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Morphological and Genetic Characterization of Local Maize Accessions from Emilia Romagna Region, Italy

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Abstract: Italian maize germplasm is particularly rich in local materials and each region is characterized by the presence of peculiar local varieties deriving from centuries of adaptation, selection and cultivation. While the introduction of hybrids, during the 1950s, led to the disappearing of many of these varieties, some have been maintained in cultivation by farmers, frequently in marginal areas, as a kind of family heritage. Local varieties were identified throughout field surveys carried out in recent years. The discovery of a traditional popcorn variety over the most common flint and semi-flint materials used for production of polenta was interesting. Since these varieties have never been adequately described and reported in scientific literature, this study was aimed to solve this lack of knowledge on recently discovered local maize populations. Characterization represents the first step of a process focused on the preservation and possible exploitation of important genetic resources. Traditional materials are a useful reservoir of genes for adaptation to local conditions and climate changes. Adequate breeding programs can use such germplasm for developing new and more resilient varieties. These local materials have been characterized at the morphological level highlighting plant, ear and kernel differences. Genetic characterization, carried out on 455 individuals by the use of 10 SSR markers, revealed 62 different alleles ranging from four for markers *phi127*, *phi076* and *phi084* to nine for marker *p-bnlg176*. The landraces are well distinguishable at genetic level since 40% of genetic variability is present among accessions. Five landraces are characterized by the presence of private alleles and heterozygosity levels are generally good. These findings support the possibility to correctly preserve local materials through in situ conservation. Phylogenetic analysis evidenced the presence of varietal clusters, the clearest one formed by three red-pigmented accessions. STRUCTURE analysis revealed that five landraces have a well-defined genetic attribution while the remaining two (EMR04-Mais Rosso di Rasora and EMR10-Mais del Principe di Scavolino) are both constituted by two different backgrounds.

Keywords: Italian Maize landraces; in situ conservation; SSR; characterization; biodiversity; Emilia Romagna region

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1. Introduction

In Italy, the presence of maize dates back to the first seed stocks that arrived at Cardinal Sforza in 1493 directly from the ships of Cristoforo Colombo [1], but around a century was necessary to establish the species as a commercial crop [1,2]. Over the centuries, several maize samples were introduced, crossed, and adapted to the different environmental

conditions and cultures of the peninsula, developing a relevant number of landraces to define Italy as a secondary center of maize diversification [1–6]. Landraces are defined as dynamic populations of genetically diverse individuals well adapted to local conditions, associated with traditional agricultural practices and local history; moreover, they can be considered the ultimate expression of crop domestication [7]. For these reasons, landraces represent a large source of genes and traits for future breeding programs, especially in maize, where commercial hybrids account only for 5% of species biodiversity [5].

Unfortunately, since the end of the Second World War, maize landraces have experienced heavy losses as a consequence of the introduction of hybrid varieties and almost disappeared from agricultural fields [1,3,8]. Estimates consider that around 95% of agrobiodiversity has been lost in northern Italy, while 73% has been lost in the south of the peninsula [8,9]. The possibility to find new corn landraces, in isolated farms, is well-reported for Italy [2,6,8,10] as well as for other countries [4,11,12]. Giupponi et al. [8] performed an extensive search across databases of traditional genetic resources in Italy and identified inland hilly and mountainous areas as the richest places for the presence of traditional varieties. With respect to Emilia Romagna, a hotspot of biodiversity can be identified in the mountains at the border of the Provinces of Parma and Reggio Emilia with Tuscany [8,13] which overlaps with the MaB reservoir of UNESCO (http://www.unesco.it/it/RiserveBiosfera/Detail/93 (accessed on 10 August 2021)).

In Italy, the census of landraces present on the territory is mainly held at the Agrobiodiversity National Register of the Italian Ministry of Agriculture and Forestry in addition to other sources as Universities, Botanical Gardens, and Germplasm collections, as outlined by several authors [8,9,14]. Several Italian regions have organized programs for the recovery, preservation, and valorization of agrobiodiversity. Emilia Romagna established a catalogue called "Repertorio regionale delle risorse genetiche agrarie" with the Regional Law 1/2008. The catalogue is the instrument by which the Region protects agricultural biodiversity by registering and cataloguing local varieties. Concerning corn, presently, a single local variety is included (Mais di Santa Sofia Romualdi), but this variety has never been deeply described morphologically or genetically. Recently, six new local varieties, characterized by different grain and ear traits, still grown mainly as familiar heritage, have been discovered in different mountain areas of the Region.

The identification of germplasm resources requires a proper description and classification. Traditional maize is classified according to morphological characters [1], while recently, germplasm characterization has been based on molecular markers. Among molecular markers, Simple Sequence Repeats (SSRs) are the most used and have been widely applied to maize germplasm collections [4,5,15–18], allowing the construction of phylogenetic trees, investigating genetic structure, and resolving cases of homonymy [2].

The six local varieties recently rediscovered in cultivation, and the one already present in the Regional biodiversity catalogue, have never been properly described. The absence of adequate germplasm description represents a gap of knowledge both in understanding the genetic relationships between different landraces, and their genetic structure and in exploiting them for commercial purposes. Consequently, to fill this gap of knowledge, the objective of the study was to characterize these seven landraces from a morphological and genetic point of view to assess the conservation status of these materials, to verify the effectiveness of in situ conservation in maintaining a wide genetic base, to elucidate eventual genetic relatedness, and to provide new information regarding local maize biodiversity.

2. Materials and Methods

2.1. Germplasm

Seven maize accessions were identified in the Emilia Romagna region within the framework of the RICOLMA project: EMR01 "Mais Tagliolino di Vetto", EMR03 "Mais Cinquantino Rosso di Ramiseto", EMR04 "Mais Rosso di Rasora", EMR06 "Mais da Scoppio di Casola Valsenio", EMR07 "Mais di Santa Sofia Romualdi", EMR10 "Mais del Principe

di Scavolino", and EMR13 "Mais Piacentino di Coli". Detailed information on sampling location are reported in Table 1.

Table 1. Detailed information about maize germplasm used in this study.

Accession	Accession Name	Sampling Location	Plant Height (cm)	Ear Height (cm)	Tasseling (GDD)	Physiological Maturity (GDD)
EMR01	Mais Tagliolino di Vetto	Vetto (Reggio Emilia, Italy)	232	114	692	1446
EMR03	Mais Cinquantino Rosso di Ramiseto	Frazione Ramiseto, Ventasso (Reggio Emilia, Italy)	222	111	646	1446
EMR04	Mais Rosso di Rasora	Frazione Rasora, Castiglione dei Pepoli (Bologna, Italy)	190	93	677	1430
EMR06	Mais da Scoppio di Casola Valsenio	Casola Valsenio (Ravenna, Italy)	210	82	739	1398
EMR07 *	Mais di Santa Sofia Romualdi	Santa Sofia (Forlì-Cesena, Italy)	200	70	618	1349
EMR10	Mais del Principe di Scavolino	Pennabilli (Rimini, Italy)	226	73	593	1430
EMR13	Mais Piacentino di Coli	Frazione Fontana, Coli (Piacenza, Italy)	240	90	646	1430

* EMR07 is already present in the Repertorio regionale delle risorse genetiche agrarie.

The accessions are maintained at the germplasm bank of the Department of Sustainable Crop Production of Università Cattolica del Sacro Cuore, Piacenza (Italy), and at the Plant Germplasm Bank of the Department of Earth and Environmental Sciences of Università degli Studi di Pavia, Pavia (Italy).

The field trial was located at CREI-CERZOO (45.005066°N, 9.704206°E, San Bonico, Piacenza, Italy) and sown on 27 April 2018. Each plot consisted of six rows 5 m long, spaced 80 cm apart each row, and 1 m aisle on the hedge; 25 seeds were planted in each row. The field was managed according to agricultural practices for maize nurseries. Leaf samples were collected from all plants at the fifth leaf stage. Maize accessions were phenotyped according to the UPOV protocol CPVO/TP2/3. Flowering and maturity were collected as days after sowing and then converted to Growing Degrees Days (GDD) as

$$\sum_{i}^{n}[(Tmin + Tmax)/2] - 10 \quad (1)$$

where i is the sowing day, n is the day of flowering or physiologic maturity, and Tmin and Tmax are the minimum and maximum daily temperature. Daily temperatures below 10 °C or over 30 °C were substituted by the cardinal temperature for maize growing (10 and 30 °C, respectively), [19].

2.2. DNA Extraction and PCR Amplification

DNA was extracted from young leaf tissues according to the "96-Well Plate Plant Genomic DNA Miniprep Kit" (BIO BASIC Europe s.r.l, Milano, Italy) following the manufacturer's instructions with the minor modifications reported in Stagnati et al. [2]. The extracted DNA was visualized on 1% agarose gel electrophoresis stained with Midori Green (Nippon Genetics Europe, Düren, Germany). Globally, 455 single plants were analyzed: 32

for both EMR01 and EMR13, 90 for EMR03, 89 for EMR04, 77 for EMR06, 61 for EMR07, and 74 for EMR10.

Ten SSR markers were selected from Palumbo et al. (2017) and the Maize Genome Database (MaizeGDB, https://www.maizegdb.org/ (accessed on 26 January 2018)). Detailed information on primer pairs is reported in Table 2. PCR reactions were carried out in a final volume of 25 µL; the PCR mixture was composed according to Stagnati et al. [2]. The PCR cycle consisted of initial denaturation at 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 1 min, annealing at optimal primer temperature as reported in Table 2 for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 2 min. PCR reactions were carried out using GeneAmp 2700 thermocycler (Applied Biosystem, ThermoFisher Scientific, Monza, Italy).

Table 2. Detailed information about primers pairs used in this study. For each microsatellite locus, marker name, locus name, forward and reverse primer sequences, linkage group (LG), annealing temperature (Ta), and amplicon size in bp are reported.

Marker Name	Locus	Forward Primer 5′-3′	Reverse Primer 5′-3′	LG	Ta (°C)	Size (bp)	Reference
M302	*phi127*	ATATGCATTGCCTGGAACTGGAAGGA	[VIC] AATTCAAACACGCCTCCCGAGTGT	2	58	100–120	Palumbo et al., 2017
M304	*phi076*	TTCTTCCGCGGCTTCAATTTGACC	[6FAM] GCATCAGGACCCGCAGAGTC	4	58	150–200	Palumbo et al., 2017
M306	*phi031*	GCAACAGGTTACATGAGCTGACGA	[PET] CCAGCGTGCTGTTCCAGTAGTT	6	58	180–220	Palumbo et al., 2017
M308	*umc1075*	GAGAGATGACAGACACATCCTTGG	[6FAM] ACATTTATGATACCGGGAGTTGGA	8	58	130–150	Palumbo et al., 2017
M310	*phi084*	AGAAGGAATCCGATCCATCCAAGC	[PET] CACCCGTACTTGAGGAAAACCC	10	58	140–170	Palumbo et al., 2017
M24	*umc1327*	AGGGTTTTGCTCTTGGAATCTCTC	[NED] GAGGAAGGAGGAGGTCGTATCGT	8	64	100–120	MaizeGDB
M33	*p-bnlg176*	AGTTCACGTCCAGCTGAATGACAG	[6FAM] CGCGCATCGCATGCTTATCCTA	1	62	140–170	MaizeGDB
M78	*umc1941*	ACGACGAGACTCTGTTCTGGTTCT	[NED] AGGAGGATTACGTCAATCTGTTCG	5	64	110–130	MaizeGDB
M90	*umc1401*	CTCTGGTCCATCCTCATCGACT	[PET] TCTCTTGATCACATATCGATCCCA	7	62	180–200	MaizeGDB
M193	*umc1786*	ACCGTGACTTCCTCCTCATAACTG	[VIC] CATTTTTCGCATTTAGGAAATCCA	8	60	180–220	MaizeGDB

Fluorescent-labeled PCR fragments were visualized using an automated genetic analyzer ABI-Prism 3130 (Applied Biosystem, ThermoFisher Scientific, Monza, Italy) according to the manufacturer's instructions and manually scored.

2.3. Molecular Marker Data Analysis

Detected alleles were analyzed with the GenAlEx6 software [20] to compute population statistics, analysis of molecular variance (AMOVA), and Principal Coordinates Analysis (PCoA) according to default parameters. The Polymorphic Information Content (PIC) was calculated with PowerMarker software, version 3.25 [21] according to the formula already implemented in the software.

A phylogenetic tree was constructed using the Unweighted Pair Group Method with Aritmetic mean method applying the *upgma* function of the phangorn package [22] starting from a genetic distance matrix calculated by the *meandistance.matrix* available in the polysat [23] package of the R software.

Population structure of the maize collection has been examined using a Bayesian clustering algorithm implemented in STRUCTURE v.2.3.4 [24]. The "admixture model" and the "correlated allele frequency model" were selected as suggested [18,24]. Ten independent replications were run for each level of K ranging from 2 to 20 with a burn-in of 2×10^5 and 10^6 Markov Chain Monte Carlo replications. The best estimation of K was selected according to the method of Evanno [18,25].

3. Results and Discussion

Historical and Morphological Characterization of Germplasm

Morphological characters were measured and used to describe accessions; plant descriptors are reported in different active sheets of Supplementary Table S1, and ear figures are reported in Supplementary Figure S1. Historical information was retrieved by direct interviews with germplasm donors and their family.

Mais Tagliolino di Vetto (EMR01) was sampled in 2015 in Vetto (Province of Reggio Emilia, Italy). The landrace is characterized by a medium height of 232 cm, and the first ear is inserted at 114 cm with a medium ear/plant insertion ratio. Tasseling, silking, and physiological maturity occur at 692, 739, and 1446 GDD (comparable to FAO300), respectively. Tassel (glumes, glume ring, and anthers) are of absent-weak pigmentation, while silks are not pigmented. Ears are slightly conical, 15–18 cm long and with 14–18 rows of yellow dent-like kernels. The cob is red. Interesting characters of this landrace are the small angle of leaf insertion and resistance to lodging. This landrace is used for human consumption.

Mais Cinquantino Rosso di Ramiseto (EMR03) was found in Ramiseto (Ventasso, province of Reggio Emilia) with a local farmer still growing this corn for the production of polenta, a traditional dish of northern Italy. The cultivation re-started in 2016 from an ear stored in a cupboard, and the landrace has been present in the donor's family for at least three generations and has not been recently distributed to other farmers. The population is constituted by plants of more than 200 cm height and ear inserted midway on the stalk. Tasseling, silking, and physiological maturity occur at 646, 677, and 1446 GDD (comparable to FAO300). The tassel reveals some kind of pigmentation at glumes and anthers, and silk color varies from absent to medium. The ear is short and slightly conical with 12–16 rows of flint-like kernels from orange-red to red color and even the cob is pigmented. EMR03 has good leaf insertion and lodging resistance.

Mais Rosso di Rasora (EMR04) was recovered in Rasora (Castiglione dei Pepoli, province of Bologna) in 2015. According to memories, it was present at least from the period between the two world wars. At that time, yellow corn was also sown and was preferred to the red one for higher production. However, the Mais Rosso was able to grow where the yellow corn was not, being sometimes intercropped with beans. The cultivation was reintroduced from seed saved from a family migrated to Pistoia (Tuscany) in the 1960s. Plants are 190 cm tall with intermediate ear/plant ratio. Tasseling, silking, and physiological maturity occur at 677, 755, and 1430 GDD (comparable to FAO300), respectively. Anthers have a medium color, while silks are faint or not pigmented. Ears are slightly conical with 12–16 rows of intermediate kernels. Kernel color is variable from orange-red to dark red, and the cob is red too. In the area of origin, the color was more intense than in the nursery field. Good characteristics are the insertion angle, attitude of leaves, and resistance to lodging.

Mais da Scoppio di Casola Valsenio (EMR06) is also called "frumentino bianco" or "frumentin" by the donor family. The presence of the accession dates back to the mid-1930s in the memories of the grandmother's donor. This maize was grown in the past for self-consumption and production of pop-corn [9]. Traditionally, kernels were placed on the hot stones of the fireplace until popping. This landrace is characterized by 210 cm tall plants and ears inserted with a small ear/plant insertion ratio. Tasseling and silking occurred at 739 GDD, while physiological maturity occurred at 1398 GDD. Ears are very short, around 11 cm, with 16–18 white-yellowish kernel rows. This accession is the only popcorn available, to our knowledge, as a traditional landrace in the region. Popped kernels have a butterfly shape. According to Brandolini [3] this landrace could be classified as a "Risiforme" (rice shape of kernels). Cobs are white. The landrace is characterized by tillering ability, and tillers are able to develop full plants and produce ears. The main stem could bear 2–3 ears.

Mais di Santa Sofia (EMR07) has been the heritage of the family Romualdi for around 80 years, and it has been multiplied yearly mainly for family consumption. The original seed stock may derive from the village of Ridracoli, where the Romualdi family lived until

1936. At present it is cultivated for polenta preparation for local agritourism. The plants are 200 cm tall with ears inserted at 70 cm. Tasseling, silking, and physiological maturity occur at 618, 677, and 1349 GDD (earlier than FAO300), respectively. Silks have a weak coloration. Ears are of a distinctive conical shape with 12–16 kernel rows, of yellow-orange color and intermediate/dent-like type. The cob is white. A rustic plant particularly suited for areas with short favorable season that is now grown around 1000 m above sea level.

Mais del Principe di Scavolino (EMR10) was rediscovered in the 1990s thanks to a school project in Pennabilli (Rimini, Italy), aimed at the preservation of local product and traditions at risk of extinction. The center of origin of this corn is the basin of Scavolino at 800 m above sea level (in the municipality of Pennabilli). According to memories the cultivation of this landrace dates back between the two world wars, and the donor has been actively cultivating this landrace since 1979. The name of the accession is in homage to the family of Conti di Carpegna, which held the princedom of Scavolino until 1819. Plants are 226 cm tall with the primary ear inserted at 73 cm on the stalk. Tasseling, silking, and physiological maturity occur at 593, 645.5, and 1430 GDD (comparable to FAO300), respectively. Ears are cylindrical with 10–12, sometimes 14, kernel rows. Seeds are flint-like/intermediate and variable in color from yellow-orange to red, even though some farmers are selecting for red, dark-red pigmentation. The cob is white. The rustic plant is able to grow in hilly-mountain areas, with good lodging resistance, but has an unfavorable leaf insertion angle.

Mais Piacentino di Coli (EMR13) was collected by a local farmer in Coli in 2016. The accession has been grown only for familiar purposes for at least ten years. The donor received the original seed stock from another farmer of the same locality. Plants can reach 240 cm, but ears are inserted low on the plant (90 cm). Tasseling, silking, and physiological maturity occur at 646, 692, and 1430 GDD (comparable to FAO300), respectively. Ears are slightly conical with 14–18 rows of flint/flint-like kernels of orange-red color, but the cob is white.

4. Genetic Characterization of Accessions

Germplasm characterization has been performed using SSR markers because, as reported in many previous studies [2,15,18,26], SSRs are considered the marker of choice in biodiversity analysis because they have a codominant nature, they are highly polymorphic, and their analysis and interpretation can be easily automated [27].

The markers data of all the 455 samples were collected and analyzed to investigate the main population parameters. Globally, 62 different alleles were detected ranging from four for markers *phi127, phi076,* and *phi084* to nine for marker *p-bnlg176*, with a mean of 6.2 alleles per locus. The number of alleles detected in this germplasm is interesting, especially if compared with other maize landraces characterizations, which are considered a higher number of landraces and/or SSR [4,11]. This number of alleles may be a consequence of the heterogeneity of the collection itself and of the reproductive isolation experienced by these materials in the recent past, which probably allowed the preservation of the ancestral genetic constitution. All loci were polymorphic in the populations with the exception of *umc1075*, *umc1327*, *p-bnlg176,* and *umc1941* in EMR01, while *phi127* and *umc1327* were monomorphic in EMR13. The presence of highly polymorphic loci is consistent with the allogamous nature of maize and with the multi-genotype constitution of landraces. A high level of polymorphisms is reported in similar studies, even if the presence of some monomorphic loci in the entire collection or in some accessions seems to be common [2,11,18,28]. It is not possible to exclude that monomorphic loci are a consequence of the selection for particular characters appreciated by farmers. In the whole collection, 15 private alleles were detected as reported in Table 3: five for EMR04 (markers *umc1327*, *umc1786*, *p-bnlg176,* and *umc1401*); three in EMR07 and EMR13 (markers *phi031*, *umc1327*, and *umc1786* and *markers p-bnlg176* and *umc1075*, respectively), two in EMR03 and EMR10 (markers *umc1327* and *umc1786* and markers *phi076* and *phi084*, respectively). The percentage of private alleles over total alleles is intermediate between data previously

reported by other authors and possibly useful to develop methods for molecular food traceability [2,18]. The number of observed alleles (N_a) ranged from a minimum of 2.43 of *phi084* to 4.00 of *phi031*, *umc1075*, *umc1786,* and *p-bnlg176* at locus level, and from 1.8 for EMR01 to 4.3 for EMR04. The number of expected alleles (N_e) was always lower than N_a ranging from 1.46 for *phi084* to 2.45 for *umc1075* at the locus level and from 1.63 to 2.32 in EMR01 and EMR03 at the landrace level (Table 4). The Shannon's index (I) was used to characterize population diversity, and it was found to be, on average, equal to 0.76 ± 0.05 over all loci and populations (Table 4). Mean values of the observed (H_o) and unbiased expected (uH_e) heterozygosity were, across loci and landraces, equal to 0.42 ± 0.03 and 0.44 ± 0.03, suggesting a reduction in heterozygosity. The highest lack in observed heterozygosity was detected for markers *umc1786* (−0.15) and *umc1075* (−0.14) and in accessions EMR13 (−0.08), EMR01, and EMR07 (−0.06); however, these defects of heterozygosity are not particularly relevant. Similar works on corn landraces report a general lack of heterozygosity both at the locus and population levels, because smallholder growers usually renew seed stocks starting from few ears, and no precautions are taken to avoid self-pollination [2,15,18,29]. The landraces of the present study are actually cultivated on discrete surfaces for family consumption or even commercial purposes. It is interesting to note that the highest deficit in observed heterozygosity was present in EMR13, which is the less-cultivated variety among the considered ones (Table 4). A similar situation was also observed in previous studies with Mais Dencin della Martesana [2]. We can hypothesize that the cultivation of few plants leads to a reduction of genetic variability and increment of homozygosity caused by an increased selfing rate.

Table 3. List of private alleles detected in the landrace collection.

Landrace	Locus	Allele	Allele Frequency
EMR03	*umc1327*	82	0.105
EMR03	*umc1786*	148	0.006
EMR04	*umc1327*	80	0.006
EMR04	*umc1786*	144	0.012
EMR04	*p-bnlg176*	126	0.017
EMR04	*p-bnlg176*	128	0.040
EMR04	*umc1401*	157	0.006
EMR07	*phi031*	192	0.050
EMR07	*umc1327*	74	0.008
EMR07	*umc1786*	124	0.009
EMR10	*phi076*	153	0.042
EMR10	*phi084*	156	0.021
EMR13	*umc1075*	120	0.016
EMR13	*p-bnlg176*	132	0.031
EMR13	*p-bnlg176*	147	0.188

The inbreeding coefficient F_{IS} had average values of 0.03 ± 0.07 and 0.05 ± 0.12, supporting the absence of inbreeding and confirming the random-mated nature of the collection and that there is no particular lack of heterozygosity with SSR loci closed to Hardy–Weinberg equilibrium [2,29]. F_{ST} was equal to 0.30, suggesting that these landraces are characterized by a good level of genetic differentiation among the population since around 30% of genetic variation was found between varieties, especially if compared to other maize landrace collections [11,18,28,29]. Average PIC value was 0.57 ranging from 0.38 of *phi084* to 0.75 of *umc1075*. PIC provides an estimation of diversity since it evaluates the ability of a certain SSR in discriminating different genotypes. PIC values of these landraces are consistent with other studies [30,31], while higher [4,5] or lower PIC values are reported [11,18].

Table 4. Genetic parameters calculated according to the ten SSR and seven landraces object of the study. Average number of observed alleles (N_a), effective number of alleles (N_e) per locus, Shannon index (I), observed (H_o) and unbiased expected (uH_e) heterozygosity, polymorphism information content (PIC), and Wright's inbreeding coefficient F_{IS}, F_{IT}, F_{ST}, and gene flow (N_m) are reported.

Locus	N_a	N_e	I	H_o	uH_e	PIC	F	F_{IS}	F_{ST}	N_m
phi127	3.00	1.88	0.68	0.41	0.41	0.51	−0.02	−0.02	0.30	0.60
phi076	2.71	2.03	0.79	0.49	0.51	0.53	0.04	0.04	0.16	1.33
phi031	4.00	2.10	0.84	0.39	0.46	0.59	0.13	0.15	0.30	0.60
umc1075	4.00	2.45	0.95	0.39	0.53	0.75	0.28	0.25	0.34	0.48
phi084	2.43	1.46	0.44	0.28	0.27	0.38	0.05	−0.03	0.41	0.36
umc1327	3.14	1.89	0.69	0.49	0.40	0.50	−0.25	−0.25	0.19	1.07
p-bnlg176	4.00	2.11	0.89	0.36	0.50	0.59	0.29	0.28	0.28	0.63
umc1941	4.00	2.38	0.93	0.51	0.50	0.72	−0.01	−0.03	0.34	0.49
umc1401	3.57	2.12	0.79	0.61	0.44	0.6	−0.33	−0.38	0.28	0.63
umc1786	3.57	1.68	0.60	0.24	0.34	0.55	0.23	0.28	0.46	0.29
All loci	3.44	2.01	0.76	0.42	0.44	0.57	0.05	0.03	0.31	0.65
dev.st	0.16	0.09	0.05	0.03	0.03	0.11	0.04	0.07	0.03	0.10
EMR01_Tagliolino	1.80	1.63	0.45	0.24	0.30		0.17	0.19	0.52	0.23
EMR03_Cinquantino_Rosso	4.20	2.32	0.95	0.49	0.53		0.11	0.09	0.15	1.46
EMR04_Rasora	4.30	2.28	0.93	0.58	0.52		−0.09	−0.11	0.17	1.25
EMR06_Mais_da_Scoppio	3.60	2.14	0.80	0.48	0.46		−0.04	−0.04	0.27	0.68
EMR07_Santa_Sofia	3.90	1.80	0.72	0.34	0.40		0.19	0.15	0.36	0.44
EMR10_Principe_di_Scavolino	3.80	2.18	0.90	0.53	0.51		−0.05	−0.05	0.19	1.09
EMR13_Piacentino	2.50	1.72	0.56	0.26	0.34		0.14	0.23	0.46	0.29
All pop	3.44	2.01	0.76	0.42	0.44		0.05	0.05	0.30	0.58
dev.st	0.16	0.09	0.05	0.03	0.03		0.04	0.12	0.14	0.46

5. Cluster Analysis and Phylogenetic Tree

Principal coordinate analysis (PCoA) separated the landraces into two distinct groups (Figure 1). The two first principal components account for 15.06% and 10.57% of genotypic variability, leaving much variation unexplained. The low resolution of PCoA can be explained considering the high intra-population genetic variability. This finding is in agreement with previous studies [2,18]. The first group of landraces is composed by EMR06 and EMR07, which are clearly clustered apart. The remaining landraces formed a bigger cluster where EMR10 seems to be the most distinguishable while EMR01, EMR03, EMR04, and EMR13 are partially overlapped. The separation of EMR06 and EMR07 may be explained by the fact that these two materials are quite different from the other landraces: EMR06 is a white popcorn, while EMR07 is characterized by conical ears and can be ascribed to the "Conici" group [1]; moreover, it is maintained in the mountains in reproductive isolation. The other landraces were more similar at ear level and putatively ascribed to the "Ottofile derivati" (EMR10) or "Microsperma vitrei" (EMR04 and EMR03); EMR01 is included in the dent-type varieties, while EMR13 is of uncertain attribution, with intermediate characters between Ottofile derivati and Microsperma vitrei. Considering maize reproduction habitus and the diffusion of the crop in the region, we cannot exclude a past event of intercrossing between different materials, giving the origin of the actual accessions.

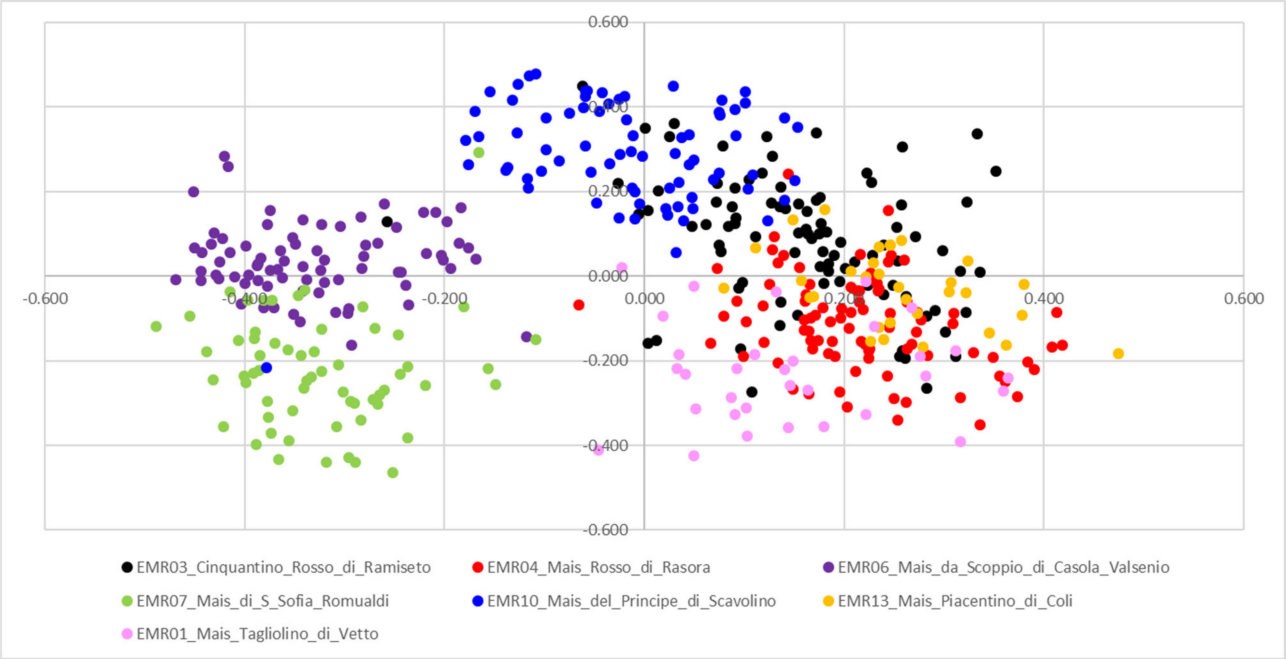

Figure 1. Principal coordinates analysis (PCoA): Coordinate 1 vs. Coordinate 2 of the 455 samples characterized by the 10 SSR set.

In PCoA analysis computed according to landrace assignment, as reported in Figure 2, the percentage of cumulate genotypic variation rose to 59.88%. In this case, the separation among landraces is clearer and, consistently with the PCoA of individuals, EMR06 and EMR07 are well separated in the same part of the graph, while EMR03, EMR04, and EMR10 are very close together, particularly the former two. Nei's unbiased genetic distance (uNei, Table 5) confirms the findings that EMR03 and EMR04 are genetically similar (uNei = 0.17), having also the lowest pairwise F_{ST} (0.074), while EMR07 and EMR13 are the most differentiated (uNei = 0.869 and pairwise F_{ST} = 0.321). Despite being clearly distinguished (Figure 2), EMR13 and EMR01 showed a low uNei (0.253), meaning that they could be more related, as further discussed. The pattern in Figure 2 can be explained considering that EMR06 and EMR07 were the most unique types; EMR03, EMR04 and EMR10 are pigmented varieties; and EMR01 and EMR13 are yellow/orange types from the western part of the region.

The AMOVA analysis revealed a very high level of differentiation between landraces since 41% of variability is among accessions and 59% within accessions. This value is very high if compared to similar researches [2,18,29], while it is reported to be similar for corn landraces from Switzerland or Former Yugoslavian Territories [4,28]. As already mentioned, such high differentiation may derive from (i) the reproductive isolation experienced by these materials for long time; (ii) the presence of a popcorn landrace, belonging to a completely different maize typology with genetic barriers to other maize [3]; or (iii) cultural and geographical separation from eastern and western part of the region where these materials have been maintained.

The phylogenetic tree of individuals, computed by the UPGMA function, revealed a clear clusterization of samples according to each landrace assignment. The tree, reported in Supplementary Figure S2, is clearly distinguished in two main branches (I and II).

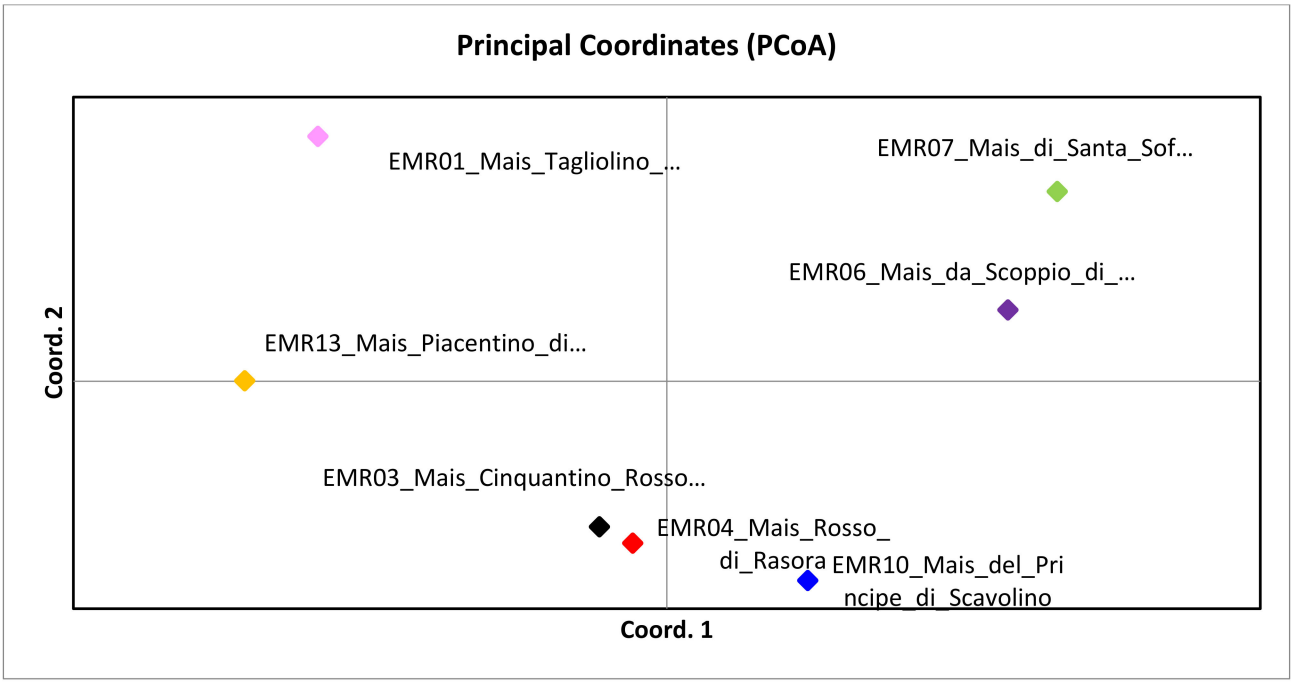

Figure 2. Principal coordinates analysis (PCoA) according to different populations.

Table 5. Summary of Nei's unbiased genetic distance between the landraces examined in the present study.

EMR03	EMR04	EMR06	EMR07	EMR10	EMR13	EMR01	
0.000							EMR03
0.170	0.000						EMR04
0.473	0.588	0.000					EMR06
0.537	0.609	0.395	0.000				EMR07
0.213	0.472	0.503	0.514	0.000			EMR10
0.270	0.457	0.742	0.869	0.542	0.000		EMR13
0.359	0.505	0.677	0.618	0.664	0.253	0.000	EMR01

Clade I is subsequently divided into three main subclades: the majority of individuals belonging to EMR04 and EMR03 are located on subclade Ia and, even if some of them are dispersed, a good separation between Mais Rosso di Rasora and Cinquantino Rosso di Ramiseto is present. Moreover, a core-set of plant of each of these landraces is identifiable on an independent branch of the subclade.

Plants belonging to EMR01 and EMR13 are clearly clustered into Subclade Ib while individuals of Mais del Principe di Scavolino (EMR10) are located on the subclade Ic. Clade II, which can be divided into Subclades IIa and IIb, locates plants of the Mais da Scoppio di Casola Valsenio and Mais di Santa Sofia Romualdi, respectively, on Subclades IIa and IIb. This distribution of samples is confirmed also by a different phylogenetic tree, shown in Figure 3, which has been computed using distances between populations instead of individuals. The different analyses of PCoA, phylogenetic trees, uNei and pairwise F_{ST} confirm a closed relationship between Mais Rosso di Rasora and Cinquantino Rosso di Ramiseto and between Mais Piacentino di Coli and Mais Tagliolino.

Summarizing the various clusterizations, the relatedness of EMR01–EMR13 and EMR03–EMR04–EMR10 is confirmed by both the phylogenetic tree (Figure 3) and PCoA (Figure 2). The proximity among EMR06–EMR07 requires further investigations. Regarding EMR06, very little is known about Italian popcorns and, in the maize sampling of 1954, they had only marginal consideration. To our knowledge, this is the first genetic study considering an Italian popcorn landrace. The presence of high intra accession variability

may explain the low resolution of PCoA (Figure 1), while EMR06–EMR07 are more differentiated in Figure 2, as suggested by plant morphology. An overlapping samples distribution is present also in Supplementary Figure S2, with the only difference of EMR10, which separates early from the group of pigmented varieties. The "Ottofile derivati", to which EMR10 has been attributed, derive from the cross of "Ottofile" and other maize types [1].

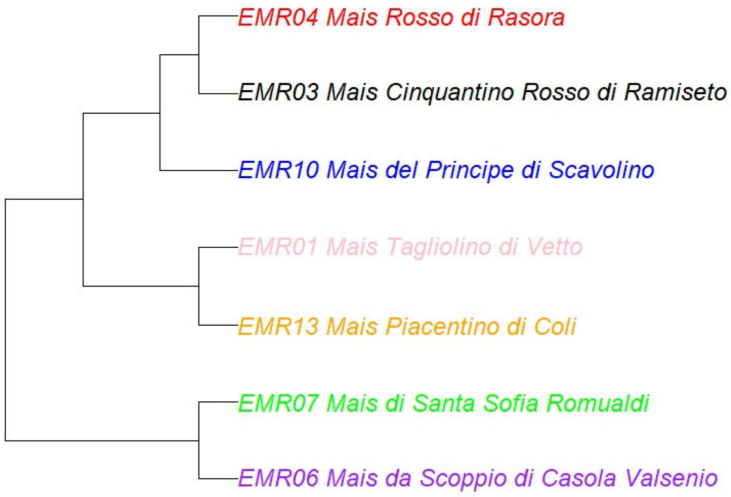

Figure 3. Phylogenetic tree of the seven maize landraces obtained by the UPGMA method on a genetic matrix derived from SSR data.

6. Structure Analysis

Population structure was investigated using the STRUCTURE software and the procedure of Evanno [25] was followed to determine the best level of K. The highest ΔK was found at K = 9 (ΔK = 127.89). STRUCTURE analysis clusterized the 455 individuals into nine genetically distinct clusters, as reported in Figure 4. The clustering of genotypes found that 265 out of 455 individuals (58.2%) showed a strong ancestry association (>90%) with one specific cluster. In details, 33% of individuals of EMR03 showed a high association with Cluster 1 while two plants were associated with Clusters 3 and 6; EMR04 revealed 43.8% and 10% of plants associated with Clusters 6 and 3, respectively. The popcorn EMR06 showed 67% of samples associated with Cluster 8; 78.7% of EMR07 plants were strongly associated with Cluster 5, while EMR10 showed similar associations with Clusters 2 and 4 with 21.6% and 17.6% of individuals, respectively. EMR13 was associated with Cluster 7 with 81.2% of plants, while 78.1% of EMR01 individuals were associated with Cluster 9. Admixed genotypes were considered with respect to the main cluster of each landrace and in the case of an association lower than 80% [18]. EMR04 and EMR03 presented the highest number of admixed genotypes (48% and 38.2%, respectively), while EMR01 and EMR13 revealed only 3% of admixed individuals. The EMR06 and EMR07 landraces were intermediate with 13% and 8.2% of admixed plants. In the case of EMR10 (Mais del Principe di Scavolino), 17.6% and 14.8% of individuals were considered admixed to Clusters 2 and 4 (level of assignation to a cluster between 0.5 and 0.8). Cluster 3 is characterized by the presence of a high number of individuals (90.3%) with assignation level to this cluster $\leq$0.3, and only 11 plants (2.5% of total) have assignation $\geq$0.9: of these, nine are of EMR04 and two are of EMR03. The presence of individuals of only these two landraces with good assignation to this cluster, and the moderate pairwise F_{ST} [12,32], suggest the presence of a certain kind of past relatedness between EMR03 and EMR04. Seventeen plants were considered as not assigned to any particular group having cluster assignation lower than 0.5: nine of EMR03, four of EMR10, and three and 1 of EMR04 and EMR06, respectively.

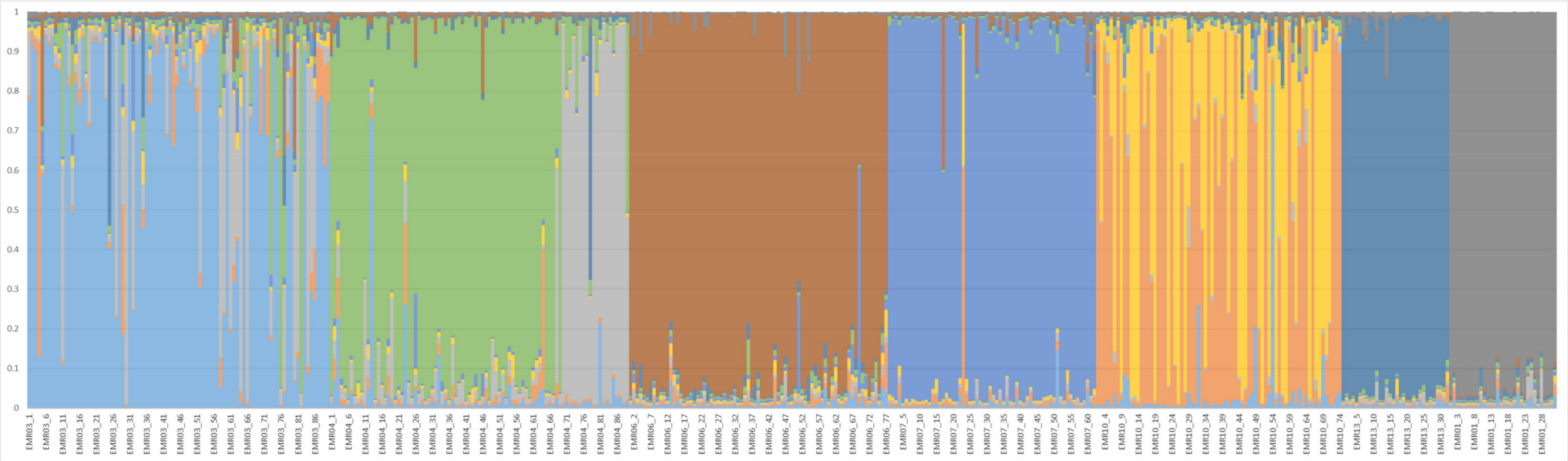

Figure 4. Population genetic structure of the seven maize accessions of Emilia Romagna as estimated by STRUCTURE. Each sample is represented by a vertical histogram partitioned in K = 9 colored segments representing the membership to each of the identified ancestral populations. The ancestry proportion (%) is reported on the *y*-axis.

What emerged from this analysis is a clear genetic differentiation of the popcorn EMR06, the conical EMR07 and the dent corn EMR01, which are the only representative of their main cluster. A certain kind of relatedness and probably gene flow or common origin between EMR03 and EMR04 was observed, as confirmed by the mixture of individuals in the phylogenetic tree, the low values of uNei, and proximity in the populations' PCoA. In the case of EMR10, it is possible to suppose that, according to STRUCTURE analysis, there is the presence of two distinct subpopulations that are actually merging or dividing during each cycle of reproduction. At the timing of collection, and with subsequent interviews with donor and landrace growers, a strong variability for kernel color was observed from yellow to dark-red, and some growers are actually performing a mass selection for kernel color according to their preferences. Strong color segregation is also reported in a famous Italian landrace as "Nostrano di Storo". Here, even though farmers are selecting, there is not the presence of sub-populations. The case of Storo is different because, in Storo, maize fields are adjacent to one another, and pollen exchange is allowed between fields and farmers' selections [33]. In the case of EMR10, growers of Valmarecchia (the area of Scavolino) are scattered on a wide territory, fields are small and isolated, and therefore genetic flux and pollen exchange is quite unlikely among different landrace strains. Further investigations with the analysis of seed stocks from several farmers will be required for the EMR10 landrace.

7. Conclusions

The seven maize accessions assessed in the present study were clearly distinguishable both at morphological and genetic levels. Pigmented maize such as EMR03, EMR04, and EMR10 presented a certain kind of relatedness as revealed by different analysis. Moreover, EMR04 and EMR10 seem to be constituted by two different subpopulations, as revealed by STRUCTURE analysis. The proximity of the popcorn EMR06 to the conical corn EMR07 requires further investigation as well with other maize landraces since Italian popcorns have never been studied until now. Globally, good levels of genetic diversity and heterozygosity have been evidenced for all landraces. The seven accessions presented high degrees of inter-accession (41%) and intra-accession (59%) genetic variability. These findings confirm that, in hilly and mountain areas of the region Emilia Romagna, and probably in also the other Italian regions, it is still possible to find in situ conserved materials and, even if conservation has been performed by unskilled people, the germplasm has been properly maintained. This material has also been propagated ex situ, and these seeds can be useful not only for research or breeding purposes but also for bringing them back in cultivation in small local production chains.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/su14010091/s1. Figure S1: full ears, ear sections, kernels and cobs of the seven maize accessions examined in the present study. Figure S2: phylogenetic tree of single plants of the seven maize accessions examined in the present study. Table S1: plant descriptors of the seven maize accessions examined in the present study.

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