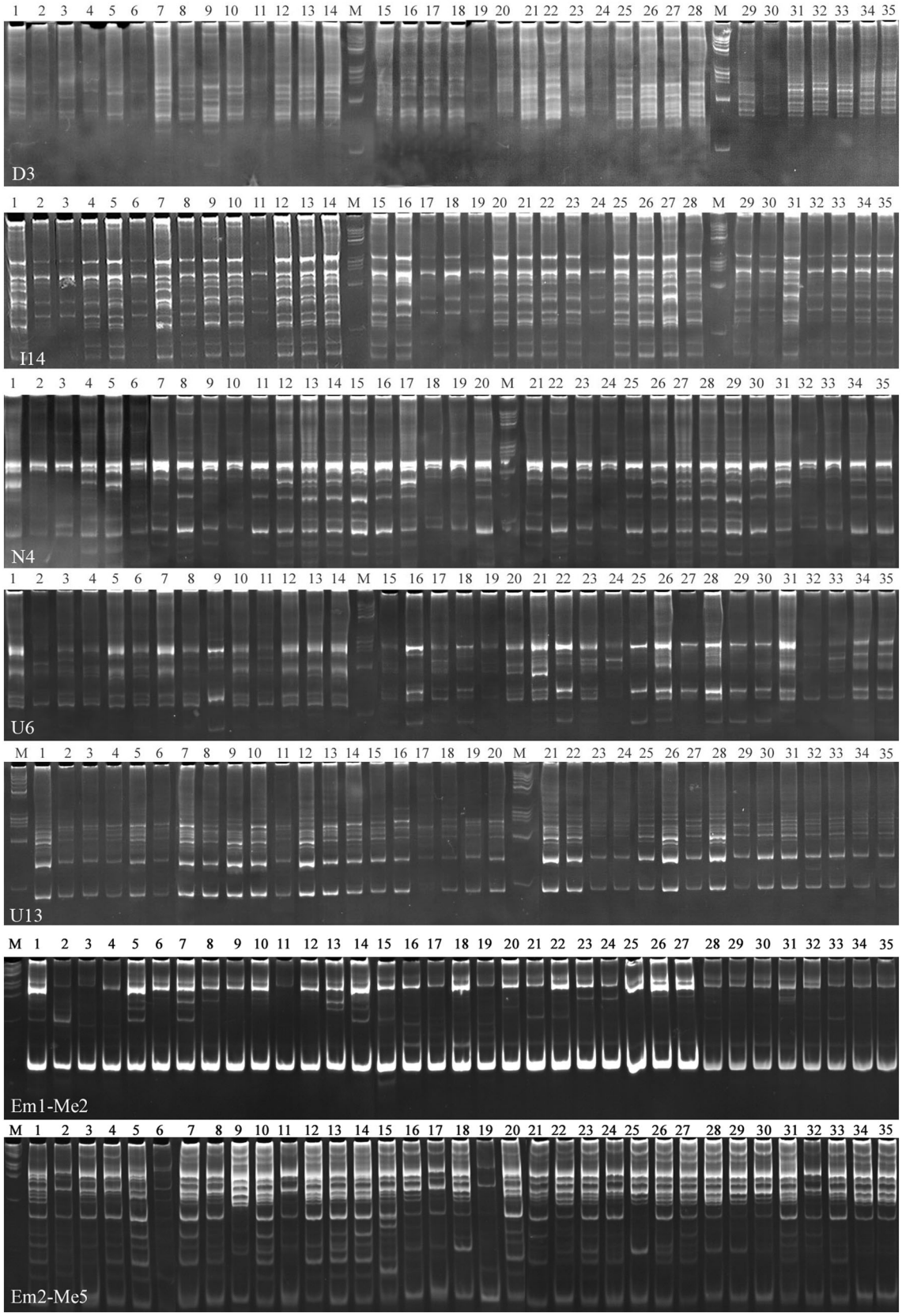

Fig. 1 RAPD and SRAP agarose gel electrophoresis profiles of 35 chestnut cultivars. Each slot marked by numbers (1, 2, 3,…, 35) represents the individuals that belong to cultivars listed in Table 1. M–1 kb ladder

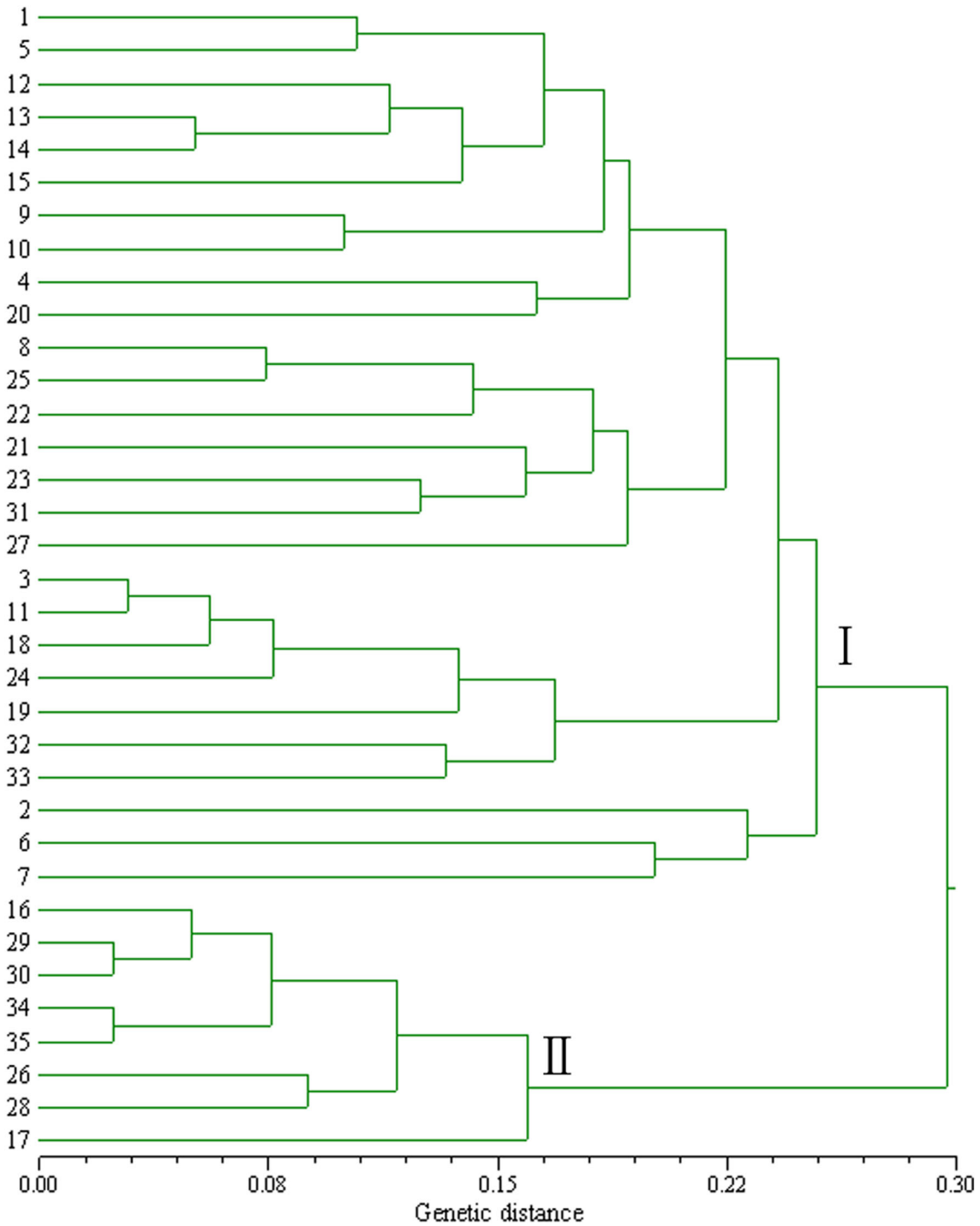

Fig. 2 UPGMA dendrogram illustrating the genetic relationships between 35 chestnut cultivars based on Nei's genetic distance

case $K = 2$. STRUCTURE generated three subpopulations in case $K = 3$, where first one included the group II in Fig. 2, the other subpopulations consisted group I in Fig. 2. Similarly, in both cases $K = 4$ and 5, all varieties were divided into four and five subpopulation respectively, with group I in Fig. 2 as an independent subpopulation.

The subpopulations were estimated based on RAPD and SRAP markers for K values for different number of

Table 3 Genetic diversity indices for 35 chestnut varieties

Species	N_{PB}	P_{PB}	n_a	n_e	h	I
C. crenata	55	82.09	1.82 ± 0.39	1.47 ± 0.36	0.28	0.41
C. mollissima	18	26.87	1.27 ± 0.45	1.18 ± 0.34	0.10	0.15

N_{PB}, number of polymorphic bands; P_{PB}, percentage of polymorphic bands; n_a, observed number of alleles; n_e, effective number of alleles; h, Nei's (1987) gene diversity; I, Shannon's information index

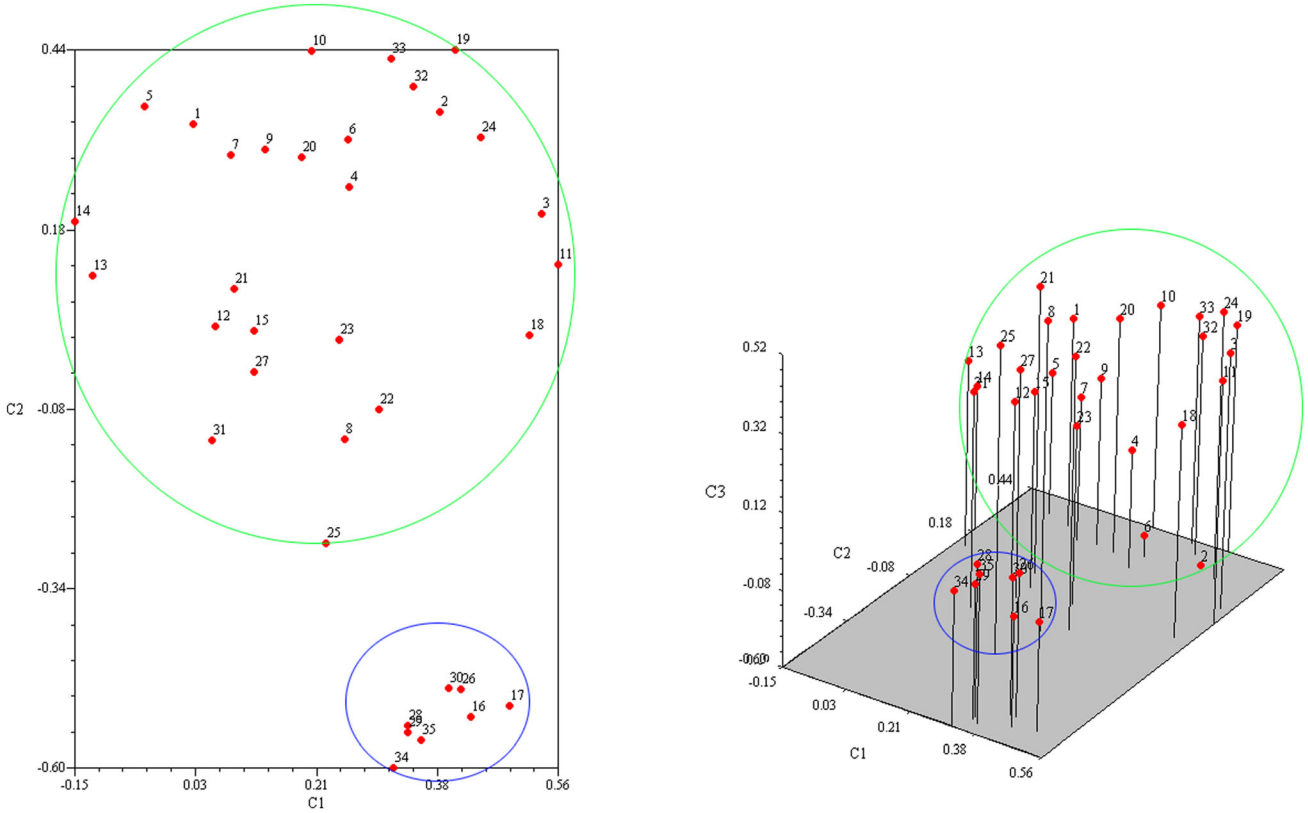

Fig. 3 2D (**a**) and 3D (**b**) PCoA plot based on RAPD and SRAP data in chestnut varieties

putative subpopulations. Maximum number of subpopulations was inferred as $K = 5$.

Based on the genetic distance between 35 varieties, the genetic variance was anlaysed using PopGene.version 1.31.

In this study, a mean Gst value of 0. 2576 for 2 chestnut species (ranging from 0.0000 to 0.9646) and an average number of migrants per generation (Nm) of 1.4410 (in the range of 0.0184 and 5.7241) among populations were obtained. And the AMOVA was performed to assess genetic stability on 35 cultivars of 2 chestnut species using Arlequin ver. 3.5.2.2 (Table 4). Of the total genetic diversity, 63.9% was attributable to differences among cultivars and 36.1% was to differences within cultivars, showing a significant varietal differentiation in newly obtained stock cultivars. The fixation index (F_{st}) was 0.631.

Discussion

Genetic Relationship Among Chestnut Varieties in DPR Korea

Studies on genetic relationship between and within several plant species have been carried out by SRAP markers solely [3] as well as other molecular markers. ISSR and SRAP markers were used to identify genetic differentiation in four Indian populations of *Simarouba glauca* with different ecological and geographical characteristics [15]. Using RAPD and SRAP markers, genetic differentiation in different populations of European chestnut species has been distinguished [13]. In addition, genetic relationship between different populations belonging to the two species of *Averrhoa* has been revealed [20].

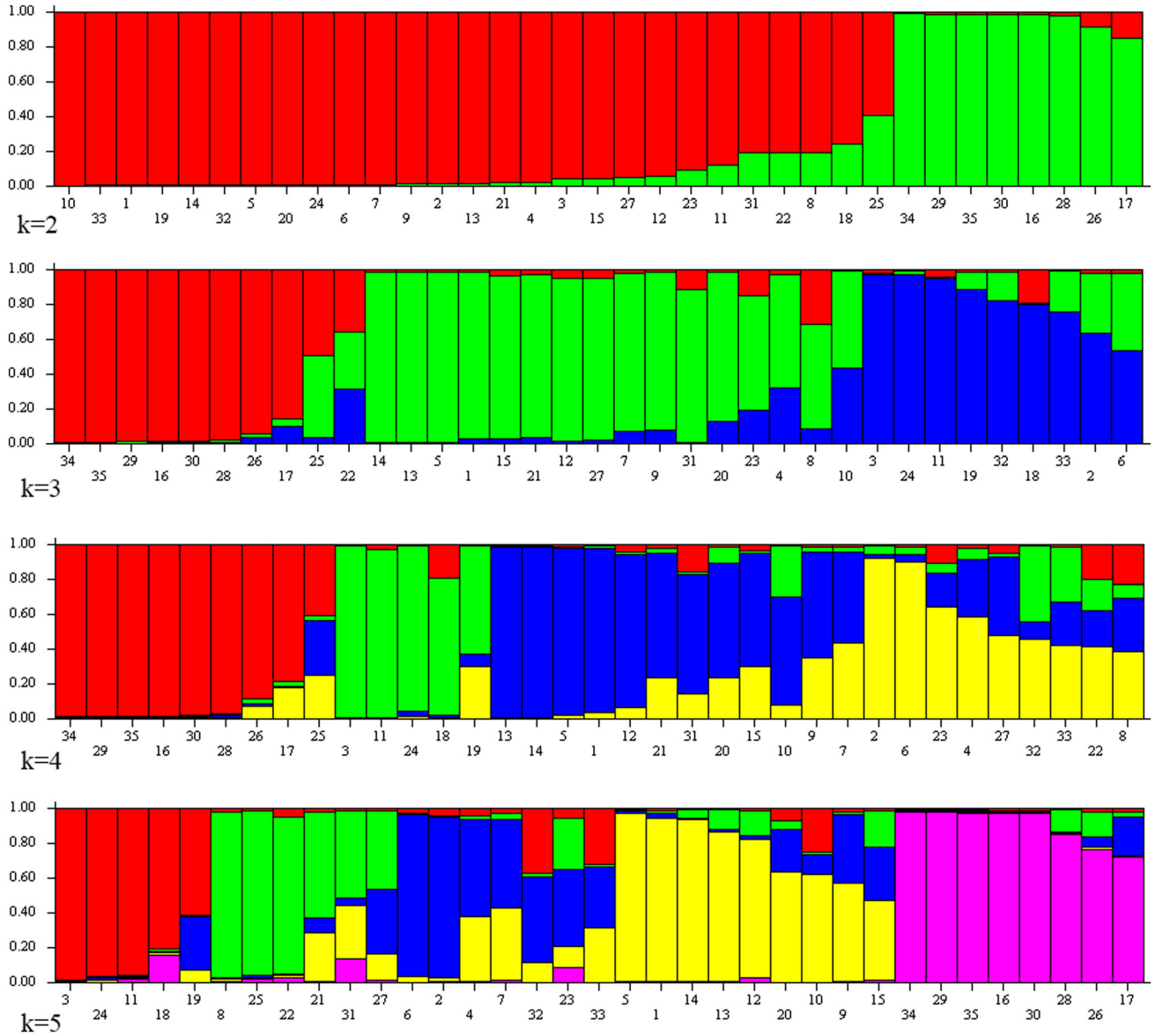

Fig. 4 Hierarchical population structure analysis using STRUCTURE HARVESTER V.2.3.4

Table 4 Analysis of molecular variance (AMOVA) based on RAPD and SRAP profiles in 35 chestnut cultivars

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation (%)	*P*	Fixation index
Among species	33	1233.380	7.47503 Vb	63.90	$P < 0.0002$	F_{CT} 0.63105
Within species	1	298.049	4.22388 Va	36.10	$P < 0.0002$	F_{SC} 1.0000
Total	34	1531.429	11.69891			

Thirty-five chestnut varieties used in this study are broadly divided into two species: Chinese chestnut (*C. mollissima*) and Korean chestnut (*C. crenata*). *C. mollissima* includes 'SongchonBam' and 'HamjongBam' cultivated from natural seedling in Songchon for a long time. The results suggested that exotic cultivars such as 'Hwagwang', 'Honggwang', 'Jungil No.1', 'Ryongbong Bam', 'Pukun' and 'OunBam' were included in the group of *C. mollissima* species. The species of *C. crenata* include 12 exotic varieties and 15 native varieties including

'KumyaWangBam' and 'KosongWangBam' exhibiting good taste, high-yield and resistance to chestnut bumblebee, drought and cold. Although accurate identification of these foreign cultivars require nuclear ITS sequence and mitochondrial DNA barcoding gene, RAPD and SRAP markers may reveal genetic background of these cultivars. In this study, cluster analysis using RAPD and SRAP markers showed that *C. mollissima* populations, including six *cultivars* such as 'SongChonBam' and 'HamjongBam', and *C. crenata* populations, including 28 cultivars such as 'KumyaWangBam' and 'KosongBam', were classified at genetic distance 0.3.

In case $K = 2$, all samples were divided into two groups corresponding to two species (*C. mollissima*, *C. crenata*) which is coincident with the clustering result (Fig. 2) and the PCoA result (Fig. 3) using NTSYSpc2.11. In cases $K = 3$–5, group II in Fig. 2 representing *C. mollissima* remained as an independent subpopulation and group I in Fig. 2 representing *C. crenata* divided into different subpopulations. These results suggest that the pattern of genetic relationship between 35 chestnut varieties revealed by SRAP and RAPD markers is likely to be associated with species differentiation.

Genetic Diversity of Chestnut Varieties in DPR Korea

It has been reported that genetic diversity at the species level was 0.150 [14], while genetic diversity of 10 natural populations of *C. mollissima* was higher than natural level of plant except Laiyang population (0.1347) and Tai'an population (0.1455) [5]. In this study, genetic diversity of *C. mollissima* at the species level was low as 0.10 while *C. crenata* was 0.28 representing higher value than results in several studies [5, 14]. These results may attribute to perennial character, cross-pollination, and existence of exotic varieties.

Breeding and Conservation of Chestnut Varieties

'SongchonBam' belonging to the *C. mollissima* is famous for its good taste and many efforts have been made to protect its germplasm. Meanwhile, studies on breeding of elite varieties belonging to *C. crenata* such as 'KumyaWangBam' and 'KosongWangBam' and use of them as starting materials for breeding new chestnut varieties have been conducted. These results provide detailed information on genetic diversity of native and exotic varieties in DPR Korea to assist in breeding and conservation strategies.

Conclusions

Chestnut varieties cultivated in DPR Korea are distinguished according to the nuts and leaf traits, which could be affected by environment and cultivation conditions. The discrimination of species and cultivars using these traits alone may lead to mistakes in the establishment of breeding and conservation strategies. Therefore, the genetic relationship between the 35 chestnut cultivars cultivated in DPR Korea revealed by RAPD and SRAP markers would contribute to the identification of more suitable cross-parents for heterosis.

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Declarations

Conflict of interest Authors declare that there is no conflict of interest.

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