Rosa Romana apple: A heritage of the apple germoplasm of the Tuscan-Emilian Apennines to be recovered and promoted

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ABSTRACT

A molecular characterization on the ancient variety 'Rosa Romana' was carried out to improve biodiversity knowledge and preserve these trees from extinction risks.

In this work, 47 accessions were collected throughout an investigation in the Emilia-Romagna region (Italy) and particularly in the mountain area of the Bologna province (19 collection sites). The analysis at molecular level by using 15 SSRs (microsatellites) identified two main genotype groups and ascertained their relationship with several phenotypic traits. This two clusters contained most of the collected accessions, while remaining genotypes differ clearly, according with the phenotypic diversity on the behavior of the trees or fruits.

This study also revealed the highest quality traits of 'Rosa Romana' apple grown in the Apennines mountain around Bologna (in a range between 400 and 1000 m.a.s.l.) if compared to each main clone produced at the lowland corresponding to the Bologna University Agricultural Experimental Station (30 m.a.s.l). Therefore, the apple quality as color, appearance, taste (flesh firmness and texture, sweetness, acidity, aroma, polyphenol soreness), were improved in the higher altitudes. In conclusion, the results of this environmental and genetic investigation on the residual cultivation of 'Rosa Romana' apple provided a genomic validation of its best identified clones (correspondent to the main two clusters), which now can be recovered and promoted as new planting, with a own brand 'Rosa Romana' produced in the Apennines mountain of north Italy.

Keywords: *Malus X domestica* Borkh., accessions, SSR, molecular characterization, qualitative parameters, Cluster analysis.

1 Introduction

Apple (*Malus x domestica* Borkh.) is the main fruit crop of temperate regions of the world such as Europe, the west area of Turkestan and the south-east and Centre of Asia (Velasco et al., 2010), in terms of production levels. It occupies a central position as nutritional value and also in culture, art and folklore (Janick, 2005; Cornille et al., 2014). Much of the genetic diversity of the old cultivated apples is currently maintained in germoplasm repositories and amateur collections (Alessandri et al., 2016).

The 'modern' apple was domesticated in Central Asia from *Malus sieversii* (Velasco et al., 2010; Cornille et al., 2012; Volk et al., 2005 and 2013) and was brought to Europe through human migrations between 6,000 and 3,000 years ago (Janick, 2005; Ross-Ibarra et al., 2007; Cornille et al., 2012). Humans have been exploiting, selecting, and transporting apples for centuries, and several thousand apple cultivars have been historically documented (Ross-Ibarra et al., 2007; Cornille et al., 2014). Over time, many of the 'old' varieties of Italian apple trees however have been marginalized and now are present only in small local area. In some cases, only single specimens of trees have survived, a memory of a glorious past, while unfortunately some genotypes have been forever lost.

The first historical quotation of a 'Rosa' apple in Emilia-Romagna region dates back to the 16th century, by the famous naturalist Aldrovandi and a first pictorial representation was released at the end of the 17th century by Bartolomeo Bimbi, a famous painter of the Medici's court, who painted more than one hundred apple varieties and reported their correct names (Fideghelli, 2017).

'Rosa Romana' was grown in the Reno Valley for its high fruit quality (flavor, taste, texture), high storability, easy harvesting, short juvenile phases, synchronicity in blooming and fruit ripening (Gregori et al., 2013). The Reno Valley represents the propagation point of the 'Rosa Romana' variety. Probably because this valley was a passage area during the Roman age since it allowed the connection between the regions of Emilia-Romagna and Tuscany.

In 1929, this variety represented the 25% of the apple production in the Bologna area. However, this apple has almost disappeared in the time frame of thirty years (Sansavini et al., 2018).

The 'Rosa Romana' fruit descriptor evidences a flattened shape, a short peduncle, a yellow ground color with bright red on 20-30% of the skin (only in the mountain areas). The fruit has a thick and slightly waxy skin when the apple is ripe. Normally the peduncle cavity is covered by russeting (Figure 1). Flesh is firm, juicy, fine, non-crispy and non-astringent. The taste highlights a well-balanced equilibrium of sweetness and acidity with a slightly bitter aftertaste. Storability without refrigeration is excellent (even till 4 months), but a controlled atmosphere it can be suggested for much longer storage. Fruits are susceptible to physiological disorders such as bitter pit, especially in young, too vigorous, over-nourished trees. The picking time is late autumn as well as the ripening time (Fideghelli et al., 2017; Sansavini et al., 2018).

The international literature on commercial, nutritional and genetic information relating to the 'Rosa Romana' variety is scanty despite its cultivation and use in the Reno Valley dates back to ancient times (at least since the Roman age).

Sansavini et al. (2018), showed like this variety currently consumed and promoted in the market by local farmers pointing out its health and gustatory qualities together with its strong link with the Reno Valley territory and history – heritage which deserves proper protection and interest.

Farneti et al., 2015 evidenced that ancient apple varieties as 'Rosa Romana' have a higher level of phenols compared to commercial apple cultivars. In particular, the organic acids and the phenolics compounds were significantly influenced and dependent by human selection. Bignami et al., 2001m carried out the only reported work on the variability of qualitative traits of the 'Rosa Romana' genotype. The analysis of nutrients and polyphenols showed the high quality of this apple.

The local germplasm of apple varieties represents a good source for breeding programs so as to guarantee the availability of a wide genetic variability (Bignami et al., 2001). To preserve this

75 genotype, in particular, it is necessary to identify and classify the possible variables that can be
76 differentiated over the long cultivation time.

77 Other two accessions grown in this area are: ‘Rosa Romana Gentile’ and ‘Rosa Nostrana’. ‘Rosa
78 Romana Gentile’ differs from ‘Rosa Romana’ for its low russeting, the smaller extension of the red
79 fruit skin overcolor (Figure 1c), the greater greasiness and for its earlier ripening while ‘Rosa
80 Nostrana’ differs from the other apple Roses for its conical fruit shape (Figure 1b), its high greasiness
81 after storage and for the not excellent sensorial traits, susceptible to scald.

82 It is important to be not confuse this apple genotypes with the other Rose apple varieties which present
83 distinctive characteristics such as different fruits and lenticelllar shape and coloration.

84 As showed by Figure 1, ‘Rosa d'Osta’ and ‘Rosa Mantovana’ differ mainly in the rounder shape
85 compared to the flat shape of ‘Rosa Romana’ (Figure 1d, e). In addition, ‘Rosa d’Osta’ is
86 characterized by a scarce over-color and absence of rust. ‘Rosa d’Oliveto’ has a longer stalk and a
87 more uniform red color diffused at lenticellar level compared to 'Rosa Romana' (Figure 1f). Lastly,
88 ‘Rosa Marchigiana’ presents a shorter stalk and more evident lenticels on the skin compared with
89 ‘Rosa Romana’ fruit (Figure 1g).

90 Molecular markers [Simple Sequence Repeat (SSR)] are fundamental for verifying the correct
91 propagation in the nurseries, the true-to-type correspondence and for reducing redundancies in
92 collections. In particular, microsatellites are considered the most suitable and useful markers for
93 exploring the genetic diversity because they are i) abundant and well distributed in the genome; ii)
94 codominant and multi-allelic; and iii) analyzed by multiplexed PCR (Polymerase Chain Reaction)
95 assays (Baric et al., 2020; Testolin et al., 2019; Larsen et al., 2017; Urrestarazu et al., 2016; Patocchi
96 et al., 2009; Hayden et al., 2008).

97 The aims of this work are: i) the phenotyping of the selected clones for the fruit quality traits and the
98 relative comparison with fruits from the lowland; ii) the identification of the genetic variability

present among the ‘Rosa Romana’, ‘Rosa Romana Gentile’ and ‘Rosa Nostrana’ accessions sampled in Reno Valley (hill around 400-600 m.a.s.l. and mountain area around 600-1000 m.a.s.l.).

The identification of historical trees and best reference plants for propagation are fundamental steps for the development of nursery activities. This will also promote and support the exploitation and protection of such ancient Italian apple cultivars. An increased interest in local products and ancient flavors is expected to follow.

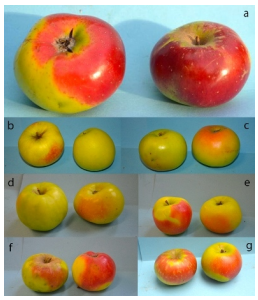

Figure 1 – Seven ‘Rosa’ varieties which differ for several fruit traits but having partially the same root name ‘Rosa’: a) ‘Rosa Romana’ apple (accession #17, cluster 1); b) ‘Rosa Nostrana’ (accession #3); c) ‘Rosa Romana Gentile’ (accession #2); d) ‘Rosa d'Osta’ (accession #15); e) ‘Rosa Mantovana’ (accession #44); f) ‘Rosa d'Oliveto’ (accession #14); g) ‘Rosa Marchigiana’ (accession #9)

2 Materials and methods

2.1 Plant material

The fruit and leaf samples were collected by historical trees in 17 different locations of the Reno Valley and in two sites of the Bologna plain (for a total of 47 accessions, Table 1; Figure S1). The sampled trees from the mountain area are grafted on non-characterized apple seedling and maintained *in situ* collections by guardian farmers and are grown following the organic farming guidelines.

The sample list includes 3 Rosa accessions from the Marche region and 9 Rosa accessions from the apple collection of the University of Bologna (Table 1). Fruit samples for quality analyses were collected in two consecutive harvesting years (2018-2019).

123 Table 1 - List of analyzed accessions and their sampling sites. BO: Bologna; PT: Pistoia.

	Accessions	Sampling Area	Altitude (m.a.s.l.)
#1	Rosa Romana	Santa Maria Villiana	643
#2	Rosa Romana Gentile	Santa Maria Villiana	643
#3	Rosa Nostrana	Santa Maria Villiana	643
#4	Rosa 1	Gaggio Montano (BO)	944
#5	Rosa 2	Gaggio Montano (BO)	944
#6	Rosa 3	Gaggio Montano (BO)	944
#7	Rosa Romana	Castel di Casio (BO)	533
#8	Rosa Romana Gentile	Castel di Casio (BO)	533
#9	Rosa Marchigiana R101	Macerata, Marche	600
#10	Rosa Marchigiana R108	Macerata, Marche	600
#11	Rosa Marchigiana R60	Macerata, Marche	600
#12	Rosa Nostrana o Locale	Bentivoglio (BO) - Villa Smeraldi	19
#13	Rosa Romana	Bentivoglio (BO) - Villa Smeraldi	19
#14	Rosa d'Oliveto	Bentivoglio (BO) - Villa Smeraldi	19
#15	Rosa d'Osta	Cadriano (BO) - UNIBO	32
#16	Rosata Russolina	Cadriano (BO) - UNIBO	32
#17	Rosa Romana	Castal dell'Alpi (BO)	737
#18	Rosa Romana	Monteacuto (BO)	915
#19	Rosa Romana	Malfolle (BO)	500
#20	Musabo Rossa	Castal dell'Alpi (BO)	737
#21	Rugginosa	Castal dell'Alpi (BO)	737
#22	Rosa Romana	Bologna	40
#23	Rosa R. Gentile	Bologna	40
#24	Rosa 1	Bologna - Villa Puglioli	270
#25	Rosa 2	Bologna - Villa Puglioli	270
#26	Rosa 3	Bologna - Villa Puglioli	270
#27	Rosa Romana	Ecchia- Prunarolo (BO)	193
#28	Rosa Romana	Ca Bortolami (BO)	334
#29	Rosa Romana	Ca Bortolami (BO)	334
#30	Rosa Romana	Ca Bortolami (BO)	334
#31	Rosa Romana	Grizzana Morandi (BO)	547
#32	Rosa Romana	Grizzana Morandi (BO)	547
#33	Rosa Romana	Veggio (BO)	550
#34	Rosa Romana	Veggio (BO)	550
#35	Rosa Romana	Pianoro (BO)	200
#36	Rosa Romana	Sambuca Pistoiese (PT)	504
#37	Rosa Romana	Capugnano (BO)	820
#38	Rosa Romana	Camparenda (BO)	800
#39	Rosa Romana	Valgattara (BO)	700
#40	Rosa Romana	Camparenda (BO)	815
#41	Rosa Romana (strain 24)	Cadriano (BO) – UNIBO	32

#42	Rosa Romana Gentile (strain 43)	Cadriano (BO) – UNIBO	32
#43	Rosa Romana (strain A23)	Cadriano (BO) – UNIBO	32
#44	Rosa Mantovana (TN)	Cadriano (BO) – UNIBO	32
#45	Mela Rosa (PD)	Cadriano (BO) – UNIBO	32
#46	Rosa d'Oliveto	Cadriano (BO) – UNIBO	32
#47	Mela Rosa (TN)	Cadriano (BO) - UNIBO	32

2.2 Apple phenotyping: qualitative parameters

After harvesting, fruits were immediately stored at cold room at 0°C with high humidity for about one month and then kept out in shelf-life for three days to ripen the fruit (Gorny and Kader, 1997). Fruit weight (g), percentage of overcolour, russeting (%), bitterness (%), soluble solid (%) and organic acid content (malic acid g/L) have been evaluated on pools of 10 fruits (Gregori et al., 2013). Firmness was measured by a penetrometer (11 mm diameter probe) on apple surfaces from opposite sides of each fruit (Kg/cm2). A pool of ten apples was analysed for each sampled tree. Soluble Solids Content (SSC) was determined by a digital refractometer (Atago, Tokyo, Japan) on filtrated apple juice obtained by homogenizing two slices taken from each of the 10 fruits. Titratable acidity (TA) was detected by automatic titrator (Crison Instruments, SA, Barcelona, Spain). Twenty millilitres of juice diluted with additional twenty millilitres of distilled water were titrated to pH 8,1 with 0,25N NaOH. Trees and fruits were evaluated with pomological descriptors in field after fruit harvesting, according to Gregori et al. (2013). Percentage of fruit skin overcolour was empirically classified. Bitterness was estimated by a sensory panel test by ranking the evaluations in classes from 1 to 9 on an empirical scale (1, absence; 9, maximum intensity). The data were processed (i) by unpaired t-test to compare means between fruits collected in mountains (the Reno Valley) vs reference those of the plains (Bologna); ii) by variance analysis (ANOVA) according to Fisher's Least Significant Difference (LSD) test at P = 0,05 to compare the single samples of different mountain areas with 4 number of replicates per sample in each of the two harvesting years.

2.3 DNA extraction, SSR genotyping and allele characterization

For each accession, genomic DNA was extracted from 50 mg of young freeze-dried leaves following the standard CTAB protocol (Maguire et al., 1994). Genomic DNA was quantified by Nanodrop™ ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and diluted to 10 ng/µl. Samples were analyzed with 15 SSR markers according to Liang et al. (2015).

The molecular data were compared and aligned with the SSR profiles of the references conserved in the collection of the Department of Agricultural Sciences and Technologies of the University of Bologna (DISTAL) in Cadriano: 'Rosa Romana' (strain 24), 'Rosa Romana Gentile' (strain 43), 'Rosa Romana' (strain A23), 'Rosa d'Osta', 'Rosata Russolina', 'Rosa Mantovana [Trento (TN)]', 'Mela Rosa [Padova (PD)]', 'Rosa d'Oliveto' and 'Mela Rosa (TN)' to better understand the variability present among the accessions collected.

The number of alleles per locus (k), the observed and the expected heterozygosities (Ho and He) and polymorphism information content (PIC) of the SSRs were estimated using the CERVUS Software Version 3.0.3 (Kalinowski et al., 2007; Marshall et al., 1998). A PIC value greater than 0.7 was considered to be highly polymorphic and informative for a certain locus. Subsequently, the dendrogram tree was calculated by using the NTSYSpc 2.0 software with the coefficient of DICE (Dice, 1945) and the software R (Project for Statistical Computing). The cluster analysis and the construction of the dendrogram related to genetic distances were obtained by the UPGMA method (Unweighted Pair-Grop Method).

3 Results

3.1 Pomology and qualitative parameters

Pomological observation and the analyses of several fruit quality parameters (fruit weight, percentage of overcolor, russeting, bitterness, soluble solid and organic acid content) was carried out on a pool of 10 representative fruits for the Rose genotypes ('Rosa Romana', 'Rosa d'Osta', 'Rosa Romana Gentile', 'Rosa Nostrana','Rosa d'Oliveto', 'Rosa Marchigiana' and 'Rosa Mantovana').

The statistical analysis was initially elaborated by comparing the pools of individual trees harvested of 'Rosa Romana' in different locations of the Reno Valley with each other and those in the Bologna plains (Experimental farm of the University of Bologna, Cadriano and Villa Smeraldi).

First of all, the fruits from accessions belonging to the 'Rosa Romana' were not statistically distinguishable for all the analyzed traits. In fact, all the qualitative parameters analyzed did not show significant differences among the samples which presented the phenotypic characteristics typical of the variety (Figure 1a).

Differences were observed by comparing samples of 'Rosa Romana' collected in the Reno Valley to those harvested in the plain (Table 3).

In particular, the apples of the plains had a greater fruit weight but with a reduced fruit overcolor (Table 3).

As reported in Table 3, the 'Rosa Romana' plain samples also presented a lower russeting in the peduncular region of the fruit. In addition, the juice of the apples of the plains had 1° Brix less than those of the mountains while the acidity was about a half. These data evidence that the fruit quality traits are enhanced in areas at medium and high altitude (400-800m). This observation was also confirmed by the analysis of the variance (ANOVA).

Finally, data collected on fruits of other Rosa accessions (such as 'Rosa Nostrana') showed difference respect the 'Rosa Romana' accessions (Table 4). In particular, 'Rosa Nostrana' is differing from the other Roses for a conical shape of the fruit, less percentage of russeting of the skin apple (1%) and less pulp firmness at harvest (4,24 Kg/cm^2), a high greasiness after storage and for the organoleptic characteristics (Figure 1, Table 4,).

Area	Mountain (Reno Valley)				Plain (Bologna)		
Accessions	#4 Rosa 1	#17 Rosa Romana	#1 Rosa Romana	**Means**	#41 Rosa Romana	#13 Rosa Romana	**Means**
Medium weight (g)	124b	167a	153a	150	230a	198b	214
Over-colored (%)	35a	35a	23b	24	3b	7a	5
Russeting (%)	20a	26a	11b	14	24a	26a	25
Bitterness (index 1-9)*	8,7a	7b	7,7a	7,8	6a	5,6a	5,8
Brix (%)	16,8a	16b	15,8b	15,1	14,1a	13,9a	14
Firmenss (kg/cm^2)	10,4a	8,39c	9,04b	9,27	6,79a	6,91a	6,85
Acidity (g/l malic acid)	4,9 b	6,5a	6,9a	7,0	3,4a	3,6a	3,5

Table 3 - Pomological profile of the 'Rosa Romana' apples from Cluster 1 and Cluster 2; separating between Mountain and Plain samples, collected in place with different altitude level [Gaggio Montano (944 m a.s.l.), Castel dell'Alpi (737 m a.s.l.) and S. Maria Villiana (643 m a.s.l.)] and in Plain (Bologna) [Cadriano (32 m a.s.l.), S. M. Bentivoglio (19 m a.s.l.)].
Analysis of variance (ANOVA) according to Fisher's LSD. *Index of empirical scale (1, absence; 9, maximum intensity)

Accessions	#3 Rosa Nostrana	#2 Rosa Romana Gentile	#15 Rosa d'Osta	#44 Rosa Mantovana	#14 Rosa d'Oliveto	#9 Rosa Marchigiana
Place	S. Maria Villiana (BO)	S. Maria Villiana (BO)	Cadriano (BO)	Cadriano (BO)	Bentivoglio (BO)	Macerata
Altitude (m a.s.l)	643	643	32	32	19	600
Fruit weight (g)	186a	148c	162b	82e	139d	135d
Overcolor (%)	22b	6c	1d	32a	30a	38a
Russeting (%)	1a	2a	3a	3a	2a	2a
Brix (%)	14,5a	13,9b	14b	13c	14,6a	14,1ab
Firmness (Kg)	4,24d	9,29a	7,30b	5,99c	7,56b	7,49b
Acidity (g/l malic acid)	5,3b	5,4b	3,8d	2,9e	6,4a	3,9c

Table 4 - Pomological profile of the other apple varieties which differ for several fruit traits from the 'Rosa Romana' phenotype but having partially the same root name 'Rosa'.
Analysis of variance (ANOVA) according to Fisher's LSD. The medium with different letters are significantly different ($P \leq 0.05$).

3.2 SSR and cluster analysis

The 47 samples collected were amplified with 15 pairs of primers already used by scientific community for their good discriminating ability (Liang et al., 2015). An average of 9 alleles per locus are observed for a total of 126 alleles. For each analyzed locus the observed and expected heterozygosity was calculated with CERVUS Software as showed in Table 2. Ho ranged from 0.333

for CN444542 to 0.917 CH01A09, CH03G07 and GD12; He ranged from 0,631 for CH02C09 to 0,861 for CH04C07 (Table 2). The highest PIC values of 0.850 and 0.803 were observed for the markers CH01H01 and GD12, respectively. Values greater than 0.7, were also observed for all the other SSRs used in the present research. More in detail, SSR loci CH01F02 and CH04C07 were able to distinguish 12 alleles (Table 2), thus showing their high discrimination power as reported by Liebhard et al. (2002) and by Cavanna et al. (2008) for apple and pear accessions.

UPGMA cluster analysis, based on DICE genetic distance, evidenced the presence of two main groups of 'Rosa Romana' (namely C1 and C2) that share a high number of alleles, confirming a high degree of similarity between the analyzed samples but also the allele differences (Figure 2).

The first cluster includes 12 accessions (#1, #5, #6, #7, #19, #27, #28, #30, #31, #35, #38, #40) with 100 % of similarity with the reference ‘Rosa Romana (strain 24)’ (#41) and ‘Rosa Romana Gentile (strain 43)’(#42) of the University of Bologna and other 3 samples (#22, #23, #39) with very low allele variations (Figure 2). The second cluster could be divided in two subgroups: the former is represented by 5 accessions (#4, #8, #13, #17, #18) that were identical to the reference of the University of Bologna 'Rosa Romana (strain A23)'(#43) and the latter one including samples collected in the area around the Grizzana Morandi site (‘Rosa Romana’ #32, #33 and #34; Figure 2). Other samples of ‘Rosa Romana’ not included in these two clusters (#2, #24, #25, #26, #29, #36, #37) should be consider as misnomer (Figure 2).

Between those groups of accessions, it should also be noted that there are three accessions of ‘Rosa Marchigiana’ that are very similar but not identical to each other and differ in 8 alleles from the ‘Rosa Romana’ samples. In addition, the two representative samples of the ‘Rosa Nostrana’ accession (#3 and #12) are clearly separated from the ‘Rosa Romana’ clusters and they are distinguishable each other for a number of allele polymorphisms. It is important to underline that the present results could not uniquely identify the ‘Rosa Romana Gentile’ accession, as the samples labelled with this name were all different. In particular, ‘Rosa Romana Gentile (strain 43)’(#42) from Bologna was found to

belong to Cluster 1 (with a few polymorphic alleles) while the 'Rosa Romana Gentile' (#8) was found in Cluster 2. The third sample 'Rosa Romana Gentile'(#2) clearly deviates from the main clusters and it represents another misnomer.

The dendrogram also included other Rosa accessions clearly separated from the 'Rosa Romana' clusters. 'Rosata Russolina' (#16), 'Rosa Mantovana (TN)'(#44) and 'Mela Rosa (TN)'(#47) presented identical allelic profile and they can be considered as synonyms (Figure 2).

Locus	K	Ho	He	PIC
CH01A09	8	0.917	0.824	0.784
CH02C09	7	0.583	0.631	0.589
CH03G07	5	0.917	0.687	0.611
CHVf1	7	0.583	0.585	0.544
GD12	10	0.917	0.842	0.803
CH01F2	12	0.833	0.838	0.799
CH02D08	9	0.875	0.820	0.781
CH04C07	12	0.833	0.861	0.828
CH01F03	9	0.875	0.810	0.767
CH01H01	10	0.708	0.883	0.850
CH01H10	8	0.833	0.834	0.794
CH01H02	9	0.542	0.773	0.729
Hi05E07	8	0.875	0.769	0.723
CH05C06	6	0.667	0.809	0.761
CN444542	6	0.333	0.714	0.652

Table 2 - Genetic variability parameters: number of alleles per locus (k); observed heterozygosity (Ho); Expected heterozygosis (He) and the PIC (Polymorphism Information Content) index.

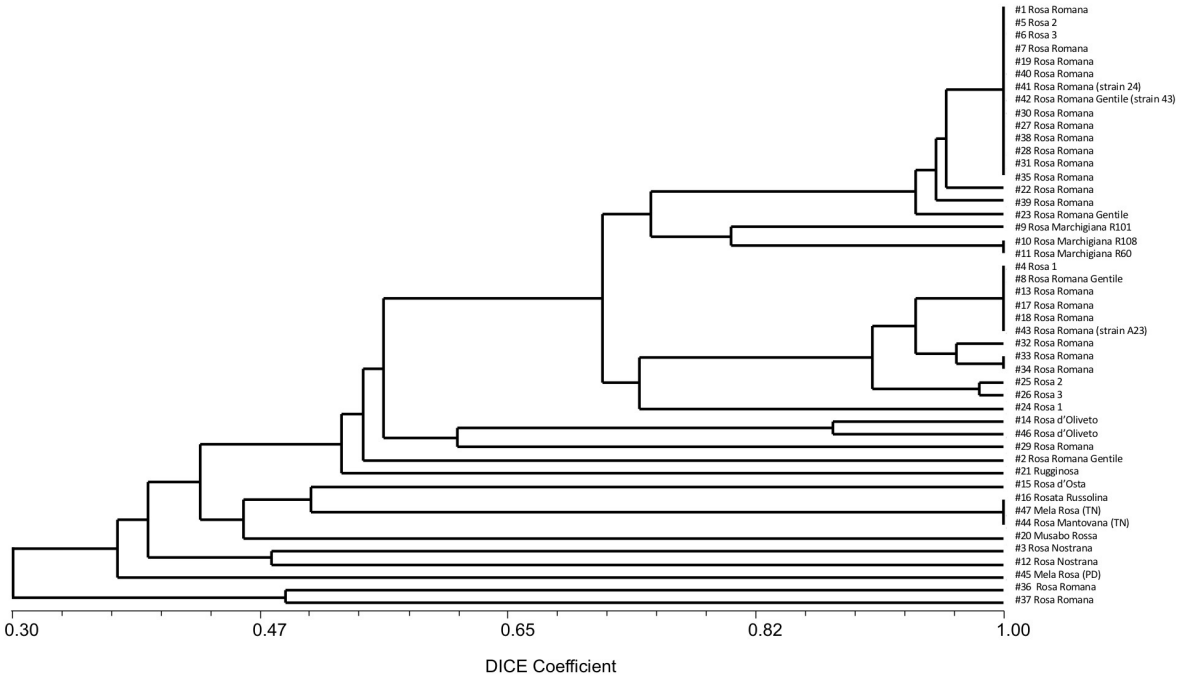

Figure 2 - UPGMA tree indicating the relationships among the 47 accessions.

4 Discussion

This study represents the first step of the re-evaluation process for the old Italian variety 'Rosa Romana'. The area between 400 and 1000 m.a.l.s within the Reno Valley has been recognized as the traditional area for cultivation of the 'Rosa Romana' apple which is particularly widespread therein since the Roman times.

Unfortunately, the cultivation of this apple was abandoned for a long time and replaced by cultivation of conventional apple cultivars. 'Rosa Romana' is currently considered of great interest for promoting apple cultivation in the Apennines area in analogy with the model studies carried out on 'Annurca', the ancient apple variety of Naples (Melchiade et al., 2007; Iannaccone et al., 2007).

The recovery of surviving trees present in this territory is the first step for the conservation and valorization of such an old variety of apple germplasm (Sansavini et al., 2018; Bignami et al., 2001).

The analysis of the different fruit quality parameters for samples of 'Rosa Romana' showed no statistically significant difference with reference to the analyzed traits. The main morphological difference can be found between fruit samples collected in the plains compared to those collected in

areas of medium and high altitude (400-800 m.a.l.s.). Due to the higher altitude the fruits of the latter area present better-quality features, such as less russeting and a more over-colored expression.

At molecular level, the high discrimination power of the 15 SSR used suggests a good differentiation of Rosa apple accessions. The average number of alleles per locus was similar to the values reported by Liang et al., 2015 (in which the CH03G07 locus also resulted less polymorphic).

With the Cluster analysis most of the 47 accessions classified as 'Rosa Romana' were divided into two main clusters that share a high number of alleles. In both groups at least a reference accession from the apple germplasm collection of the University of Bologna ('Rosa Romana (strain 24)'(#41) and 'Rosa Romana Gentile (strain 43)'(#42) for the first cluster and 'Rosa Romana (strain A23)'(#43) for the second one) was included. In both clusters only a few accessions showed a limited number of polymorphic alleles, indicating probably the presence of mutations accumulated during the ages. The first cluster included most of the oldest trees which are phenotypically correspondent to the 'Rosa Romana' descriptions. The second group, on the other hand, showed a few differences at the phenotypic level, especially in the fruits of the accessions #33 and #34 of 'Rosa Romana'.

Furthermore, the 'Rosa Romana Gentile' and 'Rosa Nostrana' accessions presented different genetic profiles that created difficulties in defining the correct genotype.

Finally, data collected on fruits of other Rosa accessions ('Rosa Mantovana', 'Rosa d'Osta', 'Rosa d'Oliveto' and 'Rosa Marchigiana') confirmed the difference with the 'Rosa Romana' cultivar in relation to molecular data and the pomological descriptions.

These resulted evidenced that 'Rosa Romana' is an ancient genotype, propagated in the area of the Tuscan-Emilian Apennines since hundreds of years being well adapted to the different pedoclimatic environments that characterize this area. The adaptation of this genotype to specific agroclimatic conditions has created allele diversity within the samples collected.

The conservation of this variety implies the discrimination of the different accessions with very similar phenotype that are present in the original cultivation area (Sansavini et al., 2018). A certain

298 degree of genetic heterogeneity is acceptable for old varieties (Sansavini et al., 2018). Molecular
299 analysis with microsatellites demonstrated to be the most efficient approach for variety fingerprinting,
300 for recognizing incorrectly labeled material (homonymy and synonymy) and, consequently, for
301 preserving the original 'Rosa Romana' genotype.
302 The identification of the most adequate reference plants is a key step for setting up the correct
303 propagation of this old variety by nurseries and for defining a business plan for its re-evaluation and
304 promotion for a new market niche. In fact, the organoleptic characteristics of the 'Rosa Romana'
305 fruits are exalted in the Apennines environmental conditions. If it will be possible to adopt organic
306 cultivation techniques and control the production costs, in all likelihood 'Rosa Romana' can represent
307 a new opportunity of income for the farmers of the mountain areas.
308

309 **5 Conclusions**

310 In this study we assessed the phenotype and molecular diversity of 'Rosa Romana' accessions
311 collected in residual cultivation of this ancient variety. The area of these old trees was located in the
312 middle and upper Reno Valley in the Tuscan-Emilian Apennines.
313 SSR results evidenced the presence of two main groups of 'Rosa Romana' accessions corresponding
314 to genomic Cluster1 and Cluster2.
315 All these accessions produce fruits that, as listed in the descriptors, are attributable to the variety
316 'Rosa Romana'. This seems to be the effect of mutations that could be probably accumulated during
317 the centuries and that produced some allelic difference between the two clusters. Clusters 1 and 2
318 represent two clones of 'Rosa Romana' and, as consequence, this should be taken into account for a
319 proper identification of reference plants for setting up the nursery propagation activity and supporting
320 the protected variety name for the market.
321

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