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The Rediscovery of Traditional Maize Agrobiodiversity: A Study Case from Northern Italy

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Abstract: Nowadays, agriculture is under the pressure of climate change and new pathogen outbreaks while farmers are requiring breeders to develop more resistant and resilient genotypes. The genetic base for breeding may be increased through appropriate conservation, description and characterization of local varieties and germplasm collections that have never been used in breeding, and which could be sources of useful alleles. In this framework, the present paper focuses on eight maize landraces of the eastern part of Emilia-Romagna, derived from the Italian maize collection sampled in 1954. Landraces are characterized by a short cycle length and different kernel types—mainly flint-like or an intermediate type of yellow or yellow–orange color—while dent landraces are less represented. Pigmented and white corns are absent even though one landrace (Va213) showed the presence of scattered blue kernels on yellow ears. Ear shape is frequently conical, a trait associated with drought-resistance and common in Italian traditional landraces. Genetic characterization was carried out on 529 individuals by using 10 SSR markers. A total of 68 different alleles, ranging from 4 for markers (*phi084* and *umc1401*) to 11 (*phi031*) and from 27 (Va217) to 50 (Va211), were evidenced at the individual and population level. AMOVA analysis revealed a small amount (19%) of variability between populations, as supported also by PCoA, with the only exception of Va217, which is different from the others, as evidenced also by phylogenetic analysis. Population structure analysis resulted in the identification of three and four population levels, which are consistent with previous results.

Keywords: Italian maize landraces; biodiversity; germplasm; SSR; genetic analysis

1. Introduction

Maize (*Zea mays* L.) is one of the most important species worldwide for agriculture. The species is native to Central America, where it was domesticated around 9000 years ago from wild relatives such as teosinte [1]. From Central America, the species spread toward North and South America, and after the trip of Columbus, maize reached the rest of the world. In Europe, maize was introduced soon after but several years were necessary to transform it into a crop [2,3]. In each country where maize was introduced, the species underwent a process of adaptation and differentiation according to different climate and soil conditions, as well as to the preferences of farmers and local populations [4–7]. In an era characterized by severe climate change, prolonged drought followed by intense rainfall, alternation between hot and cold weather and the outbreak of new pathogens and insects, the development of more resistant and resilient crop varieties is becoming an urgent necessity. Worldwide, in maize just a little part of the genetic variability of the species

has been incorporated in modern varieties, which derive from very few elite materials [8]. In the past, from the local Italian maize population, interesting open-pollinated varieties have been developed [9–14], and some hybrids have been evaluated [15–17]. The origin of Italian maize germplasm was extensively revised by Brandolini and Brandolini [3,18]. In 1954, the Stazione Sperimentale per la Maiscoltura (now CREA-Cereal and Industrial Crops; CREA-CI), located in Bergamo (Italy), promoted an intensive sampling survey on the entire nation to collect all the relevant accessions of traditional maize just before the introduction and spread of improved maize hybrids. From that sampling, 562 different accessions were collected and maintained in the germplasm collection held in Bergamo. Most of the sampled accessions were original germplasms spontaneously selected in Italy, while very few were early improved materials. Such highly differentiated germplasms allowed scientists to consider Italy as a secondary center for maize diversification [3]. However, studies aimed at the characterization of Italian maize have always been very few and frequently done in the past without the support of detailed genetic analysis. The classification of Italian maize based on morphological characters, in substitution of the traditional one of Zapparoli [9] based on cycle length, has been developed by Brandolini and Brandolini [3,18] who identified 11 groups in the traditional germplasm. Several authors have studied Italian maize, focusing on chromosome morphology [19–21], mutants [22,23] and mendelian traits [24] present in Italian maize landraces; in some cases, Italian maize was used for breeding [15–17].

In the last few years, a renewed interest toward traditional landraces has stimulated new research on maize landraces as the "Rostrata" group [25] or the "Nero Spinoso" [26], as well as the description and genetic analysis of several materials from different regions [27–32]. In the majority of the published studies, the original materials of the CREA-CI germplasm have been used in limited ways, generally as a comparison rather than as the focus of the study [27,28,30,31]. The only available genetic characterization of a wide set of Italian maize landraces is based on the AFLP characterization of varieties from Lombardy [33], leaving the remaining germplasm understudied.

Germplasm characterization can be performed at different levels according to the final objective of the study; in the past, the germplasm was described and identified by the means of morphological traits, while present-day germplasm characterization is mainly achieved by the use of DNA markers.

In biodiversity studies, among the many molecular markers that are currently available, the two most used are single nucleotide polymorphisms (SNPs) and simple sequence repeats (SSRs) or microsatellites. In maize, SSRs have been used to investigate the genetic biodiversity and structure of many germplasm collections constituted of few to hundreds of accessions [4–6,27–30,34]. In some cases, genetic characterization is accompanied by various morphological, chemical, agronomic and historic information on the characterized landraces [26,27,30–32,35], while in depth-studies are necessary to clarify several situations [36].

Emilia-Romagna is a region in Northern Italy where maize cultivation was abundant in the 1950s, with 87,000 hectares devoted to maize. In the 1954 sampling, 26 local materials were sampled from five provinces: Piacenza (8 accessions), Parma (4 accessions), Modena (4 accessions), Ravenna (1 accession) and Forlì-Cesena (9 accessions). The description of the materials was based only on those few morphological descriptors considered for the development of the new classification of Italian maize [3,37].

The present work focuses on the genetic and morphologic characterization and phylogenetic tree of eight original Italian landraces from the Emilia-Romagna region sampled in 1954 by means of SSRs.

2. Materials and Methods

2.1. Germplasm

Eight traditional maize varieties from Emilia-Romagna, deriving from the sampling of 1954, were retrieved from the germplasm collection held at the CREA-Cereal and Industrial

Crops (CREA-CI) in Bergamo. Each variety is named as Va, followed by a number. The varieties considered in the present study were: Va211 "Dente di Cavallo Nostrano", Va212 "Ottofile", Va213 "Cinquantino", Va214 "Locale Rocca di San Casciano", Va215 "Giallo Nostrano", Va216 "Giallo Comune", Va217 "Spinato" and Va218 "Nostrano". More detailed information on the germplasm sources [18,35] are provided in Table 1.

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

Accession	Accession Name	Sampling Location	Racial Complex	Local Race	Agro-Ecotype	Plant Height (cm)	Ear Height (cm)	Tasseling (GDD)	Physiological Maturity (GDD)
Va211	Dente di Cavallo Nostrano	San Lorenzo-Riccione (FC)	Conici Vitrei e derivati (Conical Flints and Derived Races)	Poliranghi (Multi Rowed Flints)	Spadone	202	105	646	1430
Va212	Ottofile	Ricò-Meldola (FC)	Ottofile Vitrei e Derivati (Eight-Rowed Flints and Derived Races)	Derivati 10–12 file (10–12 Rowed Derived Flints)	Derivati 10–12 file (10–12 Rowed Derived Flints)	230	100	646	1446
Va213	Cinquantino	Ravaldino in Monte (FC)	Conici Vitrei e derivati (Conical Flints and Derived Races)	Barbina	Barbina	220	95	646	1430
Va214	Locale Rocca di San Casciano	Rocca San Casciano (FC)	Conici Vitrei e derivati (Conical Flints and Derived Races	Barbina	Maggese	210	85	632	1366
Va215	Giallo Nostrano-Giallo Predappio	Predappio (FC)	Conici Vitrei e derivati (Conical Flints and Derived Races)	Poliranghi (Multi Rowed Flints)	Spadone	220	122	646	1446
Va216	Giallo Comune	Santa Sofia (FC)	Conici Vitrei e derivati (Conical Flints and Derived Races)	Poliranghi (Multi Rowed Flints)	Culaccione	220	90	632	1398
Va217	Spinato	Cesena (FC)	Dentati Bianchi e Gialli (Dent corn)	Dentati Bianchi Moderni (Modern White dent)	Dentato Giallo Moderno (Modern Yellow Dent)	265	122	799	1476
Va218	Nostrano	Sogliano sul Rubicone (FC)	Ottofile Vitrei e derivati (Eight-Rowed Flints and Derived Races)	Cannellino	Cannellino	245	102	662	1446

Field trial for variety characterization was located at Centro Ricerche Zootecniche (45.005066 N, 9.704206 E, San Bonico, Piacenza, Italy) and sown on 27 April 2018. Each plot was composed of five rows at 5 m long and spaced 80 cm apart, plus 1 m aisle on the hedge; 20 seeds were planted for each row. The field was managed according to agricultural practices for maize nurseries. Leaf samples were collected from all plants at the 5th 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:

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

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 substitutes by the cardinal temperature for maize growing (10 °C and 30 °C, respectively) [38].

Maize accessions were reproduced by manual random-intermating avoiding selfing. At maturity, ears were shelled, and seeds were stored at Banca del Germoplasma Vegetale of the University of Pavia within the AEGIS system, and available, upon request, for research and cultivation purposes.

2.2. DNA Extraction and PCR Amplification

DNA was extracted from young leaf tissues using the "96-Well Plate Plant Genomic DNA Miniprep Kit" (BIO BASIC) and following manufacturer instructions with the minor modifications, as previously reported [31]. The extracted DNA was visualized on 1% agarose gel electrophoresis stained with Midori Green (Nippon Genetics, Düren, Germany). Globally, 529 single plants were analyzed: 63 for Va213 and Va214, 64 for Va218, 65 for Va216, 67 for Va211 and Va215 and 70 for both Va212 and Va217.

Ten SSR markers previously reported for characterization of Northern Italian maize landraces [31,32] were selected and amplified in the present collection. PCR reactions were carried out as described in Stagnati et al. [31,32]. Detailed information on primer pairs is reported in Table 2. The multiplex of fragments, different in color and size, were separated using an ABI 3130xl Genetic Analyzer sequencer (Applied Biosystems, Waltham, MA, USA), and GeneScan™ 500 ROX™ was used as the size standard. Visualizations and sizing of the PCR fragments were performed using GeneMapper software version 4.0 (Applied Biosystems).

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	[29]
M304	*phi076*	TTCTTCCGCGGCTTCAATTTGACC	[6FAM]GCATCAGGACCCGCAGAGTC	4	58	150–200	[29]
M306	*phi031*	GCAACAGGTTACATGAGCTGACGA	[PET]CCAGCGTGCTGTTCCAGTAGTT	6	58	180–220	[29]
M308	*umc1075*	GAGAGATGACAGACACATCCTTGG	[6FAM]ACATTTATGATACCGGGAGTTGGA	8	58	130–150	[29]
M310	*phi084*	AGAAGGAATCCGATCCATCCAAGC	[PET]CACCCGTACTTGAGGAAAACCC	10	58	140–170	[29]
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

2.3. Statistical Analysis

Detected alleles were analyzed with the GenAlEx 6 software [39] to compute population statistics, principal coordinates analysis (PCoA) and analysis of molecular variance (AMOVA). The polymorphic information content (PIC) was calculated with PowerMarker software, version 3.25 [40].

Phylogenetic trees were computed considering both the 529 separate individuals as well as individuals previously aggregated according to population assignment. The phylogenetic tree of individuals was obtained using custom script of the polysat, phangorn and ape packages in R environment [41–43]. The phylogenetic tree of the population was computed using the unweighted pair group method with arithmetic mean method applying the *upgma* function of the phangorn package of R software [42], starting from a genetic distance matrix calculated by GenAlEX6.

The population structure of the 529 maize individuals was examined using a Bayesian clustering algorithm implemented in STRUCTURE v.2.3.4 [44]. The "admixture model" and the "correlated allele frequency model" were selected as suggested [29,44]. Ten independent replications were run for each level of K ranging from 2 to 15 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 [29,45]. At the first level of K, membership of single plant to the genetic cluster was considered high with membership > 0.8, while individuals with membership < 0.8 were considered as admixed and at the second level of K, membership was considered strong with values > 0.5 [29].

3. Results and Discussion

3.1. Morphological Characterization

Landraces were cultivated during the year 2018 and described according to UPOV morphological descriptors (Supplementary Material S1). According to Brandolini and Brandolini [3,18] Italian maizes are classified in a three-level system: racial complexes, local races and agro-ecotypes. Racial complexes are the highest level of classification and are constituted by several races which share discriminating characteristics at the morphological, geographical or phylogenetic level. Local races are groups of agro-ecotypes that share some common traits to be distinguished as groups, while agro-ecotypes are groups of populations/landraces which differ in adaptive characteristics but share some common genetic features. This classification is reported for each landrace (Table 1).

The varieties were followed along the crop cycle and the main characteristics scored. Subsequently, a short description of important traits for the varieties used in the present study was created. Overall, the present collection is characterized by landraces with flint—flint-like kernels while the only dent type is Va217. The prevalence of flint type in the Italian maize germplasm derives from multiple factors: (i) the massive introduction of flint maize over dent types immediately after the first explorations of the New World; (ii) the necessity of local communities to store cereals between consecutive harvesting seasons—flint maize, because of the hardness of the kernel, is better than dent maize for long-term storage; (iii) the preparation of polenta, the main dish of the rural population, which is mainly based on flint maize flour [3,9]. Kernels are generally yellow or yellow–orange, while the cob is white. Another interesting trait is the presence of conical ears. Landraces with conical cobs are suited to non-irrigated conditions since the parenchyma in the cob functions as a water reservoir [3]. Landraces showing this trait were sampled in hilly areas as well as in the mountains (Va216) or close to the sea (Va211): areas that are subjected to summer drought or where irrigation is not feasible. This trait was observed also in the characterization of landraces from Umbria [46]. Landraces from Umbria presented conical ears with a case of pine-like ears in the Locale di Norcia, which were grown up to 1000 m above sea level. Such pine-like cobs have a diameter higher than ear length, probably to increase the water storage capacity. Tassels are generally big, with a high number of ramifications, as is typical in unselected maize [47]. Another common characteristic of the collection is the earliness. This trait may be linked to the reduced favorable season in hills and mountains, to the necessity of escaping hot and drought periods during flowering and seed set or with the traditional practice of intercropping corn with vines or trees. In this last case, earliness is associated with reduced plant development, which was necessary to guarantee a sufficient space and light to the consociated species [9]. Moreover, earliness was one of the favored traits for selection, according to Zapparoli's recommendations to farmers during selection [9]

Va211 Dente di Cavallo Nostrano: Plants are 220 cm high, the ear is inserted at 105 cm and the ear/plant insertion ratio is medium. Ears are short, conical and of high diameter (>50 mm). Kernels are of dent–dent-like type, yellow and arranged in 14–18 kernel rows; the cob is white (Figure 1). Male and female flowering occurred at 646 and 723 GDD, respectively, while physiological maturity occurred at 1430 GDD. Tassels have a high number of secondary branches; silk coloration is weak. Va211 is traditionally classified in

the Conici Vitrei e Derivati (Conical Flints and Derived Races) racial complex, Poliranghi (Multi-Rowed Flints) local race and Spadone agro-ecotype [18].

Figure 1. Ears, cob and kernels of the eight maizes traditional population assessed in this study.

Va212 Ottofile: Plants of 230 cm and ear inserted at 1 m with a small ear/plant insertion ratio. Ears are short, and the shape is variable from conical to cylindrical, with a medium ear diameter. Kernels are of the intermediate type, yellow–orange in color and arranged in 12–14 rows on a white cob (Figure 1). Male and female flowering occurred at 646 and 692 GDD, respectively, while physiological maturity occurred at 1446 GDD. Tassels have a high number of secondary branches; silk coloration is absent or weak. Va212 is traditionally classified in the Ottofile Vitrei e Derivati (Eight-Rowed Flints and Derived Races) racial complex; Local Race Derivati 10–12 file (10–12 Rowed Derived Flints) and Derivati 10–12 file (10–12 Rowed Derived Flints) agro-ecotype. Materials of these groups derive from the introgression of different maizes in originally 8-rowed flints [18].

Va213 Cinquantino: Plants of 220 cm and ears inserted at 95 cm with a small ear/plant insertion ratio. Ears are short and slightly-conical in shape, with a medium ear diameter (Figure 1). Kernels are flint, orange and arranged in 12–16 rows; the cob is white. Sometimes, ears were characterized by the presence of blue–black kernels scattered within the normal orange ones. Male and female flowering occurred at 646 and 677 GDD, respectively, while physiological maturity occurred at 1430 GDD. Tassels have a high number of secondary drooping branches; silk coloration is absent or weak. Va213 is traditionally classified in the Conici Vitrei e Derivati (Conical Flints and Derived Races) racial complex; Barbina (Subconical Flints) local race and Barbina agro-ecotype [18].

Va214 Locale Rocca di S. Casciano: Plants are 210 cm tall and ears are inserted low at 85 cm on the stalk, resulting in a very small ear/plant insertion ratio. Male and female flowering occurred at 632 and 692 GDD, respectively, while physiological maturity occurred at 1366 GDD. The number of tassel branches is variable from few to many; silk pigmentation is weak. Ears are very short, of conical or slightly conical shape, with 14–16 rows of flint yellow –orange/orange kernels. The cob is white (Figure 1). Va214 is traditionally classified in the Conici Vitrei e Derivati (Conical Flints and Derived Races) racial complex, Barbina local race and Maggese agro-ecotype [18]. Saltini [48] reports that in Rocca San Casciano the cultivation of maize was present at the end of 19th century, as well as the use of polenta as the main food, relating it to the poor condition of the rural population that was affected by pellagra.

Va215 Giallo Nostrano: Plants are 236 cm tall and ears are inserted at 122 cm, resulting in a high ear/plant insertion ratio, which affected the lodging of Va215. Male and female flowering occurred at 646 and 692 GDD, respectively, while physiological maturity occurred at 1446 GDD. The number of tassel branches were high–very high, while variable in attitude; silk pigmentation is absent or weak. The short conical ears had 12–18 kernel rows. Caryopses are yellow and of the flint-like type; the cob pigmentation may be white or red (Figure 1). Va215 is traditionally classified in the Conici Vitrei e Derivati (Conical Flints and Derived Races) racial complex, Poliranghi (Multi-Rowed Flints) local race and Spadone agro-ecotype [18].

Va216 Giallo Comune: Plants are 220 cm tall and ears are inserted at 90 cm, with a very small ear/plant insertion ratio. Ears are short, conical and with a large diameter (Figure 1). There are 16–18 rows of flint yellow–orange kernels and white cob. Male and female flowering occurred at 632 and 692 GDD, respectively, while physiological maturity occurred at 1398 GDD. Tassels have a high number of secondary branches with variable attitude; silk coloration is absent or weak. Va216 is traditionally classified in the Conici Vitrei e Derivati (Conical Flints and Derived Races) racial complex, Poliranghi (Multi-Rowed Flints) local race and Culaccione agro-ecotype [18]. Preliminary morphological observation and sampling location suggested that Va216 was the ancestor of EMR07-Mais di Santa Sofia Romualdi [32]. Genetic analysis revealed that these two materials are not related as previously suspected (data not shown).

Va217 Spinato: Plants of this landrace are very tall, reaching 265 cm, ears are inserted at 122 cm on the stalk and the ear/plant insertion ratio is medium. Ears are of medium length (around 20 cm), with a high diameter, and are of cylindrical shape. Kernels are yellow, of dent type, and arranged in 16–18 rows. The cob is red (Figure 1). Va217 was found as the latest accession in the traditional maize germplasm of Emilia-Romagna. Male and female flowering occurred at 799 and 856 GDD, respectively, while physiological maturity occurred at 1476 GDD. Tassels have a medium–high number of secondary branches; silk coloration is absent or weak. Va217 is traditionally classified in the Dentati Bianchi e Gialli (Dent Corn) racial complex, Dentati Moderni (Modern Dent) local race and Dentato Giallo Moderno (Modern Yellow Dent) agro-ecotype. Such materials derive from the Corn Belt and are medium–late types [18]. The name Spinato (and related terms) is used to refer to maize with beaked kernels as those of the Rostrata group. In this case, there is no presence of an apical beak on the top of the kernel, which is, instead, wrinkled. Probably, the name refers to the roughness of the ear in contrast to the smoothness of other materials.

Va218 Nostrano: Tall plants (245 cm) with ears inserted at 102 cm. Even if the era/plant insertion ratio is not too high, Va218 is susceptible to lodging. Male and female flowering occurred at 662 and 723 GDD, respectively, while physiological maturity occurred at 1446 GDD. Ears are from 15 to 20 cm and of cylindrical or slightly conical shape; kernels are variable, flint, intermediate and sometimes of dent type, but of uniform yellow color; the cob is white. Tassels present a high–very high number of branches, and silks have a weak pigmentation. It is traditionally classified in the Ottofile Vitrei e Derivati (Eight-Rowed Flints and Derived Races) racial complex, Cannellino (Subcylindrical Flints) local race and Cannellino agro-ecotype. Variability may be explained by the traditional origin of

the Cannellino local race from the adaptive selection of crosses between the Ottofile and Conical/Agostano maize types [18].

3.2. Genetic Characterization

To investigate the main population parameters, the marker allele data of the 529 maize individuals were collected through SSR analysis. Globally, 68 different alleles were detected, ranging from 4 for markers (*phi084* and *umc1401*) to 11 (*phi031*) with a mean of 6.8 alleles per locus.

Considering populations, the number of alleles varied from 27 (Va217) to 50 (Va211), as reported in Table 3. The presence of high polymorphism is consistent with the allogamy of maize and with the heterogeneity of maize landraces, supporting the correct germplasm maintenance by the staff of the CREA-CI through manual pollination throughout the sampling year. All loci were polymorphic in all populations, with the exception of *p-bnlg176* in Va217. Some cases of monomorphic loci have been reported also in other studies [5,29,31,34] and may be linked to characters appreciated by local farmers that have selected and fixed such chromosomal regions.

Table 3. Genetic parameters calculated according to the ten SSR and seven landraces object of this study. Number of different alleles (N), 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, F_{IS}, F_{IT}, F_{ST} and gene flow (N_m) are reported.

Locus	N	N_a	N_e	I	H_o	uH_e	PIC	F	F_{IS}	F_{IT}	F_{ST}	N_m
phi127	6.00	3.25	2.07	0.79	0.50	0.49	0.54	−0.01	−0.02	0.18	0.20	0.99
phi076	5.00	3.75	2.19	0.88	0.52	0.54	0.55	0.03	0.03	0.17	0.14	1.48
phi031	11.00	5.00	2.73	1.14	0.57	0.60	0.74	0.05	0.04	0.26	0.23	0.85
umc1075	10.00	4.75	2.84	1.16	0.54	0.63	0.73	0.13	0.14	0.29	0.18	1.11
phi084	4.00	2.63	1.79	0.65	0.43	0.43	0.38	−0.03	−0.02	0.11	0.13	1.69
umc1327	7.00	3.50	2.06	0.82	0.52	0.49	0.48	−0.09	−0.07	0.06	0.13	1.74
umc1786	9.00	5.38	2.67	1.13	0.42	0.59	0.64	0.25	0.27	0.37	0.13	1.69
p-bnlg176	6.00	3.38	2.13	0.83	0.39	0.48	0.56	0.22	0.19	0.39	0.25	0.77
umc1941	6.00	3.75	2.18	0.82	0.51	0.47	0.58	−0.09	−0.10	0.22	0.28	0.63
umc1401	4.00	3.13	2.62	1.00	0.50	0.58	0.62	0.16	0.14	0.27	0.15	1.36
Mean	6.80	3.85	2.33	0.92	0.49	0.53	0.58	0.06	0.06	0.23	0.18	1.23
SE	2.44	0.17	0.08	0.04	0.02	0.02		0.03	0.04	0.03	0.02	0.13
Va211	50.00	5.00	2.38	0.98	0.41	0.52	#	0.19	0.22	0.37	0.19	1.04
Va212	47.00	4.70	2.53	1.06	0.54	0.60	#	0.10	0.10	0.16	0.07	3.36
Va213	34.00	3.40	2.28	0.90	0.50	0.54	#	0.09	0.08	0.23	0.16	1.29
Va214	35.00	3.50	2.02	0.83	0.50	0.49	#	−0.02	−0.01	0.23	0.24	0.79
Va215	39.00	3.90	2.80	1.08	0.53	0.61	#	0.13	0.14	0.18	0.05	4.65
Va216	42.00	4.20	2.55	1.01	0.53	0.55	#	0.02	0.05	0.18	0.14	1.51
Va217	27.00	2.70	1.71	0.60	0.40	0.36	#	−0.10	−0.11	0.37	0.44	0.32
Va218	34.00	3.40	2.34	0.92	0.52	0.56	#	0.05	0.08	0.20	0.14	1.60
Mean	38.50	3.85	2.33	0.92	0.49	0.53		0.06	0.07	0.24	0.18	1.82
SE	7.58	0.17	0.08	0.04	0.50	0.02		0.03	0.10	0.08	0.12	1.45

Legend: # absent values.

Sixteen private alleles were identified (Table 4): half of these were found in Va211 for markers *phi031* (2), *umc1075* (4), *umc1327* and *p-bnlg176* (1 each); six in Va212 for markers *phi127* and one for each *umc1075*, *phi084*, *p-bnlg176* and *umc1941*, while one unique allele was found for marker *phi031* for both Va213 and Va216.

Maize germplasm studies are very variable on the number, origin, size of examined population and SSR markers, therefore making comparison between different research quite complex. If the mean of the allele number per locus is considered, the allele diversity of the present collection is comparable, or higher, to other studies on Italian maize landraces [29], or the even wider germplasm collection at the national level [4,8,34].

Table 4. List of private alleles detected in the landrace collection.

Landrace	Locus	Allele	Allele Frequency
Va211 Dente di Cavallo Nostrano	*phi031*	212	0.017
		220	0.017
	umc1075	122	0.023
		124	0.031
		134	0.289
		144	0.039
	umc1327	62	0.015
	umc1796	126	0.024
Va212 Ottofile	*phi127*	102	0.015
		111	0.015
	umc1075	120	0.016
	phi084	152	0.007
	p-bnlg176	132	0.015
	umc1941	92	0.016
Va213 Cinquantino	*phi031*	196	0.032
Va216_Giallo_Comune	*phi031*	184	0.008

The number of observed alleles (N_a) ranged from a minimum of 2.63 (*phi084*) to 5.38 (*umc1786*) at the locus level, while from 2.7 (Va217) to 5 (Va211) at the population level. The number of effective alleles (N_e) was always lower than N_a, ranging from 1.79 for *phi084* to 2.84 for *umc1075* at the locus level and from 1.71 to 2.8 in Va217 and Va215 at the landrace level (Table 3). The Shannon's index (I), used to characterize population diversity, was found to be, on average, equal to 0.92 ± 0.04 over all loci and populations (Table 3). PIC evaluates the ability of a certain SSR in discriminating different genotypes, thus providing an estimation of the diversity. The average PIC value was 0.58, ranging from 0.38 of *phi084* to 0.74 of *phi031*; these results are comparable to other studies [49,50].

The mean values of the observed (H_o) and unbiased expected (uH_e) heterozygosity were, across loci and landraces, equal to 0.49 ± 0.02 and 0.53 ± 0.02, suggesting a small reduction in heterozygosity. The highest lack in observed heterozygosity was detected for markers *umc1786* (-0.17) and *p-bnlg176* (-0.10), and in accessions Va211 (-0.11) and Va215 (-0.09). This lack of heterozygosity is lower than the values reported in previous works [27,29].

The inbreeding coefficient F_{IS} had average values of 0.06 ± 0.04 and 0.07 ± 0.1 for markers and populations, respectively, 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 the Hardy–Weinberg equilibrium [8,31]. The level of heterozygosity and the absence of inbreeding may be explained by the correct management of the germplasm collection during the ex situ conservation at the CREA CI.

F_{ST} was equal to 0.18 for both markers and populations (Table 3), suggesting that these landraces are characterized by a low level of genetic differentiation among the population, since less than 20% of genetic variation was found between varieties and is consistent with previous findings [5,8,27,29]. On the contrary, the characterization of maize landraces from Emilia-Romagna, which have been maintained continuously under cultivation in the last 60 years, evidenced a higher level of genetic differentiation [32]. It is possible to suppose that the low level of F_{ST} among the varieties object of this study, with respect to the varieties characterized in Stagnati et al. [32], may be a consequence of the reduced geographic area of origin of the Va (the Province of Forlì-Cesena) compared to the entire Emilia-Romagna region analyzed in Stagnati et al. [32]. In addition, it is possible to consider that in the 50s, the years of sampling of the Va, maize cultivation was wider than now, and it is not possible to exclude the occurrence of spontaneous crosses reducing the genetic differentiation among varieties. Finally, we do not have information on how these materials were maintained before the sampling of 1954. In Italy, during the first half of 20th century,

maize seed stocks for field sowing were generally produced at the farm level. The majority of farmers were unaware of selection criteria and of the necessity of avoiding selfing in maize. Zapparoli [9] explained some easy ways to produce on-farm good quality maize seeds for sowing, underlying the necessity to maintain a certain level of morphological variation when performing selection, thus avoiding the fixation of characters–alleles. This suggestion may have contributed to preserving allele diversity until germplasm sampling.

3.3. PCoA Analysis

Detected alleles were analyzed to investigate the relationships among all the individuals by principal coordinates analysis (PCoA), as reported in Figure 2. The first two axes accounted for 13.54% and 8.21% of genotypic variability, leaving much variation unexplained. What appeared from the PCoA is a clear separation of the landraces in two distinct groups including, respectively, Va217 and all the remaining materials.
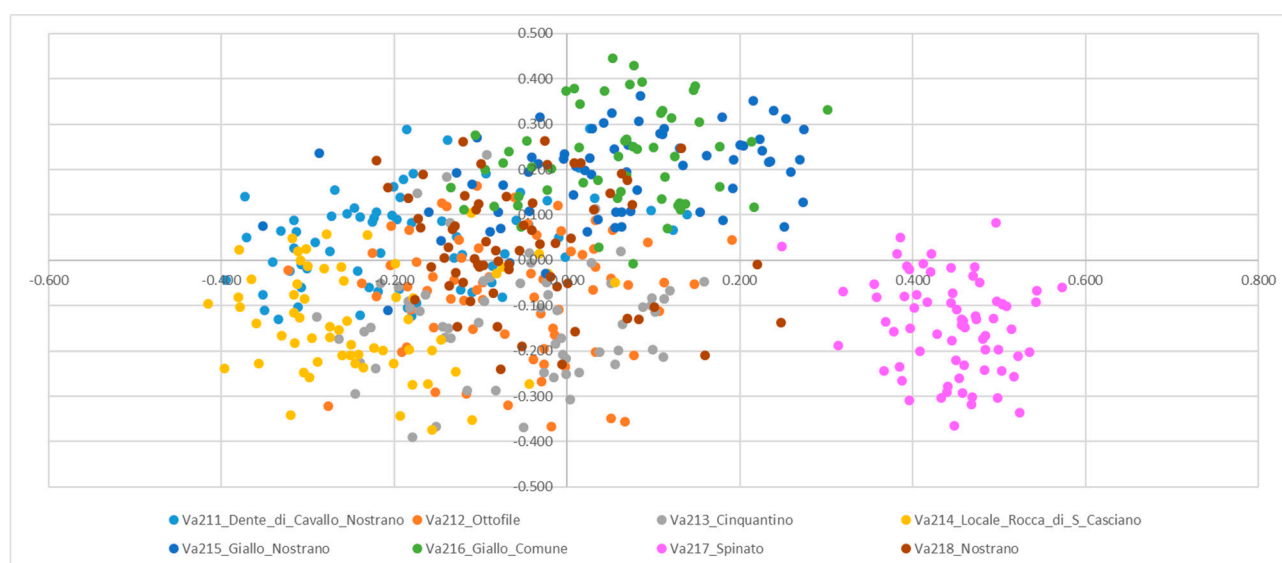

Figure 2. Principal coordinates analysis (PCoA) axes 1 vs. 2 of the 529 individuals of the eight maize landraces.

The remaining individuals are generally clustered together according to their landrace assignment, but the overlapping between different landraces is too high to have a clear separation. Plotting the first vs. the third axes, which account for 7.12% of genetic variation, it is possible to have a good separation of Va217 from all, Va211 from Va214 and Va215 from Va216, as shown in Supplementary Figure S1.

The separation of Va217 from the collection is consistent with the appearance of the plant and with the traditional classification [18]. Va217 plants are high, robust, late and with abundant leaves at the top. Ears are cylindrical, long, with a red cob and with strongly dented yellow kernels. The other landraces are earlier, less leafy, with a white cob and kernels from flint-to-dent–dent-like type; ears are often conical, as previously mentioned. Va217 is classified in the Indentata racial complex, which includes both old and recent (first half of 20th century) introductions. The complex comprises two local races: the Dentati Bianchi Antichi (Ancient White Dent) and the Dentati Moderni (Modern Dent), where Va217 is ascribed in the Dentato Giallo Moderno (Modern Yellow Dent) agro-ecotype. Va217 is the only representative of these medium late genotypes from the corn belt sampled in Emilia-Romagna [18]. Bonciarelli [46] reports the presence of dent maize with a red cob as traditional materials, describing a variety whose original name was lost and therefore re-named "Dentato di Sangemini", introduced in Sangemini in 1910. This maize was characterized by big cylindrical ears, with a red cob (trait preferred by farmers at the time of ear-seed selection) and long dented grains often wrinkled at the top. This material was

cultivated only in fertile areas at the bottom of the valleys. This description fits very well with the observed characteristics of Va217, thus supporting a wider distribution of dent maizes with red cobs also many years before the hybrid introduction.

The other landraces, which belong to other racial complexes, are of a more difficult interpretation if the PCoA is computed by keeping the 529 plant as separate samples. When the analysis is carried out by considering not the single plants for each population but grouping them according to the different landraces (Supplementary Figure S2), the separation is more evident. The level of explained variation rises to 40.46% and 19.98% for the first two axes: Va217 remains clearly separated from the other materials, Va212 and Va213 are overlapped, Va215 and Va126 are clustered together while Va211, Va214 and Va218 are alone in the plot.

3.4. Phylogenetic Analysis

Relationships existing inside the landrace collection were investigated, constructing phylogenetic trees and grouping the 529 individuals according to their population identification, as well as considering them separate entities. The phylogenetic tree, obtained by grouping individuals according to their attribution (Figure 3), presents several similarities with the PCoA (Figure 4) and Supplementary Material S2.
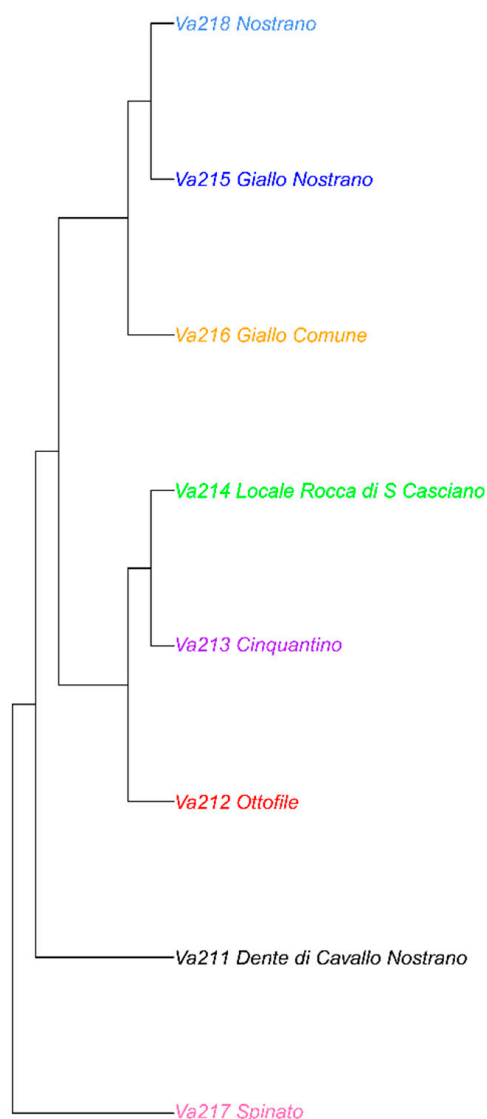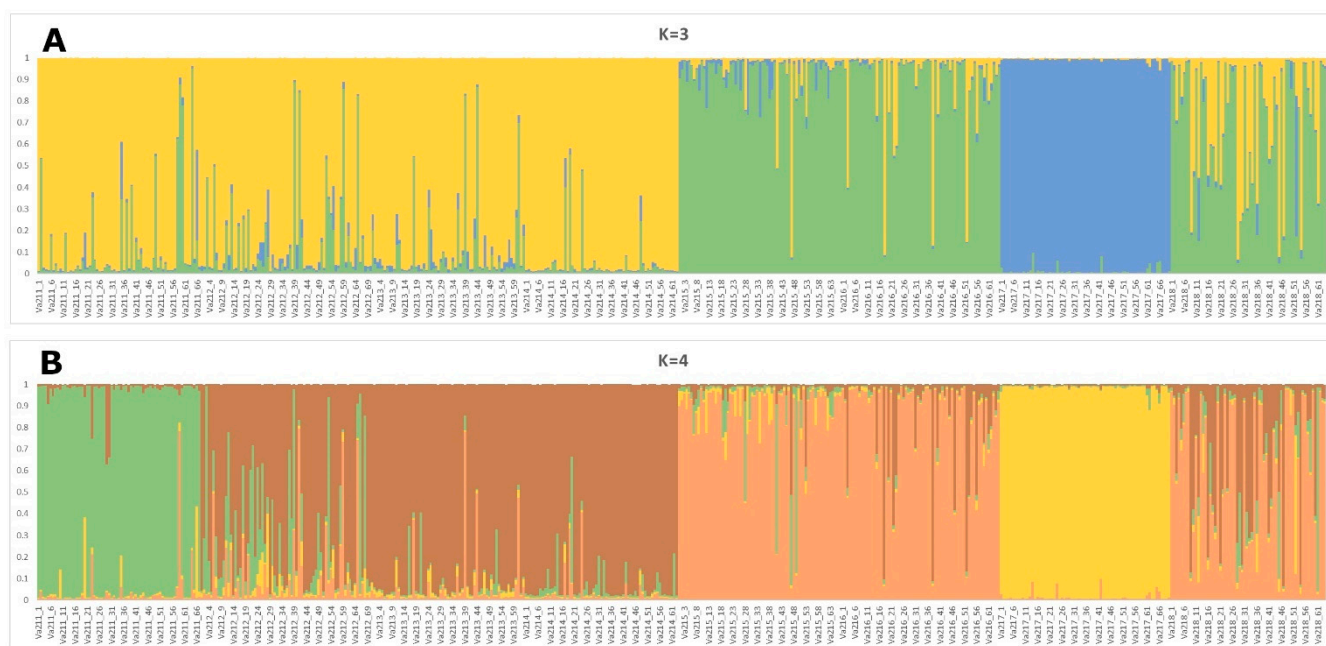

Figure 3. Phylogenetic tree of the eight landraces.

Figure 4. STRUCTURE clusterization of the collection at K = 3 (**A**) and K = 4 (**B**).

Once again, Va217 resulted completely differently from all the other varieties and, likewise in this case, Va211 is not grouped with the other landraces that are clustered in two separate groups of three populations each. The first group includes Va215, Va216 and Va218; it is possible to note that, despite a different disposition of the three landraces, the same group can be recognized also in the PCoA (Figure 4). Concerning Va216 and EMR07 [32], it was suspected that these two materials could be related. These two landraces have been sampled in the same municipality in the province of Forlì-Cesena at a distance of 50 years. Morphological observations suggested that these two maize accessions are very similar: short cycle, conical ears and yellow–orange kernels; as other UPOV descriptors are the same, this supports the hypothesis that Va216 could be considered an ancestor of EMR07. Despite this, preliminary genetic analysis supported an independent origin of these two landraces. This may be a consequence of: (i) a real difference between the two landraces, being a case of convergent evolution for similar characteristics that promote fitness in a specific environment; (ii) a difference originated from the same gene pool in consequence of the different conservation of these materials: ex situ with limited reproductions for Va216 and in situ with annual reproduction for EMR07. Moreover, the province of Forlì-Cesena could be considered a hotspot for maize landraces, since at least ten are still cultivated there [36].

The second group includes Va212, Va213 and Va214. In this case too some correspondence between the tree and PCoAs can be detected but, again, with a different arrangement of the varieties (Figure 4).

If the tree is computed with the separate individuals (Supplementary Figure S3), the separation of Va217 is still clear, but the other materials, forming anyway clear clusters, present several samples dispersed in the tree, in accordance with the high variability observed in the PCoA analysis (Figures 1 and 2).

High levels of genetic variability and overlapping between different landraces have been already reported [29,31,32]. The AMOVA analysis revealed that only 19% of variation is present in the population, leaving the remaining part at the intra-population level. Such values are not uncommon in maize, as reported by [8,29]. Pairwise F_{ST} based on AMOVA were all significant, at $p < 0.001$ and reported in Supplementary Table S1.

3.5. STRUCTURE Analysis

The population structure analysis revealed two different levels of population structure. The first, ΔK (502.54), was found for K = 3, while the second, ΔK (182.25), at K = 4.

At K = 3, the first group of landraces is formed by Va211-Va212-Va213-Va214, the second one by Va215-Va216-Va218, while Va217 is alone, as shown in Figure 4A; at K = 4 there is the separation of Va211 from the group Va212-Va213-Va214, as shown in Figure 4B.

All the individuals of Va217 were strongly associated (having a membership > 0.8) with the funding group. Higher levels of association to the main cluster were also found for Va214, Va215, Va211 and Va213, with 93.6%, 89.6%, 83.6% and 80.9%. The remaining Va212 and Va218 had only 71.4% and 50% of individuals associated to the main group, respectively. Individuals with admixed ancestry (membership < 80%) were mainly found in Va218 (42.1% of the total) and Va212 (22.8%), while Va213, Va216 and Va211 were 15.8%, 15.3% and 13.4, and Va214 and Va215 were 6.3 and 8.9%, respectively. At the second level of K = 4, the percentage of plants associated to the main cluster of the accession rose, varying from 60.9% (Va218) to 100% (Va217).

Population stratification revealed by the STRUCTRUE analysis is very consistent with the result shown in the phylogenetic tree. Particularly, different statistical analysis confirmed that Va217 is completely unrelated to the other materials, as suggested by morphology and by historical data available for this maize [18]; Va211 can be easily separated by the remaining Va, as revealed from its separation at K = 4 and in the phylogenetic tree. Va212-213-214 and Va215-216-218 constitute two distinct groups, as revealed by the phylogenetic tree and both levels of population stratification. In Supplementary Figure S4, the PCoA of 529 individuals was colored according to the results of the STRUCTURE analysis. It is possible to see a correspondence of the groups, revealed at K = 4 and the PCoA. In particular, Va211 colored in black is at a marginal position in the left part of the space, Va217 is completely separated in the right part, the population of the second group (Va212-Va213, Va214) is clustered in the lower part of the cloud (for negative values of both axes), while plants of the third group are placed in the upper part of the graph, mainly for positive values of both axes. The overlapping of samples of the second and third group is present, but, overall, the result is consistent to the phylogenetic tree.

The presence of many admixed genotypes in Va212 and Va218 is congruent with the results of Brandolini and Brandolini [18], who indicated that Va212 and Va218 are derived races from crosses between Eight-Rowed Flints and other materials. Moreover, for Va218 several kernel types were observed, thus suggesting the presence of high variability and different genetic contributions.

4. Conclusions

The eight maize accessions assessed in the present study are distinguishable both at the morphological and genetic level. Traditional maize is mainly early cycle and yellow flint type with conical ears; only Va217 is very different from the others, being a late dent corn. Earliness and conical cobs are worthy of further study due to their potential in helping the crop in escaping unfavorable conditions caused by climate change.

The genetic variability is high, both at the population as well as at the collection level. The intra-population genetic variability is very high, hampering the possibility of an easy discrimination between different varieties. Heterozygosity and inbreeding coefficient values were consistent to the allogamous nature of maize, supporting the proper preservation of the germplasm in the last 70 years. Population structure analysis supported previous ideas that Va217, even if sampled in the traditional germplasm, is something different and unrelated to ancient Italian corns.

Moreover, since landraces are experiencing a revival period, the characterization of traditional materials present in germplasm banks may stimulate interest in bringing them back into cultivation in small local production chains.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/su141912110/s1, Table S1: Pairwise FST calculated through AMOVA. All values are significative at $p < 0.001$; Figure S1: Principal coordinates analysis (PCoA) axes 1 vs. 3 of the 529 individuals of the eight maize landraces; Figure S2: PCoA analysis of the eight landraces with individuals grouped according to landrace assignment; Figure S3: phylogenetic tree of the 529 individuals of the eight maize land-races; Figure S4: PCoA analysis of the eight landraces with individuals grouped according to STRUCTURE assignment at K = 4; Supplementary Material S1: UPOV descriptors of the eight landraces of the present study. Supplementary Material S2: 3D PCoA analysis of the 529 individuals.

Author Contributions: L.S. investigation, formal analysis, writing—original draft; G.S. investigation, data curation, writing—original draft; F.D. data curation; A.L. writing—review & editing; S.Z. writing—review & editing; A.M. conceptualization, review & editing; G.R. funding acquisition, conceptualization, writing—review & editing; M.B. conceptualization, funding acquisition, investigation, writing—original draft, writing—review & editing. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement: All the relevant data of this study are presented as figures and tables in the present research.

Conflicts of Interest: The authors declare no conflict of interest.

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