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NOTES ON NEGLECTED AND UNDERUTILIZED CROPS

Microsatellite and morphological characterization of three Rostrato di Val Chiavenna (Sondrio, Italy) maize (*Zea mays* L.) accessions

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Abstract Beaked corn represents one of the most characteristics and neglected group of Italian maize landraces. These genotypes, classified in the "Rostrata" group, were mostly grown in northern Italy, on the left bank of the Pò river, until the end of the Second World War and then subjected to strong genetic erosion because of the subsequent introduction of improved genotypes and hybrids of US origin. These materials are experiencing a revival period for cultivation, commercial exploitation and an increased number of farmers is seeking particular genotypes for the production of niche products. In Valchiavenna (Sondrio, Lombardia, Italy) maize cultivation has historical importance for *polenta* preparation and is characterized by the presence of at least three accessions of "Mais Rostrato Val Chiavenna", one conserved since 1982 in germplasm bank (here named as R17_BG) and two in situ (here named as R17_M; R17_T), with distinctive morphological traits at both ear and plant level. In the present study, these accessions have been characterized at the morphological and genetic level with 10 SSR markers and compared to other landraces of the Rostrata group. SSR analysis revealed from 3 to 11 alleles per locus evidencing a good level of heterosis and absence of allele fixation for landraces. Both phylogenetic and STRUCTURE analysis evidence that the three "Mais Rostrato Val Chiavenna" are different entities with a distinct genetic origin. Historical investigation revealed that the morphology most close to the "original" Mais Rostrato di Val Chiavenna is that of R17_T, dating back to the XIX century. These observations, coupled with morphological and genetic results may corroborate that R17_T corresponds to the original "Mais Rostrato Val Chiavenna".

Keywords Maize (*Zea mays* L.) · Landraces · Rostrata group · Microsatellite · Biodiversity

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Introduction

Maize (*Zea mays* L.) is one of the most important cereal crops in Italy and worldwide. The species was introduced, in Italy, soon after the discovery of the Americas by Cristoforo Colombo (Brandolini and Brandolini, 2009; Ardenghi et al. 2018) but it was necessary around one century to become an established crop and to appear for the first time in commercial trades (Brandolini and Brandolini, 2009). During this time, several seed samples, from small to huge quantities, were repeatedly introduced in the Old World but it was only after the introduction of seeds from highlands and high latitude of the Americas that maize became a valuable crop in Europe (Brandolini and Brandolini, 2009). The introduction of photoperiod insensitive genotypes, the allogamous nature of maize that helps the possibility of hybridization, the huge differences of environmental conditions of the Italian peninsula, new and diversified uses of maize grains and cultures have stimulated the rising and selection of numerous new landraces adapted to different agro-ecological situations to define Italy as a secondary center of maize diversification (Brandolini and Brandolini, 2006, 2009; Eschholz et al. 2010; Cömertpay et al. 2012; Ardenghi et al. 2018). After the Second World War (WWII), just before dent hybrid introduction from the USA, in Italy was promoted a survey to collect all maize landraces that were grown on the territory resulting in the collection, during 1954, of 562 accessions (Brandolini and Brandolini, 2009), as well as in different countries in the same period or more recently (Eschholz et al. 2010; Cömertpay et al. 2012; Oppong et al. 2014). In northern Italy, until WWII flint corn was the staple crop connected to the human diet based on polenta. Similar to other countries after WWII traditional landraces were replaced by high-yielding dent hybrids of American breeding for animal feeding while human diet changed to be more diversified (Ardenghi et al. 2018).

Among many accessions sampled in 1954, one of the most characteristic groups of northern Italy is that of the "Rostrato" type (Brandolini and Brandolini 2006). The assemblage of this group of landraces, their history and taxonomic reallocation has been recently revised by Ardenghi et al. (2018). The authors have outlined that the Rostrata Group represents one of the most ancient and neglected gene pools among Italian maize landraces and dating its origin, and cultivation period, from the second half of the XIX century to before the appearance of the improved maize cultivars of the 1920s and 1930s. They surveyed the cultivation of maize landraces belonging to the Rostrata Group in northern Italy finding 28 entities still cultivated; among those 18 landraces were reported for the first time.

The development of commercial hybrids has drastically reduced the genetic base of the crop and pedigrees of hybrids reveal that the majority of commercial corn derives from 10–20 elite inbred. Therefore, conservation and characterization of maize biodiversity are of crucial importance to discover and maintain favorable alleles for future breeding (Qi-Lun et al. 2008). Studies on landraces characterization are reported in the literature for both Italian (Barcaccia et al. 2003; Lucchin et al. 2003; Hartings et al. 2008; Cassani et al. 2017; Palumbo et al. 2017; Giupponi et al. 2020, 2021) and worldwide materials (Qi-Lun et al. 2008; Eschholz et al. 2010; Cömertpay et al. 2012; Oppong et al. 2014). A common conclusion is that the genetic potential hidden in landraces is still unknown and may represent a powerful basin to detect favorable alleles for future breeding programs (Meseka et al. 2015; Puglisi et al. 2018).

The limited knowledge on landraces is even more scarce concerning maize belonging to the Rostrata Group. Historical records report that this group is characterized by high yield and remarkable quality of the flour (Ardenghi et al. 2018). These advantages helped the diffusion of "Rostrato" landraces with high genetic and morphologic variability in the North of Italy, in Africa Orientale Italiana, Italian Somaliland, and areas of the former Yugoslavia territories (Ardenghi et al. 2018). Having a heterogeneous origin and being open-pollinated the variability may be further emphasized by spontaneous crossing with other maize or by selection operated by farmers as reported by Ardenghi et al. (2018). These landraces are fundamental resources to be preserved for future applications and they represent unique value under heritage and cultural perspective for the sustainment of agriculture in disadvantaged or mountains area (Newton et al. 2011).

Germplasm preservation requires also the proper classification of varieties and landraces. Historical classification of Italian maize was based on sowing time and cycle length (Zapparoli 1941) while a more recent organization is based on morphological traits,

identifying the collection in nine racial complexes and 65 agro-ecotypes (Brandolini and Brandolini 2009).

Nowadays molecular markers are widely used for germplasm identification, breeding or molecular traceability purposes in various crops. Among molecular markers, SNPs and Simple Sequence Repeats (SSRs), also known as microsatellites, are the most used. SNPs are widely applied for quantitative genetic purposes while SSR is used for molecular traceability and diversity analysis because of their co-dominant nature, high informativeness, abundance in the genome, chromosome specificity, even distribution among chromosomes, repeatability and reliability (Cömertpay et al. 2012; Stagnati et al. 2020). Microsatellites have been widely used in the characterization of maize landraces (Barcaccia et al. 2003; Reif et al. 2005, 2006; Eschholz et al. 2010; Cömertpay et al. 2012; Palumbo et al. 2017) allowing the construction of phylogenetic trees elucidating the history beyond germplasm collections.

During the last few years, a process of reviving landraces has been forced by farmers and consumers, associating traditional crop varieties with food production, the cultivation of marginal areas and proper description and conservation actions for traditional genetic resources (Barcaccia et al. 2003; Lucchin et al. 2003; Cassani et al. 2017; Palumbo et al. 2017; Ardenghi et al. 2018; Bernardi et al. 2018), a process recently encouraged also by national and EU legislation. For this reason in the present study three accessions referred to "Rostrato Val Chiavenna", a landrace currently cultivated by smallholder farmers in the Alps in Valchiavenna (Sondrio, Italy), have been characterized at the genetic and morphological level and compared to other maize of the same landrace group (Rossi et al. 2019). One of the three accessions of Rostrato di Val Chiavenna was sampled in the area of Chiavenna (Sondrio) in 1982 and ex-situ conserved up to now by CREA-CI (Bergamo), sample named VA 1196 "Rostrato Val Chiavenna" as reported in Bertolini et al. (2002), while the remaining two were recently sampled in-situ and stored at the Plant Germplasm Bank of the University of Pavia. The objective of the study was to: (1) characterize from a morphological and genetic point of view the "Rostrato Val Chiavenna" landrace, (2) assess the relationships between these three accessions and other "Rostrata" related materials and (3) assess the effectiveness of in-situ conservation in maintaining a wide genetic base.

Materials and methods

Germplasm

Three different "Rostrato Val Chiavenna" maize accessions were considered and compared to four different "Rostrata" maize landraces from Northern Italy: Dencin della Martesana, Dente di Cavallo del Friuli, Rostrato di Mortara and Spinato di Gandino processed in the so-called CORE SAVE Project, in the framework of activities funded by Lombardy Region.

The "Rostrato Val Chiavenna" Va1196 (R17_BG) seeds were kindly obtained from CREA-CI (Centro di Ricerca per la Cerealicoltura e Colture Industriali, Bergamo) and originally collected in Chiavenna in 1982, according to Bertolini (2002); instead the other two accessions were sampled *on farm* and donated respectively by Anna Miracca (R17_M) and Lorenza Tam (R17_T) both from a close locality, Prata Camportaccio (Sondrio). The last two accessions are locally cultivated at the family level.

Based on interview and historical reconstructions, R17_M has been cultivated for at least 70 years by the donor family, but it may not have a local origin, as the family was coming in 1945 to Prata Camportaccio (Sondrio) from Pavia, a city located in the south of Lombardia, about 200 km far from Prata bearing their corn. Originally the ear was not beaked but, presently, kernels show a pronounced beak likely as a consequence of possible introgression from locally grown Rostrato Val Chiavenna. Concerning R17_T historical reconstruction suggested that this type has been cultivated for at least 150/200 consecutive years in different localities of the valley (Prata Camportaccio and Samolaco) by local farmers and their relatives before.

For the other accessions, Dencin della Martesana (R1), Dente di Cavallo del Friuli (R3), Rostrato di Mortara (R16) and Spinato di Gandino (R21), informations are provided in Table 1, germplasm coding is defined by Ardenghi et al. (2018). Germplasm is maintained at the germplasm bank of the Department of Sustainable Crop Production of Università Cattolica del Sacro Cuore, Piacenza, Italy and the Plant Germplasm Bank held at Department of Earth and Environmental Sciences of Università degli Studi di Pavia, Pavia, Italy.

Field trials were set up during the years 2019 and 2020, in at CREI-CERZOO (45.005066 N,

Table 1 Detailed information about maize germplasm used in this study

Accession	Accession name	Sampling location	Year of collection	Collector	Conservation
R17_BG	Rostrato di Val Chiavenna Va1196	Chiavenna (Sondrio, Italy)	1982	CREA	CREA, UNIPV, UCSC
R17_M	Rostrato Val Chiavenna	San Cassiano di Prata Camportaccio (Sondrio, Italy)	2017	UNIPV	In situ, UNIPV, UCSC
R17_T	Rostrato Val Chiavenna	Prata Camportaccio (Sondrio, Italy)	2017	UNIPV	In situ, UNIPV, UCSC
R1	Dencin della Martesana	Cernusco sul Naviglio, (Milano, Italy)	2017	UNIPV	UNIPV, UCSC
R3	Dente di Cavallo del Friuli	Dolegna del Collio (Gorizia, Italy)	2017	UNIPV	In situ, UNIPV, UCSC
R16	Rostrato di Mortara,	Mortara (Pavia, Italy)	2017	UNIPV	In situ, UNIPV, UCSC
R21	Spinato di Gandino	Gandino (Bergamo, Italy)	2016	UNIPV	In situ, UNIPV, UCSC

9.704206E, San Bonico, Piacenza, Italy). The field was sown on April 17th in 2019 and April 15th in 2020. Each plot consisted of 3 rows 5 m long, spaced 80 cm apart from each row and 1 m aisle on the hedge for Rostrato di Val Chiavenna and 1 row for the remaining accessions; 20 seeds were planted in each row. Field trials were managed according to agricultural practices for maize nurseries. Leaf samples were collected from all plants at the 5th leaf stage during 2019. Maize accessions were phenotyped according to the UPOV protocol CPVO/TP2/3 in both the experiment years.

DNA extraction and PCR amplification

DNA was extracted from young leaf tissues according to GenElute DNA Miniprep Kit (Sigma-Aldrich) following manufacturer instructions with a minor modification consisting of the addition of 5% w/v Polyvinylpyrrolidone (PVP) during the lysis step to enhance the removal of polyphenols (Stagnati et al. 2020). Crude DNA extract was visualized on 1% agarose gel electrophoresis stained with Midori Green (NipponGenetics).

PCR reactions were carried out in a final volume of 25 µL. PCR mixture was composed of: 20 ng of crude DNA extract, 1X Reaction Buffer (2.5 µL 10X Reaction Buffer), 12 pmol dNTPs (0.3 µL of 10 mM stock), 4 pmol each primer (0.4 µL of 10 µM), 1U *Taq* polymerase (0.2 µL of 5 U/µL stock), 2% PVP (5 µL from 10% stock solution) and H_2O to final volume. PVP was added also during amplification to improve PCR amplification (Stagnati et al. 2017, 2020).

Ten SSR markers were selected from Palumbo et al. (2017) and Maize Genome Database (MaizeGDB, https://www.maizegdb.org/). Detailed information on primer pairs is reported in Table 2.

PCR cycle consisted of initial denaturation at 94 °C for 5 min, 40 cycles of denaturation at 94 °C for 2 min, annealing at optimal primer temperature as reported in Table 2 for 30 s, extension at 72 °C for 1 min and a final extension at 72 °C for 2 min.

Fluorescent labeled PCR fragments were visualized using an automated genetic analyzer ABI-Prism 3100 (Applied Biosystem) according to manufacturer's instructions and manually scored.

Statistical analysis

Detected alleles were analyzed with the GenAlEx6 software (Peakall and Smouse, 2006) to compute population statistics, analysis of molecular variance (AMOVA) and Principal Coordinates Analysis (PCoA).

The Polymorphic Information Content (PIC, Botstein et al. 1980), was calculated according to the formula implemented in the PowerMarker software, version 3.25 (Liu and Muse 2005).

Collected genetic data were used to construct a phylogenetic tree using the Unweighted Pair Group Method with Arithmetic mean method applying the UPGMA function of the phangorn package (Schliep

Table 2 Detailed information about primer 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	[6FAM]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	[HEX]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	[6FAM]CACCCGTACTTGAGGAAAACCC	10	58	140–170	Palumbo et al.(2017)
M24	*umc1327*	AGGGTTTTGCTCTTGGAATCTCTC	[HEX]GAGGAAGGAGGAGGTCGTATCGT	8	64	100–120	MaizeGDB
M33	*p-bnlg176*	AGTTCACGTCCAGCTGAATGACAG	[6FAM]CGCGCATCGCATGCTTATCCTA	1	62	140–170	MaizeGDB
M78	*umc1941*	ACGACGAGACTCTGTTCTGGTTCT	[HEX]AGGAGGATTACGTCAATCTGTTCG	5	64	110–130	MaizeGDB
M90	*umc1401*	CTCTGGTCCATCCTCATCGACT	[HEX]TCTCTTGATCACATATCGATCCCA	7	62	180–200	MaizeGDB
M193	*umc1786*	ACCGTGACTTCCTCCTCATAACTG	[HEX]CATTTTTCGCATTTAGGAAATCCA	8	60	180–220	MaizeGDB

2011) starting from a genetic distance matrix calculated by the mean distance matrix available in the polys at package of the R software (Clark and Jasieniuk, 2011).

The genetic structure of the landrace collection was investigated using a Bayesian clustering algorithm implemented in STRUCTURE v.2.3.4 (Falush et al. 2003). The "admixture model" option was selected since there was no previous information on the collection under study (Palumbo et al. 2017) and the "correlated allele frequency model" because it has a better power to detect subtle population structure without affecting results if such correlation does not exist (Falush et al. 2003; Palumbo et al. 2017). Ten independent replications were run for each level of K ranging from 2 to 22 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 (Evanno et al. 2005; Palumbo et al. 2017).

Results and discussion

Morphological characterization of germplasm

Morphological characters were measured during both years of the field experiment and used to describe accessions according to CPVO/TP2/3 protocols. Plant descriptors are reported in different active sheets of Supplementary Table 1.

The Rostrato di Val Chiavenna R17_BG is a medium–high landrace with an average height of plants of 2 m and a first ear insertion of 0.88 m, ear/plant insertion rate is 0.36. Tasseling, silking and physiological maturity occurs at 707, 755 and 1476 GDD, respectively. Leaf sheaths and silks present no anthocyanin pigmentation, while anthers have a medium color. Ears are very short with an average length of 12 cm and medium diameter (43.9 mm) with a slightly conical/cylindrical shape and 14 kernel rows. Caryopsis are of an intermediate type and from yellow to orange in color as shown in Supplementary Fig. 1.

The accession Rostrato di Val Chiavenna R17_M is characterized by tall plants (over 3 m) with high ear insertion (1.6 m) and ear/plant insertion rate is 0.54. Before tasseling, plants were characterized by a transitory bending of the leaf cone, which resolves during tasseling. Tasseling, silking and physiological

maturity occurs at 826, 870 and 1507 GDD, respectively. Leaf sheaths and silks don't show anthocyanin pigmentation, while anthers have a medium intensity of color. Ears are very short with an average length of 14.7 cm and medium diameter (42.4 mm) with a cylindrical shape and 14.8 kernel rows as an average number. Caryopsis is dent-like varying from orange to red color, the beaked character is particularly prominent as shown in Supplementary Fig. 2.

The accession Rostrato di Val Chiavenna R17_T is characterized by tall plants (3 m as average) bearing the first ear at 1.7 m and ear/plant insertion ratio of 0.56. Tasseling, silking and physiological maturity occurs at 870, 929 and 1540 GDD, respectively. Leafs sheaths are green while tassel glumes, anthers and silks showed a medium color intensity. Ear types are classified as long (average length of 22.7 cm) and medium diameter (41.7 mm) with a cylindrical shape and 14 kernels rows. Grains are of the dent-like type with dark red pigmentation as shown in Supplementary Fig. 3. The beaked character is very faint and sometimes absent as a consequence of repeated selection carried out at farm level because shelling was done manually and the kernel beak stung hands, palms and fingers.

At a morphological level, it appeared evident that strong phenotypic differences characterize the three accessions grown under the same name which suggests a possible case of homonymy without excluding, a priori, huge genetic variability within these populations.

Spinato di Gandino R21 is a medium–high population with an average height of 2.66 and first ear insertion at 1.26 m, ear/plant insertion rate is 0.47. Anthesis, silking and maturity are reached at 739, 771 and 1492 GDD, respectively. Leaf sheaths are not pigmented while tassel and silks are medium-colored. The ear is short and thin with 15.3 cm length and 37.6 mm diameter. The ear shape is a slightly conical shape with beaked kernels arranged in 10–12 rows; grains are of a flint-like type. Kernel color is yellow.

Dencin della Martesana R1 is a medium landrace with an average plant height of plants of 2.02 m, first ear inserted at 0.83 m and ear/plant ratio of 0.41. Tasseling, silking and physiological maturity occurs at 707, 826 and 1492 GDD, respectively. Leaf sheaths are green, while glumes and anthers show a medium pigmentation. The ear is short with an average length of 16 cm and very thin with a diameter of 35.2 mm. Ears shape is slightly conical with a number of 10/12 kernel rows. Grains are of flint-like type, beaked and orange-red color.

Dente di Cavallo Bianco e Rosso R3 is a tall landrace with plants reaching 3.1 m, ear develops at an average height of 1.46 m, ear/plant ratio is 0.47. Anthesis, silking and maturity occur at 842, 899 and 1524 GDD, respectively. Leaf sheaths and glumes have no particular pigmentation while silks and anthers show medium pigmentation. The ear is of medium length (average 21 cm) and medium diameter (average 40.8 mm). The shape is conical with 12/14 kernel rows. Seeds are of the dent-like type of white and red pigmentation.

Spinato di Mortara R16 is a medium–high population with the plant's average height of 2.6 m and first ear inserted at 1.09 m. The ear/plant ratio is 0.42. Tasseling, silking and physiological maturity are at 707, 812 and 1492 GDD, respectively. Leaves and silks have no pigmentation, while anthers and glumes show medium pigmentation. The ear is short (average 17.8 cm) with a thin diameter (average 39 mm). The shape is conical with 12 kernels rows. Caryopsis is dent-like and orange to red-colored.

A common characteristic of these landraces is the presence of beaked kernels. This trait is more or less pronounced since the proximal cultivation, occurred in the past, with other varieties of flint and dent types may have driven the appearance of intermediate types with less pronounced beak and intermediate or dent-like kernel as R3 and R16. At the same time this has contributed to the huge vigor of the plant as R17_M, R17_T and R3 (Bertolini et al. 2005; Ardenghi et al. 2018).

Genetic characterization of accessions and population structure

The marker data of all 117 samples were collected and analyzed to investigate the main population parameters.

Globally 62 different alleles were detected with a mean number of alleles per locus of 6.2, the allele number ranged from a minimum of 3 alleles for locus *phi084* to a maximum of 11 for *umc1075* as reported in Table 3. Globally 22 private alleles were identified with an allele frequency per accession and locus varying from a minimum of 0.01 (*p-bnlg176* for R17_BG) to a maximum of 0.875 (*umc1786* for R1),

Supplementary Table 2. Va1196 (R17_BG) is characterized by the presence of 3 private alleles for *umc1075* (alleles 125, 128 and 141), 3 for maker 33 (alleles 187, 193 and 195) and one for marker 78 (allele 105). R17_M possesses 4 private alleles (159, 72, 125 and 138) at markers *phi076*, *umc1327*, *umc1401* and *umc1786,* respectively. R17_T has two private alleles (131 and 140) at locus *umc1075* while loci *phi127*, *phi031* and *p-bnlg176* are characterized by the presence of one private allele (107, 205 and 186), respectively. Two private alleles were detected at locus *umc1941* (89 and 102) and one (171) at locus *phi031* in the accession R21; two private alleles were scored for the accession R1 for loci *phi084 umc1786* (158 and 132, respectively) and one for the accession R16 at locus *p-bnlg176* (191). Private alleles make possible the differentiation of varieties and may be used for further studies aimed, for example, at the development of methods for DNA-based traceability of food productions obtained from these varieties.

The global allele number, observed in the present study, is consistent with values reported for similar studies (Cömertpay et al. 2012; Oppong et al. 2014; Palumbo et al. 2017) while the number of private alleles seems to be higher than previously reported (Eschholz et al. 2010; Palumbo et al. 2017). This difference can be a consequence of the collection itself, SSR markers used, reproductive isolation and independent ancestral origin of these landraces as further discussed.

The number of polymorphic loci varied from 80% (R17_BG and R1) to 100% (R17_M and T, R21 and R16, where monomorphic loci were not observed).

The number of observed alleles (N_a) ranged from a minimum of 2 of *phi084* to 4.43 of *umc1075* at locus level while from 2 of R1 to a maximum of 3.8 R17_T at accession level; the number of expected alleles (N_e)

Table 3 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_{IS}	F_{ST}	N_m
phi127	3.71	2.68	1.01	0.69	0.59	0.63	− 0.249	0.22	0.90
phi076	2.57	2.05	0.76	0.6	0.52	0.57	− 0.19	0.19	1.08
phi031	2.57	1.54	0.52	0.33	0.32	0.36	− 0.080	0.36	0.44
umc1075	4.43	3.14	1.20	0.64	0.68	0.80	0.02	0.21	0.93
phi084	2.00	1.80	0.60	0.41	0.44	0.41	0.04	0.18	1.12
umc1327	3.00	2.53	0.94	0.99	0.60	0.70	− 0.70	0.17	1.21
p − bnlg176	2.86	1.53	0.49	0.27	0.29	0.73	0.03	0.60	0.16
umc1941	3.29	2.39	0.94	0.71	0.58	0.71	− 0.29	0.18	1.10
umc1401	2.29	1.64	0.55	0.3	0.35	0.56	0.13	0.47	0.28
umc1786	2.57	1.97	0.7	0.18	0.45	0.64	0.57	0.44	0.31
All loci	2.93	2.13	0.77	0.51	0.48	0.61	− 0.07	0.30	0.76
St. dev	0.15	0.09	0.05	0.04	0.02	0.14	0.10	0.05	0.13
R17_BG	3.20	1.79	0.65	0.41	0.39	0.34	− 0,06	0.43	0.34
R17_M	3.20	2.28	0.86	0.51	0.51	0.47	− 0,006	0.24	0.78
R17_T	3.80	2.59	0.98	0.56	0.56	0.50	− 0,0002	0.17	1.23
R1	2.0	1.72	0.54	0.42	0.77	0.29	− 0,111	0.47	0.29
R3	2.50	2.04	0.73	0.55	0.49	0.42	− 0,121	0.31	0.56
R16	2.80	2.07	0.77	0.53	0.5	0.40	− 0,062	0.28	0.63
R21	3.00	2.40	0.87	0.59	0.55	0.44	− 0,076	0.23	0.85
All pop	2.93	2.13	0.77	0.51	0.48	0.41	− 0.06	0.30	0.67
St.dev	0.15	0.09	0.05	0.04	0.02	0.07	0.05	0.11	0.32

was always lower than N_a ranging from 1.53 for *phi031* to 3.14 for *umc1075* and 1.72 (R1) to 2.59 (R17_T) for SSR markers and landraces, respectively (Table 3).

Values observed here are lower than those reported by Palumbo et al. (2017) probably because of a smaller number of plants per landrace. The three R17 accessions showed a higher number of observed alleles with respect to the others and R17_T was found to be the richest in alleles. A higher number of observed alleles allows the origin of several different genotypes after sexual reproduction and denotes high genetic diversity within the population.

The Shannon's index (I) was used to characterize population diversity and it was found to be, on average, equal to 0.77 ± 0.05 over all loci and populations (Table 3).

Overall the SSR loci and populations, the observed (H_o) and unbiased expected (uH_e) heterozygosity were, as mean value, equal to 0.51 ± 0.039, and 0.461 ± 0.026. Observed heterozygosity was significantly lower than expected only for locus *umc1786* while for *phi031* and *p-bnlg176* values are almost equal while for locus *umc1327* H_o is extremely high (0.99) as shown in Table 3. Heterozygosity defects were not observed at the population level except R1 which is the only landrace with no active cultivation (Ardenghi et al. 2018). These results are in agreement with the reproduction system of maize whose decline inflorescences help outbreeding and cross-pollination in consecutive generations. Moreover, in the field, accessions showed phenotypic variability for many traits which is typical to open field pollinated outcrossing materials.

Similar works on corn landraces report general lacks of heterozygosity both at a locus and population-level because smallholder growers usually renew seed stocks starting from few ears (Barcaccia et al. 2003, Qi-Lun et al. 2008; Palumbo et al. 2017) and usually do not apply any precautions to avoid self-pollination. In allogamous species, like corn, homozygosity and inbreeding severely affect plant performance and the homozygous plant usually produce smaller ears. At the smallholder farmer level, seed selection is often performed by choosing seeds from larger fruit, in this case, the biggest ear (Zapparoli 1930). It is possible that such an operation selected seeds from heterozygous plants thus maintaining a good level of "hybrid vigor" in the population (Qi-Lun et al. 2008).

PIC values were considered to estimate the ability of each selected SSR marker to discriminate among the different genotypes within the collection. The average PIC value was 0.61 ranging from 0.36 of *phi031* to 0.80 of *umc1075*. PIC values calculated for this set of SSR data are above average than those reported in similar studies (Oppong et al. 2014; Palumbo et al. 2017) while lower than values calculated for Swiss and Turkish entire landraces collections (Eschholz et al. 2010; Cömertpay et al. 2012).

The inbreeding coefficient F_{IS} had average values of -0.07 ± 0.10 and -0.06 ± 0.05 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 (Qi-Lun et al. 2008).

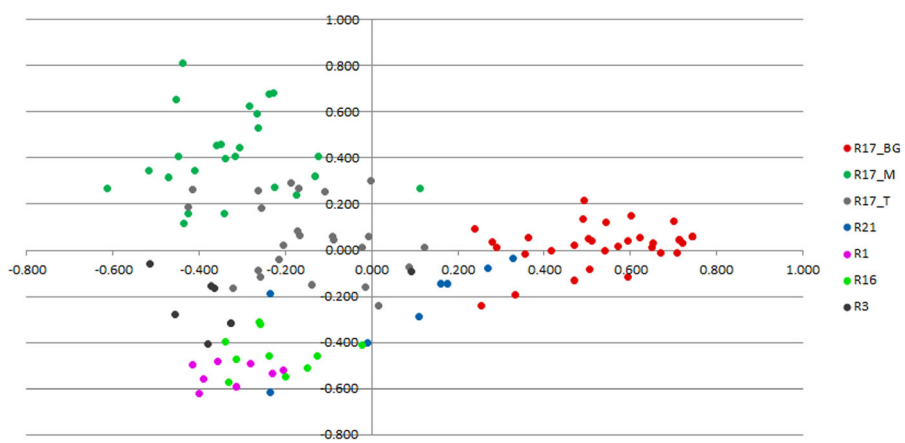

Fig. 1 Principal Coordinates Analysis (PCoA) of the 117 samples characterized by the 10 SSR set

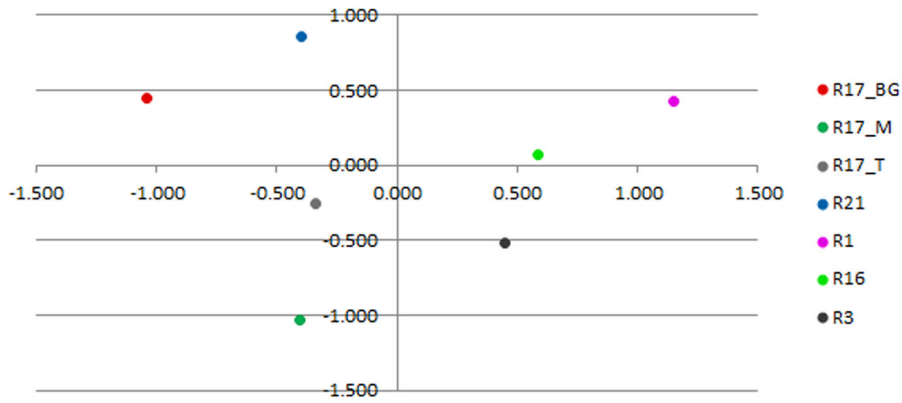

Fig. 2 Principal Coordinates Analysis (PCoA) according to different populations

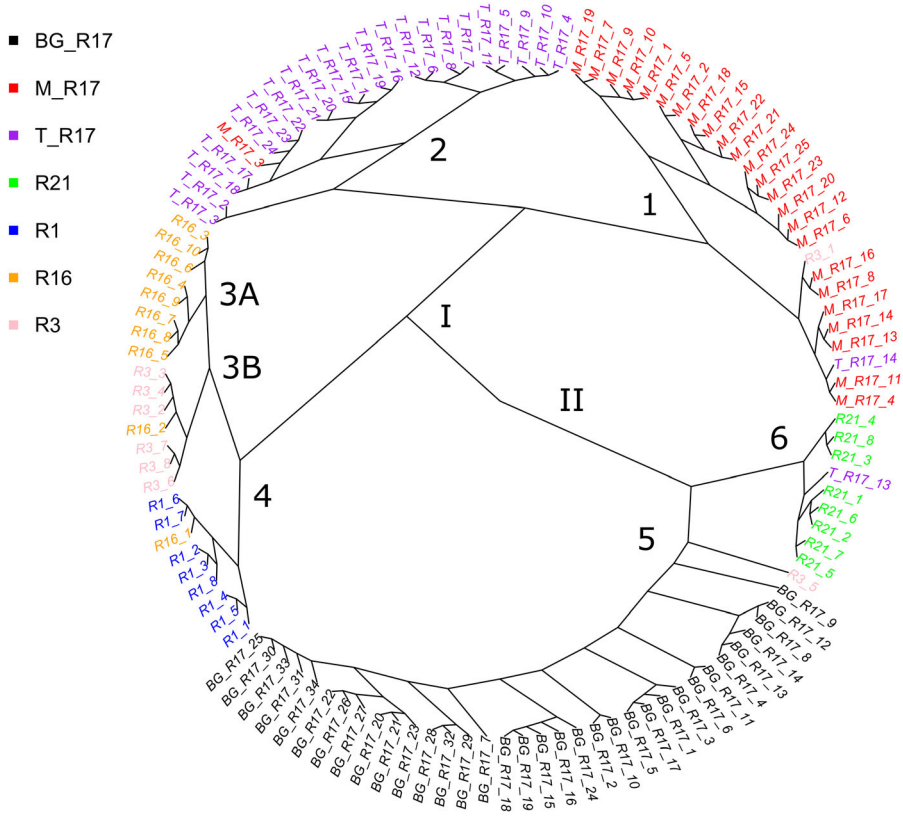

Fig. 3 Phylogenetic tree obtained by the UPGMA method on a genetic matrix derived from SSR data

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 is found between varieties, especially if compared to other maize landrace collections (Ignjatović-Micić et al. 2008; Qi-Lun et al. 2008; Oppong et al. 2014; Palumbo et al. 2017). Population statistics calculated for these landraces confirm a good level of variability within the collection and that the conservation status of landraces, with particular interest to the accessions of Rostrato di Val Chiavenna, is adequate to preserve genetic variability and maintain a sufficient level of heterozygosity.

Cluster analysis and phylogenetic tree

Principal Coordinate Analysis (PCoA) showed that samples separated according to different accessions and even if there was not a very clear separation between groups four areas can be identified in Fig. 1 corresponding to each one of the three R17 accessions and the different outgroup landraces. The only exception was represented by R21 which was more scattered and intermediate between outgroups and R17 (BG and T).

The first principal component accounted for 19.87% of the variability and separated the 3 accessions of R17 according to the sampling site and conservation strategy (in situ vs ex situ). The second component, accounting for 12.92% of variability, separated the two in situ accessions of R17. Regarding the other accessions, used as a comparison, only R21 and R3 were separated from other materials by the first and second components, respectively.

Concerning the two in situ accessions of R17, it was clear the presence of an overlapping area where several samples clustered together. This might suggest the presence of cross-pollination between these two accessions grown in the same area, however, population structure analysis did not support this consideration. Another interesting observation was the distribution of the three R17 accessions in three main clusters along with the first principal component. From PCoA it appears that R17_T was closer to the "original" R17 conserved in the germplasm bank in Bergamo than R17_M while at the morphological level the situation was the opposite with R17_M more similar, at ear level, to R17_BG.

If the genetic distance matrix was computed according to landrace assignment, it appeared that R17 accessions were well separated by the second component with the two in situ accessions more related together than the R17_BG and with the other varieties more dispersed. According to this analysis, the 7 accessions were more separated than considering the entire set of individuals, however, no clear accession clustering seemed to be evident (Fig. 2). Also at this level, R21 was more related to R17_BG and R17_T than to other landraces as in the individual-based computed PCoA. Further studies should clarify the relationships existing between these three materials.

AMOVA analysis revealed a good differentiation between different accessions explained by 29% of variability among landraces while 71% of variation lied within a single population. Such differentiation, as expressed by F-statistics, correspond to other studies (Ignjatović-Micić et al. 2008; Giupponi et al. 2021) as a result of reproductive isolation because R17_BG is still under controlled reproduction for around 40 years, R17 T and M are currently grown in areas where commercial corn is absent and corn field is dispersed between woods. The outgroups, even if recently collected, are maintained with accurate distance from other corn (R16, R21, R3) as reported by Ardenghi et al. (2018).

The UPGMA phylogenetic tree, reported in Fig. 3, revealed the presence of two main clusters and six sub-clusters. The first main cluster (I) corresponded to a group composed of R17_M, R17_T and the outgroups R16, R3 and R1 landraces while the second one (II) clustered R17_BG and R21. At a sub-clusters level it is possible to note that samples corresponding to R17_M and R17_T are defined in sub-clusters 1 and 2, respectively; sub-cluster 3 is further divided in sub-cluster 3A corresponding to R16 and sub-cluster 3B which comprise the majority of R3 samples; sub-cluster 4 comprises R1 accession while sub-clusters 5 and 6 correspond to R17_BG and R21, respectively. What emerged from this analysis is a clear separation of R17_BG from the other two R17 accessions and a closed genetic relationship between R17_BG and R21 as highlighted also in PCoA analysis where R21 was in an intermediate position between R17_BG and R17_T.

The absence of a relationship between R17_BG and other materials sampled in the province of Sondrio was confirmed also by the work of Hartings et al. (2008). Similarly, the closest relationship between R17_BG and R21 is somehow supported by the same authors which found proximity with accessions sampled in the surroundings of Bergamo despite accessions of Valchiavenna or nearby areas.

Comparing the phylogenetic tree to STRUCTURE analysis it appears that, at K = 4, R21 should group closed to R17_T while all the other outgroup landraces were clustered together. At K = 6 all populations are differentiated except R16 and R3 consistently with the grouping of these two on the same tree branch and in sub-clusters 3A and 3B.

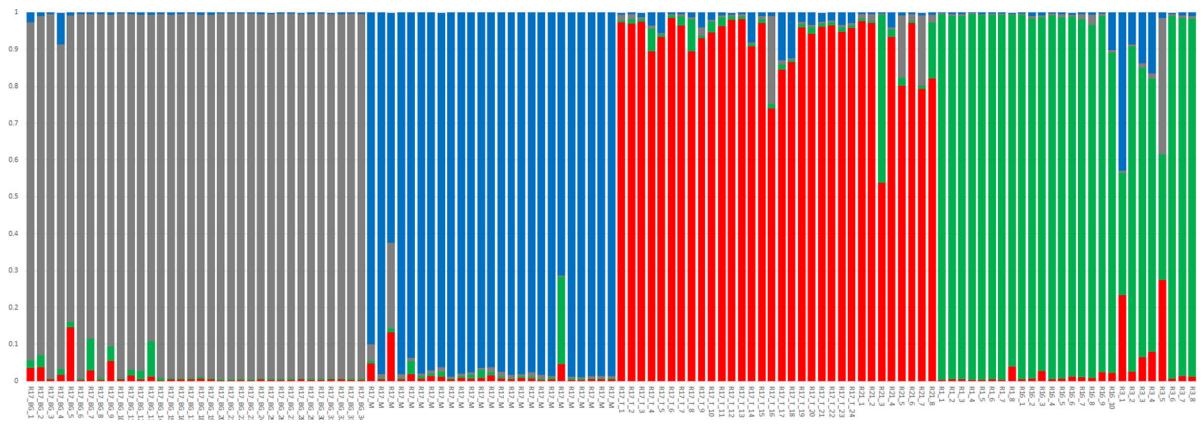

Fig. 4 Population genetic structure of the seven accessions of the Rostrata Group as estimated by STRUCTURE. Each sample is represented by a vertical histogram partitioned in K = 4 colored segments representing the membership to each of the identified ancestral populations. The ancestry proportion (%)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

The presence of some spurious samples as R17_T plant 13; R3 plant 1; and R16 plant 1 is confirmed by admixture levels of the ancestry model from K = 6.

Structure analysis

Population structure was investigated using the STRUCTURE software and the procedure of Evanno (Evanno et al. 2005) was followed to determine the best level of K. The highest ΔK was found at K = 4 (ΔK = 375.46) while at K = 6 a second ΔK (ΔK = 134.78) was identified. According to this analysis, 117 corn samples were organized into four genetically distinct clusters. The clustering of genotypes revealed that 95 out of 117 samples (81%) showed a strong ancestry association (> 90%) to their genetic cluster. From 79% (R17_T) to 92% (R17_M) of analyzed plants showed ancestry association higher than 90% to the genetic group. Admixed genotypes, showing a membership lower than 0.8, were sporadically present in R17_M (2 individuals) and R17_T (1 individual).

At this K level, the first cluster comprised R17_BG, the second R17_M while R17_T was found in the third cluster as well as R21; R1, R16 and R3 constituted the fourth cluster.

What emerged from this analysis was that the three accessions of "Rostrato Val Chiavenna" are three different genetic entities with no genomic interconnection among them (populations 1–3 in Fig. 4). At the same time, the outgroups, even if belonging to the same varietal group, are genetically distinct from Rostrato Val Chiavenna. An interesting finding was that Spinato di Gandino, at structure level, clustered within group 3 together with R17_T. All the analyses that have been carried out supported close proximity of R21 with two populations of R17. More specifically: (1) PCoA analysis placed R21 between R17_BG and R17_T; (2) UPGMA analysis placed R21 closer to R17_BG and (3) STRUCTURE analysis grouped R21 with R17_T. At the morphological level, these three accessions are very different but R21 is more similar to R17_BG.

The second K revealed an additional level of structuration of the collection with the definition of another sub-clusters corresponding, respectively to R21 (Spinato di Gandino) which was separated from R17_T and R1 which separated from other outgroups. The first three clusters corresponded, again, to R17_BG, R17_M and R17_T. The sixth cluster grouped the remaining individuals excepting one sample of R3 with 72% of the membership to cluster 4 probably because of introgression from other maize cultivated in the area or selection pressure (Ardenghi et al. 2018).

It was interesting to note that from K = 2 there was a clear separation of R17_BG from the other accessions (data not shown). It is to note that R17_BG was sampled around 40 years ago (1982) and from that time it is maintained by controlled pollination at CREA-CI in Bergamo while the other accessions, originated from recent *in loco* samplings, represented the offspring of currently evolving populations.

The two R17 still grown in Valchiavenna are two distinct materials of different origin and probably

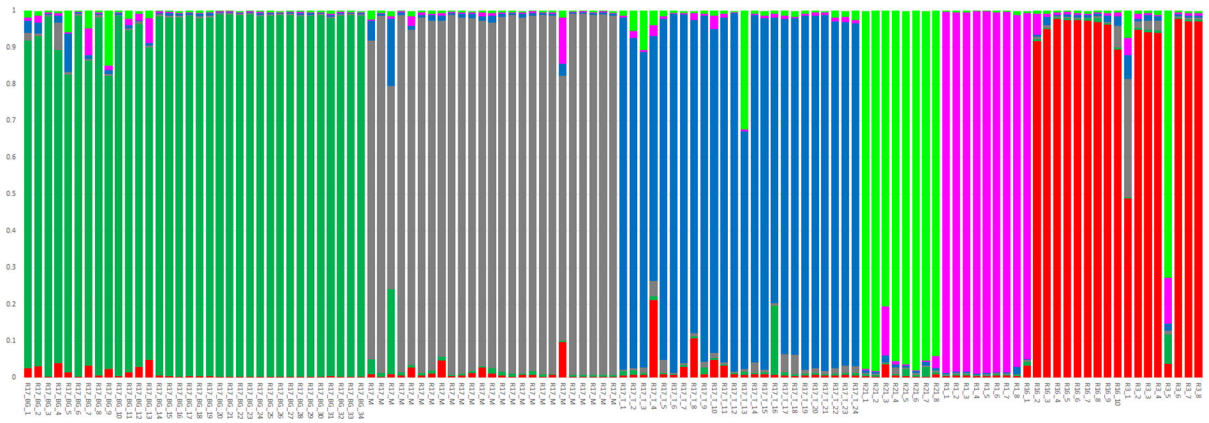

Fig. 5 Population genetic structure of the seven accessions of the Rostrata Group as estimated by STRUCTURE. Each sample is represented by a vertical histogram partitioned K = 6 colored segments representing the membership to each of the identified ancestral populations. The ancestry proportion (%) is reported on the y-axis

unrelated to the R17_BG accession as noted for plant morphology and ear appearance and kernel pigmentation. Records of maize cultivation in the area reported the presence of a second landrace named "Locale Chiavenna", sampled in Verceia (SO) in 1954, and conserved in the CREA germplasm with the code Va65. For this accession, it is available a note specifying that the cultivation area was within the municipality of Chiavenna where the variety was adapted 15 years before the sampling of 1954 (Bertolini et al. 2002). It is not to be excluded that spontaneous crosses between an original Rostrato di Val Chiavenna and Va65 might have played a role in the origin of the different R17 accessions that have been analyzed in this study. For the other accession, Rostrato di Val Chiavenna R17_BG, conserved at CREA (Va 1196), no particular information on cultivation area or origin is available (Bertolini et al. 2002). The observation that these materials are presently cultivated under the same name is a clear case of homonymy as documented for other landraces especially those under the names "Locale" or "Nostrano" (Ardenghi et al. 2018) and the location of accessions with a similar name in different ancestry groups is not uncommon (Oppong et al. 2014). In this case, the name is slightly different but it contains a strong reference to ear type and growing locations. We can not exclude that these materials are the consequence of a strong selection process, which happened in the last 40 years, that clearly differentiated the materials but, currently, this idea is not supported by any data.

A similar study (Palumbo et al. 2017) showed that at the second ΔK it is possible to highlight admixture of individuals of different populations grown under the same name. In this case, no particular admixture seems to be present regarding the two R17 still grown in situ and even if they are grown under the same name and in the same area events of hybridization and introgression could be excluded as supported by the results of structure analysis and previously suggested by the strong differences at ear level. These accessions are maintained by smallholder farmers that usually take precautions to avoid contamination with different corn and it cannot be excluded that these accessions have undergone a certain unconscious selection operated by farmers (Ardenghi et al. 2018).

Conclusion

The three Rostrato di Val Chiavenna populations assessed in the present study revealed a clear case of homonymy supported by all the genetic analysis carried out and by morphological characterization. It is likely to hypothesize a distinct origin for each one of the three accessions in particular for R17_BG; it is not to be excluded a common origin for R17_M and R17_T but, likely, they separated early many generations ago even if cultivated in the same area. Among the other landraces considered interesting is the case of R21 which share a common origin with R17 BG or T according to a different analysis. Globally good levels of genetic diversity, distinctiveness and

heterozygosity have been evidenced for all landraces. These results confirm that in situ and ex situ conservations if correctly applied, represent proper strategies for germplasm resource maintenance for different purposes.

The historical investigation, carried out *in loco* during the experiment, revealed that the morphology most close to the "original" Mais Rostrato di Val Chiavenna is that of R17_T while R17_M represents something of more recent origin and cultivated only by the same family in recent years. Finally, R17_BG represents a variety stored and conserved only ex-situ, which has not been used by local farmers in these years. These observations, coupled with the genetic results previously mentioned may corroborate that R17_T corresponds to the original "Mais Rostrato Val Chiavenna", locally cultivated in Valchiavenna (Sondrio) since 150/200 years ago up to presently.

Author contributions *Lorenzo Stagnati:* Investigation, Formal analysis, Writing—original draft; *Michelangelo Martino:* Field management, Laboratory analysis; *Giovanna Soffritti:* Data curation, Review & editing*; Alessandra Lanubile:* Review & editing; *Adriano Ravasio:* Field management, Review & editing; *Adriano Marocco:* Conceptualization, Review & editing; *Graziano Rossi*: Funding acquisition, field exploration in Valchiavenna, historical studies, review & editing; *Matteo Busconi:* Funding acquisition, Investigation, Data curation, Writing.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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