Typing European Chestnut (*Castanea sativa* Mill.) Cultivars Using Oak Simple Sequence Repeat Markers

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Abstract. Microsatellite or simple sequence repeat (SSR) markers show many characteristics of the ideal molecular marker, and recent studies have shown that loci developed in one species may allow analysis in taxonomically related species. In this study, 52 primer pairs developed in two oak species—*Quercus robur* L. and *Quercus petraea* (Matt.) Lieb.—were used to amplify DNA of 5 chestnut cultivars; 28 of them yielded amplicons and 12 polymorphic loci were selected and used to fingerprint 12 European chestnut (*Castanea sativa* Mill.) cultivars grown in the Piedmont region of northwestern Italy. The number of alleles per locus ranged from 3 to 8, mean expected heterozygosity was 0.592 (range: 0.288 to 0.868), and mean observed heterozygosity was 0.667 (range: 0.333 to 1.000). The results demonstrate the usefulness of some SSR markers isolated in *Quercus* for the fingerprinting and genetic mapping of *Castanea* cultivars.

Microsatellite or simple sequence repeat (SSR) markers show many characteristics of the ideal molecular marker: abundance in the genome, reproducibility, a high level of polymorphism, and codominant inheritance. Once an SSR-containing region has been isolated from the genome, primers can be designed from the DNA sequence of the regions that flank the repeat and used to amplify the locus using the polymerase chain reaction (PCR) and analyzed with semi-automated techniques. The DNA sequences in flanking regions are conserved within the species and often also in taxonomically related species or genera (Di Gaspero et al., 2000; Rossetto et al., 2002; Yamamoto et al., 2001). A molecular marker is considered conserved if it can be amplified in two species with the same primer pair. Conserved microsatellite loci have been reported in *Citrus* (Kijas et al., 1995), *Quercus* (Hornero et al., 2001; Steinkellner et al., 1997a), *Prunus* (Cipriani et al., 1999; Downey and Iezzoni, 2000), *Pyrus* (Akkak et al., 1998; Yamamoto et al., 2001), *Picea* (Hodgetts et al., 2001; Rajora et al., 2001), *Fagus* (Tanaka et al., 1999), *Actinidia* (Huang et al., 1998), and *Vitis* (Rossetto et al., 2002; Sefc et al., 1999). This finding may permit the analysis of different species using the same SSR loci, thus reducing the costs.

The European chestnut (*Castanea sativa* Mill.) is a tree of interest for both agriculture and forestry and represents, where the soil and climatic conditions are favorable, one of the

most common species in forests around the Mediterranean basin. Chestnut cultivation in Europe was widespread in rural as well as in mountain areas until the middle of the 20th century, at which time chestnut cultivation entered a period of decline due to socio-economic changes and mountain depopulation and to the spread of diseases such as blight caused by *Cryphonectria parasitica* (Murr.) Barr and ink disease caused by *Phytophthora cambivora* (Petri) Buis. and *Phytophthora cinnamomi* Rand.. In the last decade of the 20th century there was a renewal of interest in the growing of chestnut in Europe, thanks to the decreased virulence of *Cryphonectria parasitica* and to increased demand for chestnut nuts and wood.

In this situation there is a need for the rescue, description and identification of the once widely cultivated germplasm and for the restoration of old plantations. In fact, in spite of the abandonment of old plantations and of the widespread distribution of blight, most trees survived indicating that adult trees of *Castanea sativa* were able to tolerate attack by the fungus even before the introduction of hypovirulent strains. During the past decade, the understory has been removed from many plantations, trees 70 to 150 years old have been pruned and good productivity has been restored.

Germplasm collection and characterization has been carried out in Spain, France, Italy and Greece and genetic studies on both cultivated and wild populations have been conducted by several authors (Fineschi et al., 1993; Goulaou et al., 2001; Paffetti et al., 1999; Pereira et al., 1999; Pereira-Lorenzo and Fernandez-Lopez, 1993; Pereira-Lorenzo et al., 1996; Santana et al., 1999; Villani et al., 1991, 1993) using isozyme, randomly amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) markers, with the aim of assessing genetic variability, constructing phylogenetic

trees and fingerprinting cultivars. In addition, linkage maps for *Castanea* have been constructed (Barreneche et al., 2003; Casasoli et al., 2001; Kubisiak et al., 1997). SSR markers have only recently been isolated from *C. sativa* (Buck et al., 2003; Marinoni et al., 2003).

Preliminary research (Botta et al., 1999; Steinkellner et al., 1997a) showed that primers developed for some SSR loci in two species of oak, *Quercus robur* L. (Kampfer et al., 1998) and *Quercus petraea* (Matt.) Lieb. (Steinkellner et al., 1997b), would also amplify chestnut DNA. This paper identifies the oak SSR loci most useful for fingerprinting chestnut cultivars.

Materials and Methods

Plant material and DNA extraction. ‘Mari-goule’ (*Castanea crenata* Sieb. et Zucc. x *Castanea sativa* Mill.), 12 *C. sativa* individuals representing the cultivars grown in different valleys of the Piedmont region of northwestern Italy (Table 1), and two individuals of *Q. petraea* were used. For evaluating polymorphism in the selected SSR loci, a group of 12 cultivars was chosen. The group included ‘Marrone di Chiusa Pesio’ which is of high interest for the quality of its nuts, ‘Garrone Rosso’ and ‘Garrone Nero’, which are considered related to ‘Marrone’, and other cultivars that are genetically more distant.

DNA was extracted from 0.5 g of leaves using the procedure described by Thomas et al. (1993) in a Tris-EDTA-NaCl buffer containing 0.25 M NaCl, 0.2 M Tris-HCl pH 7.6, 2.5% PVP (molecular weight 40,000), 0.05 M Na₂EDTA pH 8.0, 3% sarcosyl, 20% ethanol and 1% β-mercaptoethanol. After purification the DNA was suspended in 70 μL Tris-EDTA buffer.

Microsatellite PCR amplification and analysis. Primer pairs designed for 41 loci from *Quercus robur* (Kampfer et al., 1998) and 11 from *Q. petraea* (Steinkellner et al., 1997b) were used to amplify DNA of five chestnut cultivars (‘Marrone di Luserna’, ‘Gioviasca’, ‘Pelosa piccola’, ‘Pelosa grossa’, and ‘Marigoule’). The primer sequences for 10 loci from *Q. robur* were unpublished and were kindly provided by Steinkellner (Zentrum fuer Angewandte Genetik, Universitaet fuer Bodenkultur, Vienna). DNA of the two *Q. petraea* individuals was included as positive controls.

PCR was performed in a volume of 20 μL containing 50 ng DNA, 0.5 U Taq-DNA polymerase (Ampli-Taq Gold polymerase, Applied-Biosystems), 2 μL 10× PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 1.8 mM MgCl₂, 200 μM dNTPs and 0.5 μM of each primer. A DNA Thermal Cycler 480 (Perkin Elmer Cetus) was used and the PCR conditions were: first a denaturation step at 95 °C for 10 min followed by 26 cycles of denaturation (50 s at 95 °C), annealing (45 s at the optimal temperature for *Quercus* for each primer pair and ranging from 45 to 60 °C, Table 3) and extension (90 s at 72 °C). The final elongation step was at 72 °C for 7 min.

The PCR products were separated by elec-

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Table 1. Allele sizes (in base pairs) at 12 SSR loci in 12 chestnut cultivars grown in the Piedmont region of northwestern Italy. The (–) sign was used when only one allele was detected, the missing allele could be of the same size (homozygous individuals) or could be a null allele (heterozygous individuals)

DNA		Allele																							
code	Cultivar	QrZAG7		QrZAG20		QrZAG75		QrZAG96		QrZAG101		QrZAG121		QpZAG15		QpZAG16		QpZAG36		QpZAG46		QpZAG110		QpZAG119	
C26	Garrone rosso	122	124	173	177	164	–	155	160	127	129	217	–	120	128	158	160	217	223	192	194	210	219	64	92
C57	Gabiana	120	124	177	–	164	166	155	160	129	–	215	217	120	128	160	–	221	223	194	–	213	219	78	92
C37	Garrone nero	122	124	173	177	112	164	155	160	129	155	217	–	128	–	160	–	217	221	192	194	210	219	64	78
C67	Gentile	122	124	161	179	132	–	155	157	129	155	217	–	120	128	160	–	217	221	192	194	210	213	64	78
C06	Gioviasca	120	124	173	179	136	155	155	–	129	–	217	223	128	–	160	162	217	221	192	194	213	219	64	86
C19	Lusench	120	–	179	–	136	166	155	162	129	–	217	–	118	128	158	160	221	223	194	–	213	219	64	86
C10	Madonna	120	124	173	–	140	155	155	166	129	155	217	–	120	128	160	–	217	221	192	196	210	230	86	92
C22	Marrone di Chiusa Pesio	120	122	173	179	112	–	155	157	127	155	217	–	128	–	160	–	217	221	192	194	210	–	64	86
C09	Pelosa grossa	120	124	173	–	140	–	155	160	129	–	217	223	120	128	158	162	217	221	192	194	213	219	64	86
C17	Pelouset	120	–	167	–	136	145	160	–	129	–	217	–	128	–	160	–	219	221	194	–	219	230	86	–
C83	Ruiana	120	124	159	179	145	166	155	–	129	–	217	–	128	–	158	160	217	221	192	194	213	–	64	76
C16	Tempuriva	120	–	173	–	136	155	155	–	127	155	217	223	128	132	158	160	211	221	194	198	213	219	64	86

trophoresis on a 8 × 10-cm polyacrylamide gel and stained with ethidium bromide to verify amplification. Loci which showed good amplification were then analyzed on sequencing gels using chemiluminescent detection.

One microliter of each sample was run, after denaturation in 1 µL of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanole) at 95 °C for 3 min, on a 50 × 21-cm sequencing gel (6% polyacrylamide 24:1, 8 M urea, 1× TBE buffer). After electrophoresis, DNA was blotted on a Zeta Probe (Bio-Rad, USA) positively charged nylon membrane and hybridized 1h at 45 °C in 15 to 20 mL of hybridization buffer (1 M EDTA, 7% SDS, 0.25 M disodium phosphate pH 7.2) containing 60–120 pmol of a biotin labeled (GA)₁₃ and (GT)₁₃ probes. Chemiluminescent detection was performed using a Phototope-Star Detection Kit by New England BioLabs (USA) following the manufacturer's instructions.

Twelve cultivars of *C. sativa* were chosen for analysis at twelve selected loci using a semi-automatic system in order to evaluate SSR marker polymorphism. Two cultivars of *C. sativa*—‘Marrone di Luserna’ and ‘Pelosa piccola’—were not analyzed because they were found to be synonyms of ‘Marrone di Chiusa Pesio’ and ‘Pelosa grossa’, respectively (Marinoni, 2000). The forward primer of each locus was labeled with one of the following fluorochromes: NED (yellow), HEX (green), or 6-FAM (blue).

One microliter of a mix containing amplification products of three differently labeled loci was added to 3 µL of a mix containing 10:2:1 parts of formamide, GeneScan-350 ROX size standard and loading buffer (25 mM EDTA, 50 mg·mL⁻¹ blue dextran) respectively. Samples were denatured at 95 °C for 5 min and analyzed on a sequencing gel (4.25 % acrylamide, 1× TBE buffer, 6 M Urea) using an ABI-PRISM 377 sequencing machine. Data were analyzed with GENESCAN software and alleles were designated by their size in base pairs.

Statistical analysis. The software IDENTITY 1.0 (Wagner and Sefc, 1999) was used to calculate the number of alleles per locus and their frequency, expected and observed heterozygosity, frequency of null alleles, paternity exclusion probability and probability of finding two identical genotypes. Expected heterozygosity (He) was calculated as $He = 1 - \sum p_i^2$ where p_i denotes the frequency of

allele i in the cultivars (Nei, 1973). Observed heterozygosity (Ho) was obtained from direct calculation. The null allele frequency was calculated as $r = (He - Ho)/(1 + He)$ where He = expected heterozygosity and Ho = observed heterozygosity (Brookfield, 1996). The probability of finding two identical genotypes was calculated as $PI = \sum p_i^4 + \sum (2 p_i p_j)^2$, where p_i and p_j are the frequencies of alleles i and j , respectively (Paetkau et al., 1995).

Results and Discussion

SSR locus analysis. In this study we wanted to evaluate SSR markers developed for oak species and to select a set of loci that could be routinely used in chestnut. Our first objective was to verify in chestnut the successful amplification by each oak primer pair. Of the 52 primer pairs, 28 (54%) yielded amplicons in five chestnut cultivars (Table 2), showing the existence of homologous regions in the genomes of *Quercus* and *Castanea*; successful amplification was obtained for 91% of loci from *Q. petraea* and 40.5% of loci from *Q. robur*. The remaining 24 (46%) were not pursued further since they were able to amplify oak DNA but not chestnut, even with lower stringency conditions during PCR.

Steinkellner et al. (1997a) found that SSR markers from *Q. petraea* were present in *Q. robur* (100%) and *Q. pubescens* Willd. (100%), while only some of them could successfully be amplified in *Q. cerris* L. (64%), *Q. rubra* L. (47%), *Q. palustris* Muenchh. (47%), and *Fagus sylvatica* L. (24%). Similar results are reported by Hornero et al. (2001) for *Q. suber* L. (54.5%). The presence of common markers across the species can be of great interest for genetic mapping (Casasoli et al., 2001; Barreneche et al., 1998) and, in particular, for comparative mapping, as demonstrated by the results of Barreneche et al. (2003).

Yet, the successful amplification of DNA does not always mean that SSR loci are present in the amplicons. Out of 28 primer pairs that yielded amplification in chestnut, 13 were not used for further analysis. Loci *ssrQrZAG4*, *ssrQrZAG46*, *ssrQrZAG87*, *ssrQrZAG88-MS*, *ssrQrZAG88-TT*, *ssrQrZAG90*, and *ssrQpZAG58* yielded a number of bands higher than the two expected. In this case either the amplification is not specific enough and more regions of the genome were amplified or the

SSR locus is present in multiple copies. These loci were discarded for use in fingerprinting, as interpretation of the results would have been difficult. In some cases, specific bands are not amplified when PCR conditions were made more stringent (e.g. higher annealing temperature) but this was not the case for the mentioned loci. Loci *ssrQrZAG59*, *ssrQrZAG73*, and *ssrQpZAG1/2* produced fragments of larger size (350–600 bp) in *Castanea* than in *Quercus*. These fragments may come from other regions of the genome and, even if they contain the SSR sequence, are not of practical use for fingerprinting due to the large size of the amplicons. Further three loci, *ssrQrZAG31*, *ssrQrZAG86*, and *ssrQpZAG3/64*, were discarded because showed weak amplification and could not be improved resulting in unclear profiles. Weak amplification may be due to mismatches in the primer annealing site.

The other 15 loci gave good amplification, the results were clear and the allele sizes were approximately the same as in oak. They were analyzed using a manual sequencing apparatus and chemiluminescent detection. Loci *ssrQrZAG5*, *ssrQrZAG65*, and *ssrQrZAG112* were the least polymorphic (≤2 alleles) and although they could be useful for mapping or other purposes, were not further considered for fingerprinting.

Twelve loci showed at least two alleles and were selected for the analysis of 12 cultivars of *C. sativa* using the automated sequencer ABI-PRISM 377 and GENESCAN software. Results (Table 1) were highly reproducible and conditions of analysis were optimized for each locus to minimize stuttering.

SSR loci polymorphism. For evaluating polymorphism in the selected set of 12 loci, a group of 12 different cultivars were chosen from the cultivated germplasm. Number of alleles, number of genotypes and expected heterozygosity are indicators used for evaluating polymorphism and discriminant power of a locus.

The number of alleles per locus ranged from 3 to 8 (Table 3). Loci *ssrQrZAG75* (8 alleles) and *ssrQrZAG20* (6 alleles) had the largest number of alleles.

The combination of profiles across all loci resulted in 12 different genotypes, one for each cultivar (Table 1). The highest discriminative power was shown by loci *ssrQrZAG75* (11 genotypes), *ssrQrZAG20* (8 genotypes) and *ssrQpZAG110* (7 genotypes).

The probability of identity (PI) for each locus ranged from 0.062 for *ssrQrZAG75* to 0.579 for *ssrQrZAG121* (mean: 0.339), whereas the total probability of identity was 4.58×10^{-7} .

The allele frequencies ranged from 0.042 to 0.833 (Table 4). The expected heterozygosity averaged 0.592 and ranged from 0.288 (for *ssrQrZAG121*) to 0.868 (for *ssrQrZAG75*), but the observed heterozygosity averaged 0.667 and ranged from 0.333 (for *ssrQrZAG121*) to 1.000 (for *ssrQpZAG36*).

Among loci from *Q. petraea*, observed

heterozygosity in chestnut was 1.00 at *ssrQpZAG36* and 0.92 at *ssrQpZAG119*, higher than in oak (Steinkellner et al., 1997b), while lower values were found for *ssrQpZAG15*, *ssrQpZAG16*, *ssrQpZAG46*, and *ssrQpZAG110* (0.58, 0.50, 0.75 and 0.83, respectively). Among loci from *Q. robur*, *ssrQrZAG7*, *ssrQrZAG96*, and *ssrQrZAG75* showed the highest level of observed heterozygosity (0.75, 0.67 and 0.67, respectively).

The possible presence of null-alleles showed positive values (0.131 and 0.108) for *ssrQrZAG20* and *ssrQrZAG75*, respectively.

Table 2. Results from amplification of *Quercus robur* and *Q. petraea* SSR loci in four cultivars of *Castanea sativa* Mill. and in hybrid 'Marigoule' (*C. crenata* x *C. sativa*).

Locus ^z	Amplification ^y	Results ^x
<i>ssrQrZAG2</i>	—	
<i>ssrQrZAG4</i>	+	>2 Bands
<i>ssrQrZAG5</i>	+	Good but monomorphic
<i>ssrQrZAG7</i>	+	Good
<i>ssrQrZAG11</i>	—	
<i>ssrQrZAG13</i>	—	
<i>ssrQrZAG15</i>	—	
<i>ssrQrZAG18</i>	—	
<i>ssrQrZAG19</i>	—	
<i>ssrQrZAG20</i>	+	Good
<i>ssrQrZAG25</i>	—	
<i>ssrQrZAG30</i>	—	
<i>ssrQrZAG31</i>	+	Weak
<i>ssrQrZAG39</i>	—	
<i>ssrQrZAG44</i>	—	
<i>ssrQrZAG46</i>	+	>2 Bands
<i>ssrQrZAG51</i>	—	
<i>ssrQrZAG52</i>	—	
<i>ssrQrZAG56</i>	—	
<i>ssrQrZAG58</i>	—	
<i>ssrQrZAG59</i>	+	Larger amplicons
<i>ssrQrZAG65</i>	+	Good but monomorphic
<i>ssrQrZAG73</i>	+	Larger amplicons
<i>ssrQrZAG75</i>	+	Good
<i>ssrQrZAG77</i>	—	
<i>ssrQrZAG82</i>	—	
<i>ssrQrZAG86</i>	+	Weak
<i>ssrQrZAG87</i>	+	>2 Bands
<i>ssrQrZAG88-MS</i>	+	>2 Bands
<i>ssrQrZAG88-TT</i>	+	>2 Bands
<i>ssrQrZAG90</i>	+	>2 Bands
<i>ssrQrZAG95</i>	—	
<i>ssrQrZAG96</i>	+	Good
<i>ssrQrZAG101</i>	+	Good
<i>ssrQrZAG102</i>	—	
<i>ssrQrZAG103</i>	—	
<i>ssrQrZAG108</i>	—	
<i>ssrQrZAG111</i>	—	
<i>ssrQrZAG112</i>	+	Good but monomorphic
<i>ssrQrZAG113</i>	—	
<i>ssrQrZAG121</i>	+	Good
<i>ssrQpZAG1/2</i>	+	Larger amplicons
<i>ssrQpZAG1/5</i>	—	
<i>ssrQpZAG3/64</i>	+	Weak
<i>ssrQpZAG15</i>	+	Good
<i>ssrQpZAG16</i>	+	Good
<i>ssrQpZAG36</i>	+	Good
<i>ssrQpZAG46</i>	+	Good
<i>ssrQpZAG58</i>	+	>2 Bands
<i>ssrQpZAG104</i>	—	
<i>ssrQpZAG110</i>	+	Good
<i>ssrQpZAG119</i>	+	Good

^zPrimer sequences, all from *Q. robur*, not previously published are *ssrQrZAG13*, *ssrQrZAG19*, *ssrQrZAG46*, *ssrQrZAG51*, *ssrQrZAG52*, *ssrQrZAG82*, *ssrQrZAG86*, *ssrQrZAG88 MS*, *ssrQrZAG88 TT*, and *ssrQrZAG113*.

^y(+)Successful amplification of chestnut DNA, (—) no amplification of chestnut DNA

^xGood = presence of 1 to 2 bands of expected size; weak = weak amplification, bands of expected size; larger amplicons = size of amplicons (350 to 600 bp) larger in chestnut than in oak; >2 bands = presence of 3 or more bands.

The estimated frequency of null alleles was a positive value for only 2 of the 12 loci: *ssrQrZAG20* (0.131) and *ssrQrZAG75* (0.108). The number of studied samples was too small to draw conclusions on the occurrence of null alleles, whose presence can be truly ascertained only by studying their segregation or their frequency in a large population (Callen et al., 1993).

On the other hand, the main purpose of this paper was to evaluate the loci for their polymorphism, ease of analysis and discriminative ability. Of the 12 tested, loci *ssrQrZAG20*, *ssrQrZAG75*, *ssrQrZAG96*, *ssrQpZAG36*, *ssrQpZAG110*, and *ssrQpZAG119* can be considered the most interesting for fingerprinting on the basis of polymorphism. Loci *ssrQrZAG75* and *ssrQrZAG20*, in particular, produced the highest number of genotypes.

The loci could be studied in multiplex analyses (Botta et al., 2001) in combinations of 5 to 6 together or in association with the loci isolated in chestnut (Marinoni et al., 2003). For the particularly low molecular weight (64 to 92 bp) of its alleles, locus *ssrQpZAG119* could be conveniently used without overlapping the other loci.

Loci showing less polymorphism, *ssrQrZAG101*, *ssrQpZAG15*, and *ssrQpZAG110* (≤ 4 alleles), should not yet be discarded, as we might expect to find new alleles if a larger number of genotypes is analyzed.

For DNA typing purposes, SSR are likely to be the most appropriate markers since they show a high level of polymorphism, are codominant and permit an easier and reliable transfer of information across laboratories (This et al., accepted). SSR show evident advantages over isoenzymes (Pereira et al., 1999; Pereira-Lorenzo and Fernandez-Lopez, 1993; Pereira-Lorenzo et al., 1996), that are much less polymorphic, and more affected by physiological and environmental conditions and by the kind of plant tissue used for extraction.

The results obtained by Goulao and Oliveira (2001) indicate that SSR markers provide a powerful tool for the fingerprinting exhibiting advantages over RAPD and AFLP. The RAPD markers, used in chestnut by Fineschi et al. (1993), Goulao et al. (2001), Paffetti et al. (1999), and Santana et al. (1999), are very sensitive to the PCR conditions and therefore are less reproducible in different laboratories. AFLP, that were used by Yamamoto et al. (1998) for the genetic characterization of Asian species of *Castanea*, are extremely polymorphic but are based on a complex technique that requires high DNA quality.

Conclusions

The present paper confirms that SSR loci are conserved across taxonomically related species in the *Fagaceae*, but that only a relatively small number (about 20%) of the loci isolated in oak can be conveniently used for the fingerprinting of chestnut. Among them, six loci (*ssrQrZAG20*, *ssrQrZAG75*, *ssrQrZAG96*, *ssrQpZAG36*, *ssrQpZAG110*, and *ssrQpZAG119*) are recommended for routine use while at least three

Table 3. Number of alleles with size range and number of genotypes found for 12 oak simple sequence repeat loci in 12 chestnut cultivars at the specified annealing temperature.

Locus	Fluorochrome	Annealing temp (°C)	N° alleles	Size range	N° genotypes
ssrQrZAG7	HEX	52	3	120–124	4
ssrQrZAG20	NED	52	6	159–179	8
ssrQrZAG75	HEX	54	8	112–166	11
ssrQrZAG96	NED	46	5	155–166	6
ssrQrZAG101	HEX	48	3	127–155	4
ssrQrZAG121	6-FAM	50	3	215–223	3
ssrQpZAG15	6-FAM	54	4	118–132	4
ssrQpZAG16	6-FAM	60	3	158–162	4
ssrQpZAG36	6-FAM	50	5	211–223	5
ssrQpZAG46	HEX	46	4	192–198	4
ssrQpZAG110	NED	50	4	210–230	7
ssrQpZAG119	NED	46	5	64–92	7

Table 4. Characteristics of 12 SSR loci studied in 12 chestnut cultivars.

Locus	Allele size (bp)	Allele frequency	Heterozygosity expected (He) and observed (Ho)	Frequency of null alleles	Probability of identity (PI)
ssrQrZAG7	120	0.500	He: 0.611	-0.086	0.378
	122	0.167	Ho: 0.750		
	124	0.333			
ssrQrZAG20	159	0.042	He: 0.726	0.131	0.196
	161	0.042	Ho: 0.500		
	167	0.083			
	173	0.417			
	177	0.167			
ssrQrZAG75	179	0.250		0.108	0.062
	112	0.125	He: 0.868		
	132	0.083	Ho: 0.667		
	136	0.167			
	140	0.125			
	145	0.083			
	155	0.125			
	164	0.167			
ssrQrZAG96	166	0.125		-0.050	0.324
	155	0.583	He: 0.587		
	157	0.083	Ho: 0.667		
	160	0.250			
ssrQrZAG101	162	0.042		-0.002	0.415
	166	0.042			
	127	0.125	He: 0.496		
ssrQrZAG121	129	0.667	Ho: 0.500	-0.035	0.579
	155	0.208			
	215	0.042	He: 0.288		
ssrQpZAG15	217	0.833	Ho: 0.333	-0.091	0.443
	223	0.125			
	118	0.042	He: 0.451		
ssrQpZAG16	120	0.208	Ho: 0.583	-0.036	0.458
	128	0.708			
	132	0.042			
ssrQpZAG36	158	0.208	He: 0.448	-0.205	0.293
	160	0.708	Ho: 0.500		
	162	0.083			
	211	0.042	He: 0.660		
ssrQpZAG46	217	0.333	Ho: 1.000	-0.132	0.443
	219	0.042			
	221	0.458			
	223	0.125			
ssrQpZAG110	192	0.333	He: 0.545	-0.073	0.254
	194	0.583	Ho: 0.750		
	196	0.042			
	198	0.042			
ssrQpZAG119	210	0.250	He: 0.708	-0.117	0.226
	213	0.333	Ho: 0.833		
	219	0.333			
	230	0.083			
	64	0.375	He: 0.715		
	76	0.042	Ho: 0.917		
	78	0.125			
	86	0.333			
	92	0.125			

more loci (ssrQrZAG101, ssrQpZAG15, and ssrQpZAG110) also show promise. The remaining three loci plus the three discarded after the first selection (ssrQrZAG5, ssrQrZAG65, and ssrQrZAG112) might be useful for other purposes such as mapping.

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