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The assessment of genetic diversity of *Castanea* species by RAPD, AFLP, ISSR, and SSR markers

Sofiane ABDELHAMID[1,2,*], Cong-Linh LÊ[3], Marco CONEDERA[4], Philippe KÜPFER[1]

[1]Olive Tree Institute, Sfax, Tunisia

[2]Laboratory of Evolutionary Botany, University of Neuchâtel, Neuchâtel, Switzerland

[3]Agroscope, Swiss Agricultural Research Station Changins, Nyon, Switzerland

[4]Swiss Federal Institute for Forest, Snow, and Landscape Research, Southern Branch, Bellinzona, Switzerland

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Abstract: *Castanea* Mill. (chestnut) is a multipurpose deciduous tree and member of the family Fagaceae, widely distributed throughout North America, Europe, and Asia. The evaluation of the genetic diversity of chestnut species is crucial for the effective conservation of this economically and ecologically valuable tree. In this study, we applied 4 DNA markers to detect the genetic variability among and within *Castanea* species and to compare the effectiveness of each system in estimating genetic variation. We amplified 106 random amplified polymorphic DNA (RAPD), 228 amplified fragment length polymorphism (AFLP), 42 intersimple sequence repeat (ISSR), and 36 simple sequence repeat (SSR) polymorphic markers using 12, 5, 4, and 5 primer combinations, respectively. The findings on the effective multiplex ratio, polymorphism information content, and marker index revealed that AFLP was the most effective molecular marker system used in this study. Each marker system classified the species under investigation into clear but incompletely separated clusters, although partial agreement was achieved with respect to species relationships when the RAPD method was employed. The comparison of the correlation coefficient of RAPD marker data and the other markers showed a higher correlation [($r = 0.69$, $P < 0.01$), ($r = 0.77$, $P < 0.01$), and ($r = 0.47$, $P < 0.01$) with AFLP, ISSR, and SSR, respectively]. When variance was partitioned among and within groups, AFLP (94.62%) showed greater variation within the groups and reverse RAPD (67.87%) yielded greater variation among the groups. Overall, the results indicate that the AFLP represents an efficient molecular marker system for the assessment of chestnut genetic diversity and, hence, the development of effective conservation strategies to preserve this valuable tree species.

Key words: *Castanea*, DNA fingerprinting, RAPD, AFLP, ISSR, SSR, genetic diversity

1. Introduction

The genus *Castanea* Mill. consists of forest trees with exceptional ecological, socioeconomic, and cultural importance. Chestnuts (*Castanea*), members of the family Fagaceae, are naturally widespread in deciduous forests of North America, Europe, and Asia (Fei et al., 2012). The genus includes 4 economically important species bearing abundant sweet nuts and timber, including the Chinese chestnut (*Castanea mollissima*), Japanese chestnut (*Castanea crenata*), European chestnut (*Castanea sativa*), and American chestnut (*Castanea dentata*). These tree species are multipurpose plants that play significant socioeconomic, ecological, and cultural roles in the lives of local communities of the region and are increasingly gaining importance as sources of food and other products (timber, etc.) worldwide.

The natural distribution of chestnut species in the 3 continental regions occurs through South and Central Europe, East Asia, and North America. The evaluation of species genetic diversity and natural populations is necessary for planning a conservation strategy and for creation of breeding programs in order to create genotypes resistant to ink disease and canker blight, caused by the chestnut blight fungus (*Cryphonectria parasitica*), one of the most severe diseases affecting *Castanea sativa* (European chestnut) and *Castanea dentata* (American chestnut) (Huang et al., 1994; Montenegro et al., 2008).

The literature indicates that the *Castanea* species have a rich array of genetic diversity and morphological and ecological variability. Sweet chestnut (*Castanea sativa* Mill.) is the only native species of the genus *Castanea* that is widely distributed from Spain, Portugal, Italy, France, and the southern part of England to the Caucasus and through Greece and Turkey (Martín et al., 2007). In fact, chestnut cultivation has a very long history; it existed in Europe during the Roman period (Pittet, 1986). Later, a group of high-quality varieties, called Marroni, was selected and cultivated for commercial purposes in specific regions of the Italian Peninsula and France.

* Correspondence: sofiane.abdelhamid@gmail.com

The existence of differentiation patterns in adaptive traits among European populations gave rise to a very rich, complex, and highly articulated structure of chestnut culture, which was marked by the wide range of chestnut cultivars in the region (Pittet, 1986). There are more than 300 different varieties in Italy (Pitte, 1986), 250 in France (Camus, 1929), 200 in Spain, and 100 in southern Switzerland.

This large number of existing varieties justifies the need for an efficient genetic identification method which, in turn, might help develop effective conservation and development strategies to preserve those valuable genetic resources and protect the quality of commercial varieties (i.e. Marroni vs. chestnut varieties).

Several studies have been performed to investigate the genetic variation in chestnut varieties, and species identifications were based on the morphopomological traits related to shoots (Valle, 1959), shape of fruit, and leaf and flower phenology (Rudow and Conedera, 2001).

The advent of molecular marker systems has thus become a reliable method to explain the genetic and adaptive diversity in cultivated varieties (Parmaksız and Özcan, 2011). Several studies were carried out using molecular markers, including those on *Castanea* species and chestnut germplasm populations (Yamamoto et al., 1998; Botta et al., 1999; Gobbin et al., 2007; Lang et al., 2007; Pereira-Lorenzo et al., 2011; Mellano et al., 2012; McCleary et al., 2013).

So far, however, only a few studies have investigated the genetic diversity of cultivars using multiple markers on the same chestnut population materials simultaneously (Goulão et al., 2001; Martin et al., 2010). Accordingly, the present study was undertaken to apply 4 DNA markers [random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), intersimple sequence repeat (ISSR), and simple sequence repeat (SSR)] to detect the genetic variability among and within *Castanea* species and to compare their effectiveness and utility in estimating the genetic variation among chestnut cultivars.

2. Materials and methods

2.1. Plant materials

The study was conducted on 73 accessions belonging to Swiss, French, Italian, and Asian varieties, which are listed in Table 1. Most of the plant materials were obtained from the Arboretum of the National Institute of Agronomic Research (INRA, Bordeaux, France), where more than 250 *Castanea* spp. trees have been grafted with accessions coming from all over the world. Different ecotypes and cultivars from different European countries and accessions from the United States, China, and Japan were collected. Trees were used both as rootstocks for superior varieties and for their own roots. The Swiss cultivated and wild varieties (coppice shoots) were collected in situ from different areas in southern Switzerland.

2.2. DNA extraction

Total genomic DNA was extracted from small leaves using hexadecyl trimethyl ammonium bromide (CTAB) according to the method described by Porebski et al. (1997). High salt concentrations and polyvinyl-polypyrrolidone were added separately to remove polysaccharides and polyphenol compounds. Extraction was performed by an extended RNase treatment and a phenol-chloroform method. DNA was purified by the Prep-A-Gene matrix (Bio-Rad) and quantified spectrophotometrically. It was resuspended in a TE solution (pH 8) and stored at –20 °C.

2.3. Amplification methodologies

2.3.1. RAPD

Twelve RAPD primers from sets OPA (02, 04, 07, 10, and 15), OPB (08), OPD (20), OPE (01, 04, 16, and 19), and OPX (17) (Operon Technologies) were used to amplify specific markers. Polymerase chain reaction (PCR) was performed in a total volume of 25 µL. The amplification reaction contained 1X PCR buffer, 1.4 mM $MgCl_2$, 0.2 mM dNTP, 0.4 µM primer, 1 U/µL Taq polymerase (Eurobio), and 20 ng/µL template DNA. PCR was carried out in a Hybaid PCR express thermal cycler (HBPX 220) with the following cycling profile: an initial denaturation at 94 °C for 4 min, followed by 38 cycles of 1 min at 93 °C, 1 min at 45 °C, and 1 min at 72 °C, with a final extension at 72 °C for 5 min. PCR products were mixed with loading buffer and separated on 1.6% (w/v) agarose gel containing 0.4 µg/mL ethidium bromide in 1X TBE at 100 V for 90 min. Finally, the DNA fragments were visualized in UV light.

2.3.2. AFLP

Four sets of selective primer combinations were used (E-AGG/M-CTT, E-AAC/M-CTT, E-AGT/M-CAT, and E-AAC/M-CAT) (Table 2) from the GIBCO BRL AFLP Core Reagent Kit to generate AFLP fragments. The DNA concentration was adjusted to 100 ng/µL, and DNA was digested in 40 µL of restriction-reaction mixture containing 5 U of *Eco*RI and 5 U of *Mse*I (Biolabs) in T4 ligase buffer. It was then incubated for 2 h at 37 °C. For the ligation reaction, a mixture containing *Eco*RI adapter (40 pmol/µL), *Mse*I adapter (40 pmol/µL), 1 U of T4 DNA ligase, and 1X T4 DNA ligase buffer was added to the restriction reaction and incubated for 3 h at 37 °C.

Preamplification was carried out in 20-µL volumes. The reactions contained 1X PCR buffer, 1.5 mM $MgCl_2$, 1 mM dNTP, 10 pmol/µL of each preselective primer *Eco*RI-A and *Mse*I-C, 1 U of Taq polymerase (QIAGEN), and 100 ng/µL template DNA. Preamplification with primers having a single selective nucleotide was performed in a Hybaid PCR express thermal cycler (HBPX 220) with the

Table 1. Studied accessions of *Castanea* cultivars and their origin.

Accession order	Accession number	Studied species	Genotype	Geographical origin
1	1	*C. sativa*	Verdanesa	Calonico 01.CH
2	2	*C. sativa*	Verdanesa	Calonico 04.CH
3	3	*C. sativa*	Verdanesa	Giornico 03.CH
4	4	*C. sativa*	Verdanesa	Giornico 06.CH
5	5	*C. sativa*	Verdanesa	Giornico 04.CH
6	6	*C. sativa*	Verdanesa	Chironico 02.CH
7	7	*C. sativa*	Verdanesa	Chironico 06.CH
8	8	*C. sativa*	Verdanesa	Chironico 10.CH
9	9	*C. sativa*	Verdanesa	Chironico 12.CH
10	10	*C. sativa*	Verdanesa	Chironico 13.CH
11	11	*C. sativa*	Verdanesa	Lodrino 04.CH
12	12	*C. sativa*	Verdanesa	Torricella 10.CH
13	13	*C. sativa*	Verdanesa	Torricella 13.CH
14	1	*C. sativa*	Lüina	Calonico 02.CH
15	2	*C. sativa*	Lüina	Calonico 07.CH
16	3	*C. sativa*	Lüina	Giornico 01.CH
17	4	*C. sativa*	Lüina	Giornico 02.CH
18	5	*C. sativa*	Lüina	Chironico 01.CH
19	6	*C. sativa*	Lüina	Chironico 05.CH
20	7	*C. sativa*	Lüina	Chironico 08.CH
21	8	*C. sativa*	Lüina	Chironico 14.CH
22	9	*C. sativa*	Lüina	Lodrino 03.CH
23	10	*C. sativa*	Lüina	Lodrino 05.CH
24	11	*C. sativa*	Lüina	Lodrino 14.CH
25	12	*C. sativa*	Lüina	Torricella 08.CH
26	13	*C. sativa*	Lüina	Torricella 09.CH
27	14	*C. sativa*	Lüina	Torricella 17.CH
28	1	*C. sativa*	Bonè negro	Calonico 03.CH
29	2	*C. sativa*	Bonè negro	Calonico 05.CH
30	3	*C. sativa*	Bonè negro	Calonico 06.CH
31	4	*C. sativa*	Bonè negro	Calonico 08.CH
32	5	*C. sativa*	Bonè negro	Chironico 03.CH
33	6	*C. sativa*	Bonè negro	Chironico 04.CH
34	7	*C. sativa*	Bonè negro	Lodrino 02.CH
35	8	*C. sativa*	Bonè negro	Lodrino 11.CH
36	9	*C. sativa*	Bonè negro	Lodrino 12.CH
37	10	*C. sativa*	Bonè negro	Lodrino 13.CH
38	1	*C. sativa*	Berögna	Lodrino 07.CH
39	2	*C. sativa*	Berögna	Lodrino 08.CH
40	3	*C. sativa*	Berögna	Prosita 07.CH
41	1	*C. sativa*	Pinca	Vezio 21.CH

Table 1. (Continued).

42	2	*C. sativa*	Pinca	Vezio 22.CH
43	3	*C. sativa*	Pinca	Vezio 31.CH
44	1	*C. sativa*: Coppice		S. Antonino.CH
45	2	*C. sativa*: Coppice		S. Antonino.CH
46	3	*C. sativa*: Coppice		S. Antonino.CH
47	4	*C. sativa*: Coppice		Bellinzona.CH
48	5	*C. sativa*: Coppice		Bellinzona.CH
49	6	*C. sativa*: Coppice		Bellinzona.CH
50	7	*C. sativa*: Coppice		Bellinzona.CH
51	8	*C. sativa*: Coppice		Bellinzona.CH
52	9	*C. sativa*: Coppice		Bellinzona.CH
53	1	*C. sativa*	CA 105. Sardonne	Ardèche (FR)
54	2	*C. sativa*	CA 106. Marron Comballe	Ardèche, Lozère (FR)
55	3	*C. sativa*	CA 107. Marron du Var	Gard (FR)
56	4	*C. sativa*	CA 109. Marron de laguépie	Dordogne (FR)
57	5	*C. sativa*	CA 135. Précoce de Vans	Ardèche (FR)
58	1	*C. sativa*	CA 511. Marrone di Chiusa Pesio CN2	Piedmont (IT)
59	2	*C. sativa*	CA 512. Garrone rosso CN7	Piedmont (IT)
60	3	*C. sativa*	CA 513. Marrubia di Bermezzo	Italy
61	4	*C. sativa*	CA 570. Pelosa grossa	Piedmont (IT)
62	5	*C. sativa*	CA 653. Castel del Rio	Italy
63	1	*C. mollissima*	CA 75	China
64	2	*C. mollissima*	CA 578	China
65	3	*C. mollissima*	CA 737	China
66	4	*C. mollissima*	CA 744. Ching-za	China
67	5	*C. mollissima*	CA 797. Mossbarger	China
68	1	*C. crenata*	CA 03	Japan
69	2	*C. crenata*	CA 04	Japan
70	3	*C. crenata*	CA 564. Iphara	Japan
71	4	*C. crenata*	CA 598. Rihei	Japan
72	5	*C. crenata*	CA 599. Ibuki	Japan
73	6	*C. crenata*	CA 600. Ishizuchi	Japan

following cycle profile: 2 min of DNA denaturation step at 94 °C, followed by 28 cycles of 45 s at 94 °C, 45 s at 56 °C, and 1 min at 72 °C, with final elongation at 72 °C for 10 min. The reaction mixtures were diluted 10-fold for selective PCR.

Selective amplification was conducted with 4 combinations of selective primers using the following nucleotides: E-AGG/M-CTT, E-AAC/M-CTT, E-AGT/M-CAT, and E-AAC/M-CAT.

Reactions were conducted in a 20-µL volume containing 1X PCR buffer, 0.75 mM $MgCl_2$, 1 mM dNTP, 0.25 µM of each selective primer *Eco*RI-ANN and *Mse*I-CNN (Table 3), and 1 U of Taq polymerase (QIAGEN). PCR was performed for 36 cycles with the following cycle profile: a 30-s DNA denaturation step at 94 °C, a 30-s annealing step, and a 1-min extension step at 72 °C. The annealing temperature was set at 65 °C for the first cycle, gradually reduced by 0.7 °C for each of the next 13 cycles, and kept at

Table 2. Details on primers used in AFLP analysis

Reaction	Primer	Sequence (5' to 3')
Ligation of adapter	*Eco*RI AdapterE1	CTCGTAGACTGCGTACC
	*Eco*RI AdapterE2	AATTGGTACGCAGTCTAC
	*Mse*I AdapterM1	GACGATGAGTCCTGAG
	*Mse*I AdapterM2	AATTGGTACGCAGTCTAC
Preselective reaction	*Eco*RI-A	GACTGCGTACCAATTCA
	*Mse*I-C	GATGAGTCCTGAGTAAC
Selective reaction (*Eco*RI-ANN and *Mse*I-CNN)	*Eco*RI-AGT	GACTGCGTACCAATTCAGT
	*Eco*RI-AAC	GACTGCGTACCAATTCAAC
	*Eco*RI-AGG	GACTGCGTACCAATTCAGG
	*Mse*I-CAT	GATGAGTCCTGAGTAACAT
	*Mse*I-CTT	GATGAGTCCTGAGTAACTT

Table 3. Comparison of information generated with various molecular markers in evaluating genetic diversity of *Castanea* species.

Molecular marker	RAPD	AFLP	ISSR	SSR
Number total of bands	169	248	53	36
Number of polymorphic bands	104	229	42	12
Percentage polymorphism (%P)	61.5	92.3	79.2	51.71
Fraction of polymorphic markers	0.38	0.48	0.44	0.25
Polymorphism information content (PIC)	0.755	0.887	0.667	0.483
Multiplex ratio (MR)	22.75	119.25	19	9.6
Effective multiplex ratio (EMR)	8.64	57.24	8.36	2.4
Marker index (MI)	6.52	50.77	5.57	1.15

56 °C for the remaining 23 cycles. One microliter of PCR product was mixed with a 12 µL of deionized formamide and 0.5 µL of Gene Scan 500 (ROX) size standard marker. The resulting mixture was heated for 2 min at 95 °C and then quickly cooled on ice. Each sample was loaded and run on an ABI-310 automated DNA sequencer (capillary electrophoresis). GeneScan and Genotyper software (PE Applied Biosystems) was used to score the AFLP profiles.

2.3.3. ISSR

Five primers were selected, namely UBC 810, 834, 836, 841, and 890 (obtained from UBC Primer Set 100/9, University of British Columbia, Canada), based on their capacity to amplify polymorphic fragments.

Amplification reactions were carried out in volumes of 25 µL. The reaction contained 1X PCR buffer, 1.4 mM $MgCl_2$, 0.2 mM dNTP, 0.4 µM of primer, 1 U/µL Taq polymerase (Eurobio), and 30 ng/µL template DNA. PCR reactions were performed with the following conditions: 4 min at 94 °C for initial denaturation followed by 35 cycles of 35 s at 93 °C (denaturation), 45 s at optimal temperature ranging from 52 to 55 °C (annealing), and 90 s at 72 °C (extension), with a final extension step at 72 °C for 5 min. PCR products were separated and revealed on denaturing polyacrylamide gels using a DNA Silver Staining Kit (Pharmacia Biotech).

2.3.4. SSR

Five primer pairs that were originally developed for oak species (*Quercus petraea* and *Q. robur*) (Steinkellner et al., 1997; Botta et al., 1999) were selected and used in this study for their usefulness in the genotyping of chestnut cultivars. Four primer pairs originally developed for *Q. petraea* (QpZag7, QpZag9, QpZag108, and QpZag110) and 1 primer pair originally developed for *Q. robur* (QrZag121) successfully amplified SSR fragments for chestnut.

The amplification reaction was used in a total volume of 25 µL containing 1X PCR buffer, 1 mM $MgCl_2$, 0.2 mM dNTP, 0.4 mM of each primer, 0.75 U/µL Taq polymerase (Eurobio), and 50 ng/µL template DNA. Amplification was performed in a Hybaid PCR express thermal cycler (HBPX 220) under the following cycling conditions: a denaturation procedure at 93 °C for 3 min, followed by 35 cycles of denaturation (1 min at 93 °C), annealing (1

min at optimal temperature ranging from 47 to 54 °C), and extension (90 s at 72 °C), and a final elongation step at 72 °C for 10 min. PCR products were separated and revealed with the DNA Silver Staining Kit electrophoresis system (Pharmacia Biotech).

2.4. Data scoring and analysis

For the primers that produced a clear pattern, the polymorphic DNA fragment detected by the 4 types of markers were scored as present (1) or absent (0).

The generation of data for the 4 markers involved the construction of dendrograms by unweighted pair-group method with arithmetic averages (UPGMA) cluster analysis based on Jaccard's coefficient (Sneath and Sokal, 1973) using cluster analysis software (http:www.biology.ualberta.ca/jbrzusto), which were then visualized using the TREEVIEW program (Page, 1998).

In order to test the species discrimination of each marker, Jaccard's coefficient of similarity (Sneath and Sokal, 1973) was calculated. Afterwards, the similarity matrices were converted into distance matrices and principal coordinates analysis (PCoA) was performed.

Jaccard's similarity indices calculate the genetic distance in an adequate and simple way as they do not take the double absence of a band into account, which reflects what actually happens in biological reality. Similarity was determined by the SIMIL and PCOORD modules of the R4 (beta version) package (Philippe Casgrain and Pierre Legendre, Department of Biological Sciences, University of Montreal) and calculated as follows:

Jaccard = Nab / (Nab + Na + Nb),

where Nab is the number of polymorphic bands shared by samples a and b, Na the number of bands present in a and absent in b, and Nb the number of bands present in b and absent in a.

To assess the discriminative potential of each locus and each marker, the polymorphism information content (PIC) values (Lynch and Walsh, 1998) were calculated based on the number of alleles expressed and their relative frequencies according to the formula $1 - \Sigma(p_i)2 - \Sigma\Sigma\ 2\ (p_i)^2\ (p_j)^2$ using CERVUS v.2 software (Marshall et al., 1998).

Effective multiplex ratio (EMR; number of polymorphic loci and nonpolymorphic loci from a single amplification reaction), multiplex ratio (MR; dividing the total number of bands amplified by the total number of assays), and marker index (MI; the product of EMR and PIC) values were calculated as indicated by Powell et al. (1996) and Varshney at al. (2007).

The correlations between RAPD, AFLP, ISSR, and SSR techniques of genetic distance matrices were investigated by the Mantel test of matrix correspondence (Mantel, 1967), based on Jaccard's similarity coefficient. The similarity matrices generated by RAPD, AFLP, ISSR, SSR, and the combined data techniques were compared pairwise.

Mantel's tests were performed using the R4 (beta version) software package (Philippe Casgrain and Pierre Legendre, Department of Biological Sciences, University of Montreal) and statistical significance was determined by random permutation (999 permutations).

The analysis of molecular variance (AMOVA; Excoffier et al., 1992) was carried out as estimates of molecular diversity at the hierarchical level among and within group categories, using the ARLEQUIN 2.000 software package (Schneider et al., 2000). Groupings were made as follows: Group A (Swiss varieties and coppice shoots), Group B (French varieties), Group C (Italian varieties), Group D (*Castanea mollissima* varieties), and Group E (*Castanea crenata* varieties). The statistical significance of P-values was tested nonparametrically after 1023 permutations.

3. Results

3.1. Marker analysis

Table 3 summarizes the number total of bands amplified and the number of polymorphic bands and percentage of polymorphisms detected for the different marker systems in *Castanea* species. For RAPD, the 12 primers produced a total of 104 polymorphic bands. The findings revealed that the highest levels in terms of polymorphic band numbers and polymorphism percentages (87.5%) were obtained with primer OPE-01 (14 fragments), whereas the lowest rates were attained with primers OPA-15 and OPA-2 (4 and 6 fragments, respectively). An average of 8.83 bands per primer ranging from about 1000 to 5000 bp was produced. Interestingly, the number of bands produced by AFLP was so high that it was difficult to count. Moreover, the 4 primer combinations yielded 229 polymorphic fragments whose sizes ranged between 50 and 350 bp. The number of scored fragments amplified by each pair of primer set varied from 51 to 68, and the average number of polymorphic bands per reaction was 57.2: 51 from E-AGG/M-CTT, 53 from E-AAC/M-CTT, 57 from E-AGT/M-CAT, and 68 from E-AAC/M-CAT, with the latter primer set yielding the most informative primer combination and highest percentage of polymorphism detected (97.1%).

Five primers were able to amplify visible fragments on polyacrylamide gels. They detected 42 polymorphic ISSR fragments. The number of scored bands per primer ranged from 4 (with primer UBC-890) to 12 (with primers UBC-841 and UBC-834).

Furthermore, 36 polymorphic fragments were amplified using 5 microsatellite (SSR) primer pairs. The most polymorphic primer pair was QpZag9, which produced 12 polymorphic bands and detected the highest percentage of polymorphism (75%).

3.2. Statistical analysis

The effectiveness and the comparison of the 4 marker systems on the basis of different criteria are given in Table

3. The relative efficiencies of different molecular markers for detecting available polymorphisms within *Castanea* species depend on the number of detectable alleles and the distribution of their frequency.

The results indicated that the AFLP tool scored higher in terms of polymorphism detection (92.3%) compared to ISSR (79.2%), RAPD (61.5%), and SSR (51.71%). In fact, an earlier comparison between the ratios of band numbers per primer for the methods under investigation revealed that the ratios were similarly low for RAPD and ISSR (8.6 and 8.4, respectively), very high for AFLP (57.25), and low for SSR (7.2).

Furthermore, the highest value recorded for PIC was obtained by AFLP (PIC = 0.887), followed by RAPD (PIC = 0.755), ISSR (PIC = 0.667), and SSR (PIC = 0.48), respectively. Finally, the findings revealed that the value recorded in terms of MI, a measure of the overall efficiency of a marker, was very high for AFLP at 50.77 suggesting the supremacy of this marker system for application in the identification of genetic diversity in *Castanea* species.

A high correlation coefficient between matrices based on AFLP and RAPD ($r = 0.69$, $P < 0.01$) was observed (Table 4). Likewise, the results from the Mantel test showed high correlation between RAPD and ISSR ($r = 0.77$, $P < 0.01$). Low correlation coefficients were, however, observed among the molecular markers based on the SSR distance matrix and the 3 different sets of data ($r = 0.38$, $P < 0.01$; $r = 0.47$, $P < 0.01$; and $r = 0.44$, $P < 0.01$ with AFLP, RAPD, and ISSR, respectively).

3.3. Cluster analysis and AMOVA

The clustering patterns obtained by the UPGMA cluster analysis of RAPD, AFLP, ISSR, and SSR data are given in Figures 1–4, and the combined data are presented in Figure 5. The analysis of the RAPD data revealed a clear separation of the accessions into 4 groups by cutting the dendrogram at a genetic similarity value of 0.25, with greatest separation of the *C. mollissima* and *C. crenata* accessions. The data failed to differentiate Swiss, French, and Italian *C. sativa* cultivars into separate clusters according to their geographical origins.

Table 4. Mantel test correlation coefficients of a distance matrix created by AFLP, RAPD, ISSR, SSR, and combined data analysis. The difference was significant (*: $P < 0.05$, **: $P < 0.01$).

AFLP				
RAPD	0.69**			
ISSR	0.62**	0.77**		
SSR	0.38	0.47	0.44	
Combined data	0.68**	0.57*	0.50*	0.42
	AFLP	RAPD	ISSR	SSR

AFLP analysis failed to group *C. crenata* accessions into a separate cluster, but clearly separated the Swiss chestnut accessions. The results also indicated that the data obtained for ISSR in terms of species assignment to groups were identical to those of RAPD, showing the displacement of the *C. crenata* 06 accession. Moreover, and compared to that of AFLP, the SSR classification failed to separate *C. mollissima* and *C. crenata* into clear and separate groups. The results presented in this study revealed a closer association with European chestnut. As far as geographic structuring is concerned, the findings revealed that although a number of groups could be identified, the dendrograms showed little to no geographic structuring of accessions for country or for affiliation to "Marroni".

The data generated with regard to the PCoA of pairwise genetic distances, presented in Figures 6–10 can be summarized as follows. The results from PCoA analysis revealed that the RAPD system clearly separated Asiatic from European species (Figure 6). The 2 plotted axes accounted for 25.71% and 8.89% of the variation present at the molecular level, respectively. The first principal coordinate clearly separated the *Castanea sativa* Swiss cultivars from the other accessions (*C. crenata*, *C. mollissima*, and the French, Italian, and coppice members of *C. sativa*). The second principal coordinate was noted to separate accessions at the species level, with the Asiatic chestnuts (*C. crenata*, *C. mollissima*) concentrated and isolated from the *C. sativa* individuals. No further discrimination was, however, visible among the different varieties of Swiss cultivars. Conversely, the 2 Asiatic chestnut species were well separated within their group.

The PCoA results obtained based on AFLP data clearly separated *C. sativa* Swiss cultivars from the remaining ones (Figure 7). The 2 plotted axes accounted for 33.69% and 8.84%, respectively. The discrimination between the 2 groups was performed by the first principal coordinate.

The PCoA obtained by ISSR data sets showed clear separation of *C. sativa* Swiss cultivars from the other cultivars (Figure 8). Similar results were attained with AFLP data. The 2 plotted axes accounted for 21.52% and 8.04%, respectively. Moreover, the use of the first principal component with SSR yielded a clear differentiation of *C. sativa* Swiss cultivars from the other cultivars on the diagonal of the plot (Figure 9). The 2 plotted axes accounted for 17.41% and 9.80%, respectively. Last but not least, the PCoA results obtained by the combined data set showed a clear separation of Swiss cultivars of *C. sativa* from the other species and cultivars (Figure 10).

We estimated the variance components to assess which contributes more to genetic diversity: within-group variance or among-group variance (Table 5). The lack of group structure in the dendrograms was reflected

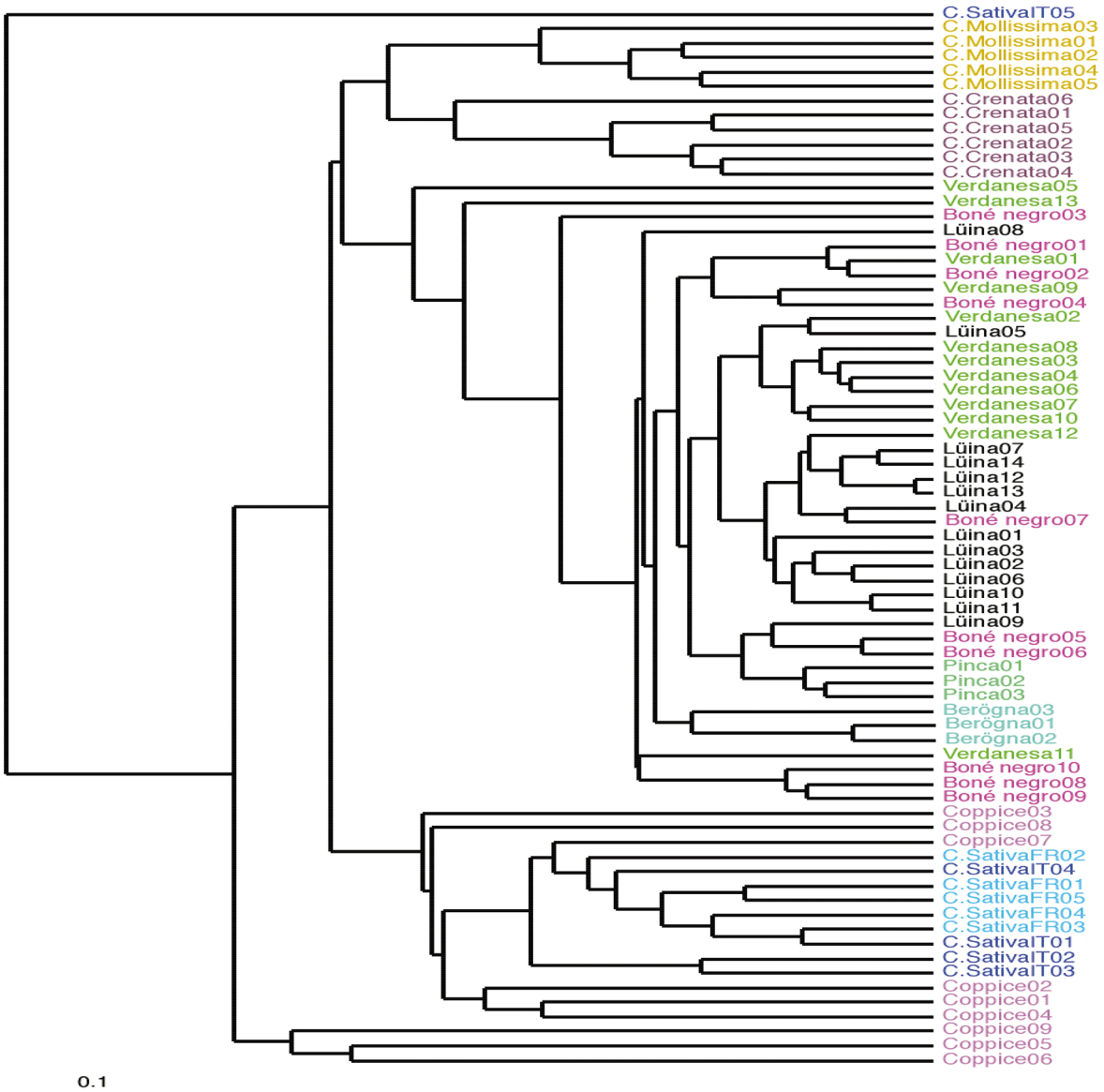

Figure 1. RAPD dendrogram based on Jaccard's genetic distance.

in the AMOVA data analysis. The among-group variance components were very low for AFLP and SSR. The respective percentages of variation were

5.38% and 30.81%. The findings also showed that the within-group variance components were low for RAPD (32.13%) and ISSR (36.73%), which indicates that the genetic background attributable to the geographical origin contributes to genetic diversity.

4. Discussion

The application of molecular marker systems has revolutionized the pace and precision of plant genetic analysis and helped to develop efficient plant conservation strategies. Although several molecular marker systems are currently available in the literature, most of the studies so far performed have employed only one marker system for the analysis and characterization of cultivated chestnuts. Recently, comparison between 2 marker methods on genetic diversity in chestnut is becoming common (Goulão et al., 2001; Martin et al., 2010). The dominant markers (RAPD, AFLP, and ISSR) used in this study provide a large number of polymorphic loci and were in general agreement with other studies of genetic diversity measurements. Microsatellites marker are typically codominant markers but showed lower congruence with dominant-marker data (Allan et al., 2008).

Accordingly, these results demonstrate that each method is useful and informative for evaluating *Castanea*

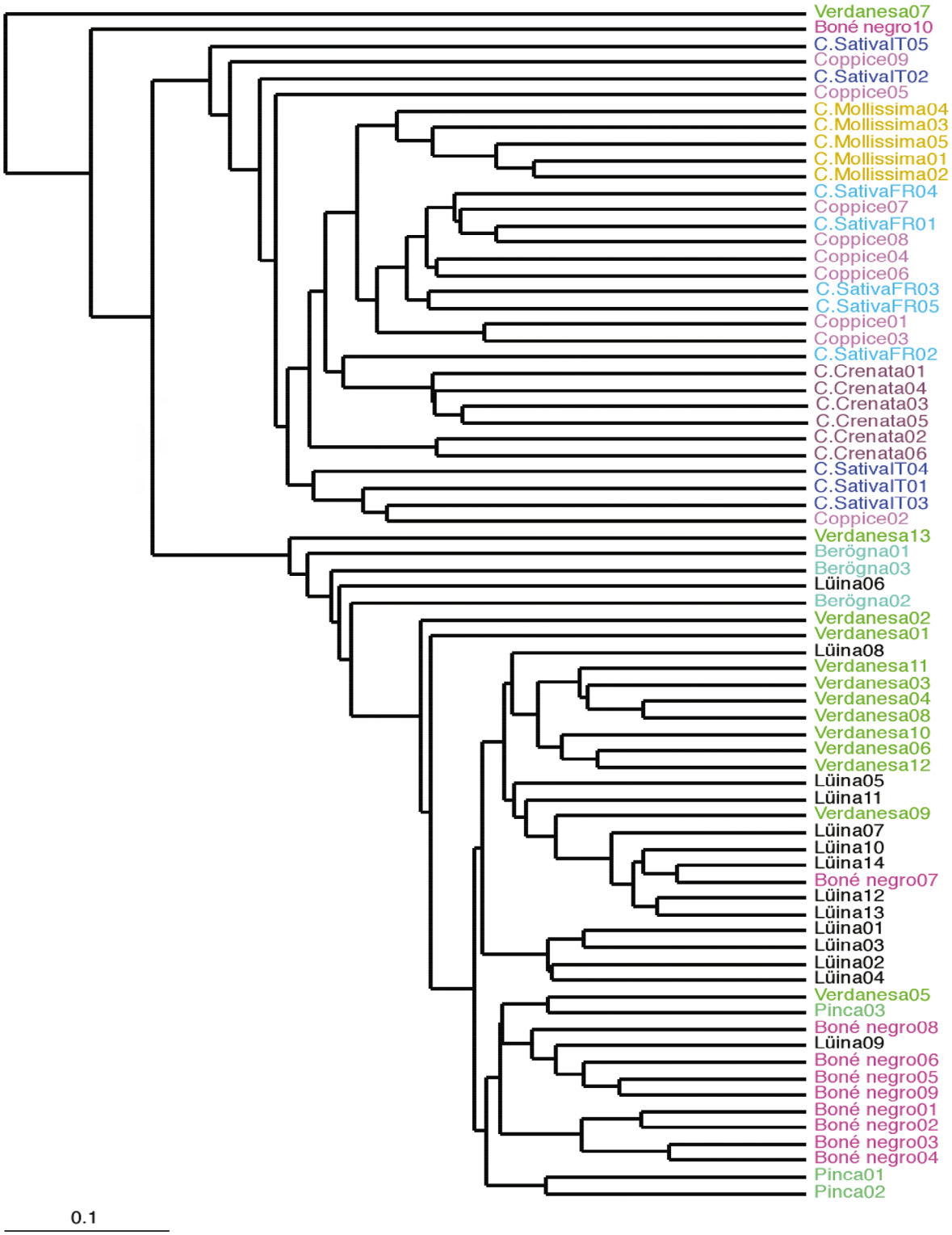

Figure 2. AFLP dendrogram based on Jaccard's genetic distance.

genetic diversity. The most useful RAPD primer was OPE-01, generating 14 banding patterns with polymorphism of 87.5%, although the most useful AFLP pair primer was E-AAC/M-CAT with 68 and a high MR (119.25%) and MI (50.77). The most useful ISSR primers were UBC-841 and UBC-834, generating 12 bands with PIC of 0.667. The

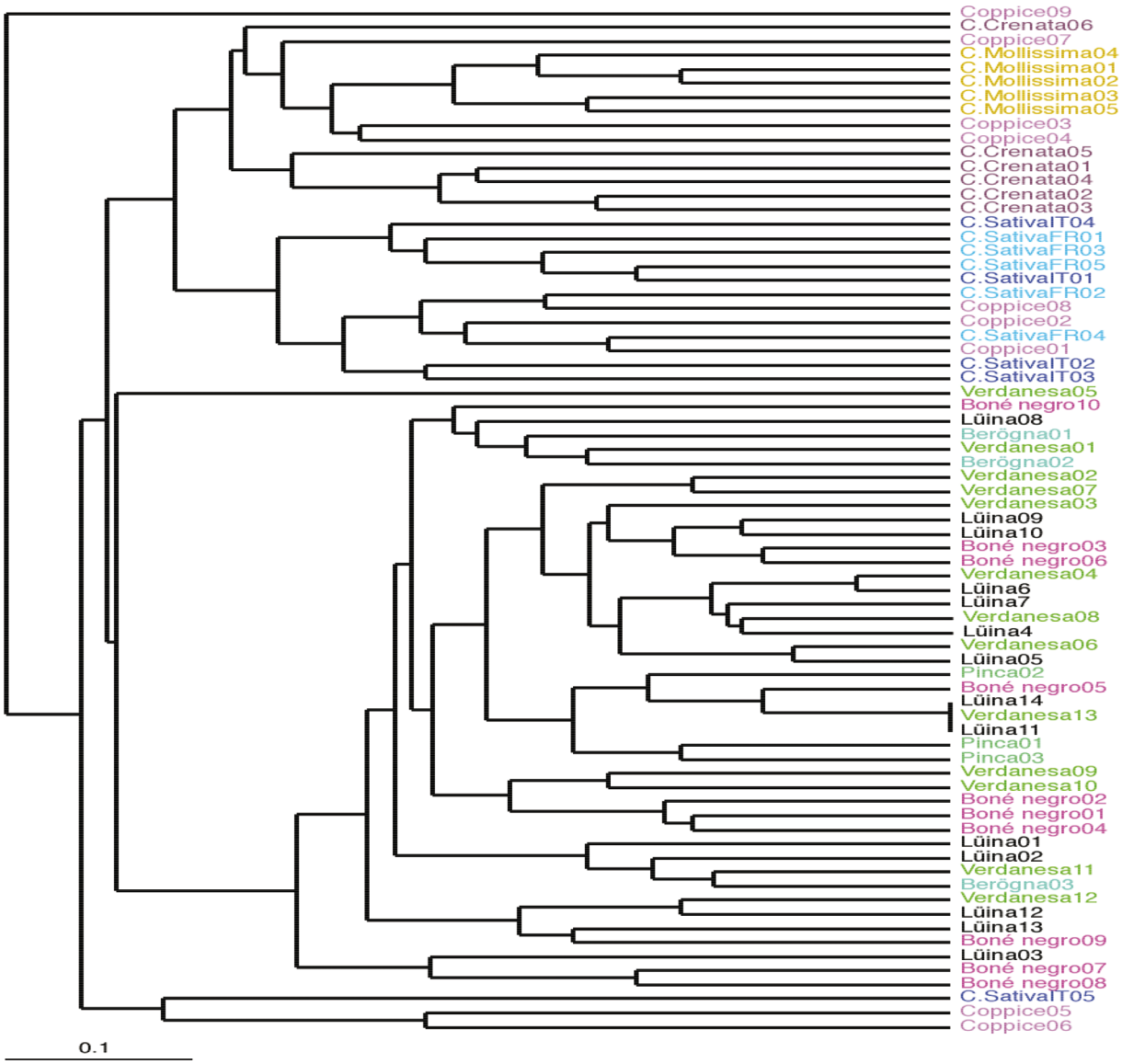

Figure 3. ISSR dendrogram based on Jaccard's genetic distance.

most useful SSR primer was QpZag9, which detected 12 polymorphic bands with PIC of 0.483.

Among these markers, the AFLP marker was considered to generate the greatest number of polymorphic loci (248). The efficiency of AFLP markers and their capacity to reveal a high number of polymorphic bands per amplification and per primer has previously been reported in several studies (Russel et al., 1997; Coart et al., 2002; Fernandez et al., 2002).

However, the number of polymorphic bands was lower for ISSR (42) and SSR (36) overall than those described for Portuguese chestnut (Goulão et al., 2001). These results suggest a low genetic diversity in this chestnut population, which might be caused by its different domestication levels, as chestnut has undergone natural and artificial selection pressures, which have shaped the actual genetic and phenotypic traits, or because Japanese chestnut, Chinese chestnut, and European chestnut are thought to share the same origin, located in eastern Asia (Lang et al., 2007).

In addition, AMOVA analysis indicated that 94.62% of the total genetic diversity by AFLP is distributed within groups, although only 5.38% of the diversity is attributed to differences between regions. This low variability between regions was also reported by Fei et al. (2012) and showed that phylogenetic analysis of chloroplast DNA sequence data indicates an origin for chestnut in East Asia and migration to North America from Europe.

A recent study by Marinoni et al. (2013) on the genetic and morphological diversity among various local populations of Italian chestnut (Piedmont) reported that

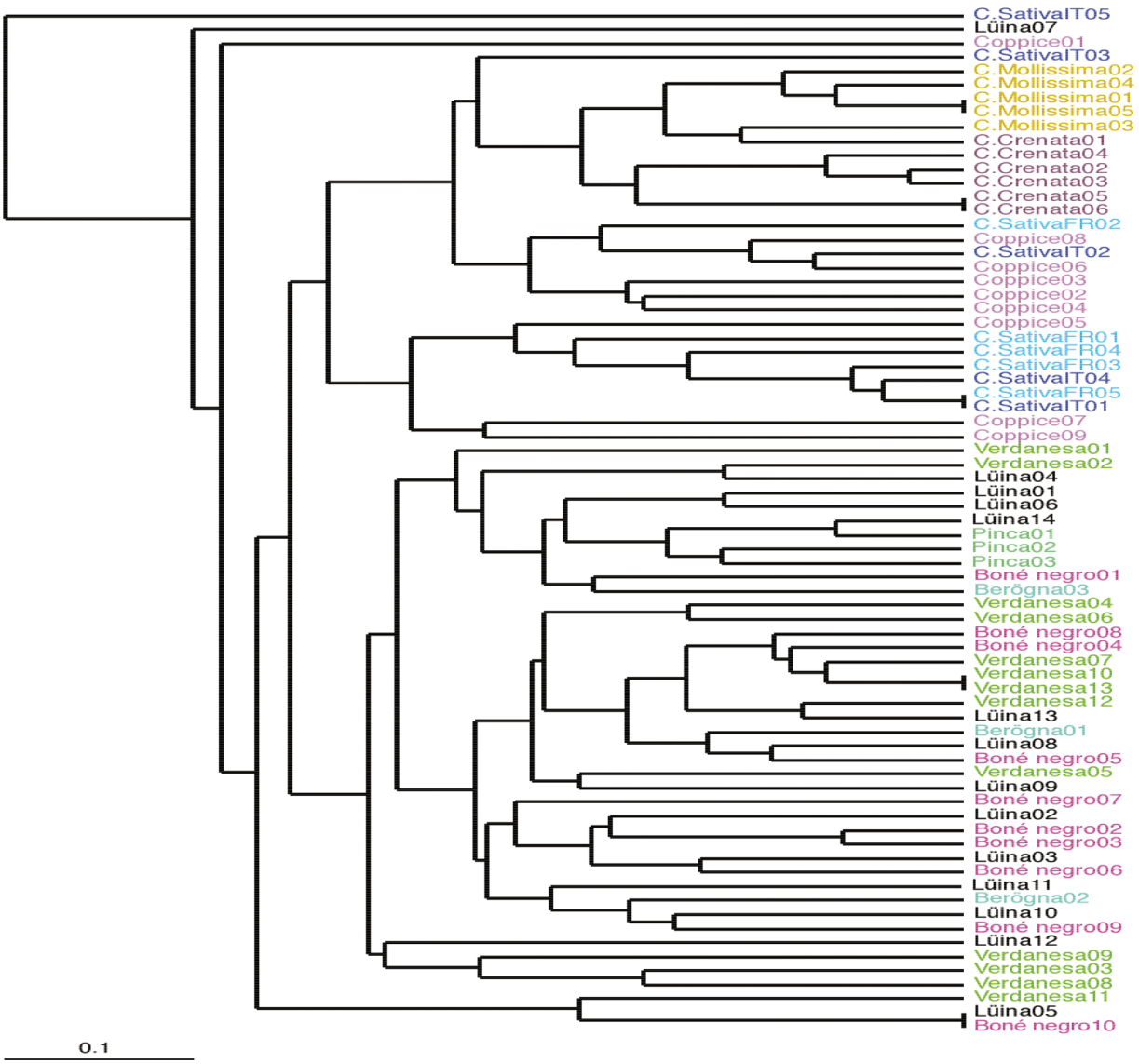

Figure 4. SSR dendrogram based on Jaccard's genetic distance.

genetic intracultivar homogeneity was observed for some of the most valuable cultivars.

On the other hand, RAPD represent 67.87% of among-group variation, showing that RAPD reflects slightly more variation depending on geography. According to Mellano et al. (2012), the low divergence between species can be explained by the wide diversity of *Castanea* species and the good adaptation of the genus to different environmental conditions. It shows variability for morphological and ecological traits, vegetative and reproductive habits, nut size, wood characteristics, adaptability, and resistance to biotic and abiotic stresses, and the burden between natural biodiversity and human selection is very weak and sometimes unclear.

For the accessions in our study, consistent patterns of clustering according to the *Castanea* species were not found, and no consensus grouping was generated in the 4 dendrograms for the 4 markers. This result was supported by the low correlation coefficient among the 4 markers. Yamamoto et al. (1998) also reported the failure of clustering according to species in chestnut accessions.

There were some differences between the marker techniques in terms of clustering. The choice of an appropriate method of genetic analysis generally depends on the nature of the study that the method will be used for, since the methods differ in their fittingness to sample different parts of the genome. In fact, the RAPD method is easier and faster than SSR and AFLP, but comparable to

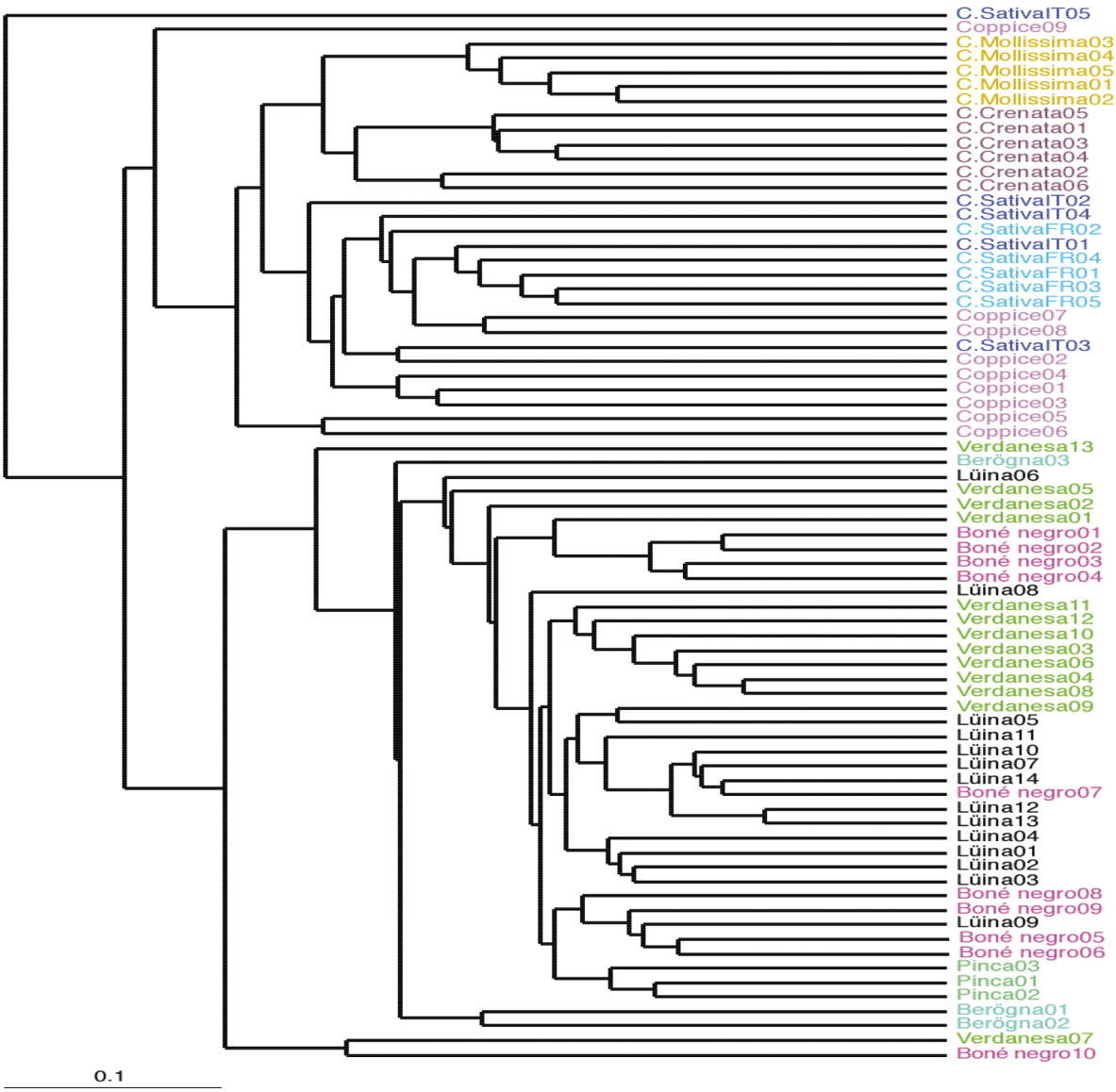

Figure 5. Combined data dendrogram based on Jaccard's genetic distance.

ISSR. However, RAPD reproducibility is a weak aspect of this technique.

The comparison of data obtained with SSR and the 3 other types of markers revealed low correlation coefficients. Several works have previously used SSR as a tool to study genetic variation, and the microsatellite results obtained were often highly discordant with other molecular data (Russell et al., 1997; Pejic et al., 1998). Despite the small number of SSR bands found in this study (36 bands), the higher specificity of amplifications with SSR primers (microsatellites amplified DNA repetitive regions and those regions were hypervariable due to the slippage mechanism) could explain the discordance among the other molecular data sets (Schloss et al., 2002).

Microsatellites loci may be particularly sensitive to inbreeding effects; are suitable to perform mating-system analyses in small and isolated populations where dominant AFLP, ISSR, and RAPD molecular markers are less appropriate; and are able to discriminate between very closely related genotypes as previously shown elsewhere (Russel et al., 1997).

Principal coordinates analyses and cluster analysis performed on the distance matrices showed a close similarity between Asian species *Castanea mollissima* and *Castanea crenata*. This result is in agreement with the findings previously reported in the literature (Huang et al., 1994; Morimoto et al., 1997; Yamamoto et al., 1998), showing that *Castanea mollissima* might be considered as

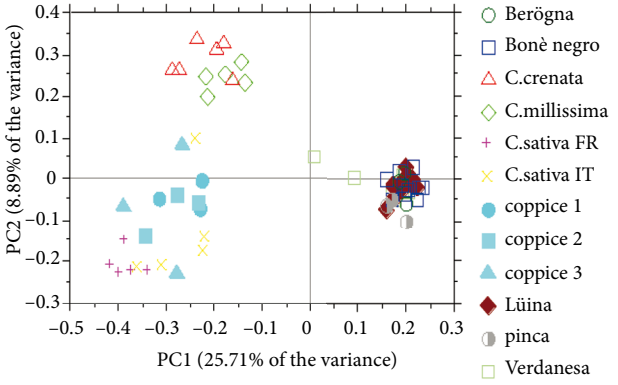

Figure 6. Principal coordinates analysis (PCoA) based on RAPD genetic similarity matrix.

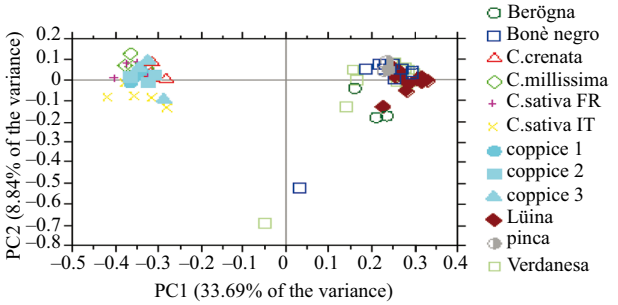

Figure 7. Principal coordinates analysis (PCoA) based on AFLP genetic similarity matrix.

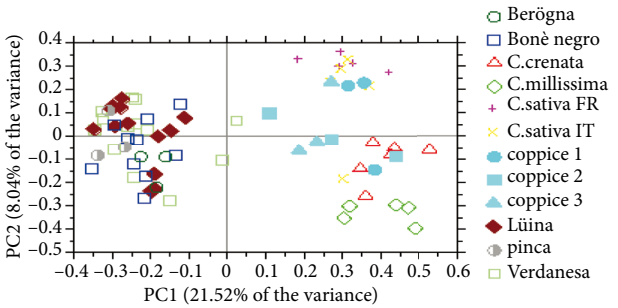

Figure 8. Principal coordinates analysis (PCoA) based on ISSR genetic similarity matrix.

the progenitor of all other *Castanea* species, and genetic relationships between Korean *C. crenata* varieties and Chinese chestnuts were rather complicated.

The presence of unexpected genetic associations among *Castanea* species was already reported by Sawano et al. (1984), who unsuccessfully tried to find species-specific markers among the Japanese, Chinese, Japanese–Chinese hybrid, and European chestnut using 3 enzyme systems.

The highest correlation was recorded between RAPD and ISSR marker types (Mantel's r = 0.77, P < 0.01). These results can be explained by the fact that both molecular

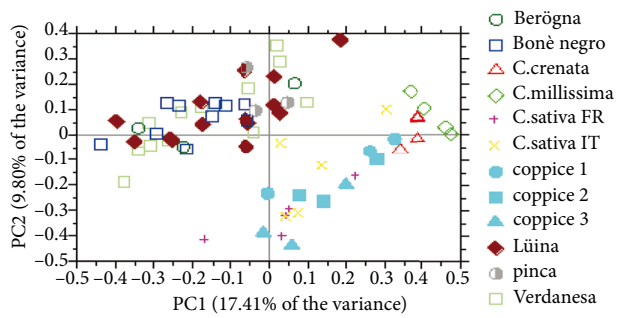

Figure 9. Principal coordinates analysis (PCoA) based on SSR genetic similarity matrix.

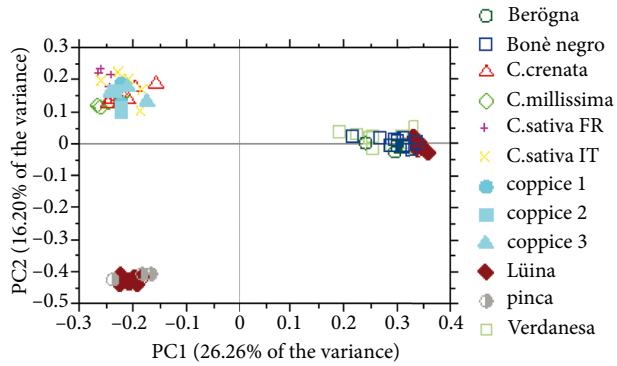

Figure 10. Principal coordinates analysis (PCoA) based on combined data genetic similarity matrix.

types are dominant markers and that each marker system samples a very small fraction of the genome that was arbitrarily amplified (Fahima et al., 1999).

Virk et al. (2000) previously performed a comparative study involving the use of different classes of DNA markers for the identification and classification of variation in rice germplasm. They reported that AFLP and isozyme data were more suitable for the differentiation of rice groups than RAPD and ISSR.

The results presented in this study with regard to the more informative nature of ISSR as compared to RAPD are in disagreement with the findings previously reported by Casasoli et al. (2001). This disagreement may be attributed to differences in the number of primers used in the experiments, which is considered as a source of variation in molecular data (Lefebvre et al., 2001).

The presence of accessions from different countries of origin in the group that contained Swiss, French, and Italian cultivars, as illustrated in dendrograms and PCoA, could presumably be attributed to their common origin and their diffusion into Europe by human activity (Pereira-Lorenzo et al., 2001). This existence of substantial genetic uniformity within European cultivars reflects the long history of chestnut cultivation, and the reduction of

Table 5. Comparative AMOVA statistics for RAPD, AFLP, ISSR and SSR.

Source of variation		d.f.[a]	% of variation	P-value[b]
RAPD	Among group	3	67.87	<0.001
	Within group	5	32.13	
AFLP	Among group	3	5.38	<0.001
	Within group	5	94.62	
ISSR	Among group	3	63.27	<0.001
	Within group	5	36.73	
SSR	Among group	3	30.81	<0.001
	Within group	5	69.19	

[a]: Degrees of freedom.
[b]: Significance test after 1023 random permutations.

diversity produced by grafting may have been compensated for by the use of seedlings, as reported by Pereira-Lorenzo et al. (2011).

Cluster and PCoA analyses showed a unique genetic structure in the Swiss *C. sativa* group, showing genetic intracultivar homogeneity for some of the most valuable cultivars. This homogeneity could presumably be due to the selection pressure made by local breeders in southern Switzerland a long time ago or to the adaptation of cultivars to appropriate soil and microclimate conditions (Abdelhamid et al., 2004). In fact, the close relatedness among individuals of 'Verdanesa', 'Bonè negro', 'Lüina', 'Berögna', and 'Pinca' could be explained by the existence of polyclonal varieties and, hence, the possible agametic propagation of 'Verdanesa' by seeds and compatibility for single trees (Gobbin et al., 2007).

The findings from the molecular marker analyses presented in this study indicated that the application of 4 types of molecular markers for genotyping purposes is a promising approach that offers valuable possibilities for the exploration of the genetic diversity and variability among *Castanea* species. DNA analysis provided consistent information about chestnut species' genetic variability, which may open new and promising opportunities for the development of germplasm collections. The higher levels of efficiency recorded for AFLP as a molecular marker system for the assessment of chestnut genetic diversity can also enhance the pace and precision with which effective conservation strategies can be developed to preserve this valuable tree species. Accordingly, further research, some of which is currently underway in our laboratories, is needed to investigate the genetic diversity of other European chestnut cultivars and to sample a large number of Asian varieties so as to identify their genetic background in relation to European cultivars. The attainment of better levels of discrimination among the 3 studied species of chestnut also requires the testing of more primers via ISSR and SSR methods as well as other molecular analysis techniques.

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