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Characterization of a flint maize (*Zea mays* var. *indurata*) Italian landrace, II. Genetic diversity and relatedness assessed by SSR and Inter-SSR molecular markers

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Abstract

A comparative characterization of 10 field populations of the maize (*Zea mays* var. *indurata*) landrace ''Nostrano di Storo'' was carried out using different types of PCR-based markers. The inbred line B73 and three synthetics (VA143, VA154 and VA157) selected from as many landraces were also used. Genetic diversity and relatedness were evaluated over 84 SSR and 53 I-SSR marker alleles using a total of 253 individual DNAs. Up to 23 alleles per SSR locus were scored while the average effective number of alleles per population was 6.99. Nei's total genetic diversity as assessed with SSR markers was $H_T = 0.851$ while the average diversity within populations was $H_S = 0.795$. The overall Wright's fixation index F_{ST} was as low as 0.066. Thus, more than 93% of the total variation was within population. Unique alleles over all SSR loci were found for six populations. An average of 17.7 marker alleles per I-SSR primer were scored with an effective number of marker alleles per locus of 1.34. The Shannon's diversity information index over all populations and I-SSR loci was 0.332, varying from 0.286 to 0.391. The extent of differentiation between populations was as low as $G_{ST} = 0.091$. Dice's genetic similarity matrices were estimated for both SSR and I-SSR markers. The mean genetic similarity coefficients within and between populations were respectively 0.269 and 0.217, for SSR markers, and 0.591 and 0.564, for I-SSR markers. UPGMA dendrograms displayed all field populations but one clustered into a distinct group, in which the synthetic VA154, selected from the ''Marano Vicentino'' landrace, was also included. One field population and the other two synthetics were clustered separately as well B73. The matrix correlation assayed by the Mantel's correspondence test was as high as 0.908. Findings suggest that, although a high variability can be found among plants, most plant genotypes belong to the same landrace called ''Nostrano di Storo''. Although gene flow from commercial hybrids might have occurred, the large number of polymorphisms and the presence of both unique alleles and alleles unshared with B73 and synthetics are the main factors underlying the value of this flint maize landrace as a source of genetic variation and peculiar germplasm traits. Because of its exclusive utilization for human consumption, such a molecular marker characterization will be a key step for obtaining the IGP mark and so promote the *in situ* conservation and protection of the landrace ''Nostrano di Storo''.

Introduction

The introduction of maize (*Zea mays* L.) in the Italian cultivation system goes back to approximately four centuries ago. Over this time, new landraces have originated from the original populations introduced, through adaptation to local conditions as well as hybridization brought about by continuous exchange and trade (Trifunovic 1978). Farmers have continued to cultivate and maintain open-pollinated varieties of maize until the mid of this century when hybrids were introduced in Europe. Subsequently, the Italian agricultural scenery has radically changed, with subsistence, mixed farming replaced by commercial monoculture, intensive farming (Bertolini et al. 1998).

Unless in the last few decades conservation of

landraces in gene-banks has taken place, their gradual replacement by hybrids has resulted in genetic erosion. Many maize breeders are now concerned that genetic diversity within this species has been decreasing at an alarming rate as a consequence of modern hybrids and other agricultural changes. For instance, most inbreds have been developed from a limited number of elite lines and synthetics, a practice that heightens the risk of genetic uniformity in commercial maize production fields (Hallauer et al. 1998). Thus, maize breeders have recently become more aware of the need for both maintaining genetic diversity among hybrid varieties and improving the management of genetic resources through the conservation of landraces (Goodman 1994).

Very little is known about the original (pre-hybrid) Italian maize (Lanza 1961; Brandolini et al. 1967; Brandolini 1970). The *Z. mays* var. *indurata* landrace ''Nostrano di Storo'', object of this study (Part I), is a surviving sample – perhaps one of several – and it could be used as a model for understanding issues of *in situ* conservation in Europe and elsewhere. Information on the genetic structure of a landrace could help limiting genetic erosion as well as conservation of landraces while also allowing the possible exploitation of genes for traits like resistance or tolerance to biotic and environmental stresses.

Landraces are populations with high genetic variability and fitness to the natural and anthropological environments where they have originated (Zeven 1996). They represent not only a valuable source of potentially useful traits, but also an irreplaceable bank of highly co-adapted genotypes (Brush 1999). Knowledge of germplasm diversity among local populations and breeding stocks is expected to have a significant impact on the improvement of crop plants. In maize, this information is known to be useful in planning crosses for hybrid development, assigning lines to heterotic groups, maintaining genetic variability of landraces, and protecting inbreds and varieties. It can be obtained by surveying both qualitative and quantitative morphological traits or using molecular markers for investigating polymorphisms at the DNA sequence level (Barcaccia et al. 1999).

Despite their importance, the genetic characterization of landraces as a key tool for their conservation has been largely ignored until very recently. Genetic variability in maize landraces has been primarily characterized by using morphological traits (Bonciarelli 1961; Camussi 1979; Camussi et al. 1980; Azar et al. 1997; Bosch et al. 1997; Louette and Smale 2000) and isozymes (Goodman and Stuber 1983; Bretting et al. 1987; Pfluger and Schlatter 1996; Sanau et al. 1997; Revilla et al. 1998). Evidence of *in situ* conservation as an effective strategy for genetic diversity maintenance of landraces has been reported (Brush 1995), and the importance of molecular markers as a main method for monitoring genetic diversity and relatedness among elite breeding maize materials has been assessed (Melchinger et al. 1991; Kantety et al. 1995; Smith et al. 1997; Senior et al. 1998; Pejic et al. 1998).

Besides linkage mapping, gene targeting and assisted breeding, the plant DNA polymorphism assays are powerful tools for characterizing and investigating germplasm resources and genetic relatedness (Powell et al. 1996). These techniques include restriction fragment length polymorphism (RFLP) markers and PCR-based molecular markers, such as simple sequence repeat, SSR or microsatellite (Tautz 1988; Morgante and Olivieri 1993) and inter-microsatellite, I-SSR (Zietkiewicz et al. 1994) markers.

Molecular markers detect polymorphism by assaying subsets of the total amount of the DNA sequence variation in a genome. Polymorphisms detected by RFLP assays reflect the variation of restriction fragment sites. PCR-based polymorphisms result from DNA sequence variation at primer binding sites and from DNA length differences between primer binding sites. The SSR assay utilizes pairs of primers flanking each simple sequence repeat and polymorphisms differ for the number of repetitive di-, tri- or tetranucleotide units present at one locus. The I-SSR assay detects polymorphisms between two inversely oriented microsatellites and involves the use of a single primer anchored at one end of the target repeat.

Although RFLPs have provided useful estimates of the genetic diversity and relatedness in maize, there is some concern about their discriminatory power. Increasing the number of probe-enzyme combinations may improve the number of RFLP marker loci detectable, but the level of polymorphism that can be revealed by PCR-based markers still remains higher. In fact, owing to their own genetic nature, SSRs usually detect multiple alleles at a given locus while I-SSR assays detect multiple loci randomly distributed in the genome.

Results of a comparative molecular characterization of populations and synthetics belonging to or derived from old landraces of *Z. mays* var. *indurata* still locally cultivated in Northern Italy are here reported. Genetic diversity and relatedness were

evaluated with SSR and I-SSR markers using single plant DNA samples. Information for an appropriate *in situ* conservation and management of this valuable flint maize germplasm is reported and discussed.

Materials and methods

Plant material

Ten maize field populations of an Italian landrace named ''Nostrano di Storo'' (shortened NSt) were collected in the Chiese Valley, Trento (Italy) in 1997. Each population was represented by 20 individuals from as many kernels randomly sampled in four different ears selected by farmers on the basis of their morphology. Three maize synthetics, coded as VA143, VA154, and VA157, selected from three distinct Italian landraces (''Spino Bresciano'', ''Marano Vicentino'', and ''Dente di Cane Piemontese'', respectively) by the Istituto Sperimentale per la Cerealicoltura of Bergamo (Italy), were also used. These varieties were represented respectively by 19, 17 and 16 individuals. The inbred line B73 was used as tester. Polymorphism degree and genetic relationships among all 253 individual DNA samples were evaluated with SSR (microsatellite) and I-SSR (inter-microsatellite) markers. Detailed information on germplasm stocks and collection sites are reported in part I.

Genomic DNA isolation

Approximately 0.5 to 0.75 g of maize leaf tissues were collected from healthy plants and frozen in liquid nitrogen. Total genomic DNA from leaf samples was isolated according to the protocol described by Dellaporta et al. (1983). The DNA pellet was washed twice with 70% ethanol, dried and redissolved in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The concentration of DNA samples was determined by optical density reading (DU650 spectrophotometer, Beckman) at 260 nm (1 O.D. = 50 μg/ml) and the purity calculated by the $O.D._{260}/O.D._{280}$ ratio and by the $O.D._{210}-O.D._{310}$ pattern (Sambrook et al. 1989). An aliquot of genomic DNA was also assayed by electrophoresis on 1% agarose gels.

Molecular markers

SSR markers

Microsatellite (SSR) loci analysis was performed according to Taramino and Tingey (1996) in a 20-μl volume containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 5 pmol of each primer, 1 U *Taq* DNA polymerase (Pharmacia Biotech) and 25 ng of genomic DNA. Forward and reverse primers flanking AG (locus phi031), and AC (loci dupssr1, dupssr7 and dupssr10) sequence repeats are reported in Table 1. (Additional information on SSR loci is available at:

- http://www.agron.missouri.edu).

Polymorphisms were visualized by labeling one of the primers with γ-[^{33}P] ATP (Amersham, Life Science) using T4 polynucleotide kinase (Pharmacia Biotech). Amplifications were performed using a 9700 Thermal Cycler (Perkin Elmer) with the following temperature conditions: start with 4 min at 94 °C followed by 30 cycles of 1 min at 94 °C, 1 min at 56 °C and 1 min at 72 °C, and end with 7 min at 72 °C. PCR products were added to an equal volume of stop solution (98% deionized formamide, 2 mM EDTA, 0.05% bromophenol-blue and 0.05% xylene-cyanol) and heated for 5 min at 95 °C. A 3 μl aliquot of each reaction mixture was analyzed by 6% denaturing polyacrylamide gel (Acrylamide/Bis 19:1 solution) electrophoresis run with 1 × TBE (45 mM Tris-HCl, 45 mM ortoboric acid and 1 mM EDTA) buffer using a Sequi-Gen GT Sequencing Cell (BIO-RAD) apparatus. Gels were blotted on Whatmann 3 MM paper, dried at 75 °C for 1 h and visualized by autoradiogram (BIOMAX MR-1 film, Kodak) after 12 hrs exposure at − 80 °C using intensifying screens (Amersham, Life Science).

I-SSR markers

Inter-microsatellite markers were assayed by using four different I-SSR primers (synthesized by Life Technologies, Inc.) anchored at the 3′ or 5′ terminus of the simple CA repeat and extended into the flanking sequence by two or three nucleotide residues, i.e. $(CA)_8AG$, $(CA)_8GC$, $CAG(CA)_8$, and $CGT(CA)_8$ (Table 1). The PCR protocol used for detecting I-SSR polymorphisms was that reported by Kantety et al. (1995), with some changes (Barcaccia et al. 2000). The reaction constituents were 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 150 μM each of dCTP, dGTP, dATP and dTTP, 1 μM of a single primer, 1.5 U of *Taq* DNA polymerase (Pharmacia Biotech) and 30 ng of genomic DNA, in a 25-μl volume. Amplification reactions were performed in a 9700 Thermal Cycler (Perkin Elmer) under cycling conditions resembling a touchdown profile: an initial denaturation

Table 1. Primers used to detect SSR and I-SSR markers.

Primers		Sequence (5′ to 3′)
SSR		
p-phi031	forward	GCAACAGGTTACATGAGCTGACGA
	reverse	CCAGCGTGCTGTTCCAGTAGTT
p-dupssr1	forward	TGTTCTCAACAACCACCG
	reverse	CGTTTAGCGATATCATTTTCC
p-dupssr7	forward	GAAGCTTAATCTGGAATCTGG
	reverse	TGTTGCTTCCTTGTAAAATCT
p-dupssr10	forward	AGAAAATGGTGAGGCAGG
	reverse	TATGAAATCTGCATCTAGAGAAATTG
I-SSR		
p-issr1	unique	CACACACACACACACAAG
p-issr6	unique	CACACACACACACACAGC
p-issr13	unique	CAGCACACACACACACACA
p-issr14	unique	CGTCACACACACACACACA

at 95 °C for 3 min was followed by 2 cycles of 1 min at 95 °C, 1 min at 63 °C and 2 min at 72 °C. Then, the annealing temperature was reduced by 1 °C every two cycles until a final annealing temperature of 56 °C was reached. The last cycle was repeated 26 times and was terminated by a final step at 72 °C for 10 min. Inter-microsatellite DNA fragments were separated by electrophoresis in 2% agarose gels run with 1 × TBE buffer (45 mM Tris-HCl, 45 mM ortoboric acid and 1 mM EDTA) at 150 V for 3 hrs. Photographs (K65HM video copy processor paper, Mitsubishi) of the polymerized genomic fragments were taken after staining of the agarose gels with ethidium bromide.

Data analysis

Microsatellite and inter-microsatellite data from each single plant DNA sample were recorded according to the multiallelic and dual nature of the marker system by assigning the molecular weight to each polymorphic SSR and I-SSR band identified by comparing sample lanes with known DNA sequences and DNA ladders, respectively. Different measures of diversity and differentiation were used to estimate the levels of polymorphisms within and between different maize populations.

For SSR markers, the average number of alleles observed per locus (n_o) was computed as the arithmetic mean over loci of the total number of alleles observed at each locus. The effective number of alleles per locus (n_e) was computed as: $n_e = 1/(\Sigma p_i^2)$, where p_i is the frequency of the i^{th} allele (Kimura and Crow 1964). The parameter n_e is a measure of diversity and indicates the size of an ideal population in which, given the existing allele frequencies, all individuals are totally differentiated.

Genetic diversity (H) and populations differentiation (D_{ST}) statistics of Nei (1973), which are related to Simpson's measure of ecological diversity, were used to summarize the data of SSR alleles. Let p_{ji} denote the frequency of the i^{th} allele at the j^{th} locus. The genetic diversity computed as $H = 1 - \Sigma p_i^2$ is equivalent to the expected heterozygosity (H_e). This value provides an estimate of the discriminatory power of a locus by taking into account not only the number of alleles, but also their relative frequencies. For a single locus, H ranges from 0 (monomorphic) to 1 (very highly discriminative with many alleles in equal frequencies). The average diversity over all r loci for each population is:

$$H = 1 - \sum_{j=1}^{i=r} \sum_{i=1}^{i=n} p_{ji}^2 / r$$

In measuring the extent of genetic differentiation, either at a single locus or averaged over all loci, the total genetic diversity (H_T) is first computed. For a single locus, the quantity H_T is the probability that two individuals taken at random from all sampled populations will have different alleles at that locus. When based on several loci, the value for the total diversity is the average of the H_T values for the loci. From this, the proportion of diversity expressed between populations (G_{ST}) was estimated as D_{ST}/H_T, where D_{ST} is the among population genetic differentiation computed as H_T-H_S and thus $G_{ST} = 1$-H_S/H_T. In the case of a single locus, H_S is the average over all populations of the within population diversity at the locus. For estimates based on several loci, H_S is the within population diversity over loci and populations. Descriptive statistics at each locus and over all loci for each single population and over all landrace populations were conducted using the software Genetic Data Analysis (GDA) version 1.0 (Lewis and Zaykin 1999).

A hierarchical analysis of variance with estimation of Wright (1965) F-statistics was also performed. Heterozygosity within population (F_{IS}) and between populations (F_{IT}) were determined as well the fixation index (F_{ST}) according to Wright (1978). F_{IS} values were computed also for single SSR loci to measure the deficiency or excess of heterozygotes at each locus and in each population. F_{ST} measures the genetic effect of landrace subdivision as the proportional reduction in overall heterozygosity owing to variation

in SSR allele frequencies among different landrace populations. Values of F_{ST} were averaged across populations of the landrace and over all loci.

The Smouse et al. (1983) coefficient was adopted as a general measure of departure from random union of gametes and so from the Hardy-Weinberg equilibrium. It is equivalent to the average within population fixation index (F_{is}) over all r loci and was calculated as follows:

$$\theta = \sum F_{is}/r$$

The bootstrapping procedure of numerical resampling was employed with the purpose of providing sampling variances and determining realized confidence intervals for F-statistics for allele frequencies over all SSR loci. It provides a convenient way of making inferences when there is no evolutionary basis for a distribution of allele frequencies over populations. Two landrace populations were judged to have different allele frequencies if the estimated fixation indices have nonoverlapping confidence intervals. The calculations were performed using a number of replicates of 1000 and a nominal confidence interval of 95% with the software Genetic Data Analysis (GDA) version 1.0 (Lewis and Zaykin 1999).

Gene flow was estimated from F_{ST} (which, for multiallelic loci, is equivalent to G_{ST} according to Nei (1973)) as follows: $Nm = 0.25(1 - F_{ST})/F_{ST}$. The result is independent of population size because the force of gene flow, which is measured by the fraction of migrants in a population (denoted as m), is counteracted by the force of genetic drift, which is proportional to the inverse of the population size (N). $Nm < 1$ indicates a local differentiation of populations, while $Nm > 1$ when a little differentiation among populations occurs (McDermott and McDonald 1993).

Genetic distances between populations were estimated for SSR markers using Nei (1978) unbiased genetic distance coefficient. This parameter is defined as:

$$GD_{ij} = -\ln \sum p_i p_j / \left(\sum p_i^2 \sum p_j^2 \right)^{1/2}$$

p_i and p_j being the frequencies of a given allele in populations i and j. For multiple loci these values are calculated by summing over alleles at all loci studied. $GD_{ij} = 0$ if no alleles are shared between populations i and j while a $GD_{ij} = 1$ indicates that the two populations have identical allele frequencies. The resulting genetic distance matrix was used for a cluster analysis according to the unweighted pair-group arithmetic average method (UPGMA).

Associations between frequencies for alleles at different loci were investigated for each pair of loci in each population and for the whole data set. Linkage disequilibria at individual loci and for all pair-wise comparisons of loci were estimated and tested by Fisher's exact test. A probability less than 0.05 was chosen to indicate a statistically significant amount of disequilibrium. All calculations and analyses were conducted using the software Genetic Data Analysis (GDA) version 1.0 (Lewis and Zaykin 1999).

I-SSR markers were scored as present or absent over all individuals of the landrace populations and synthetics. The average number of bands per primer was computed as the arithmetic mean over all primers of the total number of bands obtained at each assay. The individual I-SSR marker allele frequency for each primer and its relative standard error were calculated for each single population and over all landrace populations. Once supposed that gene frequencies of landrace populations do not deviate significantly from Hardy-Weinberg expectations, the frequency of a given band (F_b) was used to calculate the allele frequencies at dominant I-SSR loci adopting the following derivation of the Hardy-Weinberg equilibrium equation: $p_i = 1 - (1 - F_b)^{1/2}$, where $1 - F_b$ is equal to q_i^2.

The observed number of alleles (n_o), the effective number of alleles (n_e) according to Kimura and Crow (1964), the number and % of polymorphic loci (n_{pl}) were calculated. Nei (1973) genetic diversity statistics were performed for each single population and averaged over all I-SSR loci in order to evaluate H'_T, H'_S, D_{ST} and G_{ST} parameters. Let the average over all n populations of the within population diversity be $H'_S = 1/n\Sigma H'$, the total diversity (H'_T) is quantified from the allele frequencies in all the populations considered together. Then the proportion of diversity present within populations, H'_S/H'_T, can be compared with that between populations, $1 - H'_S/H'_T$. Gene flow was estimated from G_{ST} as follows: $Nm = 0.5(1 - G_{ST})/G_{ST}$ (McDermott and McDonald 1993). All statistics were computed using the software POPGENE version 1.21 (Yeh et al. 1997).

The Ewens-Watterson's test for neutrality was performed on the basis of the observed gene identity values computed as sum of the squared frequencies of alleles over all I-SSR loci, $F = \Sigma p_i^2 + p_j^2$ (Manly 1985).

The polymorphism degree was calculated over all n

populations using Shannon's information index (I) of phenotypic diversity (Lewontin 1972). Let p_i be the frequency of the i^{th} marker phenotype, the average diversity for the n^{th} population can be written as follows:

$$I = -\sum_{j=1}^{j=r} \sum_{i=1}^{i=n} p_i \ln p_i$$

All calculations and analyses were conducted using the software POPGENE version 1.21 (Yeh et al. 1997).

Dice (1945) genetic similarity estimates between individuals, based on the probability that an I-SSR marker from one accession will also be present in another, was calculated in all possible pair-wise comparisons using the following formula: $GS_{ij} = 2M_{ij}/(2M_{ij} + M_i + M_j)$, where M_{ij} represents the number of shared amplification products scored between the pair of samples/fingerprints (i and j) considered, M_i is the number of products present in i but absent in j and M_j is the number of products present in j but absent in i. Thus, $GS_{ij} = 1$ indicates identity between i and j, whereas $GS_{ij} = 0$ indicates complete diversity. The between-populations mean genetic similarity estimate was obtained by averaging individual GS estimates using the whole set of plants belonging to the populations being compared. As well the within-population mean genetic similarity estimate was calculated taking into account all plants representing that population. The cluster analysis was performed according to the unweighted pair-group arithmetic average method (UPGMA), and dendrograms of all populations were constructed from the symmetrical mean genetic similarity matrix. All calculations and analyses were conducted using the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) Version 1.80 (Rohlf 1993).

The Nei's genetic distances and Dice's genetic similarities from the SSR and I-SSR matrices of 13 × 13 elements each were subjected to the analysis of variance using the CoStat software (CoHort Software, Minneapolis, MN) to estimate the significance of differences for each pair-wise comparison between entries and for each entry tested against the rest of entries. The model included as source of variation entries between groups (df = 12) and entries within groups (df = 143). Statistical differences among means were tested by LSD at the 5% level.

Population-specific alleles (i.e. private alleles) and common alleles were scored for all SSR and I-SSR loci detected. Moreover, a classification between rare and common alleles was done according to marker allele frequencies ($p < 0.05$ and $p > 0.05$, respectively).

The degree of differentiation within populations (δ_T) for each single SSR and I-SSR marker across all types of molecular markers used was computed as: $\delta_T = (1 - \Sigma p_i^2)\,[N/(N-1)]$, where N is the population size and p_i is the frequency of the i^{th} marker allele in the population studied (Gregorius 1987). The δ_T averaged over all loci was calculated for each single population and for the total set of landrace populations. A value of 0 indicates that none of the markers is able to identify specific genotypes while 1 means that the marker allows to discriminate each of the genotypes within that population or set of populations. The arithmetic mean of δ_T provides an information index for each specific class of molecular markers.

To compare the efficiency among the two methods applied at the single plant level, where co-dominant SSR primers analyze single loci and detect one or two marker alleles per assay, whereas dominant I-SSR primers analyze several loci and detect multiple marker alleles per assay, an assay efficiency index (Ai) was calculated. This index combines the effective number of alleles identified per locus and the number of the polymorphic bands detected in each assay as: $Ai = \Sigma n_e/P$, where Σn_e is the total number of effective alleles detected over all loci and P is the total number of assays performed (i.e. primers used) for their detection (Pejic et al. 1998).

An additional parameter, the marker index (MI), which is the product of expected heterozygosity and multiplex ratio, was used to evaluate the overall utility of each marker system (Powell et al. 1996). It was calculated as $MI = H_{pl}\ \beta\ n$, where H_{pl} is the total genetic diversity computed over all polymorphic loci, β is the percentage of polymorphic loci and n is the number of loci detected per primer.

A number of matrix comparisons were performed in order to measure the goodness of fit of cluster analyses, determine the correspondence between dendrograms of different molecular marker classes and assess the relationship between genetic and geographic distances. For each dendrogram the cophenetic coefficients between the matrix of genetic similarities and the matrix of cophenetic values were computed. The degree of relationship for all pair-wise comparisons of genetic similarity matrices was assessed by plotting one matrix against each other, element by

element, exception made for the diagonal values. The comparison between genetic and geographic distance matrices was also performed. All data irrespective of the multiallelic or dual nature of the marker system were scored in the form of a binary matrix. The mean genetic similarity (MGS) estimates from single plant DNA-derived SSR and I-SSR markers were used. GS values were determined using both the Dice's coefficient reported above and the similarity coefficient of Nei and Li (1979): $GS_{ij} = 2M_{ij}/(M_i + M_j)$, where M_{ij} represents the number of shared amplification products and M_i and M_j is the sum of total products scored in both plant fingerprints i and j considered. The significance of the correlation observed was then assayed by the Mantel (1967) matrix correspondence test. Let X_{ij} and Y_{ij} be the off-diagonal elements of matrices X and Y, then the coefficient of correlation between matrices (equivalent to normalized Mantel statistic, Z) is computed as:

$$Z=\sum_{i<j}^{n} X_{ij} Y_{ij}$$

All calculations were conducted using the appropriate options of the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc) Version 1.80 (Rohlf 1993).

Results

Characterization by SSR and I-SSR markers using single plant DNAs

A high variability was detected among plants of this landrace as can be seen from banding pattern profiles of Figure 1. Both SSR and I-SSR markers were able to detect molecular polymorphisms within and between populations selected by farmers.

SSR descriptive statistics and genetic variability estimates

Descriptive statistics over all SSR loci along with information on the amount of genetic variability found in the landrace populations and synthetics are reported in Table 2.

A mean number of 20.75 observed alleles (n_o) per SSR locus was recorded over all entries. In the landrace populations the assayed loci scored a mean number of alleles equal to 19.75, almost twice that observed in the synthetics, 10.25 (Table 2). Loci phi031, dupssr1, dupssr7 and dupssr10 showed a total number of alleles of 20, 24, 15 and 25, respectively. The mean number of effective alleles (n_e) per polymorphic SSR locus was 6.87 (Table 2). The mean

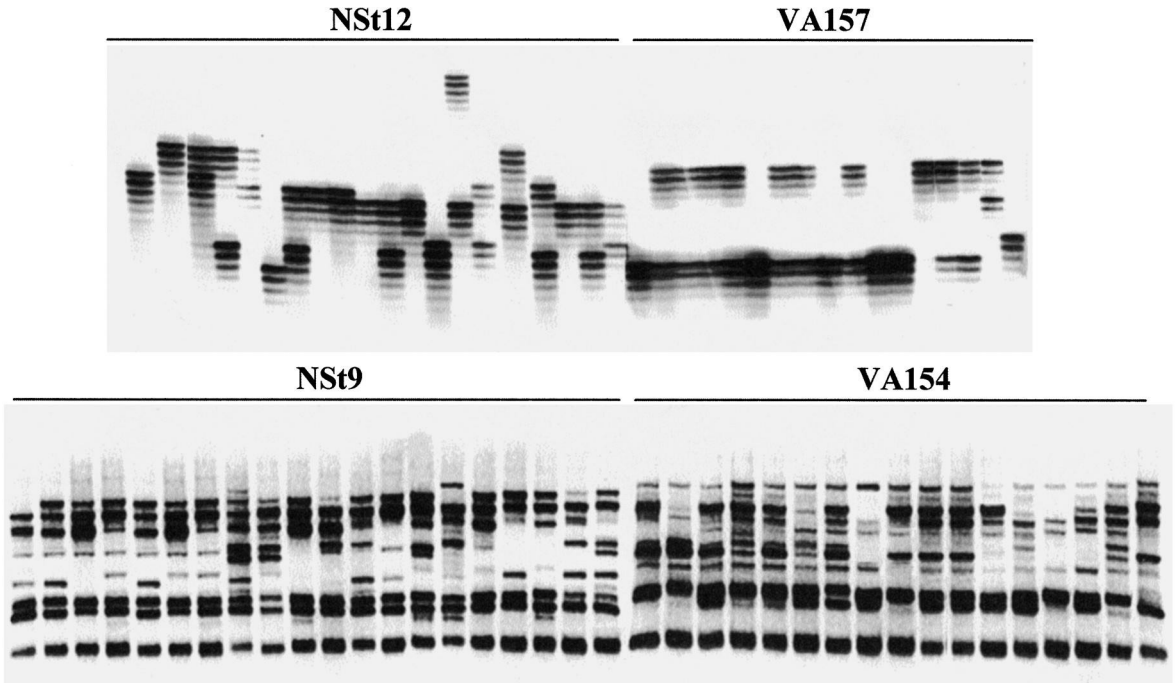

Figure 1. Banding patterns generated by SSR (top) and Inter-SSR (bottom) marker alleles using primers p-dupssr10 and p-issr14.

Table 2. Descriptive statistics over all SSR loci including mean sample size (s), mean number of observed (n_o) and effective (n_e) alleles per locus, mean allele frequency over loci (P_i) with standard error, Levene (1949) observed (H_o) and expected (H_e) heterozygosity, inbreeding coefficient estimate (f), Shannon's (Lewontin 1972) information Index (I) and Dice (1945) genetic similarity (GS).

Entries	s	n_o	n_e	$p_i \pm$ s.e.	H_o	H_e	f	I	GS
NSt2	19	12.25	7.11	0.083 ± 0.012	0.802	0.874	0.084	2.169	0.202
NSt3	19	8.25	4.12	0.118 ± 0.023	0.539	0.741	0.278	1.627	0.317
NSt4	18	11.25	5.44	0.089 ± 0.015	0.695	0.811	0.136	1.977	0.250
NSt5	19	9.25	5.53	0.105 ± 0.016	0.760	0.838	0.095	1.902	0.251
NSt6	20	9.50	5.85	0.105 ± 0.014	0.722	0.847	0.168	1.976	0.249
NSt8	20	8.75	4.65	0.114 ± 0.021	0.700	0.780	0.105	1.753	0.328
NSt9	20	8.75	5.59	0.118 ± 0.017	0.838	0.833	− 0.015	1.904	0.301
NSt10	20	10.50	5.72	0.095 ± 0.015	0.694	0.835	0.171	1.979	0.281
NSt11	19	8.50	4.43	0.121 ± 0.021	0.639	0.784	0.188	1.739	0.284
NSt12	18	8.50	5.07	0.114 ± 0.017	0.652	0.823	0.226	1.818	0.234
Overall NSt	193	19.75	6.99	0.105 ± 0.007	0.705	0.851	0.144	2.334	0.222
s.d.		3.59	1.48		0.161	0.037	0.057	0.187	0.031
VA143	17	6.25	3.88	0.160 ± 0.032	0.589	0.705	0.169	1.464	0.390
VA154	19	6.25	3.68	0.160 ± 0.028	0.641	0.736	0.132	1.487	0.367
VA157	16	3.00	2.17	0.333 ± 0.060	0.585	0.553	− 0.061	0.840	0.687
Overall VA	52	10.25	5.21	0.194 ± 0.022	0.608	0.794	0.080	1.876	0.287
s.d.		2.50	1.67		0.025	0.062	0.021	0.257	0.172
Overall	244	20.75	6.87	0.117 ± 0.006	0.685	0.848	0.132	2.333	0.223
s.d.		4.65	1.69		0.129	0.037	0.048	0.209	0.051

values were 6.99 for landrace populations and 5.21 for synthetics, ranging between 2.17 of VA157 and 7.11 of NSt2 (Table 2).

The allele frequency also showed great variability. Marker alleles were highly polymorphic in the landrace populations, with frequencies that individually varied between 0.003 and 0.416 (mean p_i value 0.105) and were on average lower than those of the synthetics (mean p_i value 0.194) (Table 2). In particular, NSt2 showed the highest total number of alleles over all loci (49) and the lowest mean allele frequency (0.083) while the opposite was true for VA157 that manifested the lowest total number of alleles (12) and the highest mean allele frequency (0.333).

The mean observed heterozygosity was $H_o = 0.705$ in the landrace populations, ranging from 0.539 of NSt3 to 0.838 of NSt9, while it was 0.608 in the synthetics. The expected heterozygosity (H_e) scored for landrace populations was always higher than that computed for synthetics (0.851 vs. 0.794), with a maximum of 0.874 found in NSt2 and a minimum of 0.533 found in VA157 (Table 2).

The inbreeding coefficient estimate (f) was highly variable over all entries analyzed. In the landrace populations it ranged from 0.084 of NSt2 to 0.278 of NSt3 and showed a mean value of 0.144 (Table 2). Two of the three synthetics had estimates comparable to those computed in most of the landrace populations. Moreover, negative inbreeding coefficients were found in VA157 and NSt9 (Table 2).

The Shannon's information index (I) varied between 1.627 and 2.169 at the single population level, while it was equal to 2.334 for the landrace as a whole (Table 2). This information index was as low as 0.840 in the synthetic VA157. The Dice's genetic similarity (GS) ranged from 0.202 to 0.328 in the landrace populations, being 0.222 on average (Table 2). Synthetics always showed a higher genetic similarity compared to landrace populations. VA157 confirmed to be the most genetically uniform entry, showing a similarity estimate as high as 0.687 (Table 2).

Genetic diversity statistics and gene flow estimates based on SRR loci

Nei's genetic diversity statistics and gene flow for all SSR loci over single, grouped and multi-populations are reported in Table 3.

The total genetic diversity as assessed by SSR markers was $H_T = 0.848$, being the average diversity of landrace populations equal to 0.851 and that of synthetics of 0.794 (Table 3). The within population genetic diversity was $H_S = 0.795$, ranging from 0.722 of NSt3 and 0.850 of NSt2 (Table 3). This index ranged from 0.535 to 0.716 in the synthetics (with an average value of $H_S = 0.645$). The extent of differentiation between landrace populations was $D_{ST} = 0.056$. Thus, more than 94% of the total variation was within population (Table 3). The proportion of the among population genetic diversity was as low as G_{ST}

Table 3. Summary of single-, grouped- and multi-populations Nei (1973) genetic diversity statistics and gene flow for all SSR loci.

Entries	s	H_T	H_S	D_{ST}	G_{ST}*	Nm
NSt2	38		0.850	0.001	0.001	236.139
NSt3	38		0.722	0.129	0.152	1.399
NSt4	36		0.789	0.062	0.073	3.165
NSt5	38		0.816	0.035	0.041	5.829
NSt6	40		0.825	0.026	0.030	8.061
NSt8	40		0.760	0.091	0.107	2.096
NSt9	40		0.812	0.039	0.046	5.191
NSt10	40		0.813	0.038	0.045	5.363
NSt11	38		0.763	0.088	0.103	2.173
NSt12	36		0.800	0.051	0.060	3.905
Mean NSt			0.795	0.056	0.066	3.556
Overall	386	0.851				
VA143	34		0.684	0.110	0.138	1.558
VA154	38		0.716	0.078	0.098	2.295
VA157	32		0.535	0.259	0.326	0.518
Mean VA			0.645	0.149	0.187	1.084
Overall	104	0.794				
Total	490	0.848	0.761	0.087	0.103	2.180
s.d.		0.037	0.080	0.065	0.082	2.791

* For multiallelic loci G_{ST} is equivalent to F_{ST} according to Wright (1965) terminology.

$= 0.066$. The little genetic differentiation among landrace populations was confirmed by the gene flow estimate that resulted as high as 3.556 (Table 3). On the whole, NSt2 was the population that displayed the highest genetic diversity. It showed no differentiation from the landrace as a whole and so an elevated gene flow. NSt3, NSt8 and NSt11 were the most genetically uniform and differentiated populations.

Fixation indices and heterozygosity measures

The amount of heterozygosity as measured within entries for each single locus and over all loci investigated is reported in Table 4.

At locus phi031, seven of the ten landrace populations showed a negative F_{IS} value that varied between -0.181 and -0.024 indicating a heterozygosity excess. An opposite situation was found at locus dupssr7 for which nine of the landrace populations showed a positive F_{IS} value which ranged from 0.692 of NSt6 to 0.241 of NSt5. Heterozygosity deficiency was also found at the locus dupssr1, but it was much less accentuated (Table 4). At locus dupssr10, five populations had heterozygosity excess and five heterozygosity deficiency (Table 4). When estimated over all loci, all landrace populations but one (NSt9) showed heterozygosity deficiency with a mean $F_{IS} = 0.114$. A significant Hardy-Weinberg's disequilibrium was found only for NSt12 being the non-random union of gametes measured by the Smouse's coefficient as high as $\theta = 0.457$ ($\chi^2 = 10.97$, $P = 0.0269$, df $= 4$). Among synthetics, VA157 displayed heterozygosity excess at three of the four loci investigated (mean $F_{IS} = -0.097$) while the other two synthetics showed heterozygosity deficiency (Table 4). A heterozygosity deficiency was always observed between entries. Mean F_{IT} values were 0.170 and 0.230 in landrace populations and synthetics, respectively. The overall Wright's fixation index was $F_{ST} = 0.103$. It was as low as 0.066 in the landrace populations and equal to 0.187 in the synthetics (Table 4).

The bootstrapping analysis across loci evidenced that inbreeding coefficients F_{IS} and F_{IT} are statistically different from zero (realized upper and lower confidence intervals were 0.345 and 0.003 for F_{IS}, and 0.379 and 0.043 for F_{IT}). For landrace populations a strong influence on the final result was determined by locus dupssr7 which showed a marked heterozygosity deficiency both within and between populations (Table 4). Moreover, because of the 95% bootstrap confidence interval for F_{ST} did not overlap zero (the confidence interval ranged from 0.053 to 0.033), also the fixation index was found significant. Thus, based on SSR data alone a significant amount of genetic divergence has occurred among the 10 landrace populations.

Linkage disequilibrium

Significant ($P < 0.05$) pair-wise linkage disequilibria between SSR alleles were found for all landrace populations (data not shown). The number of linkage

Table 4. Wright (1978) measures of heterozygosity deficiency (+ values) or excess (− values) within population (F_{IS}) and between populations (F_{IT}) and fixation index (F_{ST}).

	F_{IS} over loci				
	phi031	dupssr1	dupssr7	dupssr10	Mean
Landrace populations					
NSt2	− 0.133	− 0.064	0.472	− 0.016	0.065
NSt3	0.130	0.219	0.511	0.146	0.251
NSt4	− 0.024	0.048	0.378	0.042	0.111
NSt5	0.081	0.045	0.241	− 0.078	0.072
NSt6	− 0.148	0.078	0.692	− 0.101	0.130
NSt8	− 0.061	0.060	0.304	− 0.010	0.073
NSt9	− 0.096	0.111	− 0.076	− 0.075	− 0.034
NSt10	− 0.118	− 0.034	0.673	0.023	0.136
NSt11	0.030	0.162	0.430	0.070	0.173
NSt12	−0.181	0.076	0.480	0.370	0.186
Synthetics					
VA143	0.081	− 0.070	0.155	0.341	0.127
VA154	0.172	0.070	0.073	0.112	0.107
VA157	0.163	− 0.129	− 0.185	− 0.236	− 0.097
Over NSt					
F_{IS}	− 0.058	0.068	0.409	0.036	0.114
F_{IT}	0.006	0.118	0.459	0.096	0.170
F_{ST}	0.060	0.053	0.085	0.063	0.066
Over VA					
F_{IS}	0.132	− 0.028	0.013	0.116	0.058
F_{IT}	0.267	0.105	0.257	0.292	0.230
F_{ST}	0.155	0.129	0.247	0.199	0.187
Overall					
F_{IS}	− 0.017	0.051	0.336	0.052	0.105
F_{IT}	0.070	0.129	0.422	0.154	0.194
F_{ST}	0.086	0.083	0.130	0.108	0.103

disequilibria ranged from 10 for NSt10 (1.52%) to 1 for both NSt9 (0.22%) and NSt12 (0.23%) with an average of 4.9 (0.87%) in the landrace populations. Of the synthetics, VA154 scored 5 (2.17%) pair-wise linkage disequilibria whereas VA143 e VA157 showed no or 1 (1.89%) significant linkage disequilibria. In the whole sample 55 (0.91%) significant pair-wise linkage disequilibria were found. The highest number of linkage disequilibria (14) was found between loci dupssr7 and dupssr10 located, respectively, on chromosomes 10 and 5 while the lowest one (3) between dupssr7 and phi031, which is located on chromosome 6.

I-SSR descriptive statistics and genetic variability estimates

Descriptive statistics over all I-SSR markers for single and grouped entries and for the population as a whole are given in Table 5.

An average of 17.4 marker alleles per I-SSR primer were scored with a mean value of 17.7 in the landrace populations and 16.3 in the synthetics (Table 5). The average number of observed marker alleles per locus (n_o) was 1.74 while the effective number of marker alleles (n_e) was 1.34 (Table 5). The proportion of polymorphic loci was 71.9% on the whole and was much higher for landrace populations than for synthetics (75.73% vs. 59.12%, respectively). Moreover, it was highly variable among landrace populations, ranging from 88.68% of NSt2 to 49.75% of NSt12 (Table 5).

The Nei's unbiased genetic diversity was $H' = 0.222$ in the landrace populations. The highest diversity was scored by NSt9 (0.269) and the lowest diversity by NSt4 (0.189) and NSt6 (0.187). Genetic diversity estimates of synthetics as assessed by I-SSR markers were comparable to those of landrace populations (Table 5).

The Shannon's diversity information index over all landrace populations and I-SSR loci was $I = 0.332$, varying from 0.286 of NSt6 to 0.391 of NSt9 (Table

Table 5. Summary of single-, grouped- and multi-population genetic variation statistics for all I-SSR markers including sample size (s), observed (n_o) and effective (n_e) number of alleles per locus (Kimura and Crow 1964), number of markers per primer (nm/p), number (npl) and % of polymorphic loci, mean marker allele frequency (P_i) with standard error, Nei (1978) unbiased genetic diversity (H′), Shannon's information index (I) (Lewontin 1972) and Dice (1945) genetic similarity (GS).

Entries	s	nm/p	n_o	n_e	npl	%	$p_i \pm$ s.e.	H′	I	GS
NSt2	20	19	1.89	1.32	47	88.68	0.237 ± 0.037	0.223	0.343	0.564
NSt3	20	18	1.77	1.35	41	77.36	0.259 ± 0.037	0.227	0.335	0.584
NSt4	20	17	1.79	1.29	42	79.25	0.193 ± 0.033	0.189	0.291	0.511
NSt5	20	16	1.77	1.32	41	77.36	0.260 ± 0.041	0.210	0.314	0.602
NSt6	20	16	1.70	1.27	37	69.81	0.284 ± 0.047	0.187	0.286	0.621
NSt8	20	17	1.77	1.36	41	77.36	0.292 ± 0.042	0.232	0.341	0.624
NSt9	20	19	1.83	1.43	44	83.02	0.278 ± 0.033	0.269	0.391	0.591
NSt10	20	19	1.79	1.38	42	79.25	0.300 ± 0.041	0.244	0.360	0.610
NSt11	20	17	1.75	1.33	40	75.47	0.251 ± 0.038	0.211	0.311	0.609
NSt12	20	19	1.79	1.35	42	49.75	0.271 ± 0.038	0.233	0.346	0.597
Mean NSt	200	17.7	1.79	1.34	41.7	75.73	0.262 ± 0.012	0.222	0.332	0.591
sd		1.3	0.41	0.33	2.6			0.173	0.241	0.033
VA143	17	19	1.53	1.31	28	52.83	0.294 ± 0.041	0.191	0.269	0.623
VA154	19	16	1.75	1.36	40	75.47	0.432 ± 0.057	0.233	0.340	0.726
VA157	16	14	1.49	1.34	26	49.06	0.612 ± 0.054	0.193	0.262	0.763
Mean VA	52	16.3	1.59	1.34	31.3	59.12	0.435 ± 0.031	0.205	0.290	0.704
sd		2.5	0.48	0.39	7.6			0.203	0.286	0.073
Overall	252	17.4	1.74	1.34	39.3	71.90	0.297 ± 0.012	0.218	0.322	0.617
sd		1.6	0.43	0.34	5.9			0.180	0.251	0.064

5). Among synthetics, VA143 and VA157 showed the highest genetic uniformity, being the information index as low as 0.269 and 0.262, respectively.

The Dice's genetic similarity (GS) ranged from 0.511 to 0.624 in the landrace populations and it was on average equal to 0.591 (Table 5). Synthetics displayed a mean genetic similarity as high as 0.704. VA157 confirmed to be the genetically most uniform entry, showing a GS = 0.763 (Table 5).

Genetic diversity statistics and gene flow estimates based on I-SSR loci

Nei's genetic diversity statistics and gene flow for all I-SSR loci over single, grouped and multi-populations are reported in Table 6.

The total genetic diversity as assessed with I-SSR markers was $H_T = 0.241$, being the average diversity of landrace populations equal to 0.232 and that of

Table 6. Summary of single-, grouped- and multi-populations Nei (1978) genetic diversity statistics and gene flow for all I-SSR loci.

Entries	s	H_T	H_S	D_{ST}	G_{ST}	N_m
NSt2	20		0.212	0.021	0.088	5.166
NSt3	20		0.216	0.017	0.072	6.414
NSt4	20		0.180	0.052	0.226	1.717
NSt5	20		0.199	0.033	0.142	3.020
NSt6	20		0.178	0.055	0.235	1.627
NSt8	20		0.220	0.012	0.051	9.261
NSt9	20		0.255	− 0.023	− 0.099	− 5.528
NSt10	20		0.232	0.000	0.002	289.875
NSt11	20		0.200	0.032	0.139	3.107
NSt12	20		0.221	0.011	0.049	9.779
Mean NSt	200		0.211	0.021	0.091	5.025
Overall	200	0.232				
VA143	17		0.179	0.063	0.259	1.432
VA154	19		0.221	0.021	0.088	5.178
VA157	16		0.181	0.061	0.254	1.470
Mean VA	52		0.194	0.048	0.200	1.997
Overall	52	0.242				
Total	252	0.241	0.207	0.034	0.141	3.037
sd		0.023	0.017	0.026	0.107	4.086

synthetics of 0.242 (Table 6). The within population genetic diversity over all entries was $H_S = 0.207$, ranging from 0.178 of NSt6 to 0.255 of NSt9 (Table 6). Among synthetics H_S ranged from 0.179 to 0.221 with an average value of 0.194. The extent of differentiation among landrace populations was $D_{ST} = 0.021$. Thus, most of the total variation (about 98%) was within populations (Table 6). The proportion of genetic diversity distributed among populations was as low as $G_{ST} = 0.091$. The little genetic differentiation among landrace populations was confirmed by the gene flow estimate that resulted as high as 5.025 (Table 6). On the whole, NSt4 and NSt6 were the populations with the highest genetic differentiation and consequently their gene flow estimates were low (Nm = 1.717 and 1.627, respectively) compared to the other populations. Among synthetics, VA154 showed the highest genetic diversity and, consequently, the lowest genetic differentiation ($G_{ST} = 0.088$).

Common vs. rare alleles

Rare alleles were found over all SSR loci for seven landrace populations and two synthetics (Table 7).

The 120 bp allele at locus phi031 found in NSt9 was the rare allele with the highest relative frequency (0.125) within the landrace populations. In the synthetics, the rare alleles of 184 bp and 194 bp at locus dupssr10 showed very high relative frequencies (0.375 and 0.206, respectively).

Table 7. Private SSR and I-SSR alleles defined by set of populations.

Locus	Allele	Frequency	Entry
phi031	120	0.125	NSt9
	125	0.053	NSt10
	143	0.028	NSt4
	118	0.026	NSt10
	129	0.025	NSt2
dupssr1	171	0.094	VA143
	175	0.059	NSt4
	85	0.025	NSt2
dupssr7	150	0.075	NSt9
	148	0.050	NSt6
	130	0.025	NSt3
dupssr10	184	0.375	VA157
	194	0.206	VA143
	200	0.083	NSt2
	198	0.075	NSt6
	190	0.029	VA143
	132	0.028	NSt2
	162	0.026	NSt12
issr13	900	0.051	NSt2

Only one rare allele was found over all I-SSR loci assayed, that is the allele of 900 bp detected by primer p-issr13 in NSt2 showing a frequency as low as 0.051 (Table 7).

Among the common SSR alleles of the landrace populations, the number of common alleles ($p_i \geq 0.05$) ranged from 21 (NSt3) to 31 (NSt6) with an average number of 27 and an overall frequency of 0.1413 while the number of rare alleles ($p_i < 0.05$) ranged from 5 (NSt6 and NSt9) to 19 (NSt2) with an average number of 13 and an overall frequency of 0.0265 (Table 8). Over the landrace as a whole, the total number of common and rare alleles were comparable (56 vs. 54). Synthetics showed a lower total number of both common and rare alleles, but a higher overall common allele frequency (0.2310). The vast majority of alleles scored a frequency higher than 0.05 (35 vs. 10).

Four alleles (one for each SSR locus) highly shared by all entries were also found with a relative frequency of 0.407 (107 bp allele at locus phi031), 0.351 (91 bp allele at locus dupssr1), 0.218 (136 bp allele at locus dupssr7) and 0.165 (176 bp allele at locus dpussr10). Of the 77 alleles totally detected over all landrace populations and over the four loci, 8 (10.4%) were present in all ten populations.

The number of SSR alleles at each single locus, and hence the number of different combinations arising over the four loci, was very large (552 on average). In the landrace populations, about half of the alleles (37 out of 77) were in the 0.01–0.05 frequency range, 21 alleles were found to have a frequency > 0.05 and 19 were very rare (frequency < 0.01). Of these, 15 were actually private alleles.

A total of 26 I-SSR alleles of the 53 scored were shared among the landrace populations and 21 of them were found also in the synthetics. On the whole, their frequencies ranged from 0.835 of the 180 bp marker allele of p-issr1 to 0.010 of the 680 bp marker allele of p-issr13.

The number of common alleles ($p_i \geq 0.05$) of those widespread among landrace populations, varied from 26 (NSt11) to 36 (NSt9) with a mean number of 31 and an overall frequency of 0.2690 while the number of rare alleles ($p_i < 0.05$) varied from 7 (NSt10) to 15 (NSt4) with an average number of 11 and an overall frequency of 0.0399. On the landrace as a whole, the total number of common and rare I-SSR alleles were 54 vs. 31. Synthetics showed a lower total number of both common and rare alleles (40 vs. 14) with a comparable overall common allele frequency

Table 8. Frequencies ($p_i \pm$ s.e.) of widespread alleles for SSR and I-SSR markers over all entries.

Entries	Widespread SSR alleles				Widespread I-SSR alleles	
	rare (< 0.05)		common (≥ 0.05)		rare (< 0.05)	common (≥ 0.05)
	no. $p_i \pm$ s.e.		no. $p_i \pm$ s.e.			
NSt2	19	0.0263 ± 0.0003	26	0.1294 ± 0.0182	14	0.0372 ± 0.0039	33	0.2853 ± 0.0390		
NSt3	12	0.0260 ± 0.0003	21	0.1744 ± 0.0322	10	0.0360 ± 0.0053	31	0.3224 ± 0.0374		
NSt4	16	0.0283 ± 0.0003	28	0.1257 ± 0.0212	15	0.0388 ± 0.0034	27	0.2671 ± 0.0370		
NSt5	12	0.0254 ± 0.0004	26	0.1421 ± 0.0193	14	0.0386 ± 0.0042	27	0.3359 ± 0.0413		
NSt6	5	0.0255 ± 0.0003	31	0.1209 ± 0.0155	9	0.0455 ± 0.0041	29	0.2879 ± 0.0440		
NSt8	12	0.0250 ± 0.0000	23	0.1609 ± 0.0264	11	0.0396 ± 0.0044	30	0.3291 ± 0.0354		
NSt9	5	0.0250 ± 0.0000	27	0.1361 ± 0.0191	9	0.0455 ± 0.0061	36	0.3430 ± 0.0356		
NSt10	11	0.0254 ± 0.0002	28	0.1290 ± 0.0192	7	0.0439 ± 0.0062	35	0.2755 ± 0.0306		
NSt11	8	0.0273 ± 0.0005	23	0.1587 ± 0.0261	14	0.0360 ± 0.0045	26	0.3574 ± 0.0402		
NSt12	11	0.0289 ± 0.0013	24	0.1534 ± 0.0197	9	0.0431 ± 0.0053	33	0.3242 ± 0.0401		
Overall NSt	54	0.0265 ± 0.004856	56	0.1413 ± 0.1030	31	0.0399 ± 0.0016	54	0.2690 ± 0.0249		
VA143	4	0.0274 ± 0.0004	21	0.1852 ± 0.0309	9	0.0415 ± 0.0056	31	0.3377 ± 0.0380		
VA154	4	0.0303 ± 0.0005	20	0.1892 ± 0.0375	5	0.0488 ± 0.0091	23	0.3529 ± 0.0364		
VA157	2	0.0312 ± 0.0000	9	0.4306 ± 0.0500	3	0.0320 ± 0.0066	23	0.5747 ± 0.0402		
Overall VA	10	0.0293 ± 0.0203	35	0.2310 ± 0.1780	14	0.0183 ± 0.0026	40	0.2649 ± 0.0209		

(0.2649) and a lower overall rare allele frequency (0.0183) (Table 8).

Matrices of SSR genetic distances and I-SSR genetic similarities

Nei's unbiased genetic distance and Dice's genetic similarity matrices were estimated over all entries for SSR and I-SSR markers, respectively (Table 9).

The genetic similarity estimates between landrace populations ranged from 0.497 (NSt5 vs. NSt11) to 0.592 (NSt4 vs. NSt11, and NSt3 vs. NSt4 and NSt5), with an average value GS = 0.563 while the genetic distance varied between 0.083 (NSt10 vs. NSt12) and 0.504 (NSt2 vs. NSt11), with an average value GD = 0.268. The genetic similarity and the genetic distance between synthetics were on average 0.520 and 0.816, respectively (Table 9). On the whole, NSt11 was the most differentiated from the rest of landrace populations based on its lowest mean genetic similarity (0.537). Moreover, NSt11 showed the highest similarity estimate with the synthetic VA143 (0.619). Based on genetic distances, NSt3 appeared to resemble most of the genetic traits of VA154 (0.109), as well as NSt11 and NSt8 seemed to share genetic traits of VA143 (0.208) and VA157 (0.316), respectively. Of the three synthetics, VA154 was the one genetically nearest to landrace populations being the mean genetic similarity equal to 0.568 and the genetic distance as low as 0.364 (Table 9).

UPGMA dendrograms as defined by SSR and I-SSR markers displayed all field populations but one (NSt11) clustered into a distinct group, in which the synthetic VA154 was also included. In particular, VA154 was closely grouped with NSt3 and NSt11 with VA143 (Figure 2). The synthetic VA157 was clustered separately from all landrace populations (Figure 2) as well the inbred line B73 (data not shown).

Based on both SSR and I-SSR marker alleles, B73 had mean estimates of genetic dissimilarity as high as 0.906, with landrace populations, and 0.954, with synthetics.

Analysis of variance for genetic distances and similarities

Both differences between Nei's mean genetic distances and between Dice's mean genetic similarities were significant at the 95% confidence level.

The Duncan's test based on SSR data revealed that the mean genetic distances of synthetics VA143 (0.632) and VA157 (0.608) were significantly different from the rest of entries, including VA154 (0.347) which did not differ significantly from landrace populations (0.319 ± 0.014 on average). Landrace populations formed a homogeneous group where a significant difference was found only between NSt9 and NSt10 (0.144*). Similarly, no significant difference

Table 9. Matrices of genetic distance (above diagonal) and genetic similarity (below diagonal) over all populations based on 84 SSR markers and Nei (1978) unbiased coefficient and 53 I-SSR markers and Dice (1945) coefficient, respectively.

	NSt2	NSt3	NSt4	NSt5	NSt6	NSt8	NSt9	NSt10	NSt11	NSt12	Mean NSt	VA143	VA154	VA157	Mean VA	Overall
NSt2		0.174	0.386	0.299	0.279	0.263	0.301	0.309	0.504	0.344	0.318	0.747	0.265	0.657	0.556	0.377
NSt3	0.571		0.365	0.269	0.232	0.217	0.276	0.188	0.320	0.276	0.257	0.648	0.109	0.492	0.416	0.297
NSt4	0.563	0.592		0.136	0.337	0.162	0.334	0.276	0.469	0.256	0.302	0.710	0.369	0.698	0.592	0.375
NSt5	0.552	0.592	0.568		0.129	0.204	0.317	0.109	0.266	0.219	0.216	0.479	0.346	0.495	0.440	0.272
NSt6	0.550	0.588	0.578	0.582		0.239	0.365	0.134	0.139	0.227	0.231	0.249	0.287	0.630	0.389	0.271
NSt8	0.537	0.580	0.568	0.564	0.585		0.226	0.143	0.434	0.163	0.228	0.734	0.204	0.316	0.418	0.275
NSt9	0.568	0.558	0.590	0.554	0.577	0.545		0.336	0.469	0.377	0.333	0.891	0.227	0.564	0.561	0.390
NSt10	0.562	0.568	0.586	0.582	0.584	0.572	0.544		0.203	0.083	0.198	0.522	0.235	0.418	0.392	0.246
NSt11	0.521	0.535	0.592	0.497	0.524	0.513	0.558	0.558		0.316	0.347	0.208	0.396	0.874	0.493	0.383
NSt12	0.563	0.573	0.549	0.562	0.582	0.558	0.590	0.591	0.534		0.251	0.552	0.279	0.545	0.459	0.303
Mean NSt	0.554	0.573	0.576	0.561	0.572	0.558	0.565	0.572	0.537	0.567		0.662	0.364	0.770	0.472	0.319
VA143	0.548	0.560	0.509	0.537	0.550	0.585	0.573	0.544	0.619	0.548	0.557		0.578	0.995	0.919	0.632
VA154	0.555	0.601	0.586	0.570	0.555	0.555	0.578	0.573	0.538	0.564	0.568	0.843		0.609	0.726	0.347
VA157	0.502	0.508	0.516	0.532	0.527	0.526	0.485	0.511	0.511	0.512	0.513	0.485	0.498		0.802	0.608
Mean VA	0.535	0.557	0.537	0.546	0.544	0.555	0.546	0.543	0.556	0.541	0.546	0.532	0.538	0.492		0.529
Overall	0.549	0.569	0.566	0.558	0.565	0.557	0.560	0.565	0.542	0.560	0.559	0.535	0.563	0.509	0.542	

LSD values for mean genetic distances and mean genetic similarities were 0.139 and 0.021, respectively.

was observed among landrace populations for the mean genetic similarities based on I-SSR data (0.563 ± 0.08 on average). The only exception was NSt11 (0.542) which differed significantly from four populations (NSt3, NSt4, NSt6 and NSt10). Synthetic VA157 (0.509) differed significantly from the rest of entries while VA154 (0.563) did not differ significantly from anyone of the landrace populations.

The difference between landrace populations and synthetics was highly significant both in terms of mean genetic distance (0.319 vs. 0.529) and mean genetic similarity (0.563 vs. 0.542).

It is interesting to note the mean SSR genetic distances had coefficients of variability much higher than those related to the mean I-SSR genetic similarities (51% and 46% vs. 5% and 6% of landrace populations and synthetics, respectively).

Marker system utility

The computation of the assay efficiency index (Ai) and marker index (MI) parameters revealed a marker system utility of I-SSRs much higher than that of SSRs. Ai was 23.3 for I-SSRs and 4.4 for SSRs whereas MI was 3.0 for I-SSRs and 0.85 for SSRs.

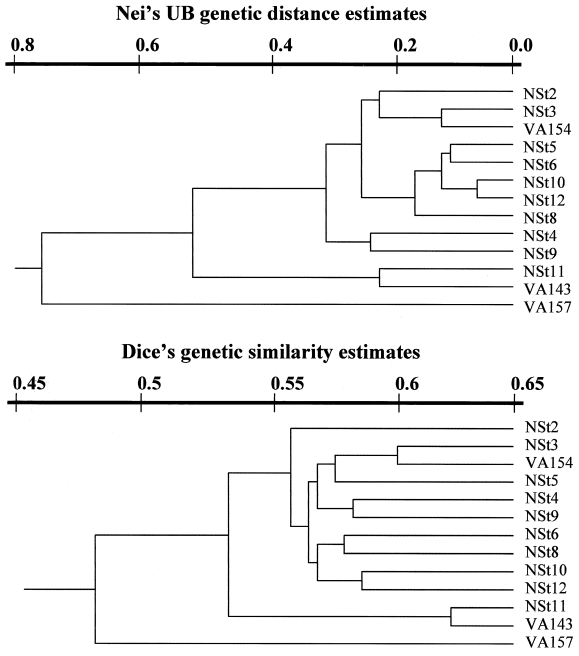

Figure 2. Dendrograms of the ''Nostrano di Storo'' farmer populations based on SSR (top) and I-SSR (bottom) data and constructed according to the UPGMA method with Nei's (1978) and Dice's (1945) coefficients, respectively.

Although the mean expected heterozygosity measured by I-SSRs was actually much lower than that scored by SSRs, the relative information content of the two marker systems was strongly influenced by the higher multiplex ratio component of the I-SSR assay compared to that of the SSR assay.

The correlation between SSR and I-SSR matrices assayed by the Mantel's correspondence test was as high as 0.908.

Primer discriminatory ability and information content of SSR and I-SSR loci

Genetic differentiation degrees and gene flow estimates were chosen to ascertain the information content of the set of SSR single-locus and I-SSR multiple-locus primers.

On the whole, the degree of differentiation of SSR loci ranged from 0.804 of phi031 to 0.894 of dupssr10. The four SSR loci had comparable levels of discriminatory ability, the best being dupssr10 that gave the highest degree of differentiation (δ_T = 0.886) among landrace populations. This locus was also highly informative for synthetics (δ_T = 0.884). Locus dupssr1 gave the highest estimate of gene flow for both landrace populations (Nm = 4.458) and synthetics (Nm = 1.694).

I-SSR primers evidenced lower degrees of differentiation and consequently higher estimates of gene flow. Among all entries the highest degree of differentiation was recorded by primer p-issr1 both for landrace populations (δ_T = 0.293) and for synthetics (δ_T = 0.328). In the landrace populations, δ_T values ranged from 0.138 to 0.293 whereas in the synthetics from 0.151 to 0.328. Despite the smaller sample size, synthetics were consistently more differentiated than populations for each of the primers used. Therefore, gene flow (Nm) values were always higher for landrace populations (where it ranged from 3.927 of p-issr6 to 8.666 of p-issr13) than for synthetics (between 1.153 of p-issr14 and 2.849 of p-issr13). Among primers, p-issr13 was able to detect the highest estimate of gene flow for both set of entries.

Discussion

Molecular markers enabled us to ascertain the level of genetic uniformity in the landrace populations maintained by farmers who select on the basis of morpho-phenological traits. Moreover, they allowed measure-

ment of the genetic diversity of the landrace as a whole as well as assessment of its structure and partition both within and among field populations. Finally, molecular markers showed the degree of genetic differentiation between field populations and landrace-derived synthetics.

The most relevant result of this study is that the flint maize germplasm cultivated at Storo in the Chiese Valley (Province of Trento, North-Eastern Italy) belongs, with few exceptions, to a single population. It means that the plant material grown for a long time in this area and maintained by local farmers through yearly selection has to be taken as only one landrace population identifiable with the name ''Nostrano di Storo''. This is supported by the low SSR genetic distances (0.261 on average) and the high I-SSR genetic similarities (0.563 on average) found among pair-wise population comparisons. This result further agrees with the high number of common alleles (21, equal to about 33% of the total number of SSR marker alleles scored, not including private alleles) shared among populations and occurring in the landrace as a whole with a frequency greater than 0.05. Moreover, 26 I-SSR marker alleles out of the 53 totally scored (49%) were shared by the landrace populations.

The selection carried out over the years by each farmer according to his own criteria produced little differentiation within the original population. Although a significant divergence among populations was found for the 4 SSR loci based on Wright's fixation indices, no significant differences were detected among populations in terms of pair-wise comparisons of Dice's mean genetic similarity matrices calculated using polymorphisms from the 53 I-SSR loci. This can be explained by taking into account the gene flow among farmer populations which is likely to have occurred in two ways: through either dispersion of pollen to neighboring cultivated fields, successful fertilization of eggs and final establishment of the resulting seeds within the farmer site or through exchange of seed among farmers who reproduce their own seed stocks, and the successful establishment of exchanged seeds within a different field population. The level of seed exchange among populations could also have been recently increased by the replacement of traditional hand harvesting with combine harvesting performed by contractors which is likely to facilitate the mixture between seed lots of neighbor farmers.

On the whole, highly significant differences were found between landrace populations and synthetics both in terms of mean genetic distance (GD = 0.319 vs. 0.529) and mean genetic similarity (GS = 0.563 vs. 0.542). However, of the three synthetics, VA154 did not significantly differ from the landrace populations either for SSR or I-SSR polymorphisms and it was the one genetically nearest to ''Nostrano di Storo'' field populations. It is worth mentioning that VA154 has been developed from the original ''Marano Vicentino'' landrace (Bertolini, pers. comm.). This finding suggests that the landrace ''Nostrano di Storo'' derives from the original ''Marano Vicentino'' and proves that all but one NSt farmer populations tightly resembles its genetic traits. One population (NSt11) is genetically close to synthetic VA143, selected from the landrace ''Spino Bresciano'', since most of the SSR and I-SSR marker alleles found were shared between them. Moreover, the synthetic VA157, developed from the ''Dente di Cane Piamontese'', a landrace which has its originary source in North-Western Italy, shows a distinct genetic background. Till the 1940s-1950s ''Spino Bresciano'' and ''Dente di Cane Piemontese'' were two of the most cultivated ''*maggenghi*'' (i.e. May maize) types of Piedmont and Lombardy irrigated plains and Venetian estuary (Northern Regions of Italy) as well the ''Marano Vicentino'' among the ''*agostani*'' (i.e. August maize) types (Lanza 1961).

The vast majority of individuals were shown to be different genotypes. All landrace populations but one scored 100% of different haplotypes (the only exception was NSt8 that scored 90%) whereas synthetics scored 92% on average. Thus, the overall molecular marker data confirm the high variability that can be found within each landrace population which strengthen the hypothesis that all populations belong to the same landrace and agree with the results of the characterization based on morpho-phenological and agronomic traits (Part I). These results also explain why local farmers, who are unable to discriminate among their own populations, use the same name (i.e. ''Nostrano di Storo'') for all of them.

As expected on the basis of the breeding system of maize, the genetic variability was ample, distributed among individuals of the whole landrace and as large among as within populations. In the landrace as a whole each individual proved to be heterozygous at a large number of loci. Each sampled population is actually a mixture of a large number of distinct genotypes that casually intercross at each generation. However, most populations seem to share a common gene pool which belongs to the landrace as a conse-

quence of the local adaptation, which might be ascribed to a combination of climatic conditions and agronomic practices. Both the type and the frequency of genes or gene sets in the common gene pool are logically determined by the fate of the genotypes in which they are assembled: if the genetic traits contributing to yield genotypes displaying high fitness in that particular environment, their frequency would have been maintained or increased over the years.

In a highly outcrossing species as maize, a high occurrence of different multilocus genotypes and a low frequency of significant linkage disequilibria among pairs of alleles as has been observed are expected (Frankel et al. 1995). In our case, two main factors might explain low genetic differentiation among populations and low accumulation of linkage disequilibria. First, the very large population, which can be up to several thousands individuals at the farm level, is mixed in the field at every generation. Second, the presence of high levels of seed exchange, which can prevent the differentiation among populations and increases the probability of outcrossing between different genotypes.

In the case of the landrace ''Nostrano di Storo'', gene contamination caused by pollen dispersal from commercial varieties has probably occurred and has been accentuated by seed exchange among farmers. Some of the landrace populations resembled genetic polymorphisms observed in the synthetics used as standard. They proved also to be characterized by genetic traits that were rare in the ''Nostrano di Storo'' materials and that more likely could derive from modern hybrid varieties. The introgression of genes from commercial hybrids might thus result in the genetic erosion of the landrace.

The large field population size and random mating, the genetic structure of each population may change over generations owing to the presence of disturbing factors such as genetic drift, migration, and selection. From this point of view, the cross of genetically similar individuals (inbreeding) seems to have a certain significance in disturbing the Hardy-Weinberg equilibrium as it is shown by populations which displayed homozygosity excess at the loci investigated. This could be the case when farmers select a very small seed stock every year and so apply a high selection intensity at each generation.

The two types of molecular markers used here assessed different levels of diversity within and among populations. The differences in terms of absolute estimates of genetic diversity within populations and differentiation among populations can be explained by the contrasting genetic nature of these markers (co-dominant vs. dominant and single-locus vs. multiple-loci) suggesting that much care should be taken in extending the inferences on the amount of genetic variability obtained with a given type of marker to the global genetic variability present in a population.

The assay efficiency index (Ai) and the marker index (MI) revealed that I-SSRs represent a marker system with a utility much higher than that of SSRs. Although the mean expected heterozygosity measured by I-SSRs was actually much lower than that scored by SSRs, the higher multiplex ratio component of the I-SSR assay compared to that of the SSR assay may have enhanced the information content of the former.

The lower estimates of the within-population diversity showed by I-SSR data in comparison to SSR data (H_S = 0.211 vs. 0.795 on average) matched with their opposite genetic nature, while the dendrograms explaining the between-population genetic relatedness were very similar for the two types of markers. The correlation between SSR and I-SSR similarity matrices was found to be highly significant (r = 0.91**). Moreover, the extent of differentiation estimated by SSR and I-SSR markers was comparable (G_{ST} values of 0.066 vs. 0.091, in the populations and 0.187 vs. 0.200, in the synthetics) as well as the gene flow (Nm of 3.556 vs. 5.025 and 1.084 vs. 1.997 respectively for populations and synthetics). SSR and I-SSR markers also detected comparable percentages of the total variation present within populations (94% and 98%, respectively).

Finally, this study reports that some marker alleles are more useful than others for diversity analysis related to *in situ* conservation of maize germplasm. Genomic loci dupssr10 e issr1, which respectively detect CA-repeat microsatellite and CA-anchored inter-microsatellite polymorphisms, proved to be those having the best discriminatory ability and retaining the highest information content within the respective marker systems.

On the whole, our findings suggest that, although a high molecular variability can be found among plants, most plant genotypes belong to the same landrace called ''Nostrano di Storo''. In addition to the common original gene pool, pollen dispersal and seed exchange among farmers could be taken into account for the low genetic differentiation among populations. The identity of the landrace as a whole seems to have been preserved and the large number of polymor-

phisms and the presence of both rare alleles and alleles unshared with B73 and synthetics potentially make this flint maize landrace a valuable source of genetic variation and unique germplasm traits. Because of its exclusive utilization for human consumption, the use of such molecular marker characterization could play a key role in obtaining the IGP (Protected Geographic Indication) mark and so promote the *in situ* conservation and protection of the landrace ''Nostrano di Storo''.

This information could be used to identify the core populations suitable to become the basic nucleus for the maintenance of the ''Nostrano di Storo'', to set a system of molecular reference for the identification of the landrace and to check the level of both genetic erosion and contamination in the area of cultivation where hybrid varieties are simultaneously adopted.

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