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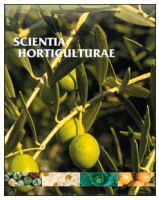

Research Paper

Guardians of quality: advancing *Castanea sativa* traceability using DNA analysis from seed to processed food

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ABSTRACT

Castanea sativa is one of the most appreciated species in Italy, due to timber and fruit quality; nuts are usually intended for fresh consumption, but also for the production of industrial foods. The demand for an efficient traceability system to identify *Castanea* species and cultivars along the supply chain, from the chestnut orchard to the processed product, is crucial to protect consumers and producers from frauds. Here we present a traceability protocol to identify the species of the *Castanea* genus and trace the cultivars of *Castanea sativa*, based on DNA markers (SSRs and SNPs) analyses. Microsatellites were applied to identify the cultivar in plant material (leaves, seed, and episperm) and processed food (creams, beverages, flour, and cookies). Although SSRs made it possible to trace cultivar starting from somatic tissues (e.g.: leaves), the presence of foreign paternal DNA from pollenizer plants in seed and processed food, interferes with the identification of the maternal one. To overcome this issue, we Illumina sequenced six *C. sativa* cultivars to highlight maternal-inherited markers. A set of SNP/INDEL markers in two chloroplastic regions were identified, able to effectively distinguish and trace *C. sativa* from *C. mollissima* and *C. crenata.* The *High Resolution Melting* (HRM) analysis was performed as a practical and efficient method for SNP/INDELvalidation and to develop a fast, reliable, and reproducible protocol for chestnut traceability in food.

1. Introduction

European chestnut or sweet chestnut, *Castanea sativa* (Mill.), plays a crucial economic and ecological role in Europe (Conedera and Krebs, 2008; Pavese et al., 2021a). It is spread to more than 25 European countries from the northwest Iberian Peninsula to the Caucasus region, with some individuals in Algeria and Tunisia (Fernández-López et al., 2021; Corredoira et al., 2017). In Europe, sweet chestnut has been cultivated for centuries, due to the importance of its wood and its nut quality, usually used for human consumption or preparation of many processed products such as flours, creams, and marrons glacés (Massantini et al., 2021). According to the FAOSTAT data (FAOSTAT, 2023), Italy has a relevant economic position in the global market, producing 50,000 t with 36,440 ha harvested in 2020. Indeed, in the last few years, the Italian chestnut sector has grown thanks to significant investments in the supply chain (Borrello et al., 2023).

Italian chestnut production is based on traditional cultivars selected for their adaptability to the soil and the quality of their nuts; each Region uses and cultivates varieties of sweet chestnuts depending on their traditional value for local producers (Poljak et al., 2021; Torello Marinoni et al., 2020).

The major producing regions are Campania, Calabria, Latium, Tuscany, followed by Piedmont and Emilia Romagna. However, following the emergence due to the spread of the gall wasp (Sartor et al., 2015; Pavese et al., 2021b, 2022b), Italy has become a major importer of chestnuts from third countries.

Asian chestnuts from *C. mollissima* (Chinese chestnut) and *C. crenata* (Japanese chestnut) threaten European chestnut productions from *C. sativa*, considered of higher quality in terms of flavor (Massantini et al., 2021). In addition, *C. sativa* nuts are almost morphologically undistinguishable from the others and consumers are unlikely to be aware of the existence of multiple *Castanea* species and hybrids between them.

In France, hybrids between *C. sativa* and *C. crenata* grown in Europe

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are sold as 'Marrone type', due to their large size and low incidence of double seeds. In Italy the term 'Marrone' should be commercially assigned only to specific cultivars of *C. sativa*. The differences in market price for the diverse types of chestnuts may encourage fraudulent practices. For these reasons, the consumer needs to be safeguarded (Pavese et al., 2022a; Avanzato, 2009).

An efficient traceability and tracking system is crucial for identifying *Castanea* species and cultivars of *C. sativa* along the supply chain from the chestnut orchard to the processed product.

DNA-based analyses could be reliable tools for food traceability (Fanelli et al., 2021). DNA can be successfully PCR amplified, even if extraction from raw materials of woody plants and from processed foods can be challenging (Bojang et al., 2021). DNA extraction needs to be set up for the diverse matrices testing different extraction protocols (Galimberti et al., 2013), such as the cetyltrimethylammonium bromide (CTAB) protocol (Stefanova et al., 2013; Onache et al., 2021; Singh et al., 2021), the phenol-chloroform-isoamyl alcohol (PCI) method (Piskata et al., 2019), or using commercial kits (Rodríguez-Riveiro et al., 2022).

Among the plethora of DNA molecular markers, Simple Sequence Repeats (SSRs or microsatellites) are typically used for germplasm identification in routine analyses because of their high level of polymorphism, reproducibility, and co-dominant inheritance (Ramesh et al., 2020). SSR markers have been successfully utilized for food authentication and the identification of cultivars and species in processed food matrices such as coffee beans (Zhang et al., 2020), olive oil (Chedid et al., 2020; Pasqualone et al., 2007), wine (Avramidou et al., 2023; Zambianchi et al., 2021), musts (Boccacci et al., 2012; Zambianchi et al., 2021), apple juice (Torello Marinoni et al., 2022), pear juice (Yamamoto et al., 2006), sweet cherry jam (Ganopoulos et al., 2011), and chocolate liquor (Stagnati et al., 2020). The genetic traceability of processed foods containing chestnuts has yet to be extensively studied or documented. However, cultivar identification of nuts in processed food using SSR markers is critical since the edible part is the seed. Chestnut is a monoecious species with a gametophytic self-incompatibility system (Xiong et al., 2019). Consequently, the embryo is derived from cross-pollination (Nishio et al., 2019) and contains maternal and paternal DNA; therefore, it is hard to identify the maternal cultivar using nuclear DNA molecular markers. To overcome this problem, it is necessary to study and validate novel molecular marker typologies, with maternal inheritance (Talucci et al., 2022).

Over the past years, SNPs/INDELs have emerged as novel classes of markers and have become crucial tools in genomics, because of their use as molecular markers for genotyping (Morgil et al., 2020). SNPs markers could represent a new frontier in food traceability, with the first report on meatballs (Zhao et al., 2020) and some examples in plants, including apricot (Hürkan, 2021) and grapevine (Gambino et al., 2022).

Several genotyping techniques are available for the detection of SNPs/INDELs. Genotyping technologies require an amplification step to target and amplify specific regions of the DNA-containing SNPs. This amplification step is essential to introduce specificity and achieve accurate and reliable results (Kim and Misra, 2007). Several detection methods are available, including: TaqMan assay (Boccacci et al., 2020), loop-mediated isothermal amplification (LAMP-PCR) (Gill and Hadian Amree, 2020), SNaPshot approach (Zhang et al., 2022), KASP (Kompetitive Allele Specific PCR) (Kaur et al., 2020), high-resolution melting (HRM) (Raizada and Souframanien, 2021; Quintrel et al., 2021), and sequencing technologies, such as Sanger sequencing (Pati et al., 2004), or NGS (Next Generation Sequencing).

High-Resolution Melting (HRM) is a powerful and rapid molecular technique for SNP/INDEL identification. The benefits of HRM include cost-effectiveness, reduced laboratory workload, and simplicity compared with other analytical methods (Lagiotis et al., 2020). HRM analysis detects variations between sequences based on their different melting temperatures (Tm), where 50 % of the DNA is single-stranded (Pereira et al., 2018) without sequencing or hybridization processes. Therefore, it is one of the simplest methods for high-throughput genotyping of mutation scanning (Qian et al., 2023). Consequently, the HRM technique is an effective and advanced tool in food analysis, especially in food authentication, such as the genotyping of peach genotypes and authenticity testing of the Turkish apricot cultivar (Hürkan, 2021), grapevine, wine, and olive oil (Pereira et al., 2018).

In this study, the development of a traceability method based on DNA analysis was carried out to enable the identification of species and cultivars from the chestnut orchard to the industrial product. In particular, we: i) evaluated the efficiency of SSR markers for cultivar identification in chestnut food products; ii) identified and selected chloroplast SNP/INDEL markers to distinguish *Castanea* species in chestnut-processed foods; iii) developed a HRM-based protocol and validated it as an accurate, fast, and reliable method for plant genotyping in the food industry (Osathanunkul and Madesis, 2019).

2. Material and methods

2.1. Plant and food material

For SSR analysis, 14 different cultivars of *C. sativa* were analyzed (Supplementary Table 1).

Three types of plant-derived materials were used for DNA analysis: i) *episperm* - the episperms from ten nuts were collected after soaking the seed for three days in deionized water; ii) *kernel* - for each cultivar, one single seed was collected and dipped in deionized water for three days. The episperm was then removed, and the kernel was ground; iii) *monovarietal flour* - ten nuts were collected from each cultivar and immersed in deionized water for three days. The kernels were then peeled and roasted for three days at 50 °C. The roasted kernels without episperm were chopped using a mixer.

From all these samples, an aliquot (0.1 g) was collected and stored at −80 °C until DNA extraction.

In addition, analyses were carried out on food matrices, including a commercial cream, a commercial vegetable drink made from chestnuts, and marrons glacés (candied chestnuts preserved in sugar syrup and glazed). Each sample was prepared according to the matrix analyzed (see below) and DNA aliquots were placed at −30 °C after DNA extraction.

Chestnut commercial cream (made by kernels, sugar, pectin and vanilla flavor) - Three different pre-treatments were performed before DNA extraction: i) No pretreatment: two aliquots (0.1 g and 0.2 g) were directly collected from the cream. ii) 0.7 Vol of isopropanol was added to 30 g of cream, following protocol described by Boccacci et al. (2012). The solution was then centrifuged at 7000 g for 40 min at 4 °C. Subsequently, the supernatant was discarded, and two aliquots were collected from the pellet. iii) Isopropanol (0.7 Vol) was added to 10 g of the cream. The solution was then centrifuged at 7000 g for 60 min at 4 °C. The supernatant was discarded, and two aliquots were collected from the pellet.

Marrons glacés - A single marrons glacés was ground, and four aliquots were collected; in 2 of these, 200 microlitres of 0.7 Vol isopropanol was added.

Commercial chestnut drink - 0.7 V of isopropanol was added to 30 ml of chestnut drink, following the protocol described by Boccacci et al. (2012). Then an aliquot (0.1 g) was collected.

The analyses on SNP/INDELs markers were carried out on the cultivars of *C. sativa*, previously reported in Supplementary Table 1, on 4 cultivars of *C. mollissima* ('Jujiazhong', 'Jiaozha', 'Aioi', and 'Hakuri'), and on 4 cultivars of *C. crenata* ('Shihou', ' Toyogin', 'Ginyose', and 'Tanzawa') (Supplementary Table 1).

Analyses were conducted on different plant and food materials: leaves, episperms, kernels without episperm, flours, and pastes (kernels without episperm chopped to obtain a finely paste). To discriminate the three different species of *Castanea* genus, monovarietal flours and pastes were prepared in the laboratory. Episperms, kernels and monovarietal

flours were prepared as described above. The pastes were formulated as follows. Three different creams were obtained using the cultivars 'Marrone di Castel del Rio' (*C. sativa*), 'Jiaozha' (*C.mollissima*), and 'Ginyose' (*C. crenata*). 20 g of seeds were placed in 500 ml of water and cooked in a microwave for 10 min. After cooking and peeling, the kernels were ground using a mortar and pestle to obtain a homogeneous compound. At the same time, 5.1 g of sugar was dissolved in 6 ml of water. The chestnut puree was subsequently added to the water and sugar solution and cooked until a creamy consistency was achieved.

2.2. DNA extraction

Authentication through DNA analysis necessitates high-quality DNA, as it plays a crucial role in polymerase chain reaction (PCR)-based assessments. The DNA obtained from food products subjected to extensive heat processing is very often of low quality and may hinder the authentication procedure (Sajali et al., 2018).

DNA extraction was performed using the Doyle and Doyle (1987) for all samples following Talucci et al., 2022 results; however, it was necessary to make some adjustments depending on the plant/food materials (Lo and Shaw, 2018). The incubation time in isopropanol and sodium acetate differs for plant material (60 min at −30 °C) and the processed food (20 h at −30 °C) following Boccacci et al. (2012). The concentration and quality of the extracted DNA were checked using agarose gel and NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA).

2.3. SSR PCR amplification

Nine nuclear SSR loci, CsCAT 1, CsCAT 3, CsCAT 6, CsCAT 8, CsCAT 14, CsCAT 16, CsCAT 17, CsCAT 41, and QpZAG110 (Marinoni et al., 2003; Steinkellner et al., 1997) were selected for cultivar identification (Supplementary Table 2). PCR was performed using a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) in a total volume of 20 μL. The components of the reaction and the conditions were changed depending on the matrix analyzed.

Episperm and processed food materials - 1 μL concentrated DNA, 0.5 U Taq-DNA polymerase (KAPABIOSYSTEMS, Wilmington Massachusetts, USA); 1.5 μl 10X PCR buffer (1.5 mM MgCl2); 200 μM dNTPs, 0.5 μM of each primer, 0.5 μg/μl BSA (Bovine Serum Albumin). The PCR conditions included an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of denaturation (30 s at 95 °C), annealing (30 s at 55 °C), and extension (30 s at 72 °C). The final elongation step was carried out at 72 °C for 5 min.

Kernels and monovarietal flours - 4 μL diluted DNA (10 ng/ μL), 0.5 U Taq-DNA polymerase (KAPABIOSYSTEMS, Wilmington Massachusetts, USA); 1.5 μl 10X PCR buffer (1.5 mM MgCl2); 200 μM dNTPs, 0.5 μM of each primer, 0.5 μg/μl BSA (Bovine Serum Albumin). PCR conditions included an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of denaturation (30 s at 95 °C), annealing (30 s at 55 °C), and extension (30 s at 72 °C). The final elongation step was carried out at 72 °C for 5 min.

The amplification products were analyzed using a 3130 Genetic Analyzer sequencer (Applied Biosystems, Foster City, California, USA). The results were analyzed using GeneMapper software v. 4.0 (Applied Biosystems, Foster City, California, USA), and the GeneScan™ 500 LIZ® Size Standard was used to estimate allele sizes.

2.4. Chloroplast snp/indel markers: bioinformatic analyses

The DNA of six cultivars of *C. sativa* ('Gabbiana', 'Garrone Rosso', 'Gentile', 'Giacchettara', 'Marrone di Chiusa Pesio', and 'Riggiola'), was extracted using Doyle and Doyle extraction protocol (1987). BGI genomics (https://www.bgi.com/global/company/about-bgi) performed the library preparation and sequencing through Illumina technology with a 20X coverage depth. The raw reads were initially trimmed using the Schyte software (https://github.com/vsbuffalo/scythe) to remove the adapters sequence at the 3′ end of the fragments, and then the Sickle software (https://github.com/najoshi/sickle) was utilized to remove the low-quality reads. Burrows-Wheeler Aligner (BWA) software (Burrows and Wheeler, 1994) was used for the reads alignment: the trimmed and cleaned reads of the six cultivars were aligned separately to the reference *C. mollissima* chloroplast genome (MW322901.1, https://www.ncbi.nlm.nih.gov/nuccore/MW322901.1/) and the *C. crenata* chloroplast genome (MN402457, https://www.ncbi.nlm.nih.gov/nuccore/MN402457), which are present in the database. The command bwa index was used to create a reference FASTA file index, and the command bwa mem produced the alignment file. Aligned reads from each genotype were compared to the reference genome using samtools mpileup (http://samtools.sourceforge.net/samtools.shtm), adopting the following parameters: - Q 20 as the mapping quality and -D 1000, as the max read depth. The bcftools command (http://samtools.github.io/bcftools) was used to perform Variant Calling and vcfutils.pl (https://github.com/samtools/bcftools/issues/30) was applied to detect variants with the best robustness according to their coverage and quality. The generated vcf file was then inspected for the presence of informative SNPs/ INDELs.

2.5. Chloroplast SNP/INDELs validation

Polymorphic regions in the chloroplast genome were scanned and chosen due to their ability to discriminate *C. sativa* from *C. mollissima* and *C. crenata*. Candidate SNPs/ INDELs were validated by PCR amplification and Sanger sequencing. For each region, one pair of primers was designed using the software Primer3 (https://primer3.ut.ee/) (Table 1). PCR amplification was performed using 1 μL DNA (10 ng/μL), 5 U/ μl PCRBIO Classic Taq (Resnova), 2 μL 1x Buffer (3 mM), 1 mM dNTPs, and 400 nM of each primer in a 20 μL reaction volume.

The amplification conditions were as follows: initial denaturation for 5 min at 95 °C, followed by 35 cycles of denaturation (30 s at 95 °C), annealing (30 s at 59 °C), and extension (1 min at 72 °C), with a final extension step for 5 min at 72 °C.

After verifying the presence of PCR products on agarose gel, the amplicons were purified using Omega Bio-tek E.Z.N.A..® Gel Extraction Kit - Enzymatic Reaction Protocol (Omega Bio-tek). The purified amplicons were sent to BMR Genomics (http://www.bmr-genomics.it/) for Sanger sequencing. Sequence alignment was performed using Clustal Omega software (https://www.ebi.ac.uk/Tools/msa/clustalo/).

2.6. High resolution melting (HRM) analysis

For HRM analysis, all samples were required to reach an equal concentration of DNA. In addition, for each region, forward and reverse primers were specifically designed for HRM analysis using Primer3 (https://primer3.ut.ee/) with an annealing temperature of 60 °C, product size of 80–200 bp and GC% content of approximately 30–50 % (Table 2). PCR amplification and HRM analysis were performed using StepOne™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The PCR reaction mixture contained 5 ng/μL of DNA, 0.5 X MeltDoctor™ HRM Master Mix (Thermo Fisher Scientific), and 0.15 μM of each primer in a final volume of 10 μL. PCR amplification and the following HRM were performed with an initial denaturation step of 10 min at 95 °C, followed by 40 cycles of denaturation (15 s at 95 °C), annealing and extension (10 s at 60 °C), and a final extension of 2 min at 72 °C. The melting curves were obtained at the following conditions: denaturation for 10 s at 95 °C, annealing for 10 s at 60 °C, rising 0.3 °C/s, and final step at 95 °C for 15 s. During constant temperature increments, the fluorescence data were continuously acquired. The melt curves were analyzed using High Resolution Melt Software 3.1 (Applied Biosystem).

Table 1
Primer sequences used for the amplification of the two regions to validate the SNP/INDELs.

Region	Primer forward sequence	Primer reverse sequence
Region 1	TTGTGGATTACGAACAGCTATAAT	ATAAGTCAACTAGGCAAGTCGAAA
Region 2	TTGATGTAGGTACAGCTATTGGAC	CCATGACTGAAGAATATGAGCTT

Table 2
Primer sequences utilized in the High Resolution Melting analysis.

Region	Primer forward sequence	Primer reverse sequence
Region 1	CCCATGGACCGTATTCTTCG	GGAAGTCGGCCAACATTTCT
Region 2	AAACCGCATTCCTTTCGAGC	TCAAATTGCCTTGGGTCGTT

3. Results

3.1. DNA extraction

Extracted DNA integrity and quantity were evaluated by agarose gel electrophoresis and through spectrophotometric analysis, checking the ratios 260/230 nm and 260/280 nm (Supplementary Table 1 and in Fig. 1.A and 1.B).

DNA extracted from leaf, episperm, kernel, and flour showed good integrity and a clear band was visualized. The quality and quantity of DNA obtained from commercial products, such as marrons glacés, chestnut drinks, and commercial chestnut cream, were low and DNA bands could not be visualized on the agarose gel, due to the highly degraded DNA, in part masked by the presence of inhibitors. For every matrix, the optimal incubation time in CTAB was set to 60 min (Boccacci et al., 2012). An extended incubation time (20 h) in isopropanol and sodium acetate was used for the processed food matrices, resulting in successful DNA recovery, allowing for a higher DNA concentration and quality.

3.2. SSR genotyping

Nine SSR loci were used: CsCAT 1, CsCAT 3, CsCAT 6, CsCAT 8, CsCAT 14, CsCAT 16, CsCAT 17, CsCAT 41, and QpZAG110 (Marinoni et al., 2003; Steinkellner et al., 1997). The SSR profiles of the DNA extracted from leaves and included in the database developed at DISAFA were used as reference profiles for identification. The results are illustrated according to different matrices. DNA extracted from episperm revealed the correspondence to the cultivars included in the database at all nine SSR loci, two of which (CsCAT-14 and CsCAT-8) are reported in Fig. 2. The SSR profiles for the kernel and marrons glacés matrices were clear-cut, and the fluorescence signals were high. However, the SSR profiles revealed the presence of pollenizer alleles that hampered the correct identification of the maternal cultivar. Indeed, in all samples from the kernel, one allele is always derived from the maternal cultivar and one from the paternal cultivar (Fig. 3). The SSR profiles of the DNA extracted from the monovarietal flours showed a variable number of alleles compared with the SSR profiles from the leaves of the corresponding cultivar. For each cultivar, monovarietal flours were obtained using ten nuts. Therefore, we observed the presence of multiple alleles derived from different pollenizers (Fig. 4). Similarly for processed food: nuclear SSR profiles of chestnut drinks and commercial creams were characterized by multiple alleles for each SSR locus (Fig. 5).

3.3. Chloroplastic SNP/INDELs identification

In this study, we used the Illumina platform to sequence six cultivars of *C. sativa* with 5X coverage depth. The raw paired-end reads

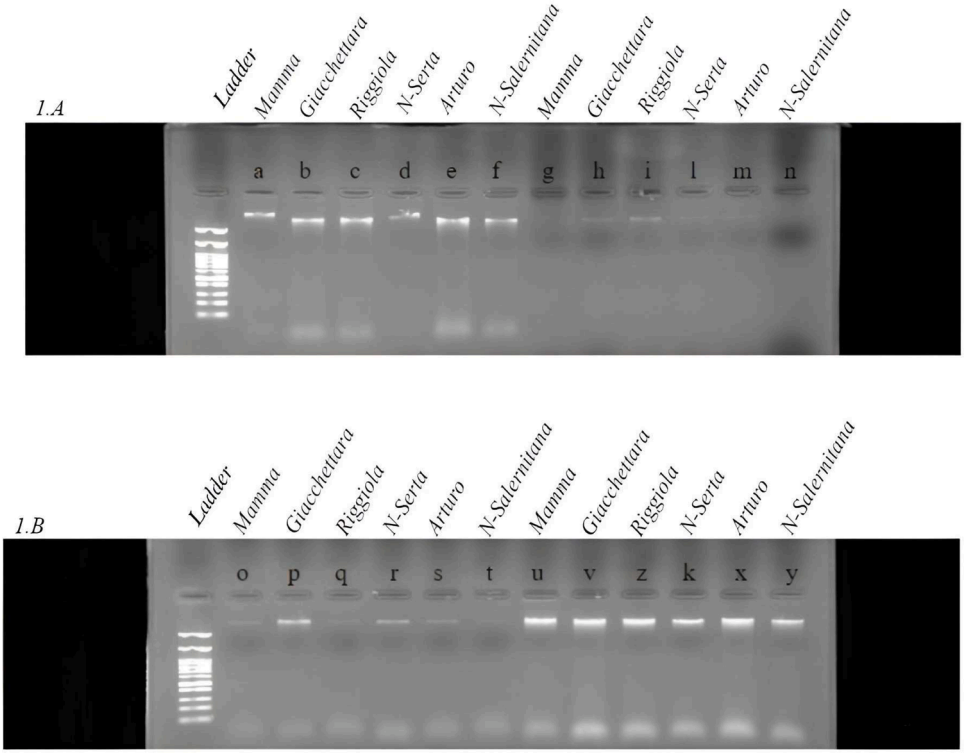

Fig. 1. Integrity check of DNA extracted from Castanea sativa cultivars from Southern Italy. 1.A: a-f) DNA extracted from leaves. g-n) DNA extracted from episperms. 1.B: o-p) DNA extracted from kernels. u-y) DNA extracted from flours. Ladder (100–3000 bp) is highlighted in 1.A and 1.B Figures.

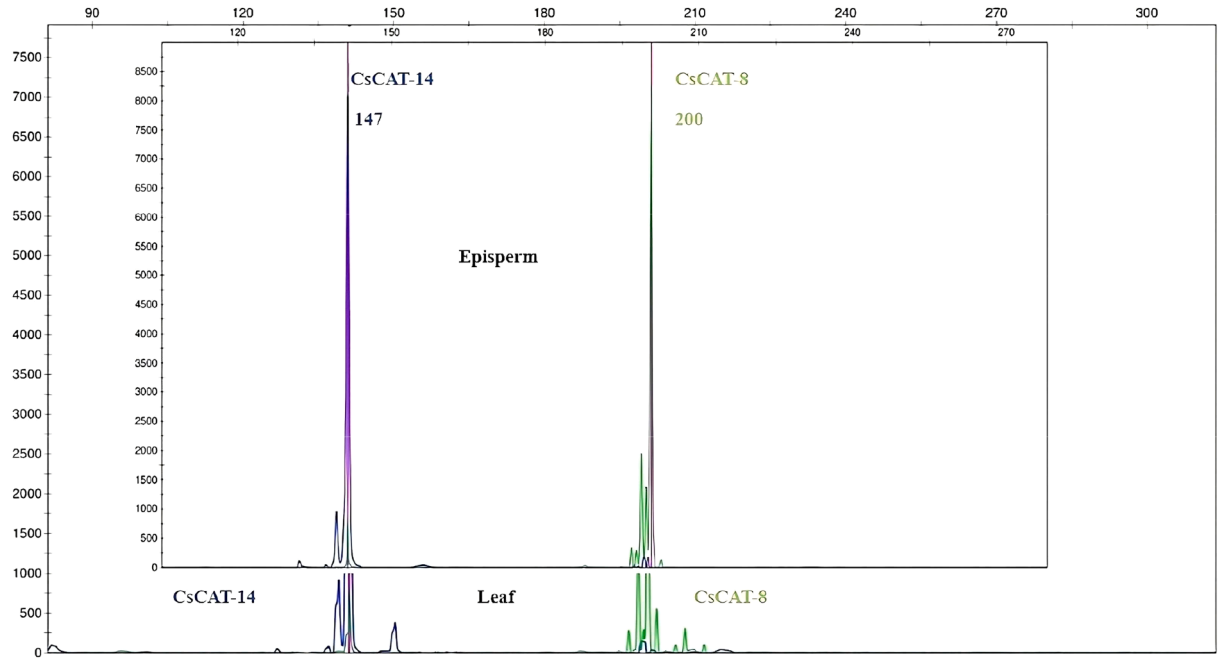

Fig. 2. Comparison of DNA genetic profiles extracted from leaf and episperm of *C. sativa* cultivar 'N-Salernitana.' The electropherogram shows alleles and size (bp) for the 2 SSR loci (CsCAT-14 and CsCAT-8). The figure indicates a complete correspondence of the episperm profile with the genetic profile of the leaf.

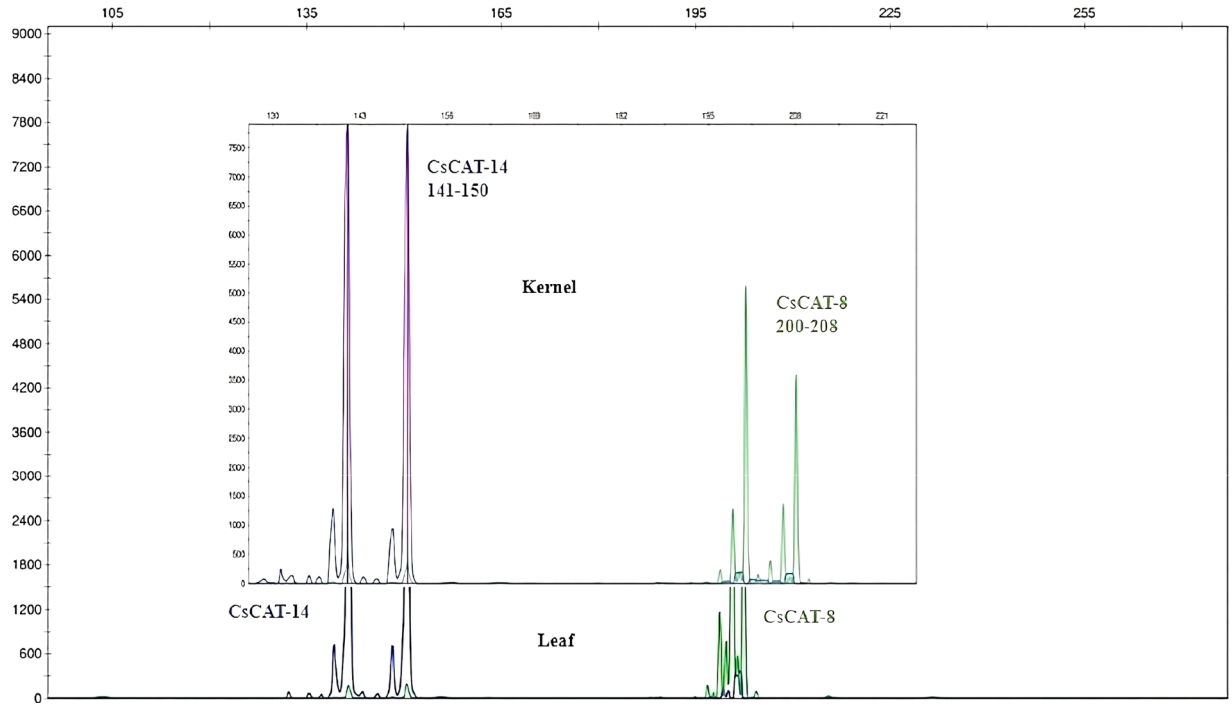

Fig. 3. Comparison of DNA genetic profiles extracted from the leaf and single kernel of *C. sativa* cultivar 'N-Serta.' The electropherogram shows alleles and size (bp) for the 2 SSR loci (CsCAT-14 and CsCAT-8). The electropherogram shows a complete correspondence between the alleles of CsCAT-14 of the kernel and of the leaf. For CsCAT-8 this correspondence is observed for one allele only, due to the presence of a different allele in the kernel derived from the paternal genotype.

(27,471,410) underwent a series of preprocessing steps, resulting in the generation of cleaned reads (27,260,302), with a slight decrement (0.8 %). The cleaned reads were then aligned successfully to the chloroplast genomes (98.82 %), enabling the variant calling analysis. SNP mining analysis revealed 214 SNPs/INDELs, indicating a relatively low mutation rate, consistent with findings by Li et al. (2020). In particular, two regions (HQ336406.1:17,302–17,593; 125,410–125,905), carrying SNPs and enough sequence to design primers, were selected for further analyses.

The first region (Region 1) of *C. mollissima* chloroplast (17,302–17,593) contained one SNP at position 17,402 bp and a deletion at the 17,493 position in *C. sativa*.

The second region (Region 2) of *C. mollissima* chloroplast (125,410–125,905) presented two SNPs at the position respectively 125,539 and 125,905 in *C. sativa*. In addition, this region contained two deletions at the position 125,410 and 125,752 in *C. sativa*. These

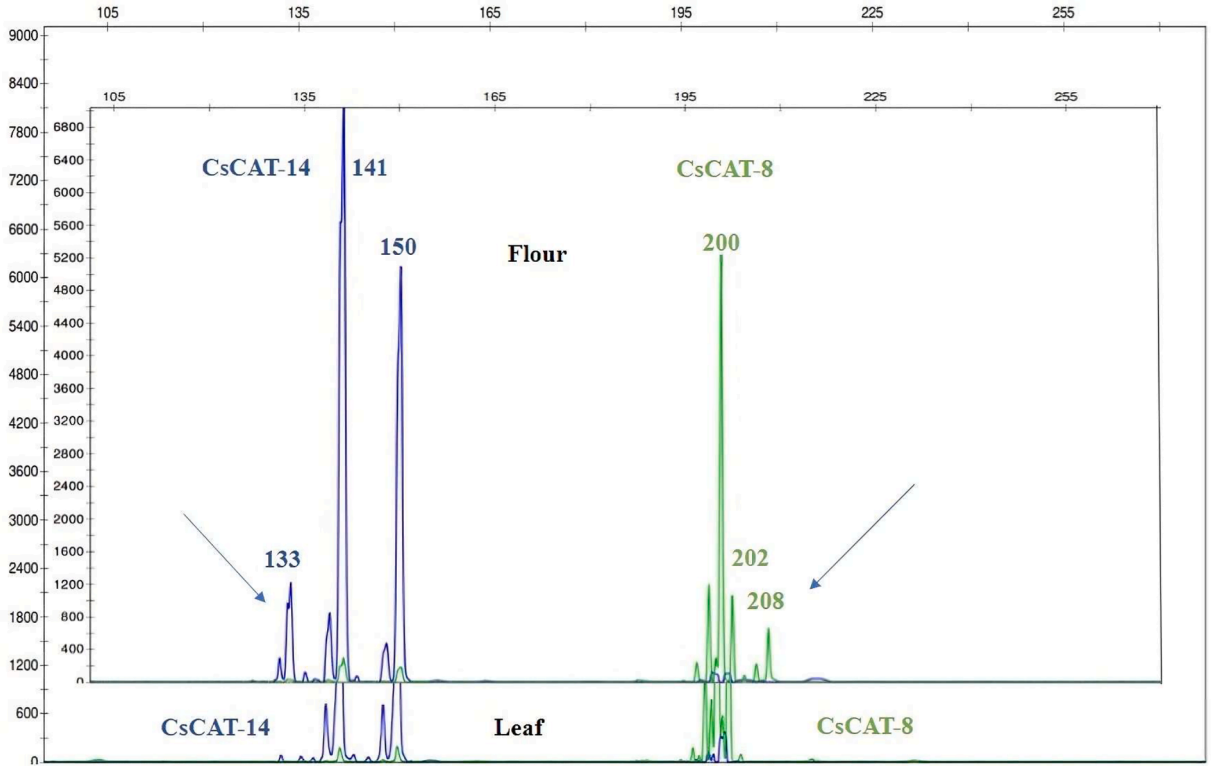

Fig. 4. Comparison of the genetic profiles of DNA extracted from leaf and flour of C. *sativa* cultivar 'N-Serta.' The electropherogram shows the alleles and the size (bp) of 2 loci SSR (CsCAT-14 and CsCAT-8). In this case, multiple alleles are present at both loci. The arrows indicate the alleles that differ from the reference genetic profile of the leaf.

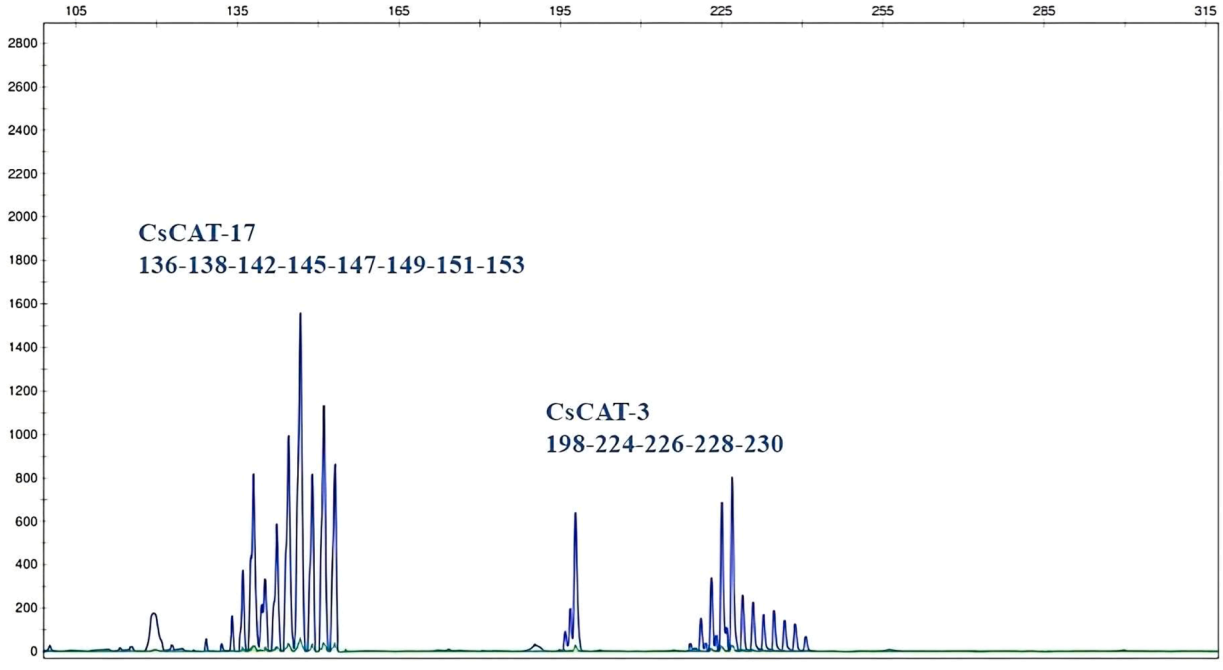

Fig. 5. The electropherogram shows the genetic profile of a commercial chestnut cream, in which CsCAT 3 and CsCAT 17 *loci* have multiple alleles.

identified polymorphisms hold promise as potential molecular markers for identification purposes.

3.4. SNP/INDELs validation

The regions containing SNPs/INDELs were analyzed by Sanger sequencing, to validate data acquired using bioinformatic approach. Leaves of the chestnut plants belonging to the species *C. sativa* (cv. ' Mamma', ' Marrone di Chiusa Pesio', and ' Garrone Rosso'), *C. mollissima* (cv. ' Aioi'', ' Connecticut Yankee', and ' Hakuri') and *C. crenata* (cv. ' Shihou' and ' Toyogin') were used. DNA analysis revealed the ability of SNP/INDELs, founded by bioinformatic analysis,

to distinguish the most commercialized *Castanea* species and were thus used as reliable molecular species-specific markers. Indeed, several SNPs/INDELs allowed to pairwise discriminate *C. sativa* from *C. mollissima* and *C. crenata*.

In the first region (Region 1) (HQ336406.1:17,302–17,593), *C. sativa* and *C. crenata* were characterized by a cytosine-thymine transition (17,402), while in *C. mollissima,* a cytosine was present (Fig. 6. B). In the same Region 1, a deletion of one thymine (17,493) was present in *C. sativa* and *C. mollissima* in contrast to *C. crenata* (Fig. 6. B). In the second region (Region 2), *C. mollissima* was marked by a cytosine-adenine substitution (125,539) compared with *C. mollissima* and *C. crenata*. In addition, *C. sativa* and *C. mollissima* showed the deletion of 2 thymines (125,752) compared to *C. crenata*.

3.5. SNP/INDELs analyses using high resolution melting (HRM)

We developed a rapid identification procedure for species-specific SNP/INDEL markers using high-resolution melting analysis (HRM). Markers were able to identify the three *Castanea* species in plant material, such as episperm, leaves, and nuts, and in processed foods including monovarietal flours and pastes. To perform HRM, we used the SNPs/INDELs found in two regions, which required the design of primers able to amplify short regions. In Region 1, the new set of primers amplified a 180 bp fragment, while in Region 2 the new primers amplified a fragment of 156 bp. While HRM usually requires a single polymorphism for genotyping analyses, a combination of two SNPs was necessary to discriminate the three *Castanea* species.

The combination of these markers in Region 1 allowed us to discriminate *C. sativa* from the other species in plant materials and processed foods, as shown in Fig. 7.

The samples from *C. mollissima* were grouped in the green cluster, *C. sativa* samples in the red group, and *C. crenata* samples in the blue cluster (Fig. 7.B). The clustering of the three species depends on the different SNP/INDELs combinations; indeed, *C. mollissima* has one guanine and one cytosine (G/C), while in *C. sativa* the cytosine is substituted with the thymine (G/T). On the other hand, *C. crenata* differs from the other two species due to the presence of adenine and thymine (A/T). The melting temperature is linked to hydrogen bonds within the sequences (Palais et al., 2005), and the species are split due to their different melting temperatures, which depend on the different substitutions. Indeed, *C. mollissima* has a melting temperature of 77.50 °C, while *C. sativa* has a melting temperature of approximately 77.40–77.25 °C, and *C. crenata* has a lower melting temperature of about 77.20–77.00 °C compared to the other two species.

The analyses carried out in Region 2 demonstrated the ability of the two SNPs to distinguish *C. sativa* from *C. mollissima* and *C. crenata*. Indeed, *C. crenata, C. sativa*, and *C. mollissima* samples are grouped into different clusters, as previously demonstrated for Region 1. The clustering pattern relies on the melting temperature of the amplicons, which is different for each species due to the different SNP/INDEL combinations. Indeed, *C. mollissima* has one thymine and one cytosine (T/C) in its sequence, while in *C. sativa* the thymine is deleted, and the cytosine is substituted with thymine (deletion/T). On the contrary, *C. crenata* presents the deletion of the thymine and the cytosine (deletion/C). Due to these substitutions and deletions, the melting temperatures of the three species are different: *C. mollissima* has a melting temperature of 73.00 °C, *C. crenata* samples have a melting temperature around 72.50 °C, and the amplicons of *C. sativa* cultivars present a melting

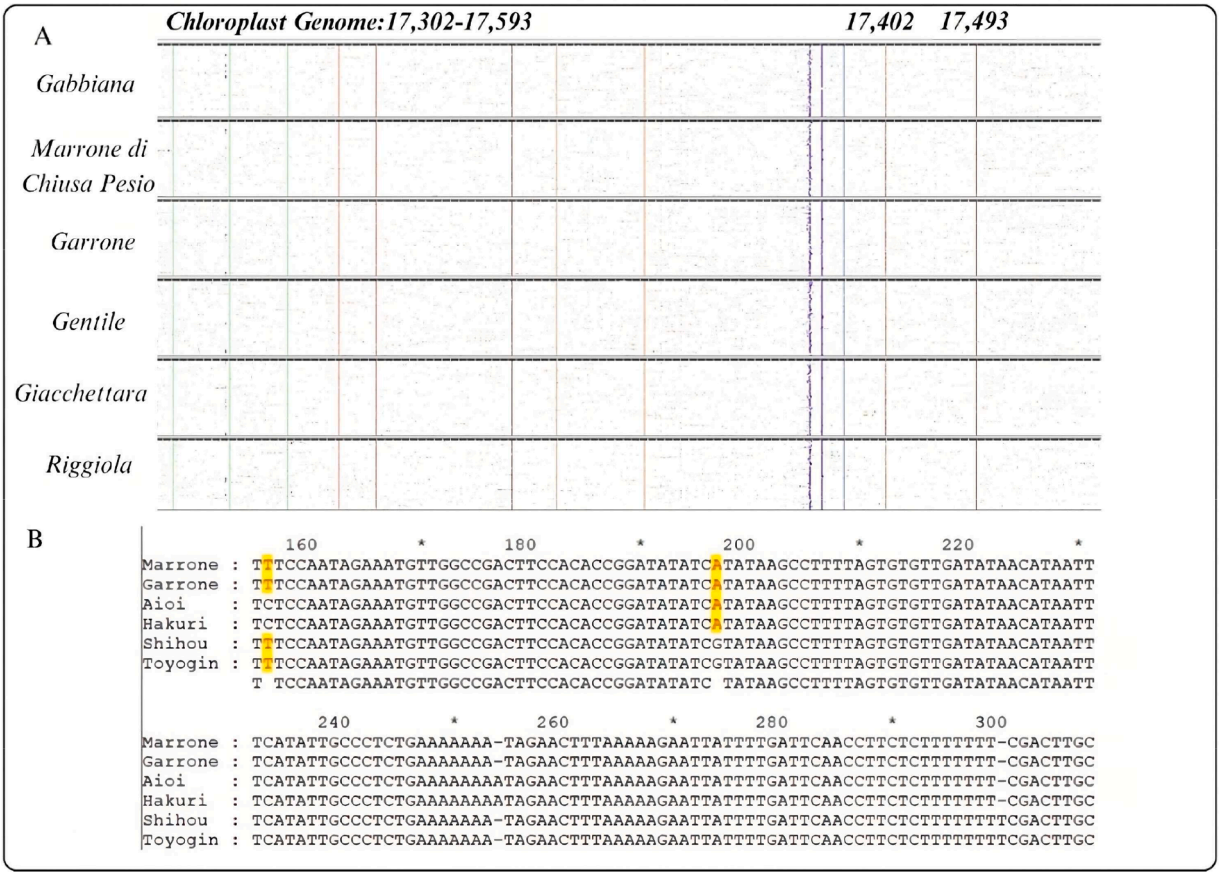

Fig. 6. A) IGV graphic representation of the SNP/INDEL markers detected in Region 1. Six cultivars of *C. sativa* were aligned on the referance chloroplast genome of *C. mollissima*. B) Alignment of the Sanger sequences of *C. sativa, C. mollissima*, and *C. crenata* Region 1 to conferm the SNP/INDEL presence. The SNP/INDELs among the three *Castanea* species are shown in yellow.

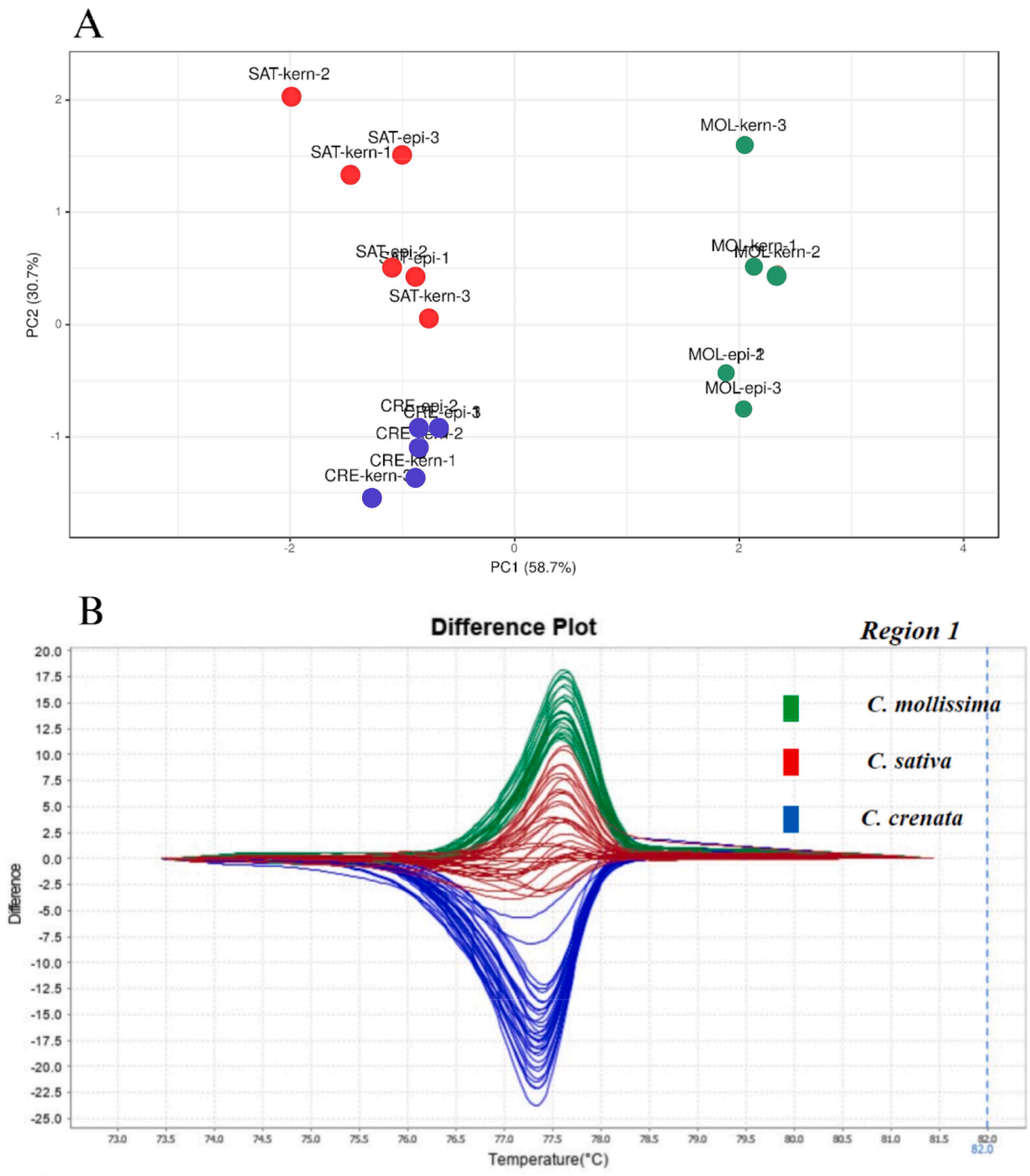

Fig. 7. (A) Principal components analysis (PCA) of the samples targeting the SNPs present in Region 1. Genotypes clustered for *C. sativa* (red), *C. mollissima* (green), and *C. crenata* (blue). (B) High resolution melting analysis of three chestnut species targeting the SNPs identified in Region 1, allowing the identification of *C. sativa, C. mollissima,* and *C. crenata*.

temperature around 72.30 °C. Thus, the difference in the nucleotide sequences influences and modifies the melting temperature, making possible to distinguish *C. sativa, C. mollissima*, and *C. crenata* using High Resolution Melting.

4. Discussion

4.1. DNA extraction protocol

In this study, we evaluated the efficiency of genomic DNA extraction protocols on specific plant materials (episperm and kernel) and food matrices (flour, cream, paste, chestnut drink, and marrons glacés). Extracting high molecular weight (HMW) DNA from plant material represents a considerable challenge. Plant cells are encased in a cell wall composed of several polysaccharide polymers, including cellulose, pectin, glycoproteins, and lignin (Zhang et al., 2021), rendering the cell wall exceptionally rigid and resistant to disruption. Consequently, the implementation of effective mechanical disruption methods is essential. In addition to cell wall, plants have evolved chemical defenses against herbivores, resulting in the production of polysaccharides and phenols, primarily accumulating in their leaves. Upon cell lysis, these compounds can bind to DNA, potentially impacting downstream molecular analyses

(Russo et al., 2022). Notably, the presence of polysaccharides has been demonstrated to inhibit the activity of restriction enzymes. As a result, the purification of DNA from plant material demands meticulous optimization. Moreover, DNA extraction from processed food is challenging and tricky because DNA is degraded by the chemical and physical transformations that occur along the processing chain, such as baking, chopping, or homogenization (Wang et al., 2022; Galimberti et al., 2019). Therefore, the preparation of samples before extraction is of great importance (Piskata et al., 2019).

The original Doyle & Doyle protocol (1987) was adapted and slightly modified to obtain DNA of reliable quality/quantity as previously reported by Talucci et al., 2022. In particular, the processed food samples were treated with isopropanol and incubated at −30 °C for 2 weeks (Boccacci et al., 2012). Subsequently, DNA was extracted from the pellet obtained by centrifugation of the same matrix, as described by Boccacci et al. (2012) and Torello Marinoni et al. (2022). To increase the concentration and the quality of the extracted DNA from processed food, the incubation time of the samples in CTAB buffer (Singh et al., 2021) was increased, as well as the incubation time in isopropanol and sodium acetate (Lade et al., 2014; Viljoen et al., 2022). The prolonged incubation time in CTAB lysis buffer and the extended incubation period in isopropanol enhanced the amount and quality of the extracted DNA. Nevertheless, the overall quality of DNA extracted from food matrices was low, indicating contamination with polysaccharides, proteins, lipids, and phenolic compounds (Silva et al., 2023). The low quality is linked to industrial processes during the preparation of the products; the high temperatures caused DNA degradation in smaller fragments, as demonstrated by Olexova et al. (2004). Notwithstanding, also modest DNA concentration and low quality did not affect the use of DNA for PCR analysis, as reported by Piskata et al. (2017).

4.2. SSR analysis

Nine SSR markers were tested for cultivar identification in different plant materials and products containing chestnuts. The BSA reagent was added to the amplification mixture to counteract the presence of inhibitors, which could hamper the amplification process (Schrader et al., 2012). Indeed, in processed food materials, the levels of inhibitors, such as polysaccharides, proteins, phenolics, and tannins, were much higher; therefore, the addition of BSA was pivotal to counteract their negative effects, avoiding interfering with Taq DNA Polymerase activity or hindering primer annealing (Hedman and Rådström, 2013). The allelic profiles obtained from the DNA isolated from the leaves of different cultivars of *C. sativa* and included in a database developed by DISAFA were used as reference. The SSR profile of raw episperm matched perfectly with the profile of the leaves of the corresponding cultivar at all nine loci tested; this is not surprising since episperm is a maternal tissue (Talucci et al., 2022). Therefore, food companies and control authorities could use the raw episperm as plant material to identify the cultivar of origin and consequently discover frauds.

Clear-cut SSR profiles were obtained from DNA extracted from kernels. However, the interpretation of SSR profiles is more difficult because of the presence of the alleles from the pollenizer's DNA, which hinders the identification of the maternal cultivar (Talucci et al., 2022). Chestnut species are characterized by floral self-incompatibility, which is studied in several woody plants of economic importance, such as pear (Sassa et al., 1992), and olive (Díaz et al., 2006). Indeed, nuts are derived from cross-fertilization between two different individuals; consequently, alleles from both parents are present in the seed.

SSR profiles at all loci were also obtained from DNA isolated from processed food products such as flour, commercial cream, and chestnuts drinks. In this case, the interpretation of SSR profiles was complicated by the presence of multiple alleles from pollenizers or other cultivars when the used chestnuts are a mixture of cultivars. Thus, even though SSR molecular markers were robust tools in germplasm analyses (Powell et al., 1996; Amar et al., 2011; Vidak et al., 2021), they did not allow for cultivar discrimination in our food-processed matrices, as observed elsewhere (Talucci et al., 2022; Caramante et al., 2011).

4.3. Chloroplastic SNP identification

To overcome the limits of microsatellites in genotype identification in processed foods, we evaluated the possibility of using maternal-inherited markers located in the chloroplast genome for the implementation of a genetic traceability protocol. As mentioned above, the chloroplast genome is maternally inherited (Zhou et al., 2021) and chloroplast markers represent a valuable tool for cultivar identification from nuts and processed food containing nuts (Teske et al., 2020). Besides, the SNP/INDELs markers allow the identification of cultivars from smaller DNA fragments, which is a notable aspect in the food authentication from processed food, such as paste, cookies and flours, that undergo physical and chemical changes (Uncu and Ozen, 2015).

The result of the sequencing pointed out how the chloroplast SNP/INDELs (214) were conserved among the *C. sativa* tested varieties while they were highly efficient in the discrimination of the three *Castanea* species. For this reason, they can be used as species-specific markers, after further validation with a larger number of cultivars. This result is consistent with previously published studies, where the chloroplast SNP/INDELs were not able to discriminate among cultivars in species such as larch (*Larix* spp.) (Bondar et al., 2019), bell pepper (*Capsicum* spp.) (Jeong et al., 2010), and rice (*Oryza* spp.) (Gouda et al., 2021).

4.4. SNP/INDELs analyses using high resolution melting (HRM)

We proposed and tested the HRM analysis as an avant-garde instrument to detect the plastidial SNPs/INDEL markers among the three *Castanea* species in both processed food and plant material. The HRM technique is less time-consuming and expensive than Sanger sequencing and other comparable methods, such as KASP (Kompetitive allelic specific PCR) or SNaPshot (Pereira et al., 2018). In addition, HRM is a sensitive method that detects sequence differences between species without the need to sequence them (Jeong et al., 2010). For these reasons, HRM has been widely utilized in the biomedical and food sector, particularly for the food authentication of olive oil (Batrinou et al., 2020). Moreover, HRM has been used recently for the traceability of cultivars and species, such as grapevine (Gomes et al., 2018), almond (Wu et al., 2008), and apple (Heo and Chung, 2020).

The melting temperature of the samples plays a fundamental role in the genotyping analyses using HRM. Due to the high sensitivity of this technique, it is necessary to standardize the DNA concentration in all specimens to ensure similar cycle threshold (Ct) values (Sun et al., 2016). In addition, all DNA samples must be prepared using the same extraction and purification protocol to minimize the ionic strength effect on the melting temperature (Erali and Wittwer, 2010). Indeed, the increase of the ionic strength reduces the repulsion between the negatively charged phosphate groups in the two DNA strands, leading to an increase in melting temperature. Therefore, the samples must be prepared using the same reaction buffers at the same concentration to avoid the generation of divergent melting temperatures, not due to SNP differences, altering the HRM results (Słomka et al., 2017).

The HRM approach also requires great attention in primers design, to obtain robust and reliable results (length: 18–20 bp; Tm: 58 °C-60 °C). Another practical tip regards the amplicon length: for the gene scanning, the primers have to amplify fragments of 200–300 bp, while an amplicon length of 60–180 bp is generally recommended for genotyping using the HRM analysis (Vossen et al., 2009). In Słomka et al. (2017)study, primers designed for 108 bp fragment amplification were unsuitable for identification. Subsequently, the decrease of the amplicon length to 74 bp improved the results of the HRM analysis. Moreover, the primers must be specific to the region of interest since the fluorescent dyes bind to all double-stranded DNA products without distinction (Wojdacz et al., 2008). In the present work, the results showed the effectiveness of the

HRM technique for genotyping *Castanea* species in all matrices. Two regions of interest were studied for species identification, allowing to clearly distinguish *C. sativa, C. mollissima*, and *C. crenata,* thus exploitable as species-specific markers in food traceability.

Indeed, in Region 1, it was possible to discriminate the *C. sativa* samples from *C. mollissima* and *C. crenata* by HRM analysis due to the different SNP/INDEL combinations in that region. These differences in the sequence are substantial for distinguishing the three species because the melting process entails the temperature rise to dissociate the double-strand DNA amplicons, leading to the decay of the fluorescence signal (Dang et al., 2012). This mechanism is sensitive to the presence of SNP/INDELs in the sequence; indeed, different SNP/INDELs modify the melting temperatures of the samples, creating different melting curve patterns for each species (Heo and Chung, 2020). Thus, the generation of divergent melting curves for each species provides an important tool to differentiate the *Castanea* species in all matrices.

5. Conclusion

The present work concerned the development of a genetic traceability protocol based on DNA analysis to be used along the supply chain of chestnut, from orchard to processed products.

Nuclear SSR markers have proven to be an effective tool for *C. sativa* cultivars identification, using leaf and episperm as plant material. However, SSR markers showed limitations in cultivars identification using processed food such as kernels, flours, creams, pastes, and chestnut drinks. The presence of the paternal DNA of the pollenizer hindered the correct identification of cultivars.

Chloroplast SNP/INDELs markers showed their attitude in traceability analyses of food along the supply chain as well as in processed food matrices, discriminating the 3 major species of the *Castanea* genus. Future goal will be to identify a chloroplast SNP set able to discriminate *C. sativa* x *C. crenata* hybrids.

Overall, results make a meaningful contribution to the genetic traceability system along the entire supply chain of the European chestnut, protecting the interests of growers and nurseries and safeguarding consumers and the productions of *C. sativa* from counterfeiting groceries.

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– Enhancing high value-added nuts and processed fruits supply chains" aimed at developing Key Enabling Technologies (KETs) to add value to typical nuts and processed fruit supply chains of South Italy. In this paper, we present the results of "OR1-plant material", aimed at searching for cultivars with characteristics appropriate to market needs, characterizing local varieties and developing innovative technologies for the management and optimization of the supply chain traceability. Cultivars of *C. sativa* were selected in the Calabria Region and DNA data were compared with data from other Italian cultivars and samples from Chinese and Japanese chestnuts.

Compliance with ethics requirements

This article does not contain any studies with human or animal subjects.

CRediT authorship contribution statement

Lorenzo Antonio Marino: Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Vera Pavese:** Conceptualization, Formal analysis, Investigation, Writing – original draft. **Paola Ruffa:** Conceptualization, Formal analysis, Investigation. **Martina Ferrero:** Formal analysis, Investigation, Writing – review & editing. **Alberto Acquadro:** Conceptualization, Writing – review & editing. **Lorenzo Barchi:** Conceptualization, Writing – review & editing. **Roberto Botta:** Conceptualization, Writing – review & editing, Funding acquisition, Supervision. **Daniela Torello Marinoni:** Conceptualization, Writing – review & editing, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data are available in the paper, only the DISAFA SSR database is confidential

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.scienta.2023.112713.

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