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Determination of S-Allele Combination in an Italian Apple (*Malus × domestica* Borkh.) Germplasm Core Collection

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ABSTRACT

An S-RNase-based gametophytic self-incompatibility (GSI) system, a mechanism that forces outbreeding by preventing self-fertilization, characterizes the genus *Malus*. Knowledge of the self-incompatibility (S) genotypes of apple cultivars is crucial for choosing pollen donors for fruit production and breeding. Even though the S-allele of most commercial apple cultivars has already been identified, limited information is available about the S-allele diversity within local germplasm collections. In this study, 67 S-allele combinations of local apple accessions were identified. The allele S3 was the most common among local apple accessions followed by S1 and S7. The main aim of this study is to provide new information on cultivar compatibility, and these results will be used to set up new parent selection in apple breeding programmes as well as pollinator selection for the commercial orchard.

1 | Introduction

The gametophytic self-incompatibility (GSI) in apple and numerous other Rosaceae is a mechanism evolved to prevent self-fertilization and therefore maintain genetic diversity in the populations (Steinbachs and Holsinger 2002). The S-locus controls the GSI recognition system, which relies on multiple males and a single female determinant, the latter being the well-known S-RNase expressed in the pistil. The S-RNase acts as a specific cytotoxin in incompatible pollen tubes, and rejection occurs when the pollen grain and pistil have the same S-haplotype (De Franceschi, Dondini, and Sanzol 2012). Therefore, diploid cultivars bearing two identical S-haplotypes are incompatible because they cannot fertilize one another. The discovery and characterization of different S-RNase allele sequences (Broothaerts, Van Nerum, and Keulemans 2004; Sanzol 2009; Matsumoto et al. 2011; Kim et al. 2016; Larsen et al. 2016; De Franceschi et al. 2018; Sheick et al. 2018) led to the availability of the S-allele composition of major apple cultivars in online databases, such as the Nagoya dataset (first report in Matsumoto et al. 2011). At present, data about S-allele combinations on more than 1100 apple varieties are available in Nagoya dataset (last update: 2021.9.6; https://www.agr.nagoya-u.ac.jp/~hort/apple/it/).

Several molecular studies have been conducted using specific allele primers to amplify the S-RNase allele or restriction enzymes (CAPS) with universal primers (Kitahara and Matsumoto 2002; Broothaerts, Van Nerum, and Keulemans 2004; Nybom, Sehic, and Garkava-Gustavsson 2008; Kim et al. 2009; Kim et al. 2016; Larsen et al. 2016; De Franceschi et al. 2018). The availability of robust molecular techniques for identifying the S-alleles of the apple tree supports the identification of the best pollinating varieties (De Franceschi et al. 2016; Sheick et al. 2018). For this reason, in recent apple orchards, a 'mixed' field scheme is adopted, in which different apple cultivars are placed alternately on the same row, thus favouring cross-pollination

by pollinating insects (Carisio et al. 2021). From a practical standpoint, understanding cultivar S-genotypes is essential for both establishing fruitful cultivar combinations in orchards and planning appropriate crosses in breeding. For several reasons, the interest in traditional, local cultivars has increased in the last years. Given its unique features, including the frequency-dependent balancing selection (Wright 1939), the S-locus retains a much greater variability than any other locus in the genome of self-incompatible species (Ioerger, Clark, and Kao 1990); nevertheless, the uneven distribution of S-alleles in modern commercial cultivars highlights the bottleneck associated with apple domestication and breeding, with a small number of alleles being much more frequent than others (De Franceschi et al. 2018). The narrowing of the genetic base of a species is an inevitable footprint of human selection; however, a wider genetic base empowers the possibility of breeding programmes to face new challenges, such as those posed by climate change or by the spread of diseases that have led to the near extinction of some plants (Begna 2021). Nowadays, we are witnessing a process of recovery and enhancement of ancient local varieties, as evidenced by national (Law 194/2015) and European (EU Regulation 2018/848) initiatives. Local varieties are of fundamental importance in light of their valuable genetic characteristics both in terms of fruit quality and genetic variability, and they can become a source of important new alleles for breeding programmes. To this end, we hereby present an investigation of S-genotypes of a previously established (Liang et al. 2015) Italian germplasm core collection.

2 | Materials and Methods

2.1 | Plant Material

Sixty-seven apple accessions were used in this work: 59 accessions forming the core collection of the Italian germplasm, maintained at the Department of Agricultural and Food Sciences (DISTAL) of the University of Bologna (Liang et al. 2015), and 8 local ancient varieties from Emilian-Tuscany Apennines. In addition, eight breeding lines with known S-locus allelic combinations were used as references for confirmation of specific S-alleles (Table 1).

TABLE 1 | Eight breeding lines used as references for their allelic composition.

Accession	S-allele combination
Reference 1	S2 S5
Reference 2	S9 S3
Reference 3	S5 S28
Reference 4	S1 S5
Reference 5	S23 S24
Reference 6	S3 S25
Reference 7	S3 S10
Reference 8	S5 S16b

TABLE 2 | Length of amplicons generated with allele specific primers and references.

Allele	Amplicon length	Forward primer	Reverse primer	Reference primers
S1	1500 bp	5′ gcctcgcacttgaatcaatatt 3′	5′ ccgtgtataggccatcgact 3′	De Franceschi et al. 2018
S3	800 bp	5′ aagtgaatgtcggctagcctt 3′	5′ ctgcaaaggacctcaaccaattc 3′	De Franceschi et al. 2018; Sanzol 2009
S5	517 bp	5′ aaacatttataaatagggaccggatc 3′	5′ ctgcaaaggacctcaaccaattc 3′	De Franceschi et al. 2018; Sanzol 2009
S6	544 bp	5′ attttcaatttacgcagcaatatcagc 3′	5′ agtcttccaatttatgcattagaa 3′	Sanzol 2009; De Franceschi et al. 2018
S10	933 bp	5′ atttcaatttacgcagcaatatcagc 3′	5′ tggctctgataccaaattgtca 3′	Sanzol 2009; De Franceschi et al. 2018
S16	483 bp	5′ acccaattgctacacacttacg 3′	5′ ctgcaaaggacctcaaccaattc 3′	De Franceschi et al. 2018; Sanzol 2009
S25	559 bp	5′ tagggtgttgttgcatgtgtg 3′	5′ ctgcaaaggacctcaaccaattc 3′	De Franceschi et al. 2018; Sanzol 2009
S42	683 bp	5′ attttcaatttacgcagcaatatcagc 3′	5′ tcaatgaagtaataaaaggaaggatga 3′	Sanzol 2009; Dreesen et al. 2010

2.2 | DNA Extraction

Genomic DNA was extracted from freeze-dried leaves for each accession, following the standard CTAB protocol (Maguire, Collins, and Sedgley 1994). Genomic DNA was quantified by NeoDot UV/Vis Nano-Spectrophotometer (Neo Biotech, Nanterre, France) and diluted to 50 ng/μl.

2.3 | Amplification and Identification of Apple S-Alleles by Using Consensus Primers

Primers for polymerase chain reaction (PCR) amplification recognized the most conserved regions of the S-RNases, upstream and downstream of the unique intron of the apple gene (Sanzol 2009). PCR with the consensus primers PycomC1F1 (5′ ATTTTCAATTTACGCAGCAATATCAGC 3′) and PycomC5R1 (5′ CTGCAAAGGACCTCAACCAATTC 3′) were carried out according to Nikzad et al. (2014). All samples were visualized in a 1.2% agarose gel in SB buffer (AppliChem) staining with GelRed nucleic acid stain (Biotium, Fremont, CA, USA).

The most common 'light' alleles (< 500 bp), which cannot be unambiguously identified by their consensus product size, were discriminated with CAPS analysis (Kim et al. 2009). Three restriction endonucleases, namely, *TaqI*, *RsaI* and *AluI* (Fermentas), were used to digest the PCR products (Table 3), and the digested samples were separated on 5% polyacrylamide gels and silver-stained (Kim et al. 2009). The restriction profiles predicted by the in silico sequences digestion are reported in Table 3 and include some alleles (S21, S23 and S29), which could potentially be discriminated but were not found in the analysed collection.

2.4 | Amplification of S-Alleles With Allele-Specific Primers

Allele-specific primer pairs (Table 2) were used to screen the core collection for the presence of the alleles S1, S3, S5, S6, S10, S16, S25 and S42 (Sanzol 2009; Dreesen et al. 2010; De Franceschi et al. 2018). PCR were adjusted in a final volume of 17 μL containing 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.3 μM of forward primer, 0.3 μM of reverse primer, 0.036 U/μL Taq DNA polymerase, 100 ng of DNA and 1 × PCR reaction buffer 10×. A touchdown PCR amplification protocol was used for the same samples to enhance amplification products (according to Sanzol 2009) to discriminate between the variants S16a, S16b and S16c.

TABLE 3 | Restriction pattern of CAPS markers (*TaqI*, *AluI* and *RsaI*); the restriction was carried out on amplicons obtained with consensus primers, with the exception of S16a/b/c for which the allele-specific primer pairs was used.

Fragment length (bp)		*TaqI* Cuts	Pattern	*AluI* Cuts	Pattern	*RsaI* Cuts	Pattern
S1	461	2	402-34-25	0	461	1	338-123
S2	270	1	240-30	0	270	0	270
S4	260	1	226-34	0	260	1	195-65
S6	290	0	290	0	290	0	290
S7	240	1	216-24	0	240	0	240
S9	267	0	267	1	241-26	0	267
S11	295	2	209-52-34	1	271-24	2	121-109-65
S16a	483[a]	4	204-150-89-36-4	—	—	4	119-116-107-77-64
S16b	483[a]	5	160-150-89-44-36-4	—	—	4	119-116-107-77-64
S16c	483[a]	4	204–150–89-36-4	—	—	3	180-119-107-77
S20	435	2	376-34-25	0	435	3	123-122-112-78
S21	297	1	263-34	1	273-24	1	188-109
S23	270	2	134-102-34	0	270	0	270
S24	456	2	397-34-25	0	456	1	344-112
S26	282	0	282	1	237-45	0	282
S28	292	0	292	0	292	1	165-127
S29	349	1	315-34	0	349	2	213-93-43
S30	300	1	266-34	1	276-24	2	121-112-67
S31	394	2	335-34-25	0	394	2	159-123-112
S32	274	0	274	0	274	0	274

[a]Amplicon obtained with allele-specific primers (Table 2).

2.5 | Cloning of S-Locus Fragments and Sequence Analysis

When requested for result validation, PCR products were cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA) and sequenced (according to De Franceschi et al. 2018) by Eurofins Genomics EU sequencing GMB (Colonia, Germany). Sequence chromatograms were analysed using 4Peaks software (v.1.8) (http://nucleobytes.com/4peaks/index.html). The sequences were then compared to known S-RNase alleles with BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) and re-aligned with reference allele sequences by Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

In particular, the alleles S17, S20, S26, S33 and S50 were detected by a gel purification with a Mix2seq kit (Eurofins Genomics EU), and the results were BLAST in the NCBI database.

3 | Results and Discussion

The present work aimed at identifying the S-alleles in an apple germplasm collection and in local varieties of the Tuscan-Emilian Apennines. Such information is relevant not only for breeding programmes as an agronomic trait but also to preserve and to re-evaluate the local apple varieties, selected through the centuries for their agronomical traits (such as resistance to abiotic and biotic stress and fruit quality). In the present paper, a large number of different S-alleles (with different S-RNase allele sizes) was expected as a consequence of the large genetic variation represented in the core collection (Liang et al. 2015). The first screening was made by using consensus primers (Nikzad et al. 2014), resulting in amplicons sized from about 600 bp to >2 kbp; for S-RNase alleles with relatively long introns, such as S3, S10 and S25, allele-specific primers were used. 'Short' alleles, which could not be unambiguously identified by only their amplicon size, have been analysed by the use of restriction enzymes, which could differentiate S2 from S9 and S26 from S32 with good accuracy, due to their peculiar restriction patterns in PAGE electrophoresis (Table 3). Through different studies, we know that neutral variants of the same S-RNase allele coexist within a species; an example of this is the apple allele S16 (Matsumoto and Furusawa 2005; Morita, Abe, and Matsumoto 2009; De Franceschi et al. 2018). The S16-RNase presents three polymorphic sequences (S16a/b/c) with identical coding sequences that are functionally the same allele. A restriction pattern compatible with the S16b allele was observed for all accessions digested with restriction enzymes *RsaI* and *TaqI*. In the panel of the Italian accessions, 19 different S-alleles have been detected (Figure 1 and Table 4). The most frequent alleles were S3, S1, S7 and S6, which were found in 17, 15, 12 and 11 accessions, respectively.

Similar results for S3, S1 and S7 were found by Nybom, Sehic, and Garkava-Gustavsson (2008) and Larsen et al. (2016) studying cultivars from Northern Europe. The S3 allele is known to be among the most frequent in modern varieties from European, American and Japanese cultivars (Broothaerts, Van Nerum, and Keulemans 2004; Dreesen et al. 2010; Larsen et al. 2016); although the selection of a specific S-allele can be due, in some cases, to its linkage with favourable genes (De Franceschi et al. 2016), its higher frequency can be as well the mere consequence of its presence in some of the most used founders of current cultivars. Accordingly, our study detected the presence of S3-RNase in some of the most relevant ancient Italian varieties, such as 'Durello di Forlì', 'Calvilla Bianca d'Inverno', 'Renetta Grenoble', 'Renetta Grigia Torriana', 'Mela Rozza' and 'Renetta Ruggine'. The last three varieties also shared the fruit russeting.

Remarkably, S6 was found to be one of the rarest alleles in a previous study conducted on a pool of worldwide apple

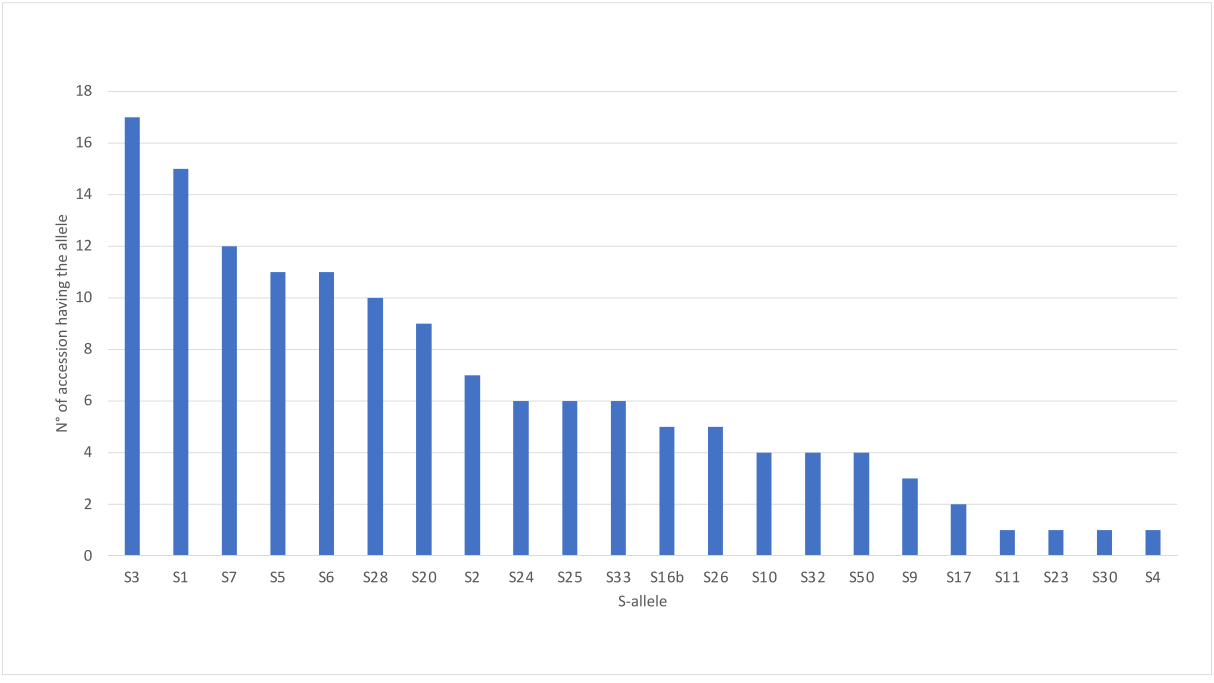

FIGURE 1 | Distribution of the S-alleles found in the analysed Italian apple germplasm.

TABLE 4 | Lists analysed accessions, their sampling sites and their S-allele determination.

Accession	Field position/GPS coordinates	Place	S-allele combination
APPICCADORZA	B29D88	UNIBO	S16b S26
ASTRAKAN BIANCO	B21A53	UNIBO	S1 ?
BACCAIANA	B29D85	UNIBO	S1 S28
BELLA SCARLATTA	B21A50	UNIBO	S3 S28
BENONI	B29A94	UNIBO	S5 S11
BIANCHI	B29L07	UNIBO	S2 S3
BIANCUCCIA	B29G37	UNIBO	S25 S33
BOMMINO	B29G61	UNIBO	S7 S26
BUSIARD	B47I095	UNIBO	S1 S5
CALVILLA BIANCA D'INVERNO	B29A88	UNIBO	S2 S3
CALVILLA SAN SALVATORE (MI)	B29A82	UNIBO	S3 S28
CARBONE	B29L19	UNIBO	S1 S50
CARLA	B29H28	UNIBO	S28 ?
CAVECH	B29C70	UNIBO	S1 S28
CAVICCHIO	B13 A19	UNIBO	S5 S24
DI LUGLIO	B29C91	UNIBO	S7 S16b
DOLCE	B20G46	UNIBO	S25 ?
DOLCINO	B29L94	UNIBO	S5 S33
DUNIZZA GIALLA	B29G10	UNIBO	S1 S7
DURELLA	B29H10	UNIBO	S2 S9
DURELLO DI FORLI'	B1 G49	UNIBO	S3 S50
FOIASSA	B29D01	UNIBO	S3 ?
GAROLA	B29L13	UNIBO	S2 S6
GRENOBLE (PC)	B29C13	UNIBO	S6 S10
GROSSA VINO ROSSO	B47I019	UNIBO	S1 S9
LAVINA	B14 A44	UNIBO	S1 S7
LAZZERUOLA	B29E10	UNIBO	S2 S17
LIMONCELLA (CB)	B20F19	UNIBO	S32 ?
LOSA D'GIAVENO	B29I22	UNIBO	S3 S20
MADONNA	B29H49	UNIBO	S24 ?
MAIOLINO (PA)	B29F64	UNIBO	S5 ?
MALUS ALBA	44.197; 11.119	Burzanella (BO)	S6 S7
MELA GIALLA I	B6E43	UNIBO	S24 ?
MELA MAGGIAIOLA	B29H13	UNIBO	S5 ?
MELA ROSA (PD)	B29A25	UNIBO	S3 S25
MELA ROZZA	B6E31	UNIBO	S3 S6
MELA TELLINA	B29F85	UNIBO	S6 S25

(Continues)

TABLE 4 | (Continued)

Accession	Field position/GPS coordinates	Place	S-allele combination
MELO DI METZECHI	B47I150	UNIBO	S16b S33
MELO FERRO (PC)	B29A70	UNIBO	S26 ?
MORELLA	B6D58	UNIBO	S2 S10
MOSCARELLO	B29G55	UNIBO	S1 S3
MUSABO' VERDE	44.144; 11.149	Lagora (BO)	S6 S7
MUSONA MUSABO'	B47 I49	UNIBO	S28 ?
NESTA	B29G34	UNIBO	S7 ?
PAOLUCCIA (VT)	B20F13	UNIBO	S30 ?
PASAROT	B29G82	UNIBO	S6 S7
POM SANDRI	B47I028	UNIBO	S20 ?
PUMA OLIO	B6E28	UNIBO	S3 S16b
PUM PERSEG	C76I55	UNIBO	S17 S33
RENETTA DI GRENOBLE	B29C19	UNIBO	S3 S6
RENETTA RUGGINE	C6L28	UNIBO	S3 S32
RENETTA WALDER (FO)	B29C31	UNIBO	S1 S7
ROSA GENTILE	44.325; 11.158	Malfolle (BO)	S20 S50
ROSA INVERNALE DI MONTEFELTRO	43.728; 12.622	Maiolo (RN)	S20 S33
ROSA ROMANA C1	44.246; 10.996	Pietracolora (BO)	S20 S26 S33
ROSA ROMANA C2	44.198; 10.931	Gaggio Montano (BO)	S20 S33 ?
ROUS D'BORSETTA	B47I013	UNIBO	S7 S24
ROUS GIAIET	B47I007	UNIBO	S6 S10
RUGGINOSA	44.299; 11.078	Vigaia (BO)	S1 S20
RUS CAVALLOTTA	B29I88	UNIBO	S3 S6
S. GIUSEPPE	B47H031	UNIBO	S4 S5
SEL. MARZABOTTO 1	B29D52	UNIBO	S24 S28
SERPENTE	B47I036	UNIBO	S28 ?
SONALIO	B29H85	UNIBO	S7 S32
TINELLA	B47H041	UNIBO	S25 S26
TONNORELLA	B20G13	UNIBO	S20 S32
VIRTICCHIARO	B29H52	UNIBO	S1 S50

cultivars (De Franceschi et al. 2018), highlighting the distinct genetic base of the analysed Italian germplasm. The full-length coding sequences are essential for characterizing well the S-alleles, as evidenced by the case of the accession 'Mela Rozza' with S6a/S6 and S6b/S17 (De Franceschi et al. 2018). The S6 allele reported in this study was analysed as reported by De Franceschi et al. (2018). Other frequent alleles are S28 and S20, identified in a total of 10 and 9 accessions, respectively. The alleles S4, S11, S23 and S30 are rare and have been found only once ('S. Giuseppe', 'Benoni' and 'Paoluccia VT', respectively). Similar results for S4 were also described by Broothaerts, Van Nerum, and Keulemans (2004), who considered S4 a rare allele. Larsen et al. (2016), on the contrary, found that S4 and S11 alleles were more widely distributed in North European cultivars.

The S9 allele was found only in two accessions ('Durella', 'Grossa Vino Rosso'). This allele is present in famous varieties such as 'Fuji' (S1 and S9; Sassa et al. 1994) and the 'Red Delicious' group (S9 and S28; Matsumoto et al. 1999), but its highest frequency

seems to be in the American, Chinese and Japanese gene pool (Nagoya database). A similar consideration can be done for the S10, S24 and S25 alleles: (i) S10 is frequent in apple cultivars from other origins ('McIntosh', 'Discovery', 'Prima', 'Priscilla', 'Spartan', 'Summerred' and others) but that it is present only in three accessions with Piedmont origin ('Grenoble PC', 'Morella' and 'Rous Giaiet'); (ii) S24 and S25 are shared by American varieties such as 'Honeycrisp' (S2 and S24; Sakurai, Brown, and Weeden 2000) and 'McIntosh' (S10 and S25; Kitahara and Matsumoto 2002) and by crabapple accessions such as 'Frettingham' (S24 and S25) and 'Snowdrift' (S24 and S45b), used for cross-pollination (Sheick et al. 2018). The S20 and S33 alleles of the 'Rosa Romana' accessions are also shared by 'Rosa Invernale del Montefeltro' as expected considering the genetic relationships among these accessions (Alessandri et al. 2016) and by 'Rosa Gentile', 'Tonnorella' and 'Pom Sandri'. S33 is also present in 'Biancuccia', 'Dolcino' and 'Melo di Metzechi'.

These S-allele combination was consistent with population structure results discovered by Liang et al. (2015), in which these accessions clustered together; the presence of a common S-allele reinforces the hypothesis of their relatedness.

Unfortunately, for 15 accessions, only one S-allele could be identified, whereas the genetic variants C2 of the 'Mela Rosa Romana' (Alessandri et al. 2021) presented only two alleles even if it is known as triploid genotype. The detection of an incomplete set of S-RNases from a sample is generally due to the different amplification efficiency between coexisting alleles, especially due to large differences in intron sizes. 'Short' amplicons are produced with greater efficiency and reach PCR plateau before 'large' ones can reach detectable concentrations. Nevertheless, other possibilities such as the presence of new uncharacterized alleles cannot be ruled out, especially considering the large diversity of the analysed panel. Further studies will be needed to integrate and expand the knowledge of S-genotypes within the Italian apple germplasm; however, the data hereby described can be readily employed for the set-up of new orchards of local apples, as well as the plan of novel mating schemes for breeding programmes. This analysis is also the ideal completion of the phenotypic and genotypic evaluation of the apple germplasm described by Liang et al. (2015) and Alessandri et al. (2021), which collectively pose the base for a rediscovery of valuable traditional Italian cultivars and the rescue of their genetic base for use in breeding programmes.

Author Contributions

Sara Alessandri, Nicolò Alvisi, and Paolo De Franceschi: methodology, figure curation, investigation, formal analysis, software, writing – original draft, writing – review and editing. **Luca Dondini:** project administration, validation, review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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