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Development and characterization of EST-SSR markers for mapping reaction to *Phytophthora cinnamomi* in *Castanea* spp.

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ABSTRACT

Utilizing recently released transcriptome data, we developed 43 simple sequence repeat (SSR) markers from expressed sequence tags (ESTs) differentially expressed in European chestnut (*Castanea sativa*) and Japanese chestnut (*Castanea crenata*) in response to inoculation with the most severe chestnut pathogen in Europe, *Phytophthora cinnamomi* (*Pc*). We used 24 parent and progeny trees – representing *Pc* susceptible, European and American chestnut (*C. dentata*), and *Pc* resistant, Japanese and Chinese chestnut (*C. mollissima*), species and some of their inter-species hybrids – to evaluate the EST-SSR markers' polymorphism and transferability rates within and among species, respectively. The set of EST-SSR markers showed a remarkably high interspecific transferability rate among the four *Castanea* species tested, ranging from 90.7% for Chinese chestnut and 100% for European chestnut. Only three EST-SSR markers were monomorphic (7%) and the average value of expected heterozygosity was 0.61, higher than that in other studies using EST-SSRs in chestnut.

The novel EST-SSR markers developed and characterized here are useful for constructing genetic linkage maps, conducting QTL analyses of phenotypic traits, genotype–phenotype association studies (especially in relation of resistance to *Pc*), high-throughput genotyping for clonal identification or marker-assisted selection, and comparative genomics.

1. Introduction

The European chestnut (*Castanea sativa* Mill.), also known as sweet chestnut and widely distributed around Europe, is a multipurpose tree species of great economic importance not only for fruit and timber but also for its contribution to landscape aesthetics and environment protection. The distribution of European chestnut has declined considerably in southwest Europe essentially as a consequence of the introduction of the oomycete *Phytophthora cinnamomi* Rands (*Pc*), a severe pathogen responsible for root rot, also known as ink disease. In Portugal, ink disease has become widespread, since *Pc* was first recorded in 1838. In North America, *Pc*, although known since the 18th century, was recognized recently as a serious threat to the American chestnut (*Castanea dentata*) restoration program (Jacobs et al., 2013) based on breeding lines resistant to chestnut blight caused by the fungal pathogen *Cryphonectria parasitica* (Murrill) Barr (*Cp*) (Anagnostakis, 1987; Russell, 1987).

Within the genus *Castanea*, the Japanese chestnut (*Castanea crenata* Sieb. et Zucc) and the Chinese chestnut (*Castanea mollissima* Bl.) show substantial levels of resistance to both *Pc* and *Cp*. These east Asian species have been used for chestnut breeding programs to the referred diseases in Europe and United States. For example, since 1989, The American Chestnut Foundation (TACF)

Abbreviations: *Cc*, *Castanea crenata*; *Cp*, *Chryphonectria parasitica*; *Cs*, *Castanea sativa*; EST, expressed sequence tag; DEG, differentially expressed gene; H_e, expected heterozygosity; H_O, observed heterozygosity; *Pc*, *Phytophthora cinnamomi*; QTLs, quantitative trait loci; SNP, single nucleotide polymorphism; SSR, simple sequence repeat; TACF, The American Chestnut Foundation.

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has been carrying out a breeding program to introduce resistance from Chinese chestnut into American chestnut (Diskin et al., 2006; Hebard, 1994). To support this breeding effort, several genomic resources were developed for *Castanea* and deposited at the *Fagaceae* Genomics Web (http://fagaceae.org/) (Nelson et al., 2014a,b). These include a large set of expressed sequence tags (ESTs) from Chinese chestnut and American chestnut obtained by high-throughput 454 sequencing of 20 cDNA libraries (Barakat et al., 2012, 2009). More recently, root transcriptomes of European chestnut and Japanese chestnut inoculated and non-inoculated with *Pc* were achieved in Europe (Serrazina et al., 2015) constituting an important increase in available genomic resources that provide an advantage for the identification of candidate genes for ink disease resistance.

Simple sequence repeat (SSR) markers are widely used in plant genetic research because of their co-dominant inheritance, relative abundance, polymorphism, high reproducibility and ease of detection and scoring (Powell et al., 1996). Earlier, small sets of SSR markers were developed from enriched genomic libraries of European chestnut (Buck et al., 2003; Marinoni et al., 2003) and Japanese chestnut (Inoue et al., 2009; Yamamoto et al., 2003). So far, genomic SSR markers have been applied for many studies of molecular characterization in *Castanea* (Dinis et al., 2011; Fernández-Cruz and Fernández-López, 2012; González et al., 2011; Marinoni et al., 2013), including genetic diversity (Beghè et al., 2013; Liu et al., 2013; Pereira-Lorenzo et al., 2011, 2010), landscape genetics and species conservation (Lusini et al., 2013; Martín et al., 2012; Martin et al., 2010; Mellano and Beccaro, 2012) and genetic mapping (Barreneche et al., 2004; Bodénès et al., 2012; Scalfi et al., 2004). More recently, the Chinese chestnut and American chestnut transcriptome datasets were used as a source of robust and reliable markers for extending the species genetic maps. In particular, SSR and single nucleotide polymorphism (SNP) markers were developed from both transcriptomes, and used for the construction of a highly informative genetic linkage map for two full-sib families of Chinese chestnut (Kubisiak et al., 2013). More recently, a large set of EST-SSRs were developed from Japanese chestnut (Nishio et al., 2011a). However, the development of EST-SSRs from datasets targeting a specific phenotypic trait, such as *Pc* resistance, has not been addressed in European or Japanese chestnuts so far.

In Portugal, since 2006, inter-specific hybridization has been on-going to introgress *Pc* resistance genes from Japanese chestnut into the susceptible species European chestnut (Costa et al., 2011). A primary goal is to use these chestnut hybrids to perform DNA marker:trait association for QTL identification affecting *Pc* resistance. As a first step, the Chinese chestnut transcriptome-derived molecular markers were used for linkage map construction and QTL detection. Additionally, a reliable phenotyping method was established to evaluate the *Pc* resistance of each individual progeny plant (Santos et al., 2014). The first European × Japanese chestnut genetic linkage map was constructed using a set of SSRs and SNPs mapped in Chinese chestnut (Kubisiak et al., 2013) revealing two QTLs for *Pc* resistance (unpublished data). Unfortunately, the transferability and polymorphism levels of the Chinese chestnut markers used by Kubisiak et al. (2013) were not high enough to be generally useful for mapping in European chestnut × Japanese chestnut families.

In this study, sequences of differentially expressed genes (DEGs) from European chestnut and Japanese chestnut inoculated with *Pc* were used to develop 43 new SSR markers potentially related to the pathogen response. Markers were characterized for PCR amplification and their transferability among four *Castanea* species was evaluated. Molecular markers related to *Pc* resistance in chestnut were identified and characterized for the first time. Transferable EST-SSRs reported here should be useful for both European and American breeding programs aimed at improving resistance to ink disease or even for other woody crop species threaten by *Pc*.

Table 1
Chestnut samples used in this study. Twenty-four chestnut samples were used to characterize the candidate EST-SSR markers, and representatives of four *Castanea* species were used to investigate cross-species transferability.

Progenitor tree	Species	Origin
CSAV	European chestnut	Portugal
CSDE	European chestnut	Portugal
CSMT	European chestnut	Portugal
CSSOU	European chestnut	Portugal
CSTRI	European chestnut	Portugal
CSVER	European chestnut	Portugal
C. crenata 1	Japanese chestnut	Portugal
C. crenata 2	Japanese chestnut	Portugal
C. crenata 3	Japanese chestnut	Portugal
Cranberry	American chestnut	TACF, USA
AD98	American chestnut	TACF, USA
NCDOT	American chestnut	TACF, USA
Pryor-79	American chestnut	TACF, USA
Pryor-180	American chestnut	TACF, USA
Ted Farm A	American chestnut	TACF, USA
Mahogany	Chinese chestnut	TACF, USA
Vanuxem	Chinese chestnut	TACF, USA
Nanking	Chinese chestnut	TACF, USA
Cataloochee-70	Japanese chestnut × American chestnut (F1)	TACF, USA
Adair KY115	Chinese chestnut (Mahogany) × American chestnut (F1)	TACF, USA
JB197	Chinese chestnut (Mahogany) × American chestnut (BC2)	TACF, USA
Cliffs GL-5	Chinese chestnut (Nanking) × American chestnut (F1)	TACF, USA
Cliffs GL-61	Chinese chestnut (Nanking) × American chestnut (F1)	TACF, USA
WWC70	Chinese chestnut (Nanking) × American chestnut (F1)	TACF, USA

2. Material and methods

2.1. Plant material and DNA extraction

Newly designed (see below) transcriptome-based SSR primer pairs were characterized with a test set of 24 individual trees. Specifically, the test set consisted of 6 European and 3 Japanese chestnuts from the Portuguese breeding program; 6 American, 3 Chinese used in TACF's breeding program, and 5 Chinese × American and 1 Japanese × American F1 hybrid trees used as parents in BC1 crosses by TACF (Table 1). Thus, all trees in the test set are progenitors used in either European or American breeding programs providing immediate implementation of our results in breeding. For Portuguese plant materials, genomic DNA was extracted from 100 mg of fresh leaves using the DNeasy™ Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. DNA extraction of U.S. plant material was done as described in Kubisiak et al. (2013).

2.2. Sources of ESTs

The root transcriptomes of *C. sativa* and *C. crenata* inoculated and non-inoculated with *Pc* were used as sources of ESTs for development of the SSRs. Experimental design of transcriptome analysis allowed comparative evaluation of DEGs in inoculated roots of European chestnut and Japanese chestnut in the background of non-inoculated controls (cDNA libraries Cci, Ccn, Csi and Csn, where "Cs" and "Cc" indicate *C. sativa* and *C. crenata*, respectively, and "i" indicates inoculated roots and "n" indicates non-inoculated control) (see details in Serrazina et al., 2015). The four transcriptome data sets are publicly available on the Fagaceae Genomics web site (http://fagaceae.org/) and in the Short Read Archive at NCBI (http://www.ncbi.nlm.nih.gov/) with the reference PRJNA215368.

Two types of DEGs were selected as *Pc* resistance candidate genes: (a) Japanese chestnut genes, given by the comparison Cci–Ccn and (b) European chestnut genes given by the comparison Csi–Csn. At a *p*-value threshold of $1E^{-3}$, 283 and 305 DEGs of the Cci–Ccn and Csi–Csn types were identified, respectively.

2.3. SSRs data mining and primer design

The *Pc*-resistant candidate ESTs sequences were extracted from European chestnut and Japanese chestnut root transcriptome data. Candidate SSR sequences were located and primer pairs were designed with the SSR Locator program (Maia et al., 2008). The criteria used for locating candidates were SSR motifs with 14 or more repeat sequences: that is, >6 repeats of di-nucleotide motifs, >5 repeats of tri-nucleotide motifs, >4 repeats of tetra-nucleotide motifs and >3 repeats of penta-nucleotide motifs.

Primer selection parameters were set for 99 SSRs previously found: a product size range of 100–500 base pairs (bp); a primer size of 18–25 bp (with an optimum of 20 bp); a primer T_m of 55–65 °C (optimum of 60 °C); and an optimum primer GC content of 50% with a minimum of 35%. A set of 60 primer pairs SSRs identified by the SSR Locator were then filtered by the following criteria: exclusion of redundant primer pairs identified in both sources (Csi–Csn and Cci–Ccn transcriptomes); and exclusion of primers with inappropriate patterns, such as: SSR-like sequences, low GC content (<35%) at the 5′ and 3′ ends of primer sequences or irregular nucleotide distribution, such as SSR pattern. A finalized list of 58 SSRs was selected for PCR amplification test. The SSR names were associated to chestnut species using the prefix Cs or Cc indicating respectively *C. sativa* or *C. crenata* candidate genes associated with resistance to *Pc*, followed by PT (Portugal) and a four-digit number identifier (CsPT0001-CsPT0023 and CcPT0001-CcPT0035).

2.4. SSRs amplification and characterization

SSR marker analysis was facilitated using a M13-specific sequence (5′-CACGACGTTGTAAAACGAC-3′), which was added to the 5′ end of each forward primer (Schuelke, 2000). To favor 3′ adenylation of the forward amplified strand, a PIG-tail (5′-3′) was added to 5′ end of each reverse primer (Brownstein et al., 1996). For fluorescent detection, PCR using three primers was performed: forward and reverse primers and a 5′ dye-labeled M13-specific primer (same sequence as the M13 "tail" described above).

The PCR reactions and amplification were performed according to Kubisiak et al. (2013) and Schuelke (2000), respectively. Completed reactions were diluted with distilled water and 1 μL was loaded on an ABI PRISM 3130xl, ABI PRISM 3730xl or ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and run according to the manufacturer's protocol. Allele sizes were determined using the LIZ600 or ROX500 internal size standard and the global southern algorithm implemented by ABI PRISM GeneMapper software version 4.0 (Applied Biosystems). Alleles were named according to Deemer and Nelson (2010) using the European and Japanese chestnut as reference samples and alleles. The observed heterozygosity (H_O), expected heterozygosity (H_e) and genetic distances were calculated for all SSRs that amplified a unique fragment using GeneAlEx software v. 6.5 (Peakall and Smouse, 2012). H_O was calculated as the number of heterozygous individuals divided by the total number of individuals. H_e was calculated using an unbiased formula from allele frequencies as $1 - \sum p_i^2 (1 \leq I \leq m)$, where m is the number of alleles at the target locus and p_i is the allele frequency of the ith allele at the target locus. A dendrogram illustrating the genetic similarities between chestnut samples was constructed using MEGA (version 6.06) software (Tamura et al., 2013).

3. Results

3.1. SSR markers development and transferability across species

A total of 99 SSRs, meeting our SSRs searching criteria, were found in the EST sequences of *Pc* resistance candidate genes, in which 43 were found in the 305 European chestnut sequences and 56 in the 283 Japanese chestnut sequences. Among them, a total of 60 SSR sequences were useful for primer design and after filtering as described, 58 SSRs were selected for PCR testing and further characterization (Table 2).

Of tested 58 SSRs, 43 (74.1%) gave unique amplification products about the predicted target size with DNA samples of at least 3 progenitor-trees, whereas 15 primer pairs showed no amplification or non-specific fragment amplification. The majority of primer pairs amplified fragments longer than the expected size. The percentage of success in PCR amplification was similar for European chestnut (72.7%) and Japanese chestnut (77.1%). 43 and 41 (95.4%) SSRs showed strong and selective amplification in European chestnut and in Japanese chestnut, respectively. Regarding the parental trees from the American breeding program, almost all SSRs (97.7%) amplified in American chestnut and 90.7% amplified in Chinese chestnut individuals. Thirty-four SSRs (79.1%) amplified from DNA of four chestnut species, while twenty-five SSRs (58.1%) were found to amplify in all 24 samples (representing the four tested species and two types of hybrids). SSRs with di-, tri-, and tetra-nucleotide motifs showed no distinct differences in transferability.

3.2. SSRs markers characterization

The polymorphism of each locus was evaluated by the number of alleles and expected and observed heterozygosity. SSRs characterization results are summarized in Table 3). For all primer pairs, an average of 5.26 ± 0.39 alleles were obtained for the individuals tested. Three SSR markers (CcPT_0002, CcPT_0019 and CcPT_0026) were monomorphic. The number of alleles obtained for the polymorphic SSRs ranged from 2 (CcPT_0001, CsPT_0016 and CsPT_0017) to 11 (CcPT_0013). The observed and expected mean heterozygosity was 0.39 ± 0.03 and 0.61 ± 0.04 respectively. Marker CcPT_0020 presented the highest observed heterozygosity (0.82) while the marker CcPT_0024 showed the lowest observed heterozygosity (0.043). The expected heterozygosity ranged between 0.22 (CsPT_0017) and 0.87 (CsPT_0003).

The most abundant repeat type was the tri-nucleotide (65.1%) followed by the di-nucleotide (27.9%). There was a predominance of CT/AG and TC/GA motifs among di-nucleotide repeats and TTG/AAC and CTT/AGG for tri-nucleotide repeats. As indicated, a higher number of alleles and higher heterozygosity values, di-nucleotide SSRs are slightly more polymorphic, compared with the tri- and tetra-nucleotide SSRs (Table 3).

Dendrogram formulated on the basis of genetic distance between chestnut samples was divided into 5 main clusters A–E (Fig. 1). In general, the molecular markers presented in this study discriminated the different chestnut species from hybrids. Cluster B–D grouped American, Chinese and European chestnuts, respectively. Clusters E grouped Japanese chestnuts and also showing genetic similarity with Japanese chestnut × American chestnut hybrid (Cataloochee-70). Finally, cluster A grouped the remaining samples used in this study (Fig. 1 and Table 1).

4. Discussion

The advent of next-generation sequencing (NGS) technologies has reduced sequencing cost and significantly increased sample throughput, making genome and transcriptome sequencing a pos-

Table 2
Summary of the number of EST-SSRs found and selected using the SSR Locator software. Total number of SSRs corresponding to all candidate SSRs meeting SSR searching criteria; total number of SSRs with primers means SSRs where appropriate primers were found; total number of SSRs selected corresponding to the final set of SSRs for testing amplification and polymorphism.

Source of candidate genes	Total number of SSRs	Total number of SSRs with primers	Total number of SSRs selected
European chestnut	43	24	23
Japanese chestnut	56	36	35
Total	99	60	58

sible way for obtaining genetic information in species with long generation period, as it is the case of *Castanea* spp.

Previous studies have reported the development and characterization of genomic SSR markers in *Castanea* spp. (Buck et al., 2003; Inoue et al., 2009; Marinoni et al., 2003; Yamamoto et al., 2003). Unlike for genomic SSRs, SSRs developed from ESTs constitute functional markers located in the transcribed regions and their variation in length can cause a phenotypic effect (Li et al., 2004; Victoria et al., 2011). Taking advantage of NGS, Nishio et al. (2011a,b) developed 146 EST-SSRs (from young leaves, nuts and pellicles) and 220 genomic SSRs for Japanese chestnut. In that study, high interspecific transferability of both SSR types was observed among *Castanea* spp. More recently, Kubisiak et al. (2013) developed an extensive set of the EST-derived SSR markers from Chinese chestnut and established a saturated Chinese chestnut reference genetic map that was integrated with a physical map (Fang et al., 2013). A subset of 22 SSRs distributed across the linkage group E was used for delineation of the QTL interval for resistance to ink disease in interspecific Chinese × American chestnut crosses (Zhebentyayeva et al., 2014). However, low transferability of the Chinese chestnut-derived EST-SSRs hampered their implementation into European breeding programs focused on introducing the *Pc* resistance to European from Japanese chestnut (unpublished data).

On the other hand, large numbers of EST-SSRs have been also developed for population genetic studies in the other *Fagaceae* genera, *Fagus* (Ueno et al., 2009) and *Quercus* (Durand et al., 2010),

Table 3
Characterization of 43 new EST-SSR markers: number of alleles (Na), expected heterozygosity (He), observed heterozygosity (HO), repeat motif, primer sequences (5′–3′) and allele size average (Al. size av., in bp). Number of alleles expected and observed heterozygosities were estimated for 24 chestnut samples, comprising the four *Castanea* species used in Portuguese and TACF breeding programs.

Locus name	Na	H_O	H_e	Repeat motif	Primer forward sequence (5′–3′)	Primer reverse sequence (5′–3′)	Al. size av.
CcPT_0001	2	0.333	0.278	(CT)12	CGAGGATTTCGAACCAGAGA	ATCACAATACACGCGCAGAG	466
CcPT_0002[a]	1	0.000	0.000	(GTG)9	AGTTCTCCACGAGGCTCAAA	TCCAAGCTGGAGAATCATCA	415
CcPT_0003[a]	7	0.435	0.742	(ATC)8	GGTGCCCAGATTTACGAGAA	ATCGCTTGGAGTCACAGCTT	426
CcPT_0004[a]	6	0.273	0.643	(CT)10	GCTGCTTCACAACCTTCCT	GCAAGAGATTCCCTTTGCT	375
CcPT_0005[a]	7	0.522	0.748	(CT)1	ACACATGGGGGTGTGAACT	TTATGGGAAACGGCATCTT	175
CcPT_0006[a]	4	0.174	0.620	(TTC)	CCTGTGAGGCTAAGAGAGC	ACCACGTCGGTGCTTCTAG	339
CcPT_0008[a]	5	0.286	0.773	(TCT)1	TCGTCCCCTTCTTCATCAT	ATATGGCCAAAAACCCATC	586
CcPT_0009[a]	8	0.227	0.801	(TC)	TTCCACCCAATTGTTACCA	GATGATGAAGAAGGGGACG	316
CcPT_0010[a]	5	0.364	0.673	(GGT)	ATCCATGAGTGAAAGCCAC	TGGAACAAGAAGCCTCGAT	503
CcPT_0011[a]	5	0.391	0.754	(CAC)	TCATCCAAGAAGCCCTCAA	TTCTGCCTCTTTTGTTGCC	431
CcPT_0013[a]	11	0.696	0.871	(AG)1	AGTACGTAGTCGAAGAGAGAAGA	AGTGAAGTTTTGTCGGGGT	197
CcPT_0014[a]	4	0.250	0.695	(TC)	AGCTGATCAACTCTCGCCA	AGCTGATCAACTCTCGCCA	172
CcPT_0015[a]	4	0.435	0.475	(CAA)	GCCCATCTGAAATCCAAAA	GCCCATCTGAAATCCAAAA	429
CcPT_0019[a]	1	0.000	0.000	(TCA)	TCGTGATGCCTATCAATCC	TCGTGATGCCTATCAATCC	228
CcPT_0020	7	0.818	0.831	(AG)	CGCCATAACTTCCTTTTCC	CGCCATAACTTCCTTTTCC	497
CcPT_0021[a]	5	0.522	0.633	(CCG)	GGATGCAAAGGCTTTAGCT	GGATGCAAAGGCTTTAGCT	316
CcPT_0022	9	0.294	0.822	(TC)	TGAGATTGTCGAGGGTTTC	TGAGATTGTCGAGGGTTTC	377
CcPT_0023[a]	3	0.348	0.491	(TGT)	GAGTTTCCAAGAACCCACC	GAGTTTCCAAGAACCCACC	472
CcPT_0024[a]	3	0.043	0.434	(TTG)	GCACCCAATTTCAATGAAC	GCACCCAATTTCAATGAAC	502
CcPT_0025[a]	9	0.500	0.750	(TTG)	GCTTTGATCCAACCAACGA	GCTTTGATCCAACCAACGA	352
CcPT_0026[a]	1	0.000	0.000	(CAG)	CGGCTGAAAGGATCAAGAA	CGGCTGAAAGGATCAAGAA	204
CcPT_0028[a]	5	0.435	0.528	(TGG)	GTTTCCCTTGATGGGTTTG	GTTTCCCTTGATGGGTTTG	200
CcPT_0030[a]	4	0.435	0.651	(GGT)	GAATTGGTGGAGGCAGAAA	GAATTGGTGGAGGCAGAAA	488
CcPT_0031	4	0.100	0.535	(AAG)	CCCCAAATTCTTTTCTGGG	CCCCAAATTCTTTTCTGGG	447
CcPT_0032[a]	6	0.619	0.772	(TTG)6-(TTG)	CCACCGCCAGTACATCTTT	CCACCGCCAGTACATCTTT	663
CcPT_0034	6	0.667	0.778	(AAAG)	CAATTTCAAAGCTTTTGGGTT	CAATTTCAAAGCTTTTGGGTT	399
CcPT_0035[a]	5	0.261	0.660	(TTTC)	ACGCTCCATTACAGCTGCT	ACGCTCCATTACAGCTGCT	229
CsPT_0003	10	0.435	0.874	(AGC)	CGCCGTAGTACTGCTGATG	CTTCTCCCCCTAACCTCTC	424
CsPT_0004[a]	7	0.565	0.766	(GAG)1	ACAGGAATTGGGATCCATC	CACACCCTCTTGTTCCACC	477
CsPT_0005[a]	3	0.391	0.581	(AG)	GCTTTTGGTTGATTTGCGA	TAAGCCCTGAGAACATTGG	289
CsPT_0006[a]	10	0.478	0.849	(TC)	CCTTGCTTCGCTCAGTCAT	GATCCGACCCGTTTGAGTT	384
CsPT_0007[a]	9	0.696	0.820	(CT)1	TCGAAGAGAGAGAAGAGGAAGA	TCGAAGAGAGAGAAGAGGAAGA	184
CsPT_0008[a]	3	0.565	0.552	(ATG)	GAACTTAGGTGGCTCAAGC	GAACTTAGGTGGCTCAAGC	354
CsPT_0010[a]	9	0.182	0.858	(TGT)	TCTCAAAGCTCGTTTCCGAT	TCTCAAAGCTCGTTTCCGA	497
CsPT_0011	4	0.455	0.380	(CT)	CACCTCAAGGGTGATGGTCT	CACCTCAAGGGTGATGGTC	286
CsPT_0013	5	0.650	0.650	(GGT)	TTACCCTGTAGCCCAACCT	TTACCCTGTAGCCCAACCT	319
CsPT_0015[a]	5	0.391	0.625	(CTT)	CCCTTCATTTTTGCTGGATG	CCCTTCATTTTTGCTGGAT	455
CsPT_0016[a]	2	0.217	0.364	(AAG)	TGTTGAGAAGATGGCAGCAG	TGTTGAGAAGATGGCAGCA	307
CsPT_0017	2	0.250	0.219	(CCA)	GCAACACTGGTTGTCAAAGC	GCAACACTGGTTGTCAAAG	500
CsPT_0018[a]	4	0.522	0.593	(AGC)	CCAAGCCAACTGCTCCTAAG	CCAAGCCAACTGCTCCTAA	501
CsPT_0020[a]	4	0.400	0.644	(TCT)	TTTGTGCCGTTAATGATGGA	TTTGTGCCGTTAATGATGG	263
CsPT_0021[a]	5	0.652	0.750	(GGT)	GATCCGACCCGTTTGAGTT	GATCCGACCCGTTTGAGTT	183
CsPT_0022[a]	7	0.563	0.633	(TTAA)	TTCAATTTCCCGGATTCAAG	TTCAATTTCCCGGATTCAA	394

[a] Indicates SSRs which amplify for the four species.

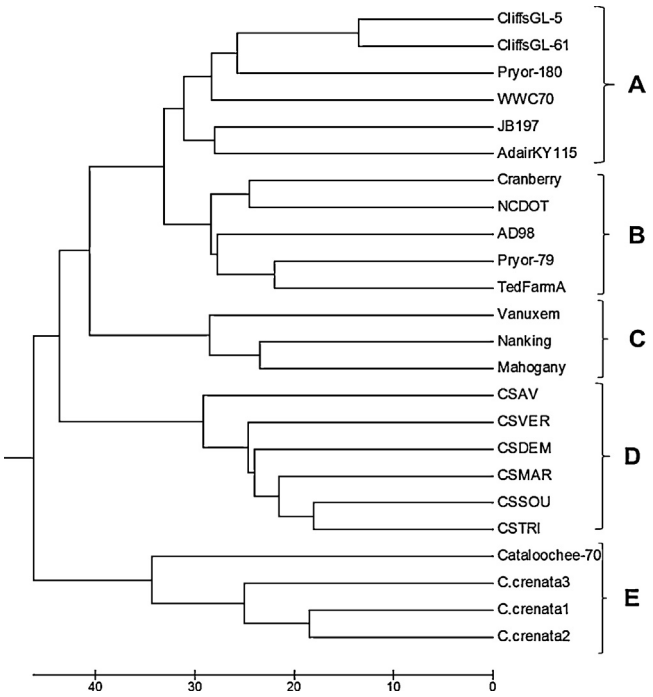

Fig. 1. Dendrogram showing genetic distance between chestnut samples used in this study.

and in several economic important woody genera, such as, *Citrus* (Chen et al., 2006; Jiang et al., 2006; Palmieri et al., 2007) *Prunus* (Vendramin et al., 2007), *Populus* (Xinye et al., 2009; Li et al., 2010), *Eucalyptus* (Ceresini et al., 2005; Faria et al., 2010; Rabello et al., 2005) and *Pinus* (Bérubé et al., 2007; Echt et al., 2011; Liewlaksaneeyanawin et al., 2004).

In the present study and for the first time in chestnut, a set of DEGs after *Pc* inoculation (283 from Japanese chestnut and 305 from European chestnut) were used for developing new EST-SSR markers. Stringent parameters were applied in order to select SSRs with specific amplification and high polymorphism rates. As a result, 43 novel EST-SSR markers were developed and characterized. The frequency of motifs for di-nucleotide and tri-nucleotide was in agreement with previous results in other vascular plants (Victoria et al., 2011). The success rate of amplification was 74.1% which is consistent with previous studies in Japanese and Chinese chestnut (Kubisiak et al., 2013; Nishio et al., 2011a). For all markers in our study, clear PCR products were obtained with strong amplification and distinct allelic peaks. As noted above, the PCR products were longer than expected. Once primers were designed for ESTs and PCR reactions were performed with genomic DNA, presence of introns and small insertions in genomic sequence is the most likely explanation of this discrepancy (Varshney et al., 2005).

From the set of EST-SSRs presented in current study, 25 were transferable among the four chestnut species, using a set of 24 diverse individuals from the American and Portuguese chestnut breeding programs. In general, high transferability was observed among *Castanea* spp. ranged from 90.7% for Chinese chestnut and 100% for European chestnut. Similar observations were made in other studies in chestnut (Nishio et al., 2011b; Kubisiak et al., 2013) and in oak (Bodénès et al., 2012; Durand et al., 2010). A noted advantage of transcriptome-derived SSR markers is the high transferability among related species, therefore allowing analysis of genetic diversity across species (Li et al., 2004; Varshney et al., 2005). Overall, the SSRs developed in this study may discriminate chestnut species and hybrids. However, extended population genetic analysis as genetic maps among *Castanea* spp. and map QTL for *Pc* resistance should be coordinated to ensure that discrimination power. Nevertheless, EST-SSRs should be very useful for comparative mapping, genetic diversity and evolutionary studies.

In contrast to genomic SSRs, EST-SSRs generally show lower polymorphism levels than genomic SSRs, because of greater DNA sequence conservation in the coding regions (Varshney et al., 2005; Ohyama et al., 2009; Kong et al., 2014). Nevertheless, in this study, only three SSR markers (7%) were monomorphic and the average values of expected heterozygosity were higher than that in other studies in chestnut using EST-SSRs (Martin et al., 2009; Nishio et al., 2011a,b).

4.1. Conclusions

The present study provides an original set of high-quality (high polymorphism, transferability and easy detection and scoring) chestnut SSRs located in Pc resistance candidate genes potentially involved in regulation of plant immune response, anti-pathogenic enzymes and metabolite synthesis, cell wall strengthening, stress adaptation and recovery. Moreover, we present an exclusive list of transferable SSR markers that cover the four chestnut species involved in both American and Portuguese breeding programs. Furthermore, these SSRs are potentially functional genetic markers that may contribute to: (1) marker-assisted selection for the facilitation of chestnut breeding; (2) establishing, more accurately and rapidly, marker-trait associations for identification of QTLs for Pc resistance; and (3) developing universal gene-specific markers for genome synteny studies within Fagaceae and related taxa. As *Pc* also has devastated important food crops and native forest (Hardham, 2005), these results may be also useful for other woody species such as *Eucalyptus* spp., *Quercus* spp., *Fagus* spp., *Juglans* spp. and many ornamental trees and shrubs.

These results are encouraging and should be validated in a larger set of chestnut samples and in other related taxa. Our next stage of work comprises the use of these novel EST-SSRs for genotyping Portuguese and American chestnut progenies from the respective breeding programs. In particular, these new markers will contribute for the saturation of the first European chestnut × Japanese chestnut genetic linkage map (in progress) and expedite QTL identification for *Pc* resistance, which may lead to a better understanding of the genetic response of different chestnut species to the pathogen.

Conflict of interest

The authors declare that they have no conflict of interest.

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