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Chapter 2

Use of Microsatellites to Study Agricultural Biodiversity and Food Traceability

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Additional information is available at the end of the chapter

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Abstract

Molecular markers are useful tools for measuring the genetic diversity among agricultural species. In plants, microsatellites are still the most used markers for germplasm characterization, conservation, and traceability purposes, while in the livestock sector, although having represented the standard for at least two decades, they are still used only for minor farm animal species. In this work, together with a review on the use of microsatellites in livestock, we also illustrate the use of these markers for the characterization of agricultural diversity and food traceability through two case studies: (i) the analysis of genetic diversity in ancient fruit tree cultivars of apple (*Malus* × *domestica* Borkh.), pear (*Pyrus communis* L.), sweet cherry (*Prunus avium* L.), and sour cherry (*Prunus cerasus* L.) from Northern Italy and (ii) the molecular authentication of wheat food chain. In the former case, a high genetic variability as well as the presence of different ploidy levels were detected, while in the latter microsatellite markers were shown to be useful for traceability and product authentication along the whole food chain. Overall, the presented evidence confirms the versatility of microsatellites as markers for both agrobiodiversity characterization and food traceability in cultivated plants and farm animals.

Keywords: agrobiodiversity, fruit tree, livestock, microsatellites, traceability

1. Introduction

Molecular characterization has various purposes in plant and animal genetic resource management, such as elucidating relationships between breeds/varieties, characterizing new genotypes, monitoring shifts in population genetic structure, and exploiting associations

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among traits and markers [1–3]. A well-recognizable molecular profile is a key factor for the protection and conservation of any genetic resource. Researchers can properly exploit plant and animal genetic resources if the materials are well characterized. Low assay cost, affordable hardware, throughput, convenience and ease of assay development, and automation are important factors when choosing DNA-based technology.

Microsatellites, or simple sequence repeats (SSRs), are polymorphic loci that derive from the repetition of short sequence motives of one to six base pairs in length. Microsatellites are among the most useful markers mainly because they are single locus co-dominant markers [4]. In the plant field, the availability of co-dominant markers is important in the analysis of hybrids. Furthermore, with respect to some categories of multi-locus markers (e.g. RAPD), microsatellites are characterized by higher reproducibility. Microsatellites have been largely used for DNA fingerprinting in several species, both wild and domesticated, although in recent years they have been increasingly replaced by single nucleotide polymorphisms (SNPs), particularly in the livestock genetic field [5].

Microsatellites have a series of characteristics that make them ideal to analyze plant genomes: (1) co-dominance that makes possible the analysis of hybrids of plant commercial varieties; (2) the amplified fragments are usually small in size (100 and 300 base pairs) resulting in positive PCR amplifications even in highly degraded DNA; (3) because of the polyploid nature of the genome of several important crop species, a small number of selected SSRs are able to provide a high discrimination capacity, as reported in the section on plant biodiversity; (4) SSRs are automatable, reproducible between different laboratories (provided that some precautions are taken to uniform allele size scoring, such as sharing of standard samples between labs), easily multiplexed, and easy to score; (5) SSRs usually show a high level of polymorphism and several alleles can be detected for a single SSR locus. This latter aspect makes SSRs extremely useful also for organisms with limited or no information on the genomic sequence because a small number of markers can be enough to clearly discriminate between a large number of samples. Compared to SNP markers, SSRs are less numerous in the genome but present a higher number of alleles per locus (SNPs are usually bi-allelic); therefore, a small number of SSRs can result in a discrimination capacity similar to that obtained with a large number of SNPs [6].

Biological diversity—or biodiversity—is a term used to describe the variety of life on Earth. It refers to the wide variety of ecosystems and living organisms: animals, plants, their habitats, and their genes [7]. While biodiversity can be considered as the foundation of life on Earth, it is crucial for the functioning of ecosystems providing us with products and services without which we could not live. Biodiversity is also the foundation of agriculture. In presence of biodiversity, men can select the genetic material available and gradually improve varieties and breeds. Preservation of biodiversity is, therefore, recognized worldwide as a topic of great concern both in wild and agricultural species, and with respect to the latter, recently, there has been an increasing interest in preserving local plant germplasms. Local varieties as breeds, landraces, ecotypes, and ancient varieties, which have been rarely subjected to breeding, are usually characterized by high genetic variability and genotypes. These germplasm resources are well adapted to both local needs and environmental conditions with good fitness for the anthropic and natural environments in which they have evolved [2, 3].

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Local germplasms, as ancient fruit tree cultivars or traditional livestock breeds, frequently face strong genetic erosion starting from the twentieth century. Genetic erosion refers to "the loss of individual genes and the loss of particular combinations of genes (i.e., gene complexes) such as those maintained in locally adapted landraces" [8]. Therefore, the term "genetic erosion" refers to both the loss of genes or alleles and the loss of varieties. Conservation of genetic materials, both using *in-situ* or *ex-situ* strategies, is expensive and needs infrastructure not always available. Because of these constraints, correct management of the different agricultural resources strongly relies on molecular information that can be generated using molecular markers.

Microsatellites have been used to evaluate crop germplasm and genetic diversity in several species, including rye [9], grape [10], sugarcane [11], rice [12], and olive [13]. Agrobiodiversity of fruit tree is of increasing concern mainly because repositories still remain a valuable source of allelic variation for many traits and can be exploited for breeding in the near future. Studying the genetic diversity of germplasm resources is not only significant for the protection of species, but also necessary for the development and utilization of germplasm resources for crop improvement and to face existing and future biotic and abiotic constraints with respect to sustainable production in the context of global environmental change [14]. Examples include apple landraces (*Malus* × *domestica* Borkh.) that represent the main fruit crop in temperate regions. It is not surprising that many studies concerning apple biodiversity were performed, both in Europe [3, 15] and in Asia [16].

In the livestock sector, microsatellite markers have been widely used for more than a decade for the characterization and conservation of livestock biodiversity and for the traceability of food products. In livestock, current genotyping standards are represented by standardized SNP panels that allow the characterization of tens or hundreds of thousand markers per sample [5]; but due to the low costs and to the possibility of in-house implementation of genotyping protocols, microsatellite markers still represent a useful resource to characterize livestock breeds in several developing countries, in which the access to SNP typing or other high throughput technologies can be difficult or too expensive [17–19]. Some years ago, FAO published recommendations for standardized sets of microsatellite loci to be used for studying diversity in the major livestock species [20] in order to make possible the comparison of results across different research projects [17–19].

The good resolution power and frequent occurrence of SSR within plant and farm animal genomes make this type of marker very useful in the food sector also. Food traceability is a milestone of EU food safety policy. The European Commission has agreed to establish a 'Reference Centre' to combat food fraud and ensure the "authenticity and integrity" of the EU food supply chain [21]. EU enhances and supports projects related to food safety as the recently approved project Food Integrity, comprising 38 participants from 18 European countries and one from China [22]. Furthermore, the addition of products without prior declaration on the label, besides representing fraud and adulteration, can also bring health risks, in particular to allergic consumers. In recent years, food traceability has become a topical field mainly to prevent fraud, adulteration, and sophistication. A database of food ingredient fraud issues was developed by [23]. The food products more subject to fraud are, in order, olive oil, milk, honey,

saffron, orange juice, coffee, apple juice and wine [23]. Most of the processed foods contain very low quality and quantity of DNA, because thermal or chemical treatments determine its degradation. Being microsatellites short repeats of 1–6 nucleotides, they are the most useful markers for DNA recovered from a treated food matrix and combined with *in vitro* DNA amplification (PCR); they allow the analysis of low amount of starting material. Indeed, as the amplified fragments are short, they can also be obtained from highly fragmented DNA.

Apart from adulteration and fraudulent procedures, traceability is of great importance to authenticate the quality and integrity of European high value food. A biochemical and genetic approach using microsatellites was useful to discriminate the geographical origin of Italian red wines obtained from Campania region native red grape varieties [10]. Several DNA-based analytical methods have been developed and applied to identify and quantify cereal species and to fingerprint and identify varieties to verify their authenticity [24, 25] developed a microsatellite-based method to verify the presence of the four required durum wheat cultivars in "Altamura" bread, and which are cultivated in a restricted geographical area close to the town of Altamura. Altamura bread, according to its European mark of protected designation of origin (PDO), at least 80% of the total flour used for Altamura bread preparation must derive from the aforementioned traditional durum wheat cultivars used alone or in combination.

In livestock, breed discrimination is useful to detect fraud and to protect and valorize typical productions. Girgentana goat (*Capra hircus* L.), an ancient breed reared in a restricted area of Sicily (southern Italy) and its dairy products were traced by the use of a specific panel of microsatellites [26]. The potential of microsatellites for determining the origin of meat products was also important for traceability of nine Portuguese breeds with PDO products [27], while four Italian cattle breeds were identified by microsatellite markers using different statistical approaches to certify the origin of their typical products [28].

The aim of this paper is to highlight the utility of microsatellite markers to study both genetic diversity of domesticated plants and animals and food traceability. Some examples have been provided in the following sections.

2. Agrobiodiversity: the case study of fruit tree species in Northern Italy

Researchers [29] reported that 940 crop plants species are threatened globally and genetic erosion was described in different crop groups, such as cereals and grasses or fruits and nuts [8]. When a species, or the diversity within a species, is lost, the genes important for improving crops are also lost. Preserving local germplasms, landraces, ecotypes, and ancient varieties, means preserving not only our history and culture (such populations represented for centuries an important source of food for local people) but also an extremely useful reserve of genes usable to introduce new characteristics in modern varieties. In order to preserve the local germplasm of ancient fruit tree cultivars, a systematic recovering and characterization of the traditional material of the western part of the Emilia Romagna region was carried out. In this area the tradition of pear (*Pyrus communis* L.), apple (*M.* × *domestica* Borkh.), sweet and sour

cherry (*Prunus avium* L. and *Prunus cerasus* L.) cultivation is well established. Seventeen accessions belonging to ancient varieties of sweet cherry, 7 of sour cherry, 20 of apple, and 32 of pear have been sampled (**Tables 1–3**), and an example of some accessions is shown in **Figures 1–3**.

Species	Cultivar name—accessions	Origin	Microsatellite markers and size of the amplicons (bp)[a]				
			EMPA 015	EMPA 018	UDP 97/402	UCDCH 17	UCDCH 31
P. avium	Selvaticona di Magnano	PC	253/219	101/92	140/118	187/185	141
	Mora piacentina	PC	253/219	101/92	140/118	187/185	141
	Picaion acc.1	PC	238	92	118	185	141/130
	Picaion acc.2	PC	238	92	118	185	141/130
	Smirne	PC	253/219	92	118	185/187	130/123
	Pavesi acc. A	PC	253/249	101/96	118	197/183	128/125
	Pavesi acc. C1	PC	253/249	101/96	118	197/183	128/125
	Pavesi acc. C2	PC	253/249	101/96	118	197/183	128/125
	Mori	PC	221/219	92	118	187/185	141/132
	Raffaella	PC	238	101	118	185	141
	Flamengo acc.A	PC	238	92	118	185	141/130
	Flamengo acc.B	PC	238	92	118	185	141/130
	Flamengo acc.C	PC	238	92	118	185	141/130
	Duroncina della goccia	PC	221/219	92	118	197/185	141
	Prima	PC	253/249	96/92	118/114	185	145/130
	Mora di Vignola	PC	221/219	101	126/114	197/185	128/123
	Giambella	PR	251/219	96/92	126/118	187/185	128/123
P. cerasus	Marasca dal peduncolo lungo	PC	249/247/221/195	96	126/112	185/179/155	130/113
	Marasca Villanova	PC	249/238/225/195	92	126/112	195/185/175/169	141/130/123
	Marinone I acc. A	PC	251/225/195	105/92	140/126/114/112	195/185/175/167	141/130/123
	Marinone II acc. A	PC	225/221/195	92	126/118/112	193/185/175/167	141/130/123
	Marinone II acc. C	PC	251/225/195	105/92	140/126/114/112	195/185/175/167	141/130/123
	Amarena Piacentina	PC	249/247/225/195	92	126/112	193/185/175/167	141/130/123
P. × gondouini	Visciola	PC	225/211/195	105/99	126/122/118/110	197/191/187/171	130/123

Microsatellite profiles are reported for each cherry cultivar. Columns from left to right indicate: (i) the species, (ii) the local name, (iii) the accession, (iv) the origin of the accession, Piacenza (PC) or Parma (PR), and (v) the size of the PCR amplified product.

Table 1. Molecular characterization of cherry varieties.

In addition, DNA analysis was carried out using SSR markers in order to obtain a preliminary fingerprint of each sampled accession and to eventually solve controversies of synonyms (different names for a single genotype) and homonyms (a single name for different genotypes). Genetic variability of the samples was evaluated using five SSR markers for each species: EMPA015, EMPA018 [30], UDP97-402 [31], UCDCH17, and UCDCH31 [32] for sweet and sour cherry; GD96, GD100 and GD162 [33] for apple; KA14, KA16 and BGT23b [34] for pear; GD142, GD147 [33] for both apple and pear (**Tables 1–3**). DNA extraction from young leaves and PCR amplification have been carried out as previously reported [35]. Analysis of PCR products was performed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystem—Thermofisher). Expected heterozygosity and discrimination power were calculated as described in [35], while observed heterozygosity was calculated as the ratio between heterozygous genotypes over the total number of the samples (*Nh/Ntot*). Results are shown in **Table 4**.

Species	Cultivar name—accessions	Origin	Microsatellite markers and size of the amplicons (bp)[a]				
			GD96	**GD100**	**GD147**	**GD162**	**GD142**
	Ruggine acc. I	PC	178/172	230/222	150/129	219/210	138/132
	Ruggine acc. II	PC	178/172	230/222	150/129	219/210	138/132
	Fior d'acacia	PC	180/172	224	146/129	230	140/138
M. × domestica	Verdone	PC	176/172	234/224	148/135/129	228/210	144/126
	Rustaio	PC	178/174	224	135/129	228/210	131
	Rustajò	PC	176/174/168	226/224/222	135	230/228/210	154/144/126
	Restajo	PC	174/150	226/219	142/135	210	144/138
	Carraia acc. I	PC	170/168/150	234/230/224	148/146/135	234/230/222/210	140/138
	Carraia acc. II	PC	174/150	232/230	148/135	228/222/210	140/138
	Salame	PC	172	224	148/146	222/210	144
	Rosa	PR	178/174/168	230/226/224	148/137/135	230/219/210	148/144/138
	Mela Rosa	PR	187/185/164	NA	139	226/210	144/132/126
	Bella di Maggio	PR	174	226	127	219/210	144/140
	Cavic	PR	178/172	224	148/135	230/228	144
	Seriana	PR	176/170/168	226	148/137/129	230/226/210	148/144/126
	Melo Olio	PR	194/176	222	142/137	234/228	152/140
	Cucumero	PR	172	224	148	222/210	144
	Ghiacciata	PR	176/172	224	135/129	210	132/126
	Musona	PR	178/172	234/224	135/129	230/228	144/142
	Codaro	PR	172/166	224/222	135	228/210	152/126

Microsatellite profiles are reported for each apple cultivar. Columns from left to right indicate: (i) species, (ii) the local name, (iii) the accession, (iv) the origin of the accession, Piacenza (PC) or Parma (PR), and (v) the size of the PCR amplified product.
a: NA means null allele and it refers to the absence of the amplification product in a specific sample.

Table 2. Molecular characterization of apple varieties.

Species	Cultivar name—Accessions	Origin	Microsatellite markers and size of the amplicons (bp)[a]				
			BGT23b	KA16	GD147	KA14	GD 142
P. communis							
	Lauro acc. I	PC	213/195	129	132/120	194/176	166/158
	Lauro acc. II	PC	213/195	129	132/120	194/176	166/158
	Limone acc. I	PC	209	129/115	118	184/178	156/152
	Limone acc. II	PC	209	129/115	118	184/178	156/152
	Limone acc. III	PC	209	129/115	118	184/178	156/152
	Rossetto	PC	193/191	147	118	184	158/148
	Macagn	PC	213/195	145/129	128/118	188	174/160
	Sburdacen	PC	191	129/123	128/118	184	182/180/176/174
	Sburdacion acc. I	PC	NA	129/123	122/118	222/190/184	178/160/156
	Sburdacion acc. II	PC	NA	129/123	120/118	190/184	178/160/156
	Coda torta acc. I	PC	505/488	147/129	124/118	194/176	174/172/146
	Coda torta acc. II	PC	505/488	147/129	124/118	194/176	174/172/146
	Nigrò	PC	NA	129/125	134/128	194/184/166	174/164/146
	Colar	PC	213	147/129	126/120/118	194/186/184	174/160/146
	Bianchetto	PC	543/509	129	124/120	184/180	180/158
	Nobile acc. I	PR	213/195	129	132/120	194/176	166/158
	Nobile acc. II	PR	213/195	129	132/120	194/176	166/158
	Butirra Polesine	PR	235/231	145/129	138/118	190/176	166
	San Giovanni	PR	191	129/125	125/118	194/184	168/160
	San Germano	PR	209/203	147/131	118	186	160/158
	San Pietro	PR	209	139/129	122/118	184/186	164/136
	Cipolla	PR	209/193	145/129/123	132/118	186/184/176	166/150/136
	Bergamotto	PR	203	131/129	122/118	184	160/156
	Nigrer	PR	179	131/129	126/118	184	164/148
	Carlet	PR	179	139/129	128/118	194/186	148/146
	Moscato	PR	209	129	124/118	184	170/160
	Spadone	PR	179	151/115	136/126/120	184/176	178/174/164
	Ingurien	PR	169	129/125	118	NA	164/148
	Svirgolato	PR	223/213	129/119	126/120	184/176	166/158
	Colar	PR	213	147/129	120/118	194/186/184	174/160/146
	Pavia	PR	209/195	145/131/123	128/118	186	158/148
	Ducale	PR	209/195	129/125	118	184/176	164/136
	Butirra Ruggina	PR	195	129/115	128/120/118	NA	174/166

Microsatellite profiles are reported for each pear cultivar. Columns from left to right indicate: (i) the species, (ii) the local name, (iii) the accession, (iv) the origin of the accession, Piacenza (PC) or Parma (PR) and (v) the size of the PCR amplified product.
a: NA means null allele and it refers to the absence of the amplification product in a specific sample.

Table 3. Molecular characterization of pear varieties.

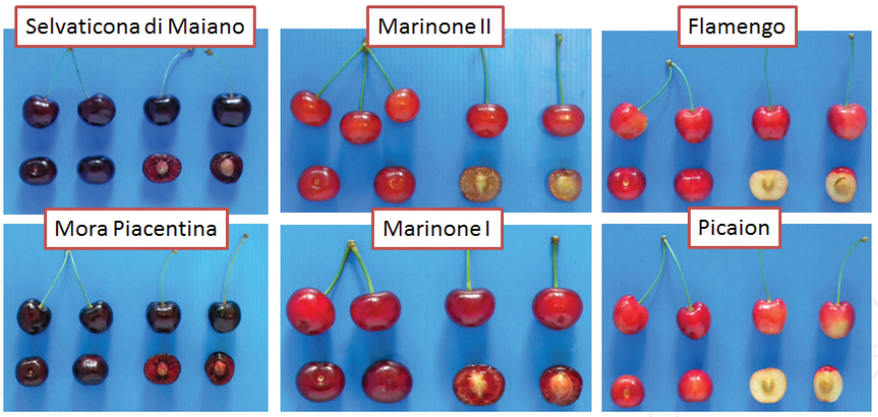


Figure 1. Fruit morphology of some ancient varieties of sweet cherry.

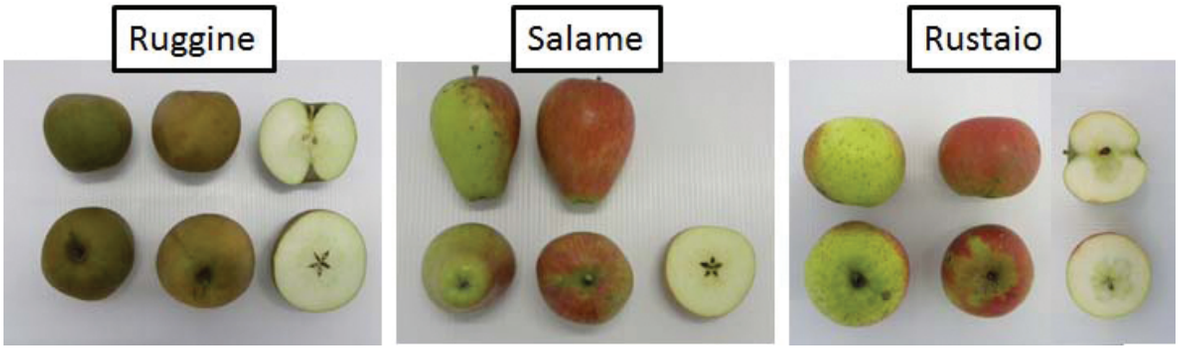


Figure 2. Fruit morphology of some ancient varieties of apple.

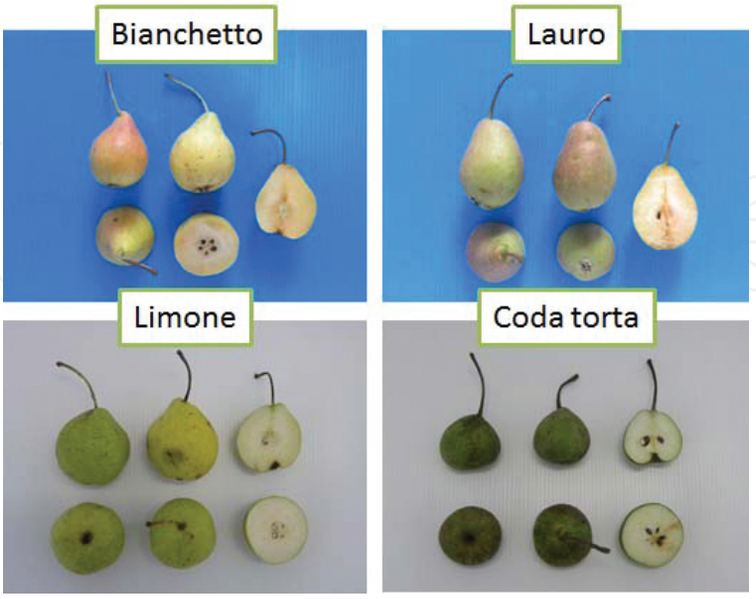


Figure 3. Fruit morphology of some ancient varieties of pear.

Species	Markers	No. of alleles	Expected heterozygosity	Discrimination power
Prunus avium Prunus cerasus	EMPA015	10	0.881	0.861
	EMPA018	5	0.668	0.743
	UDP97/402	7	0.753	0.712
	UCDCH 17	13	0.815	0.712
	UCDCH 31	8	0.775	0.854
	Average	8.6	0.778	0.776
Malus domestica	GD96	13	0.868	0.905
	GD100	8	0.788	0.867
	GD147	9	0.818	0.920
	GD162	7	0.779	0.905
	GD142	10	0.839	0.915
	Average	9.4	0.818	0.902
Pyrus communis	BGT23b	16	0.888	0.915
	KA16	10	0.723	0.898
	GD147	11	0.778	0.894
	KA14	11	0.807	0.907
	GD 142	18	0.921	0.935
	Average	13.2	0.823	0.909

Table 4. Statistical analysis of the microsatellite markers.

Sweet cherry (*P. avium* L., Rosaceae, 2n = 16) is widely cultivated in temperate regions because of the edible fruit. Likely originated in the area of the Caspian and Black Seas, sweet cherry cultivation spread through Europe during the Roman Empire. The spread of sweet cherry cultivation across Western Europe, initially, was probably the consequence of the domestication of wild individuals that were well adapted to each area of cultivation [36]. Sour cherry (*P. cerasus* L.), originated in the same area as sweet cherry, is an allotetraploid (2n = 4x = 32), that might have arisen from a cross between *P. avium* and *P. fruticosa* Pall. Finally, duke cherry is an allotetraploid species originated subsequently from natural hybridization of sweet and sour cherry. More precisely, it originated from the fertilization of sour cherry by unreduced gametes of sweet cherry [37]. In the Northern Italy, the province of Piacenza has a long history of cherry cultivation and several local varieties have been selected after centuries of use.

The microsatellite analysis revealed a different scenario regarding sour, sweet, and duke cherry accessions (**Table 1**). The number of different alleles detected is reported in **Table 4**, the average number of alleles is 8.6, the lowest number of alleles is 5 for EMPA018, and the highest is 13 for UCDCH17. The expected heterozygosity ranged between 0.668 (EMPA018) and 0.881 (EMPA015) (**Table 4**). Based on the frequencies of the different alleles, the probability to obtain a particular genotype by chance was evaluated. Despite the use of a small set of markers, we

had very low probability values ranging from 10^{-6} to 10^{-9} for diploid varieties and 10^{-12} to 10^{-19} for polyploid varieties. The smallest value was obtained for the variety Visciola, this is likely a consequence of its hybrid nature (data not shown). These results confirm what had already been shown in the case of *Vitis vinifera* L., in which a small set (six) of SSR markers was able to successfully discriminate between varieties and to identify the starting material used to produce the must [38].

The three accessions belonging to the sweet cherry cultivar Pavesi have the same molecular profile, indicating that they derived from a unique mother plant. The same could be noted for the accessions of the cultivars Flamengo and Picaion. Two cultivars, namely Mora piacentina and Selvaticona di Magnano, have the same SSR profile. This situation, with all the caution due to the small number of markers used, could be a typical case of synonymy and the two names could be two different local designations for plants anciently derived from the same genetic material and then vegetatively propagated. Concerning sour cherry, the cultivars Marasca and Marasca di Villanova, despite a similar name, had a different genetic profile suggesting that they belong to two different cultivars and they are a case of homonymy. A similar situation was found within the three accessions belonging to Marinone: Marinone I acc. A and Marinone II acc. C had the same profile while Marinone II acc. A was clearly different. Very likely, the first two accessions derived from the same mother plant while the last one had a different origin resulting in a case of homonymy. Comparing the profiles of the different markers in sweet and sour cherries, sweet cherries had a simple profile with the different loci having just one (homozygous) or two (heterozygous) alleles. On the contrary, sour cherries had a more complicated allelic combination and it was common to find, for each marker, the presence of single loci having three or four different alleles. This high number of alleles at the level of the single locus could be a consequence of local duplications of genomic regions or, more likely, of different ploidy levels. In this respect it is reported that sweet cherries are diploids while sour cherries are polyploids (such as tetraploids).

To have a better representation of the relationships among the different accessions analysis, principal component analysis (PCA) was carried out (**Figure 4**). Two clearly separated groups could be defined: the first including sweet cherry accessions and the second including sour cherry accessions. Among the sour cherry accessions, the one being closest to the sweet cherry group was the variety Visciola. The term Visciola is used to refer to a variety of duke cherry that originated by natural hybridization between a sweet and a sour cherry variety. This hybrid nature can determine the intermediate position of this sample between the sweet and sour cherry groups.

Apple and pear are among the most economically important fruit tree crops of the temperate zones. According to the FAO report on the state of world's plant genetic resources for food and agriculture, at least 97,500 apple accessions and 1140 pear accessions are present in worldwide *ex-situ* collections [35]. Moreover, apple is the most common fruit crop of temperate areas. The wild Central Asian species *Malus sieversii* (Ledeb) M. Roem was identified as the main contributor to the genome of the cultivated apple [39] but, recently, it has been demonstrated that multiple species have contributed to the genetic makeup of domesticated apples [40]. Concerning pear, there are two centers of domestication and primary origin, one located in

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China and the second in the area stretching from Asia Minor to the Middle East, in the Caucasus Mountains. Also, a third secondary center is located in Central Asia [41].

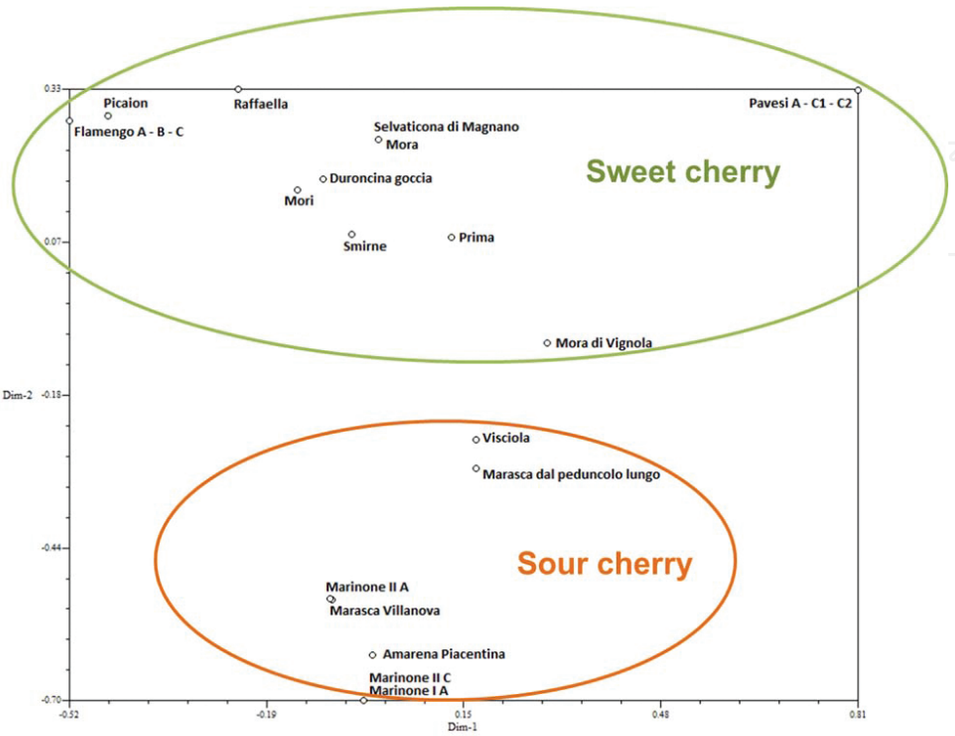


Figure 4. Principal component analysis of the cherry varieties based on the SSR profiles. The PCA based on SSR results, clearly evidence the differences between the groups of sweet and sour cherries. It is interesting to note that, in the sour cherry group, the accession of Visciola (*P.* × *gondouini*) is the closest to the sweet cherry group. This can be a consequence of the hybrid nature of the species, likely a cross between *P. avium* and *P. cerasus*.

The provinces of Parma and Piacenza have a long tradition of apple and pear cultivation, and a wide diversity of cultivars, well adapted to the local environmental conditions, was grown in this area since ancient times. In apple, as in cherry, the number of alleles highlighted at a single locus in the different samples, ranged from one to four supporting the presence of different ploidy levels (**Table 2**).

Along with cultivars having just one or two alleles at each locus, such as Ruggine, Fior d'Acacia, and Salame, there were some cultivars with three alleles per locus, such as Seriana, Rosa, and Rustajò. These results supported diploidy and triploidy as the main ploidy levels in local apple germplasm and they agree with what is generally reported in literature concerning apple varieties: most of the apples grown commercially are diploid (2n), although there are many triploid varieties (3n) [42]. The presence of four different alleles, in a single locus, was a rare event and it was found just in a single case (marker GD162, first accession of variety Carraia). While the high number of currently cultivated varieties is diploid or triploid, the presence of tetraploid forms not cultivated but useful for breeding was reported too [43]. It cannot be excluded that after centuries of vegetative propagation, some tetraploid forms could be originated and unintentionally cultivated. The number of different alleles detected by the five

SSRs is reported in **Table 4**; the average value was 9.4, the lowest value was 7 for GD162, while the highest was 13 for GD96. The expected heterozygosity ranged between 0.779 (GD162) and 0.868 (GD96). The probabilities to obtain a particular genotype by chance were very low ranging from 10^{-7} to 10^{-10} for diploid varieties and 10^{-9} to 10^{-16} for polyploid varieties (data not shown). Also in apple there were cases of homonymy: i) the two accessions of the variety Carraia were clearly different at the genetic level and, very likely, they originated from different mother plants, ii) despite very similar denominations, the varieties Rustaio, Rustajò, and Restajo had different genetic profiles, so they can be effectively considered as different cultivated varieties.

In pear, as in the previous species, it was possible to detect the presence of loci with more than two alleles (**Table 3**). As for apple and cherry, this evidence suggested the presence of different ploidy levels in the local pear germplasm. Based on the results, diploid varieties were the most diffused followed by triploids. Tetraploidy was rarer, being evidenced just a single time in cultivar Sburdacen with marker GD142. The presence of varieties of pear characterized by different ploidy level, diploids, triploids, and tetraploids was already reported in the literature [44]. The number of different alleles detected by the five SSRs is reported (**Table 4**): the average value was 13.2, the highest among the three species, while the average expected heterozygosity and discrimination power were similar to the values of apple. The lowest allele number was 10 for KA16 while the highest was 19 for GD142. The expected heterozygosity ranged between 0.723 (KA16) and 0.921 (GD142) (**Table 4**). Based on the frequencies of the different alleles, we evaluated the probability to obtain any particular genotype. Once again, the probability values were very low ranging from 10^{-8} to 10^{-11} for diploid varieties and 10^{-10} to 10^{-14} for polyploid varieties (data not shown).

A clear case of synonymy was present concerning the two names Lauro and Nobile. By comparing the genetic profiles, it was possible to see that the different accessions had the same alleles showing that they derived from a common mother ancestor. In this case, the two names are linked to the different provinces, with the name Lauro diffused in the province of Piacenza and the name Nobile in the province of Parma. The three accessions belonging to the variety Limone had the same genetic profile, confirming that they derived from the same mother plant. The same was found for the two accessions of the variety Coda torta. On the contrary, the two accessions of the variety Sburdacion were slightly different, being a case of homonymy. Probably these accessions derived from a common ancestor that encountered some genetic changes (as somatic mutations). Despite the similar names, varieties Nigrò and Nigrer and varieties Butirra Polesine and Butirra ruggina had different genetic profiles and they can be considered as different cultivars. With respect to cherry and apple, in pear a higher frequency of null alleles, i.e. five cases in pear against one case in apple and none in cherry, was observed. To verify this, the amplifications were replicated at least five independent times and the amplicons were always absent. The two accessions of the variety Sburdacion with the marker BGT23b were both characterized by the absence of amplification, supporting close genetic relationships.

This study confirmed the utility of microsatellite markers for biodiversity evaluation and for all conservation actions that can follow the preliminary analysis of genetic variability. Despite

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the use of a small number of markers, several cases were highlighted: (1) synonymy in sweet cherry (Mora piacentina and Selvaticona di Magnano) and pear (Lauro and Nobile); (2) homonymy inside the Marinone and Marasca (sour cherry), Carraia (apple), and Sburdacion (pear); (3) accessions belonging to the same cultivated variety characterized by high genetic uniformity as a consequence of the derivation from a common ancestor; (4) high biodiversity in the old local germplasm; (5) different levels of ploidy: diploidy in sweet cherry, apple, and pear; triploidy in apple and pear; tetraploidy, rare in apple and pear, and mainly present in sour cherry.

3. Microsatellite markers in the livestock sector

For more than a decade, microsatellites have been one of the most popular types of markers used in the livestock sector for various purposes [45], e.g., the characterization and conservation of diversity [46, 47], the reconstruction of the post-domestication evolutionary history of farm animals [48, 49], parentage testing [50], mapping of quantitative trait loci (QTL) [51, 52] or other causative mutations [53], and traceability of food products [26, 54, 55]. The average number of microsatellite loci used in livestock research varied between 15 and 30 [45], even if a lower number of highly informative loci have been adopted for specific purposes. For example, the International Society for Animal Genetics has established that panels of as few as 12 microsatellite loci have enough resolution for the routine identification of individuals and parentage testing in cattle and horse [56].

A large number of national and international projects aiming at the description of farm animal species diversity have relied on the use of microsatellites. These markers have been used to estimate diversity (both within and between breeds) and genetic admixture even among closely related breeds, usually by means of clustering approaches, principal coordinate analysis, or phylogenetic inference [46]. Comprehensive microsatellite-based studies of livestock diversity have been carried out in European chicken [57], goats from Europe and the middle East [58], Eurasian sheep [59], and African cattle [48], just to mention a few.

One of the major drawbacks of microsatellite genotyping is that the use of different PCR-amplification protocols and genotyping techniques may result in different allele size scoring at the same locus in different labs or experiments, thus hampering the possibility to combine microsatellite genotypes obtained from different projects. To circumvent this, the use of the same set of markers (or at least of a common subset of markers) and genotyping of standard samples across projects has been recommended [60]. In particular, to promote the use of common marker panels, the ISAG-FAO Advisory Group on Animal Genetic Diversity has published guidelines and ranked lists of microsatellite loci to be used for studying diversity in major livestock species [20]. Using these markers in order of ranking should maximize the overlap and increase the possibility of merging data from different investigations.

Concerning allele size standardization through the inclusion of standard samples, for some species (e.g. sheep and goats) the standards adopted in the course of large-scale projects have also been shared with research initiatives in different continents to permit merging of the

results. This is the case of the European project Econogene [61] whose sheep and goat standard samples have been made available to other large-scale investigations in Africa and Asia. Acknowledging the usefulness of a joint analysis of different datasets to obtain a global view of livestock diversity, as in the case of the meta-analysis performed by the EU project GlobalDiv [62, 63], a number of statistical methods have been devised that allow merging and analyzing datasets even when they have only a few breeds and/or markers in common. The method developed by [64], for example, estimates population genetics parameters (e.g., heterozygosity, allelic richness, and admixture) by means of a double regression approach and has been successfully applied to the meta-analysis of microsatellite data of cattle populations from Europe, Africa, and Asia [45]. [65], instead, have devised a method based on iterative regression to infer the contribution given by each missing allele/breed combination, which allows calculating genetic distances also on merged datasets with missing information (see [45] and figures therein).

Gaining a global view on the worldwide patterns of diversity of livestock genetic resources may allow to highlight (i) the presence of gaps, i.e., areas in which livestock characterization is incomplete or lacking, (ii) local diversity hotspots which may deserve particular attention or conservation efforts, (iii) geographical trends of clonal variation or discontinuities that can shed further light on the evolutionary history and post-domestication migration routes of farm animal species.

In livestock, current genotyping standards are represented by standardized SNP panels that allow the characterization of tens or hundreds of thousand markers per sample at the same time and at a reasonable cost. Commercial SNP chips at varying levels of marker density are already available for the major livestock species, e.g. for cattle at medium density [66] and high density [67], for sheep and goats at medium density [68, 69]. Being highly standardized, SNP panels do not suffer from allele scoring differences and thus permit an immediate comparison and merging of data produced in different labs [70]. A comparative evaluation of the effectiveness of microsatellites vs. SNP markers for individual identification and parentage assessment has recently shown that 2–3 SNPs per microsatellite were necessary to obtain a comparable exclusion power value in a highly consanguineous Angus cattle herd [71]. Therefore, in a similar context the use of, e.g. 50K SNP chip panel might be equivalent to typing of 16–25K microsatellite loci. Nevertheless, due to the low costs and to the possibility of in-house implementation of genotyping protocols, microsatellite markers still represent a useful resource, e.g. to characterize livestock breeds in several developing countries [72, 73], in which the access to SNP typing or other high throughput technologies can be difficult or just too expensive, or to set priorities for conservation at the local or regional scale [74, 75].

4. Traceability of food

Food traceability is of primary importance to avoid fraudulent procedures and to authenticate the origin of particular products. Dishonest producers may substitute, partially or totally, some food products with others less expensive to increase the profit. For this reason, certifying the

origin and composition of a certain food is becoming more and more important [76–78]. Molecular analysis is one of the most recently developed methods to trace food products. Molecular traceability is useful to distinguish traditional varieties with specific high quality traits and to protect the PDO and "Protected Geographical Indication" (PGI) marks. Italian products represent 20% of protected food in Europe and the certified "made in Italy" is important for Italian product exportation. DNA is present in every food product and its analysis makes possible to recover a lot of information about the identity of the ingredients in foods and feed. It is often reported that DNA is relatively more resistant than other classes of biological molecules (e.g. proteins) to the degradation caused by food processing. Despite this, as a consequence of processes such as cooking, fermentation etc., degradation of DNA occurs anyway and, generally, the stronger the treatment the shorter the DNA fragments become. Thus, the possibility to analyze small DNA fragments is very important for traceability purposes.

An additional problem, when working with plant-derived products is that along with the DNA, a high number of different inhibitors of polymerase reactions can be recovered from a food matrix. Plants are very rich in carbohydrates and polyphenols, which tend to be co-extracted with the DNA. Their presence can prevent the activity of polymerases hindering the analysis of DNA by PCR reaction. Different commercial kits or customized protocols can be considered to tackle this problem and usually DNA extracted from most food matrixes can be analyzed using molecular tools. Molecular markers make it possible to discriminate, not only the species from which the food is originated, but also the variety (cultivar) or population of origin [79–81]. Among the different classes of markers, some are more suitable than others for traceability purposes. Recently, the two main classes of markers that have been adopted are SNPs and microsatellites. While SNPs are becoming the most used markers for animal-based product analysis and identification, microsatellites are still the election markers for genetic traceability of plant-based products.

The final goal of DNA analysis in the agro-food sector is the comparison of the molecular profile of a sample with a reference profile to evidence the presence of congruencies or discrepancies. When the SSR profile of the sample is congruent with what is expected (similar to the reference profile), the two profiles are matching and it is possible to speculate that the sample under investigation has the same origin as the reference. However, in any final conclusion that is reached in certain cases, it is also important to evaluate the probabilities that the two profiles are identical because they derive from the same genetic material and not just by chance. This requires deep knowledge of the genetic base of the species under investigation and the probability level to obtain the same marker profile, using a set of SSRs, in two independent samples just by chance. This is very important for plant species in which it is often not enough to detect the presence of a particular species in a processed product. For several plant-derived products, as for extra virgin olive oil and wine, the final price on the market is highly dependent on the cultivated variety of the species that has been used as raw material. In this situation, a possible fraud could be represented by the substitution of a declared cultivar with another one with a smaller commercial value but with similar organoleptic properties (different cultivars of olive or of grapevine).

Sample	Xgwm 46	Xgwm 408	WMS 376	Xgwm 459	Xgwm 577	WMS5	WMS 120
Type A seed	180	97	142/96	129/113	127/150	167/165	160/129
Type B seed	180	97	142/96	113	127	154/152	160
Type C seed	180	97	142/96	117	127	154/152	129
Type A treated seed	180	97	142/96	117	127	154/152	129
Type B treated seed	180	97	142/96	129/113	127/250	167/165	160/129
Type C treated seed	180	97	142/96	113	127	154/152	160
Type A flour	180	97	142/96	117	127	154/152	129
Type B flour	180	97	142/96	117	127/150	154/152	129
Type C flour	180	97	142/96	113	127	154/152	160
Type A Pasta	180	97	142/96	113	127	154/152	160/129
Type B Pasta	180	97	142/96	129/113	127/150	167/165	160/129
Type C Pasta	180	97	142/96	113	127	154/152	160

Table 5. Molecular profile of the wheat samples and derived products for traceability purposes.

Correct identification and authentication of processed food is more challenging than that of fresh food mainly because of the presence of inhibitors and of DNA degradation. To face these problems, PCRs for food traceability are usually low template-DNA PCRs (LT-DNA PCRs), because increasing the amount of DNA may consequently increase the quantity of inhibitors and determine the failure of the amplification. These PCRs are usually carried out using very small amount of DNA (in the order of few dozens of picograms) and high numbers of amplification cycles (>35) to have a visible signal. While it is reported that PCR can theoretically work even with amounts of template DNA lower than the aforementioned ones, usually LT-DNA PCRs suffer from several limitations. Concerning SSRs, LT-DNA PCRs can be characterized by marker profiles showing a higher heterozygote peak imbalance between the signals of the observed alleles in a specific sample with respect to standard PCR or by the stochastic disappearance of some allele signals (allelic drop-out, mainly a problem for the bigger size alleles). This outcome is mainly a consequence of the small amount and of the degradation of the template DNA. In these conditions, the final result of the PCR can be strongly influenced by the effect of a random selection of the template molecules during the first cycles of the amplification. Other factors that can make the interpretation of the molecular profiles difficult are the presence of: (1) stutter bands; (2) split peaks, deriving from the incomplete adenylation of the PCR products; (3) allelic drop-in, deriving often from contamination and mainly present in the multiplexing amplifications; (4) triploid profile, deriving from the unexpected amplification of three peaks (three loci) from a diploid genome.

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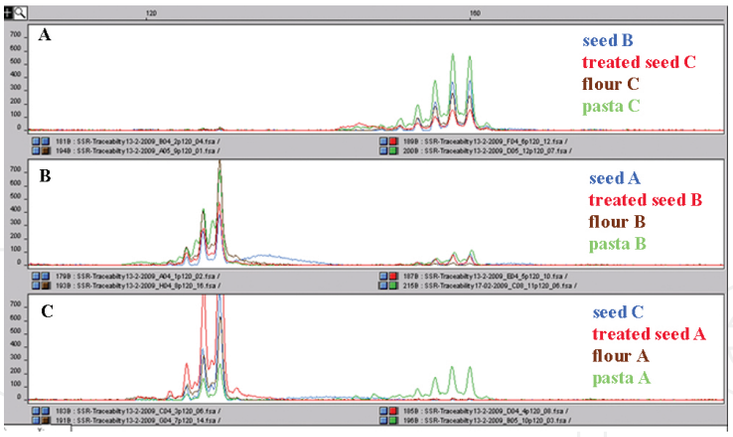


Figure 5. The electropherograms obtained with the microsatellite marker WMS120 are shown. The superimposition of the profiles has been done based on the highest level of correspondence among the different samples. In the upper panel are reported, with different color, the profile of samples seeds B (blue), treated seeds C (red), flour C (brown), and pasta C (green). In the intermediate and lower panels are reported, using the same colors as for the upper panel, respectively: samples seeds A (blue), treated seeds B (red), flour B (brown), and pasta B (green); seeds C (blue), treated seeds A (red), flour A (brown), and pasta A (green). Similarities and differences are clearly evident.

In recent years, our laboratory dealt with the extraction and analysis of DNA from different kinds of food matrices with different purposes and different markers technologies [82–86]. In this section, as an example, the results on traceability of wheat-derived products will be provided. These SSR analyses were carried out as a work under contract for which a third party commissioned us. The samples were collected from the whole supply chain of durum wheat (*Triticum durum* Desf.), starting from grain and ending with pasta and finally provided to us. In detail, DNA was isolated from seeds, vacuum-sealed (treated) seeds, flour, and pasta. Three different sample sets labeled as A, B, and C were received and analyzed in blind. Each labeled set was made of a sample of seed (seeds A, B, and C), treated seed (treated seed A, B, and C), flour (flour A, B, and C) and pasta (pasta A, B, and C). The aim of the analysis was to show the capacity and utility of SSRs to follow, along all the food chain from the raw material to the final product, the presence of a specific DNA, in this case the DNA of the cultivar used to produce the pasta. At the same time, for each labeled set, the presence or absence of correspondence among the genetic profiles of the seeds, treated seeds, flours and pasta was investigated. The DNA was extracted using different commercial kits. Some preliminary trials were carried out to determine the best kit available for our purpose, attempting to find the one providing the highest amount of PCR-grade DNA. The best results were obtained using the GenElute Plant Genomic DNA kit from SIGMA-Aldrich. As expected, high quality DNA was recovered from seeds and treated seeds; in flours some traces of degradation were present and evident as a faint smear in an agarose gel electrophoresis and, finally, from pasta, DNA was always highly degraded as evident by the more intense smear and the absence of any band indicating the presence of high molecular weight DNA. DNA with an estimated average concentration of 60 ng/μl was recovered from the first three kinds of samples (seeds, treated seeds, and flours). Because of the low amount and high degradation, it was not possible to correctly quantify the DNA in pasta. Seven SSRs were used for the analysis: Xgwm46, Xgwm186, Xgwm408, Xgwm459, Xgwm577, WMS5, and WMS120. Three microsatellites

Xgwm46, Xgwm186, and Xgwm408 were monomorphic but polymorphic signals were obtained with the remaining four markers making possible the distinction between different samples (**Table 5**).

From the results obtained, it was not possible to find correspondence between the different samples within each label. As an example, seeds A did not correspond to treated seeds A, flour A, and pasta A. On the contrary, seeds A had the same profile as treated seed B and pasta B. Similarly, seeds B had the same profile as treated seeds C, flour C, and pasta C (**Figure 5**). Concerning the last samples, the presence of correspondence between seeds C, treated seeds A, and flour A was evidenced. Absence of correspondence was found for type A pasta whose genetic profile was more similar to the genetic profile of pasta B and for flour B whose genetic profile was unique and different from the other profiles. As previously stated, samples were received in blind without any knowledge about the origin of the different labeled samples.

Based on this, it was possible to conclude that the seeds of cultivar B (the exact name of the variety was unknown) were used to produce treated seeds C, flour C, and pasta C; seeds of cultivar A were used to produce treated seeds B and pasta B; seeds of cultivar C were used to produce treated seeds A and flour A (**Figure 5**). Pasta A was likely produced by mixing flour A with flour C in almost identical percentages and this was explained by the appearance of the signal corresponding to flour C allele (**Figure 5**). The only incongruence was about flour B. This sample had a genetic profile different from the other samples: it had the same profile of flour A with just an extra allele with SSR Xgwm577. This means that flour B was obtained from a fourth and different cultivated variety, but the possibility of contamination cannot be excluded. Concerning the sample pasta B, the amplification with marker Xgm459 was replicated four times and two times just the 113 bp allele was obtained, while the other two times both the 129 and 113 bp alleles were amplified. As reported previously, working with food-derived DNA is challenging also because of the allelic drop-out: the stochastic disappearance of one of the alleles, usually the biggest one, can be observed as a consequence of DNA degradation, which can explain the results obtained for pasta B.

The results obtained were a clear indication of the utility of SSR markers in following the whole wheat chain, despite the DNA degradation determined by processing.

5. Conclusions

The recent development of high throughput genotyping methods has prompted SNPs as desired markers for several applications in agricultural research, in particular in the livestock sector. Despite this, microsatellites, because of their characteristics, can still be considered as markers of choice for numerous studies, in particular concerning plant genomes, both for biodiversity studies and for molecular traceability of plant-derived food products. In a biodiversity study of local ancient germplasm of fruit tree species, using a small number of markers, we obtained important indications as the presence of synonymy and homonymy, high biodiversity, and different levels of ploidy. Furthermore, the high polymorphism of microsatellite loci together with the different ploidy levels detected increased the probability

to link each cultivar to its corresponding genotypic profile. This is particularly interesting because it means that few properly selected SSRs can be enough to obtain robust results. In the same time, microsatellites can be very useful for molecular traceability as it was evidenced from our results of the whole production chain from durum wheat raw material to processed pasta. Indeed, despite the degradation of DNA caused by food processing, SSRs were able to find the correspondence between blind samples and genotypes highlighting some incongruences.

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