



Genetic and geochemical signatures to prevent frauds and counterfeit of high-quality asparagus and pistachio



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ABSTRACT

A fingerprinting strategy based on genetic (simple sequence repeat) and geochemical (multielement and ⁸⁷Sr/⁸⁶Sr ratio) analysis was tested to prove the geographical origin of high-quality Italian products “White Asparagus from Bassano del Grappa” and “Green Pistachio from Bronte”. Genetic analysis generated many polymorphic alleles and different specific amplified fragments in both agriproducts. In addition, a core set of markers was defined. According to variability within production soils and products, potential candidate elements linking asparagus (Zn, P, Cr, Mg, B, K) and pistachio (Mn, P, Cr, Mg, Ti, B, K, Sc, S) to the production areas were identified. The Sr isotopic signature was an excellent marker when Italian asparagus was compared with literature data for Hungarian and Peruvian asparagus. This work reinforces the use of Sr isotope composition in the soil bioavailable fraction, as assessed by 1 mol/L NH₄NO₃, to distinguish white asparagus and pistachio originating from different geographical areas.

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1. Introduction

Nowadays, food quality and safety are topics of great attention for consumers and any case of food adulteration has a strong impact on public opinion. The traceability of food products is becoming increasingly important for the global economy as a consequence of the pressure that consumers exert on knowing the nutritional value of food as well as its geographical origin and authenticity. Consumers in the European Union require guarantees on the origin of food products, which they take as a pledge for safety and quality (regulations EEC 2081/92 and EC 1898/06). Finding the appropriate tools to provide a fingerprint for geographic origin determination of food products and to establish their ‘traceability’ may be challenging (Adamo et al., 2012). The traceability system is an important tool for tracking, monitoring and managing product flows through food supply chains, potentially verifying the presence of credence attributes in consumer food purchases (Myae & Goddard, 2012). The Council Regulation (EC) N. 510/2006 established some brands with legal protection, such as Protected Designation of Origin (PDO) “that covers agricultural products

and foodstuffs which are produced, processed and prepared in a given geographical area using recognized know-how”. Among Italian PDO brands, *White Asparagus from Bassano del Grappa*, produced in province of Vicenza (north of Italy) and “*Green Pistachio from Bronte*”, produced in province of Catania, Sicily (south of Italy), represent important examples of food products with an urgent need for analytical approaches that guarantee authentication.

In recent years, there has been an increasing interest in defining analytical techniques to trace food products. Thanks to the new advances in the field of molecular biology, molecular markers have become rapid, sensitive and efficient tools to identify the origin of commercialized products (Martin-Lopes, Gomes, Pereira, & Guedes-Pinto, 2013). Of particular interest are microsatellites (simple sequence repeat (SSR) markers) because, in comparison to other DNA markers, they are faster to use and the results are more clear-cut. One additional advantage of SSR markers is that the small dimension of their target sequences may allow the amplification of DNA degraded or extracted from processed food (Pasqualone, Alba, Mangini, Blanco, & Montemurro, 2010). Molecular markers alone cannot ascertain the geographical origin of the products, thus the use of complementary techniques have been proposed (Drivelos & Georgiou, 2012). In this context, the investigation of stable isotopes has gained increasing importance. In

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particular, the isotopes of H, C, O, N and S have been widely used. Over the last years, geogenic isotopes, all above Sr isotopes, have become increasingly popular as they provide a unique link from soil to primary agricultural products (Brunner, Katona, Stefánka, & Prohaska, 2010; Swoboda et al., 2008). Sr is generally present in food at trace levels (<0.1% (w/w)) and shows a distinct variation in its isotopic composition due to the geochemical differences of soils. In addition, the Sr system does not show a significant isotopic fractionation during plant uptake. As a consequence, the bioavailable fraction in soils provides a unique system to direct link the soil to the plant. Moreover, it has been shown that the seasonal and annual variation of the $^{87}\text{Sr}/^{86}\text{Sr}$ isotope ratio is not significant, thus it represents a reliable tool that lasts over time (Swoboda et al., 2008). Examples on the use of the isotope ratio measurement to authenticate and trace the geographical origin of agricultural products are reported in cider (García-Ruiz, Moldovan, Fortunato, Wunderli, & García Alonso, 2007), grape or wine (Marchionni et al., 2013) and tomato (Trincherini, Baffi, Barbero, Pizzoglio, & Spalla, 2014).

The main objective of this study was to establish a combined analytical tool based on molecular and geochemical markers to identify the geographical provenance of high-quality protected white asparagus and pistachio downstream of the production chain to prevent fraud and counterfeiting. The genetic approach was based on the use of SSR markers. The geochemical fingerprint was built on multi-element data and the isotope ratio of $^{87}\text{Sr}/^{86}\text{Sr}$ assessed both in soil and plant materials.

2. Materials and methods

2.1. Geo-pedological properties of the cultivation areas, soil and plant sampling

2.1.1. White Asparagus from Bassano del Grappa

The geographical area devoted to the production of PDO White Asparagus from Bassano del Grappa (*Asparagus officinalis* var. "Comune", hereafter coded BSN) is the plain surrounding the town of Bassano del Grappa (in the province of Vicenza, north Italy), 129 m a.s.l. at the foothills of the Venetian Prealps, where the Brenta river flows. The soil parent material consists of river sediments and gravelly sandy deposits of alluvial fan. Soils have been classified as Cutanic Luvisols (Hypereutric, Endoskeletal, Endoarenic) (WRB, 2006). The land use is dominated by cultivation of corn,

whereas autumn-winter cereals (wheat, barley, oats) have a secondary importance. Turions and related cultivation (0–30 cm) soils were sampled from five different farms situated in the Brenta river plain. The altitude of the sampling sites ranged between 112 and 155 m a.s.l. At harvest, turions were in the full-ripe stage. A sampling strategy with three replications per farm was applied, with ten turions collected for each replication. A total of 15 soil and turion samples were collected. More details of samples and sampling sites are given in Table 1. Cultivation soils were characterized by acidic pH ranging between 5.3 and 5.6. Accordingly, soils were poor of carbonates (always below 7 g/kg), sandy-clay and exhibited a moderately high organic carbon content (values ranging from 17.3 to 25.0 g/kg) and a low cation exchange capacity as measured by BaCl_2 pH 8.1 (between 8.2 and 10.7 cmol_c/kg) (ISO 13536, 1995). In all farms, the asparagus cultivation was managed in full compliance with the PDO White Asparagus from Bassano del Grappa production guidelines (European Community Council Regulation EC 1050/2007; GUCE 240/2007; Dossier number IT/PDO/0005/0338). In order to maintain soil fertility, mature cow manure is applied in pre-plantation (60 tons per hectare) followed by yearly NPK fertilization, with nitrogen supply for at least 50% of organic nature. Phosphate and part of potassium fertilization takes place in the fall or at the end of winter, while nitrogen and the rest of potassium in the post-harvest period (no later than July). The annual supply of N, P and K never exceeds the maximum amounts of 150, 80 and 180 kg per hectare, respectively.

2.1.2. Green pistachio from Bronte

The production area of PDO Green Pistachio from Bronte (*Pistachia vera* cv. "Bianca or Napoletana", hereafter coded BRNT) is located on the north-western foot slope of the Mount Etna, an active stratovolcano on the east coast of Sicily. The altitude of the sampling sites ranges between 460 and 672 m a.s.l. The soil parent material is made of lava flows, scoria cones, spatter ramparts and pyroclastic fall deposits related to flank and summit eruptions. Lava types range from basalt to benmoreite, aphyric to highly porphyritic in texture, with phenocrysts of plagioclase, pyroxene, olivine, variable in quantity and size. Seeds of BRNT and corresponding cultivation soil (0–30 cm) were sampled from five different farms situated in the province of Catania. At the collection time seeds were at the full-ripe stage. A sampling strategy with three replication per farm was applied, with about 500 g of seeds collected for each replication. A total of 15 soil and seed

Table 1
Code and geographical location of the studied samples.

PDO	Region/Municipality	Sample code	Sample	Locality	Latitude N	Longitude E
White Asparagus from Bassano del Grappa	Veneto/Bassano del Grappa	Farm I	Soil Turions	ROSA'	45° 43' 41,734"	11° 46' 3907"
		Farm II	Soil Turions	ROSA'	45° 43' 32,879"	11° 47' 10,518"
		Farm III	Soil Turions	CASSOLA	45° 44' 28,416"	11° 45' 26,903"
		Farm IV	Soil Turions	BASSANO DEL GRAPPA	45° 44' 16,319"	11° 44' 52,996"
		Farm V	Soil Turions	CARTIGLIANO	45° 42' 40,740"	11° 42' 18,785"
Green Pistachio from Bronte	Sicily/Bronte	Farm I	Soil Nuts	ROCCARELLA	37° 44' 46,708"	14° 47' 48,463"
		Farm II	Soil Nuts	GINESTROLA	37° 42' 45,305"	14° 40' 1695"
		Farm III	Soil Nuts	MOSCARELLO	37° 43' 11,602"	14° 48' 41,357"
		Farm IV	Soil Nuts	ROCCARELLO	37° 44' 40,903"	14° 48' 29,742"
		Farm V	Soil Nuts	DAGADI	37° 45' 19,992"	14° 49' 6372"

samples were collected. More details of samples and sampling sites are given in Table 1. Cultivation soils had an acidic pH (4.7–5.9). Carbonates contents were below 4 g/kg and cation exchange capacity ranged from 17.1 to 31.5 cmol./kg. Soils were predominantly sandy with organic carbon content of 24–50 g/kg. In all farms, the pistachio cultivation was managed in full compliance with the PDO *Green Pistachio from Bronte* production guidelines (European Community Council Regulation UE 21/2010; GUUE 8/2010; Dossier number IT/PDO/0005/0305). Pistachio is cropped without fertilization.

2.2. Plant and soil preparation

Asparagus turions were cleaned from adhering soil, washed with MilliQ water, dried and cut into pieces of approximately the same size. Pistachio seeds were cleaned manually by the husk immediately after their collection and were divided into three sub-samples: whole sample, peel and pulp. All asparagus and pistachio samples were placed in Falcon and freeze-dried in a DELTA 1–24 LSC freeze-dryer (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), until complete dehydration. Asparagus turions were pulverized using agate mill jars, while pistachio materials were milled in a mortar with the use of liquid nitrogen. The freeze-dried samples were stored at -20°C . All cleaning operations and management of the plant material were performed under a glove box to avoid contamination. The geochemical surveys were conducted on samples of surface soil (0–30 cm). Soils were air-dried and 2 mm sieved and subsequently the residual moisture was determined at 105°C .

2.3. DNA extraction

DNA was extracted from asparagus turions of BSN and seeds of BRNT. Included in the analysis were also turions of four commercial varieties (*Argenteuil*; *Green Asparagus from Maremma Tosco-Laziale*; *Connovers Colossal* and a *White Peruvian Asparagus*, hereafter coded ARG, MTL, CON and PER, respectively) and seeds of four commercial pistachio products (Marketed Pistachio flour “*Sicilia perfetta*”; Pistachios from Iran; Pistachios from Turkey; Pistachios from USA hereafter coded SCL, IRN, TRK and USA, respectively). In particular, the marketed Pistachio SCL was included in the study because its geographical area of production (Bronte), corresponds to that of BRNT, and this makes it as “*Green Pistachio from Bronte*”-like. DNA was isolated from asparagus turions and pistachio seeds using the *DNeasy plant mini kit* (Qiagen, Valencia, CA, USA), properly modified to optimize the extraction from tissues different from leaves. For each variety three samples were analyzed and a reference DNA was extracted from leaves of each of them. DNA concentration and quality was estimated through NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and electrophoretic run on agarose/TAE gel 0.8%. Extracted DNA have been diluted to a concentration of 15 ng/ μL and stored at -20°C .

2.4. SSR amplification and analysis

Fifteen primer pairs for asparagus amplification were chosen from two sources: 2 (AA01, AA03) from Aceto et al. (2003) and 13 (TC1, TC2, TC7, AG2, AG3, AG5, AG7, AG8, AG10, AG11, AG12, AAT1, AGA1) from Caruso, Federici, and Roose (2008) (Supplementary Table 1). As for pistachios, 14 primer pairs (Ptms11, Ptms12 and Ptms47, Ptms3, Ptms42, Ptms41, Ptms7, Ptms31, Ptms10, Ptms33 and Ptms45, Ptms9, Ptms14 and Ptms40) were chosen from Ahmad, Ferguson, and Southwick (2003) (Supplementary Table 2). Asparagus SSR were designed on expressed sequences while pistachio SSR came from genomic sequences. The forward primer of

each set was M13-tailed to facilitate subsequently labelling with a fluorophore (Oetting et al., 1995). PCR amplification was carried out with a Veriti™ Thermal Cycler (Applied Biosystems, Foster City, CA, USA) under varying annealing temperatures (T_a), depending on the primer pair. Each PCR reaction included the M13-tailed forward primer, the reverse primer and a M13 forward primer 5'-end-labelled with a fluorophore. PCR reactions were performed in a 20 μL volume containing $1\times$ reaction buffer with 1.6 mM MgCl_2 , 0.2 mM of each dNTP, 1 unit of *goTaq* polymerase (Promega, Madison, WI, USA), 20 pM of each primer and 30 ng of genomic DNA. Cycling conditions consisted in two different cycles for the different species analyzed (Supplementary Table 3). Amplification products were checked and quantified on 2% agarose/TAE gel using 1 Kb plus ladder (Life Technologies, Carlsbad, CA, USA) and displayed at the trans illuminator. Each gel was first visually examined, and amplicons were then separated with the ABI PRISM® 3130 DNA Analyzer system (Life Technologies). Size calibration was performed with the molecular weight ladder GenScan® 500 ROX™ Size Standard (Life Technologies). Electropherograms were analyzed using the software Peak scanner ver. 1.0 (Applied Biosystems) following the factory default sizing algorithm modified in values of quality flags (pass range 0.1–1; low quality range 0–0.05). Polymorphisms of useful primer pairs were surveyed in all the individuals and several parameters of genetic diversity, including the number of alleles per locus (Na) and the number of alleles (Ne), allele size range, number of private alleles and observed heterozygosity (Ho) were calculated using GenAlex v. 6.5 (Peakall & Smouse, 2012). Also, microsatellite effectiveness for differentiating among species was based on the following parameters: Power of Discrimination ($\text{PD} = 1 - \sum p_i^2$, where p_i is the frequency of the i th genotype calculated for each SSR) and the Polymorphic Information Content (PIC, calculated according to the formula above, but with genotypic frequency replaced by the allele frequency). For each variety, data were scored for the presence or absence of each allele in all genotypes. A cluster analysis was performed based on a similarity matrix calculated using the Dice's coefficient (Sneath & Sokal, 1973) and using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) hierarchical clustering method. The analysis were carried out through a server running the program Dendro_UPGMA (<http://genomes.urv.es/UPGMA/>) (Garcia-Vallve, Palau, & Romeu, 1999).

2.5. Multielement analysis

Multielement analysis of soil and plant materials was carried out at Acme Analytical Laboratories Ltd (Vancouver, Canada) by Perkin Elmer Elan 6000 ICP-MS. As for soils, Acme's Group 1EX package (HNO_3 - HClO_4 -HF digestion) for 41 elements (Ag, Al, As, Au, Ba, Be, Bi, Ca, Ce, Cd, Co, Cr, Cu, Fe, Hf, K, La, Li, Mg, Mn, Mo, Na, Nb, Ni, P, Pb, Rb, S, Sb, Sc, Sn, Sr, Ta, Th, Ti, U, V, W, Y, Zn, Zr) was used. As for plant materials, Acme's Group 1VE – MS package (HNO_3 digestion followed by *aqua regia*) for 53 elements (Au, Ag, Al, As, B, Ba, Be, Bi, Ca, Cd, Ce, Co, Cr, Cs, Cu, Fe, Ga, Hf, Hg, In, K, Ge, La, Li, Mg, Mn, Mo, Na, Nb, Ni, P, Pb, Pt, Rb, Re, S, Sb, Sc, Se, Sn, Sr, Ta, Te, Th, Ti, U, V, W, Y, Zn, Zr, Pd) was employed.

The degree of similarity among geochemical profiles of soils and plants collected in the same production areas was evaluated by single element and overall coefficient of variation (CV), expressed as percentage, and defined as the ratio of the standard deviation to the mean.

2.6. Sr isotope ratio

For the determination of total Sr in soil, microwave (Anton Paar Multiwave 3000, Anton Paar, Graz, Austria) assisted acid (HF-HCl- HNO_3) digestion of milled soil was carried out into Teflon

vessels. The digested samples were evaporated to almost dryness. *Aqua regia* and double sub-boiled HNO_3 were added and again evaporated at 90 °C to a volume of 0.5 mL. The volume was finally filtered (0.45 μm) and filled up with 8 mol/L HNO_3 . The bioavailable Sr fraction was extracted from the soil by 1 mol/L NH_4NO_3 following the extraction procedure proposed by the German national standard DIN 19730 (Prueb, 1997). To obtain accurate results with minimum uncertainty, a Sr/matrix separation prior to measurement by MC-ICPMS was done, using a Sr specific resin (ElChrom Industries, Inc., Darien, IL, USA) with a particle size of 100 μm –150 μm according to a standard procedure (Swoboda et al., 2008). The Sr isotope ratios in the obtained solutions were measured with a double-focusing sector field MC ICP-MS instrument (Nu Plasma HR, Nu Instruments, Wrexham, Wales, UK) with a desolvating membrane nebulizer (DSN 100, Nu Instruments, Wrexham, Wales, UK) using optimized operational parameters (Swoboda et al., 2008). An SRM 987 solution (cert. $^{87}\text{Sr}/^{86}\text{Sr} = 0.71034 \pm 0.00026$) was used to calibrate the instrument for instrumental isotopic fractionation by applying a standard – sample – bracketing approach. The ‘measure zero’ method of the NU Plasma software was used for the blank correction using instrument (Nu Plasma HR, Nu Instruments, Wrexham, Wales, UK) measuring separation blanks within the measurement sequence

3. Results and discussion

3.1. Genetic signature

3.1.1. Asparagus

The initial screening for the selection of markers was conducted on 15 SSRs. Five loci were excluded because of their pattern profiles: AA03, AG11 and AG5 were affected by the presence of unamplified (null) alleles for BSN, while TC2 band profiles were not easy to interpret because of the excess of stutter bands. Finally, AGA1 amplified bands larger than 600 bp, as also reported in Caruso et al. (2008), and for this reason it was not included. The remaining 10 SSR loci (TC1, AAT1, AG2, AG3, AG7, AG8, AG10, AG12, TC7 and AA01) showed reliable polymorphisms, confirming previous data (Caruso et al., 2008). Overall, we identified 40 alleles, with an average of 4 alleles per locus (Table 2). Primers TC1, AAT1 and AG2 produced the highest number of alleles (respectively 8, 5 and 5 alleles per locus). Out of ten loci, seven were useful to identify variety-specific alleles (hereafter named private alleles). These are alleles that are found only in a single sample among a broader collection (Szpiech & Rosenberg, 2011). Private alleles are important not only in traceability studies (Adamo et al., 2012) but also in molecular ecology and conservation genetics (Kalinowski, 2004) as well as human evolutionary genetics (Schroeder et al., 2007). Our results

revealed four private alleles for BSN (three from locus TC1 and one from AG10). Private alleles were also found for the other varieties. Locus TC1 produced the highest number of private alleles (Supplementary Fig. 1), with informative results in three different varieties. AAT1, AG2 and AG10 produced two private alleles each, whereas AG3, AG7 and TC7 gave one private allele. Loci AAT1 and TC1 confirmed their power to discriminate unambiguously varieties from each other as shown in Caruso et al. (2008). The level of informativeness of SSR was estimated as PIC and PD (Table 2), two indices commonly used to evaluate informativeness degree of microsatellites. PIC is based on allele frequencies, whereas PD is based on banding pattern or genotype frequencies at a given locus. The highest PIC value for polymorphic loci was observed in TC1 (0.74) while the lowest in AA01 (0.35). The average PIC value (0.55) indicates a relatively high genetic similarity among varieties studied here. This was expected since cultivated asparagus has a narrow genetic base derived from breeding (Moreno, Espejo, Cabrera, Millan, & Gil, 2006). It is reported that nearly all existing varieties derive from the ‘Violet Dutch’ (s XVIII) (Knaflewski, 1996). In spite of the high similarity between varieties, our results did identify a core set of highly informative primers (TC7, TC1, AAT1, AG2 and AG10) with PIC values higher than 0.60. The PD index, which can range between 0 (monomorphism) and 1 (highly informative), varied from 0.91 to 0.96. It should be pointed out that all samples showed values of discriminant power (PD) higher than 0.90, indicating a good strength of the selected markers. To clarify the genetic relationship between BSN and the outgroup varieties, an UPGMA (unweighted pair groups method using arithmetic averages) dendrogram was built using the similarity coefficient of Dice (Supplementary Table 4 and Fig. 1a). According to the genetic distances, two main groups were distinguished. All varieties were grouped together with the only exception of MLT, which clustered apart. Within the main cluster, the distribution of the varieties highlighted the genetic difference between BSN and PER, the latter often confused with the former PDO.

3.1.2. Pistachio

Only few studies are available that analyse the genetic structure of pistachio varieties. For this reason, a limited number of SSR markers is available for *P. vera*, such as those identified by Ahmad et al. (2003) and Zaloğlu, Kafkas, Doğan, and Güney (2015). In our study, three loci (Ptms11, Ptms12 and Ptms47) did not produce any amplification so they were considered null alleles and as such were excluded. The remaining 11 microsatellites produced 31 alleles and gave easily interpretable and reproducible profiles. Three of them (Ptms9, Ptms14 and Ptms40) were monomorphic, the others produced polymorphisms, with a number of alleles ranging from two to five (on average 3.9 alleles per locus) (Table 3). Similarly, Ahmad et al. (2003) obtained an average

Table 2
Locus name, allele size, private alleles identified in bold and genetic parameters (Na: number of alleles; Ne: effective number of alleles; Ho: observed heterozygosity; He: expected heterozygosity; PIC: polymorphic information content; PD: discriminant power) for each analyzed locus of asparagus varieties.

Loci	Allele sizes	Na	Ne	Ho	PIC	PD
TC1	234 , 238, 241, 243, 244, 245 , 248, 250	8.00	3.84	0.77	0.74	0.96
AAT1	234, 235, 239, 240, 241	5.00	2.91	0.46	0.66	0.94
AG2	166, 169, 171, 175, 177	5.00	2.49	0.69	0.60	0.93
AG3	237, 239, 241	3.00	2.00	0.77	0.50	0.92
AG7	186, 194, 196, 198	4.00	2.27	0.62	0.56	0.92
AG8	247, 249	2.00	1.83	0.08	0.45	0.91
AG10	179, 196, 202, 296	4.00	1.74	0.54	0.43	0.92
AG12	249, 250, 253	3.00	2.70	0.54	0.63	0.92
TC7	228, 231, 235, 241	4.00	2.70	0.46	0.63	0.92
AA01	257, 258	2.00	1.55	0.00	0.35	0.91
Mean		4.00	2.40	0.49	0.55	0.93

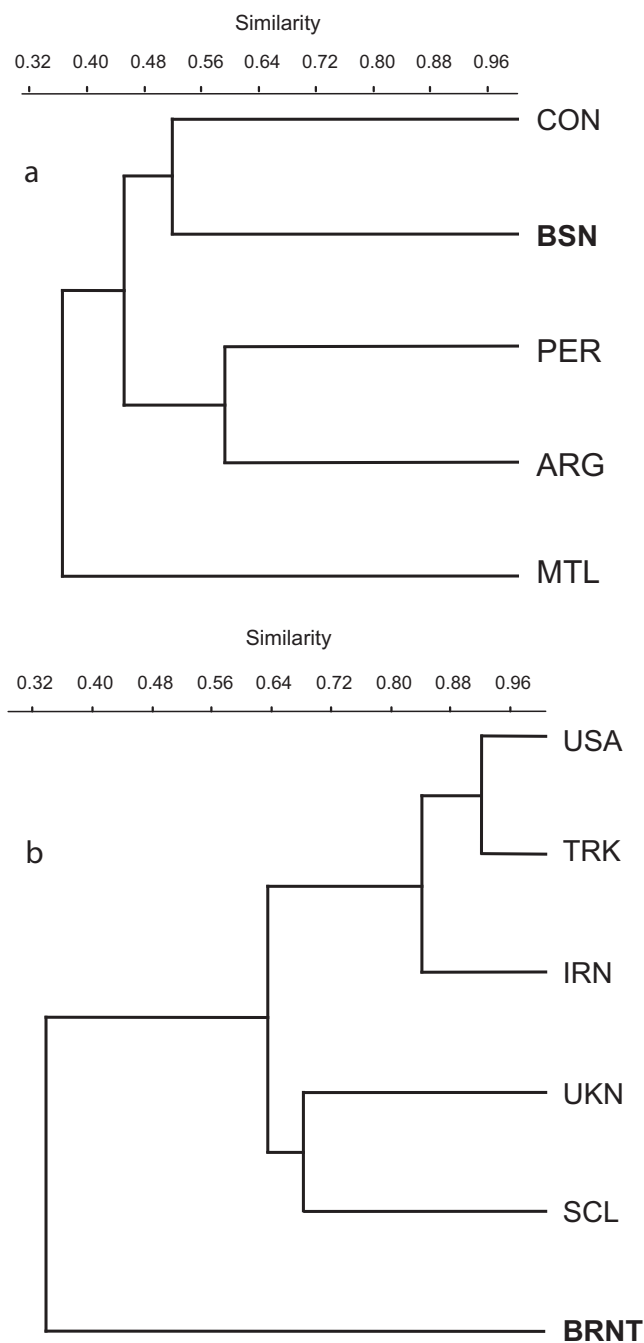


Fig. 1. UPGMA dendrogram of genetic relationship, among different genotypes of *Asparagus officinalis* (a) and *Pistacia vera* (b) using DICE coefficient. White Asparagus from Bassano del Grappa (BSN) and Green Pistachio from Bronte (BRNT) are bolded. Asparagus from Maremma Tosco-Laziale: MTL; Argenteuil: ARG; Connavers Colossal (London): CON; White Peruvian Asparagus: PER. Pistachios from USA: USA; Pistachios from Turkey: TRK; Pistachios from Iran: IRN; Unknown: UKN; Sicilia Perfetta: SCL.

of 3.6 alleles per locus from the analysis of 17 varieties with 14 SSR primer pairs. Zaloğlu et al. (2015) reported a value of 3.6 alleles per locus analyzing 47 polymorphic loci in 7 varieties. Table 3 also reports alleles identified for each analyzed marker and values of the main genetic parameters. Locus Ptms7 showed a band pattern more complex than expected, with more than two alleles for each sample analyzed. This result is in accordance with those previously reported in pistachio (Ahmad et al., 2003; Arabnezhad, Bahar, & Pour, 2011). Since pistachio is diploid ($2n = 2x = 30$), there is no

possibility of tetraploidy. Hence, amplification of more than two loci was explained by assuming the presence of two different copies of the same locus, probably as the result of a past event of duplication of the chromosome fragment containing the locus (Sánchez-Pérez, Ruiz, Dicenta, Egea, & Martínez-Gómez, 2005). The presence of two alleles in all the samples made us also assume that one of the two loci was subjected to a stronger selective pressure and therefore had no variability. These two alleles were excluded from the analysis, while the remaining alleles were considered in the count of polymorphic alleles. Primers Ptms42 and Ptms10 produced the highest number (i.e. 5) of alleles per locus. Six loci (Ptms3, Ptms14, Ptms42, Ptms41, Ptms7 and Ptms10) gave private alleles. Eight of them were identified in our PDO variety BRNT at four different loci. Among these, three loci (Ptms3, Ptms7 and Ptms10) provided the highest percentage of polymorphic fragments, allowing us to distinguish BRNT from the other varieties. The highest and lowest PIC values belonged to Ptms7 (0.66) and Ptms33 (0.46) loci, respectively. The average PIC value (0.57) indicated the presence of high genetic similarity among genotypes. However, the relatively low level of genetic diversity in the studied samples was expected because of the dioecious and outbreeding nature of the cultivated pistachio as well as the high level of heterozygosity due to the cross pollinating nature of the plant established during evolution and domestication (Kebour, Boutekrabi, & Mefti, 2012). It could also be the result of the high pressure for commercially important traits such as nut size and productivity (Ahmad et al., 2003). Table 3 also reports the percentage of polymorphic profiles (82%), the average number of alleles per locus (3.9) and the average PIC value (0.57). These values confirmed that SSR markers employed here are useful tools for genetic studies in pistachio. In spite of the low level of genetic diversity among our *P. vera* varieties, the similarity values of DNA profiles computed using Dice coefficient (Supplementary Table 5) and presented in the UPGMA dendrogram showed a clear separation of all the genotypes (Fig. 1b), with BRNT clustering apart from the other varieties. This cluster is supported by an high bootstrap value (95%). Our results are consistent with those by Hormaza, Dollo, and Polito (1994) and Zur, Heier, Blaas, and Fauhl-Hassek (2008), who reported that American pistachios are closely related to Iranian ones, while Turkish genotypes show more differences.

3.2. Geochemical signature

3.2.1. Asparagus

The total concentration of single elements in soils and turions from the five production farms of BSN are given in Supplementary Tables 6 and 7. In Supplementary Fig. 2 the chemical composition of soils and turions are compared graphically as farm geochemical profiles. Elements present in all samples with concentrations below LOD are not reported. A high degree of similarity was found among geochemical profiles of the soils collected in the different farms (overall coefficient of variation 13%). As shown in Supplementary Table 6 (and Supplementary Fig. 2), the chemical elements whose content in soils from different farms was characterized by a CV < 10% were 20; among them Bi, Ti, Na, Zr, Tl. On the other hand, a greater inhomogeneity was observed among the geochemical profiles of turions samples taken from the five different farms (Supplementary Table 7 and Fig. 2). Indeed, overall CV for turions was 36%, with only five elements showing a CV lower than 10% (P, Mg, B, K and S). While many authors dealt with element content in asparagus edible parts (Amaro-López, Cosano, Rojas, & Garcia-Gimeno, 1996; Makus, 1994), only few papers consider the element content in relation to geographical origin (Hopkins & Eisen, 1959). Moreover, most papers are restricted to several elements of known human nutritional value (Amaro-López et al., 1996) and none of them investigate the soil-plant

Table 3
Locus name, allele size, private alleles identified in bold and genetic parameters (Na: number of alleles; Ne: effective number of alleles; Ho: observed heterozygosity; He: expected heterozygosity; PIC: polymorphic information content; PD: discriminant power) for each analyzed locus of pistachio varieties.

Loci	Allele sizes	Na	Ne	Ho	He	PIC
PTMS3	153 , 159, 160, 162	4.00	2.70	0.95	0.63	0.63
PTMS7	185 , 191, 195, 200	4.00	2.97	0.55	0.66	0.66
PTMS9	147	1.00	1.00	0.00	0.00	0.00
PTMS10	174 , 163 , 153 , 158	5.00	2.04	0.20	0.51	0.51
PTMS14	123, 128	2.00	1.05	0.05	0.05	0.56
PTMS31	159, 142, 170	3.00	2.29	1.00	0.56	0.56
PTMS33	163, 174	2.00	1.88	0.75	0.47	0.47
PTMS41	225 , 230, 234, 236	4.00	2.80	0.70	0.64	0.64
PTMS42	149, 193, 203, 205, 215	5.00	2.56	0.95	0.61	0.61
PTMS40	221	1.00	1.00	0.00	0.00	0.00
PTMS45	193, 222	2.00	2.00	1.00	0.50	0.50
Mean		3.00	2.00	0.68	0.52	0.57

relationship (González, Armenta, & De La Guardia, 2011). According to González et al. (2011), a 'good' discriminant element requires large between-groups variability compared with within-groups variability. In our study, the elements P, Mg, B, K and S showed the lowest variability within production farms ($CV < 10\%$) and might be considered potential candidate as discriminant elements for authentication of BSN. All of them are essential elements for metabolic processes (Kabata-Pendias, 2010), and only few of them were considered by Mercurio et al. (2014) as good soil origin markers.

The Sr concentration in the investigated asparagus samples ranged from 1.27 and 2.37 mg/kg (dry weight), in agreement with the values reported by Swoboda et al. (2008) in asparagus samples from Slovakia, Hungary and Austria. The $^{87}\text{Sr}/^{86}\text{Sr}$ isotopic ratios of total and NH_4NO_3 extractable Sr in soils and total Sr in asparagus are given in Fig. 2. The Sr isotope ratio of asparagus ($n = 15$, range 0.7078–0.7096, 2σ 0.0006) is in good agreement with the Sr isotope ratio of bioavailable fraction in soils ($n = 15$, range 0.7064–0.7095, 2σ 0.0011). This was true in all five investigated farms (Fig. 2). By contrast, the total Sr isotope ratio in soils ($n = 15$, range 0.7265–0.7324, 2σ 0.0020) was significantly different compared to the Sr taken up by asparagus, showing much higher values than those in asparagus and consequently in the bioavailable fraction in soils. These results prove the necessity of assessing the Sr bioavailable fraction because the total Sr cannot be taken as proxy for provenance. This observation is in good agreement with previous studies (Brunner et al., 2010; Swoboda et al., 2008). In addition, similar to the observations by Swoboda et al. (2008), the variation of the Sr isotope composition of total Sr in soil was remarkably larger compared to the NH_4NO_3 extractable fraction. This result is

likely related to the geological heterogeneity of the sediments brought by Brenta river and filling the plain where soils formed, in comparison with the more homogeneous composition of the ground water of the whole area. The median ± 2 times the standard deviation of Sr isotope ratio of asparagus from Bassano del Grappa was 0.7087 ± 0.0006 . This range reflects the probability of 95% that a sample from the area can be identified as an asparagus sample from Bassano del Grappa. Values measured for Bassano del Grappa asparagus lied between the ranges measured by Swoboda et al. (2008) for Hungary (0.7069 ± 0.0011) and Marchfeld (0.7095 ± 0.0008) asparagus, with no overlapping within the total range for asparagus from Hungary but with a certain overlapping for asparagus from Austria. Peruvian asparagus, which is commonly a fraud product for BSN, could be clearly distinguished (0.7079 ± 0.0002), although only a limited number of samples were analyzed by Swoboda et al. (2008) to represent statistically relevant data. The range of the Sr isotope composition of total soil from different Bassano del Grappa asparagus farms was significantly higher than that found for soils from the Marchfeld region. This likely reflects the different elemental composition and geologic history of the two geographical areas. On the contrary, the ranges of Sr isotope composition of the NH_4NO_3 extracts were comparable. This explains the observed overlapping between the median ± 2 times the standard deviation of Sr isotope ratio of asparagus from Bassano del Grappa and Marchfeld area.

3.2.2. Pistachio

The concentration of single elements in soils and corresponding pistachio nuts (whole sample) from the five production farms of BRNT are given in Supplementary Tables 8 and 9. In Supplementary

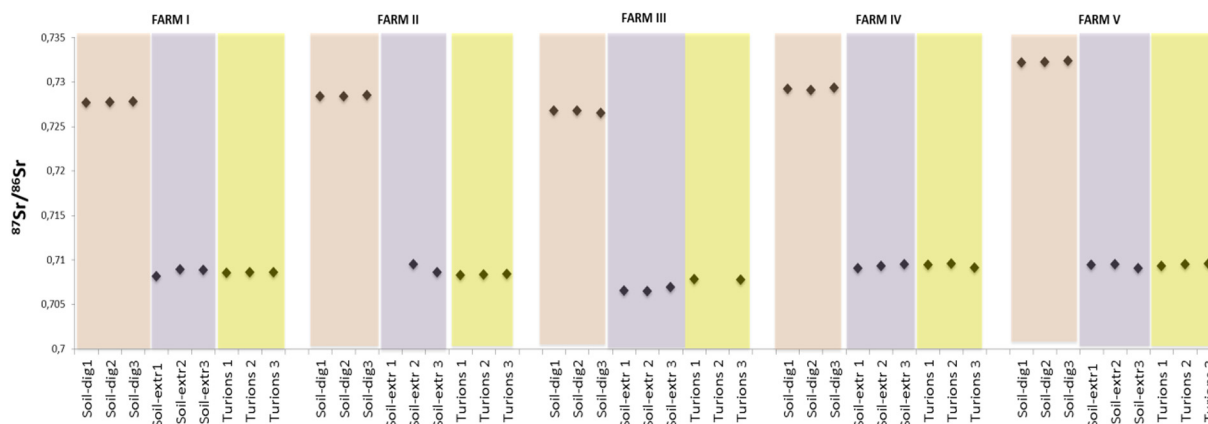


Fig. 2. Sr isotope composition in soils and asparagus turions collected from five different production farms of White Asparagus from Bassano del Grappa. Soil-dig = total Sr in soil; Soil-extr = NH_4NO_3 -extractable Sr in soil.

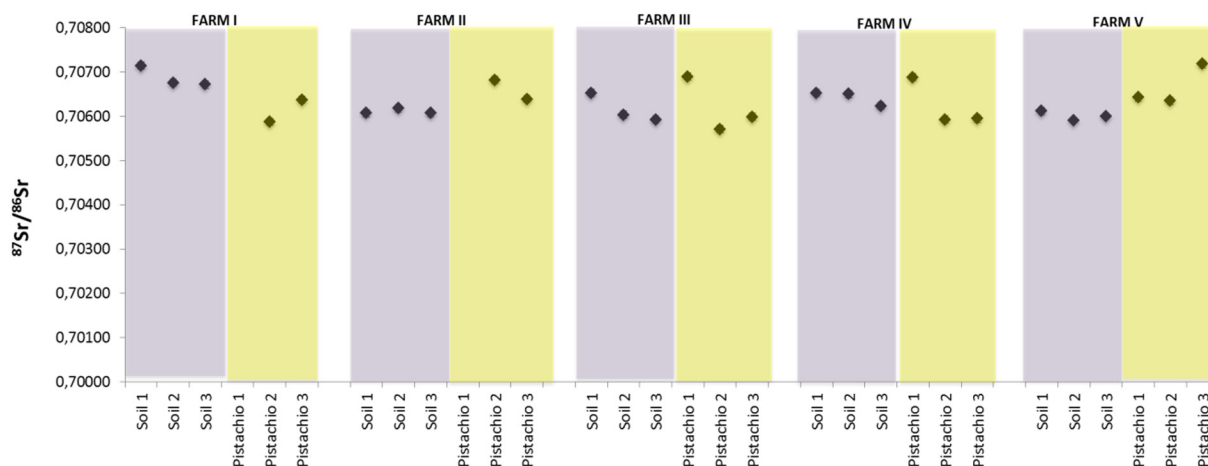


Fig. 3. Sr isotope composition in soils (NH_4NO_3 -extractable Sr) and nuts collected from five different production farms of Green Pistachio from Bronte.

Fig. 3 the chemical composition of soils and whole nuts are compared graphically as farm geochemical profiles. Elements present in all samples with concentrations below LOD are not reported. An overall 16% CV existed among geochemical profiles of pistachio soils. Chemical elements showing a CV < 10% were 12, among them Zn, Mn, Fe, V, Ti and Al. A slightly higher degree of similarity characterized the geochemical profiles of the whole nuts (overall CV 19%). Elements with CV < 10% were 10, among them P, Cr, Mg, K, Sc and S.

Differences in terms of element concentration were observed between peel and pulp nut components (Supplementary Tables 10 and 11 and Fig. 4). Some elements tended to accumulate more in the pulp than in the peel, such as Cu (pulp 15.3 ± 3.3 , peel 7.1 ± 1.4 mg/kg), Zn (pulp 26.7 ± 4.9 , peel 16.3 ± 1.8 mg/kg) and S (pulp 5004 ± 432 , peel 2513 ± 382 mg/kg). Some others accumulated more in the peel than in the pulp. Among them Mo (peel 0.97 ± 0.29 , pulp 0.40 ± 0.06 mg/kg), Ag (peel 15.1 ± 5.2 , pulp 5.5 ± 0.7 mg/kg), Fe (peel 124 ± 10 , pulp 84 ± 11 mg/kg) and Ca (peel 2005 ± 233 , pulp 1275 ± 229 mg/kg). The overall CV of pulp subsamples was similar to that of the whole nuts (21%). Only six elements showed a CV < 10% (P, Cr, Mg, B, K and S). A slightly higher dissimilarity was observed among geochemical profiles of the peel subsamples, showing an overall CV of 26%, with five elements having CV < 10% (Fe, P, Cr, K and Sc). According to Anderson and Smith (2005), Sr is the most discriminating element among the Iranian, Turkish and US (California) pistachios. Iranian pistachios had high Sr concentration (>20 $\mu\text{g/g}$); one set of samples from Turkey also had a high Sr (24.5 $\mu\text{g/g}$), whereas the other Turkey samples and all Californian samples had a Sr concentration near or below the detection limit (<1 $\mu\text{g/g}$). Our pistachios from Bronte had a very low Sr concentration (1.03–2.17 $\mu\text{g/g}$), significantly different from the Iranian and Turkish pistachios. The calcium/strontium ratio proposed by Kabata-Pendias (2010) for better understanding source and uptake of cations provided an additional discriminating power compared to Sr concentration. Indeed, the Iranian, Turkey and USA samples reported by Anderson and Smith (2005) showed a Ca/Sr ratio generally much lower than that of samples from Bronte (Iranian 42–129; Turkey 103–1038; USA 197–532; Bronte 622–1193). The comparison between data from Anderson and Smith (2005) and ours was also performed for the elements Mn, Fe, K, Ca and Mg having low variability between the production farms. Iranian, Turkey and USA pistachios had Mn concentration ranging between 6.7–17.5, 8.4–13.1 and 11.6–11.8 $\mu\text{g/g}$, respectively. Pistachios from Bronte displayed a Mn concentration of 6.7–8.7 $\mu\text{g/g}$, hence a value generally lower than all the other pistachios. Iron was generally lower in the

Iranian, Turkey and USA samples (overall range 24–55 $\mu\text{g/g}$) than in pistachios from Bronte (64.0–82.3 $\mu\text{g/g}$), suggesting a high discriminating power also of this element for Italian products. By contrast, there were not remarkable differences in the concentrations of macroelements such as K, Ca and Mg.

The Sr isotope composition in soils and pistachio nuts (whole sample) is given in Fig. 3. Since the isotopic fraction in plant proved to be highly correlated to the bioavailable fraction in soil, only the bioavailable Sr isotopic ratio was assessed. A direct correspondence between plant (nuts) and soil at each site was observed. The Sr isotope ratio for nuts ranged from 0.7057 to 0.7072 ($n = 15$; 2σ 0.0005), while for the soil bioavailable fraction the range was 0.7059–0.7071 ($n = 15$; 2σ 0.0004). This reinforces the evidence that the Sr in the NH_4NO_3 solution corresponds to the soil Sr source, which is taken up by pistachio plants. The geographic origin of pistachio from Iran, Turkey and California using stable isotope exploration ($\delta^{15}\text{N}\text{‰}$ and $\delta^{13}\text{C}\text{‰}$) was studied by Anderson and Smith (2006). Geographic regions were well separated, but seasonal differences were found to affect the discriminating power of isotopes. Sr isotope ratio has the advantage to vary only as a function of the age of the surface geology (Marchionni et al., 2013) and does not undergo significant fractionation during plant uptake due to biological processes (Baroni et al., 2011).

4. Conclusions

The results of this work show DNA fingerprints, geochemical profiling and Sr isotope ratio as promising tools for authentication studies of the high-quality Italian products “White Asparagus from Bassano del Grappa” and “Green Pistachio from Bronte” according to their geographical area of origin. We have confirmed the usefulness of microsatellites in the identification and discrimination of asparagus and pistachios varieties, even within a small sample with high genetic similarities caused by previous breeding activities. A molecular catalogue is therefore available that can help to compare the molecular patterns of PDO products investigated here with those of other samples. A representative soil and plant geochemical and Sr isotope composition database for authentic “White Asparagus from Bassano del Grappa” and “Green Pistachio from Bronte” was also produced. The high correlation between the Sr isotopic ratio in plant and in the bioavailable fraction of soil as assessed by 1 mol/L NH_4NO_3 suggests using bioavailable Sr instead of total Sr to distinguish products originating from different geographical areas. The use of trace elements may improve the discrimination power of Sr isotopic signature. In our study, Zn, P, Cr,

Mg, B and K, were identified as the less variable elements in plant samples, showing a potential for authentication of “White Asparagus from Bassano del Grappa”. For “Green Pistachio from Bronte” a similar or even better potential was observed for Mn, P, Cr, Mg, Ti, B, K, Sc and S. The validity of the multidisciplinary approach used in this study deserves future research involving a larger sample size, replicated growing seasons and more samples or data of similar agricultural products for comparisons.

Conflict of interest statement

All the authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2017.05.158>.

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