Relationships among radicchio (*Cichorium intybus* L.) types grown in Veneto and diversity between local varieties and selected lines as assessed by molecular markers

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Abstract: Red or variegated chicory (*Cichorium intybus* L., 2*n*=2*x*=18) native to, and very extensively cultivated in North-Eastern Italy as a leafy vegetable, locally called "radicchio", includes different types which represent valuable high-quality crops. The five major types of radicchio cultivated in the Veneto region were investigated by PCR-derived molecular markers (e.g. RAPDs, AP-PCRs, I-SSRs and AFLPs). The possibility of discriminating the different cultivated types was established by exploiting two different approaches: individual DNA vs. bulked DNA samples. Genetic similarity within, and differentiation and gene flow between types were estimated on the basis of the distinct marker systems. The effectiveness of phenotypic selection and the relatedness between local varieties and selected lines were also investigated. The molecular information acquired, along with morphological and phenological descriptors, will be useful for the certification of typical local products of radicchio and for the recognition of a protected geographic indication (IGP) mark.

Keywords: Chicory germplasm, landraces, DNA fingerprints, population genetics.

Introduction

The "red or variegated chicory" native to, and very extensively grown in North-Eastern Italy, called "radicchio" is acquiring more and more commercial interest. All the red types of radicchio now being cultivated derive from red-leaved individuals belonging to *Cichorium intybus* L. var. *foliosum* (Hegi) Bishoff, while the types with spotted or variegated leaves originated from spontaneous or controlled crosses between these individuals and members of the species *Cichorium endivia* L. var. *latifolium* Hegi, commonly known as broad-leaved endive. Currently, the main types of radicchio cultivated in the Veneto region are: "Rosso di Chioggia", early "Rosso di Treviso", late "Rosso di Treviso", "Variegato di Castelfranco" and "Rosso di Verona". The first of these is the most widespread, while the others represent locally valuable high-quality crops.

Radicchio reproduces mainly by out-crossing, selfing being limited by a sporophytic incompatibility system (Varotto *et al.*, 1995) and a strong gametophytic competition between self and cross-pollen. Radicchio materials grown by local farmers are usually represented by landraces known to possess a high variation and adaptation to the natural and anthropological environments where they have originated. On these populations farmers have been traditionally applying mass selection in order to improve the agronomic and commercial traits of their own populations. Although a clear-cut morphological differentiation among the five types does exist, their genetic identification is a difficult task because farmers sometimes utilize controlled hybridizations among different types to obtain recombinant superior genotypes.

The molecular investigations reported in this paper were aimed to: i) evaluate the genetic uniformity within and relatedness between local and commercial varieties; ii) assess the

relationships among the five types of radicchio and to set up a molecular reference system that would allow a precise identification of the different types grown in Veneto.

Material and methods

Plant materials

The plant materials were provided by Veneto Agricoltura, Centro Sperimentale Ortofloricolo "Po' di Tramontana" at Rosolina (Rovigo, Italy). An experimental population of "Variegato di Castelfranco" (CF-C_2) and one of "Rosso di Verona" (VR-C_2), both obtained after two selection cycles for earliness and morphological uniformity, were analyzed along with three groups of experimental lines, one of early "Rosso di Treviso" (TVP-S_4), one of late "Rosso di Treviso" (TVT-S_4) and one of "Rosso di Chioggia" (CH-S_5). Each of the experimental populations CF-C_2 and VR-C_2 was represented by 24 plants taken at random from a larger population obtained by inter-crossing in isolation phenotypically selected plants. Each of the two groups of experimental lines TVP-S_4 and TVT-S_4 was represented by 18 plants belonging to three inbreds (six plants each) for a total of 36 plants, obtained through four generations of selfing, while the group of CH-S_5 was formed by 12 plants from two inbreds (six plants each) obtained after five generations of selfing. Moreover, two experimental selections of "Rosso di Verona" (indicated by the initials VR-OP and VR-OM) were analyzed. For these selections, plant materials supplied by farmers (C_0 generation) were sampled along with those obtained after one (C_1 generation) and two selection cycles (C_2 generation) for earliness. Two experimental selections of "Variegato di Castelfranco" (CF-Q1 and CF-Q2) supplied again by farmers as C_1 and C_2 generations were also analyzed along with four local varieties (M1, M2, Rol1 and Rol2) and two commercial varieties (Badia and Corma). The experimental populations VR-OP, VR-OM, CF-Q1 and CF-Q2 were formed by inter-crossing in isolation of plants selected on a phenotypic basis. Each of these populations was represented by 40 randomly collected plants.

Molecular markers

Total genomic DNA was isolated from 0.5 g of leaf tissue according to a standard CTAB protocol. The DNA pellet was washed twice with 70% ethanol, dried and re-dissolved in 1× TE buffer. Concentration and purity of DNA samples were determined by optical density readings and by electrophoresis on 1% agarose gels using reference standards.

The molecular analysis was performed on a single-plant DNA basis with RAPD (primers OP-A1 = CAGGCCCTTC and OP-C7 = GTCCCGACGA), I-SSR (primer I-33 = AGCAGCAGCAGCT) and AP-PCR (primer M13 = TTATGAAACGACGGCCAGT) markers. Moreover, a bulk-plant DNA analysis with AFLP markers was performed by randomly grouping six or eight plants of each population within type using different Eco-RI/Mse-I primer combinations with three selective bases (E+CAC/M+ATC, E+CCA/M+AGG, and E+CAC/M+AAG). All amplification reactions of the agarose gel-detected markers occurred in a 25-µl volume with the same 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$) and were performed in a 9700 Thermal Cycler (Perkin Elmer). PCR parameters adopted for visualizing RAPD, AP-PCR and I-SSR markers were optimized in radicchio by our research group (Barcaccia *et al*., 2003). Amplification products were separated by electrophoresis in 2% agarose gels run with 1× TBE buffer at 150 V for 3 hrs. Digital photographs of the polymerized genomic fragments were taken after staining of the agarose gels with ethidium bromide and banding patterns were scored using the 1D Image software (Kodak). Restriction-ligation, pre-amplification and hot-PCR experiments were performed according to a standard AFLP protocol slightly modified to adapt it to radicchio (Barcaccia *et al*., 2003). PCR products were analyzed by 6% denaturing polyacrylamide gel

electrophoresis run with 1× TBE buffer using a sequencing cell apparatus. Gels were blotted on Whatmann 3 MM paper, dried at 75°C for 1 h and visualized by autoradiogram after 12 hrs exposure at -80°C using intensifying screens.

Data analysis

Data were recorded as a binary matrix (1=present, 0=absent) by assigning the molecular weight to each marker allele identified by comparing sample lanes with known DNA ladders.

Dice's (1945) genetic similarity estimates between plant DNA samples were calculated in all possible pair-wise molecular fingerprint comparisons. The within-type and between-type mean genetic similarity estimates were obtained by averaging individual estimates using the whole set of plants/bulks belonging to the types being compared. The ordination analysis was performed according to the UPGMA method: centroids and dendrograms of both single and bulked DNA samples were constructed from the double-centered and symmetrical genetic similarity matrices, respectively. All calculations and analyses were conducted using the appropriate routines of the software NTSYS-pc Version 1.80.

Genetic diversity and population differentiation statistics were used to summarize the data of molecular markers. The average marker allele frequency was calculated for each population and over all types taking into account the genotypic nature of plant materials. The fixation index (G_{ST}) was computed as proportion of genetic diversity expressed between types and used to derive the levels of gene flow (Nm) between types. Nei's (1978) genetic distance values between types were calculated in all possible pair-wise comparisons using marker allele frequencies over all marker loci. Calculations and analyses were conducted using the software POPGENE Version 1.21.

Results and discussion

Molecular identification of radicchio types

The RAPD, AP-PCR and I-SSR markers used in the single-plant DNA analysis allowed to obtain reproducible genomic fingerprints and to investigate a total of 27 polymorphic genomic loci in the five types. In spite of the detection of marker loci specific to one or two types, this information did not allow their discrimination from the others because of the low marker allele frequency. The cluster analysis was in fact unable to separate the 96 samples in distinct sub-groups corresponding to the five types since a number of plants of a given type were clustered within sub-groups of different types. These results were in agreement with the amount and partition of genetic diversity. The total genetic diversity was 0.217, while that calculated for single types was on average 0.149. The average value of genetic diversity between types was 0.068. The fixation index (G_{ST}=0.315) showed that about 70% of the total genetic variability observed can be attributed to within-type differences and around 30% is due to differences among the five types.

The average genetic similarities among the five types were also calculated. It was ascertained that CF and VR are very similar to one another (0.848), as are TVT and TVP (0.809). On average TVT was the least similar with respect to the other types (0.776). The calculation of the genetic distances between the different types confirmed that those genetically closer are VR and CF (0.049) and TVP and TVT (0.056), while TVT is the most distant from the other types (0.120). On the basis of both genetic similarities and genetic distances, CH was found much closer to CF and VR than to TVP and TVT.

The AFLP analysis performed on bulk-plant DNAs yielded a total of 197 amplification products, 118 (59.9%) of which were polymorphic. Fingerprint analysis enabled to identify several highly discriminant markers. Three type-specific markers were found for TVP and TVT that allow their discrimination from the other types. VR and CF shared five markers that

never appeared in the fingerprint of the other types and were differentiated from one another by seven markers present only in CF and six present only in VR. CH displays six specific markers that clearly distinguished it from the other types evaluated.

Cluster analysis was done using the similarity matrix calculated from the monomorphic and polymorphic AFLP marker data set. The bi-dimentional plotting of centroids singles out main sub-groups corresponding to the five types (Fig. 1). The most similar fingerprints were found for "Treviso" types which registered by far the highest similarity (0.865) and proved to be the less genetically differentiated types.

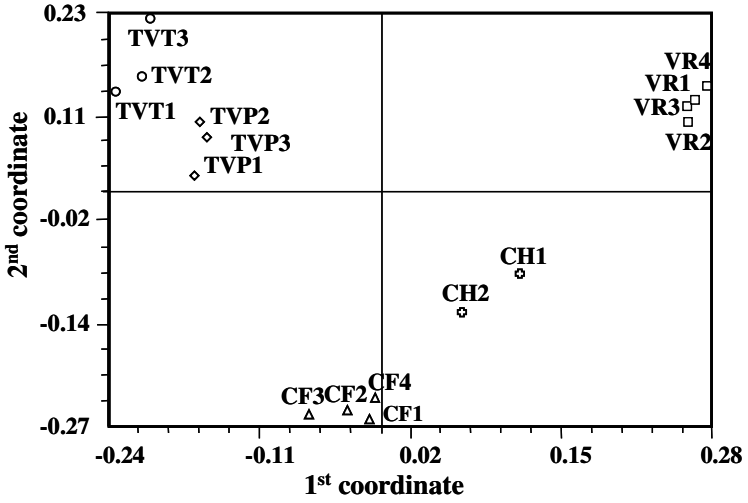

Figure 1. Centroids of the five radicchio types (CF = "Variegato di Castelfranco"; VR = "Rosso di Verona"; TVP = early "Rosso di Treviso"; TVT = late "Rosso di Treviso"; CH = "Rosso di Chioggia") constructed by the first two coordinates according to AFLP markers and based on the Dice's genetic similarity coefficients.

The observed levels of genetic differentiation tend to exclude an exchange of alleles between types, reflecting the fact that the Veneto region areas of production of each of the five types are fairly well isolated from one another. A major finding is that the sampling procedure and marker system influenced the traceability of plant materials. The different types are well distinguished from one another when analyzed by means of population bulks using AFLP markers, while they are not if analyzed at the individual level using RAPD, I-SSR and AP-PCR markers. An additional finding is that the genetic variation was shown to be much higher within than between types. Although CF and VR experimental populations were obtained by random matings in isolation among selected plants, it is likely that their gene pools did not change over the only two generations of selection applied. This is explained by the conserved amount of heterozygosity and thus of total genetic variation found within populations. The situation for experimental lines is different. Four to five generations of selfing followed by selection within each line have led to high degrees of homozygosity by the fixation of distinct alleles at a high number of loci and so to the formation of genetically differentiated inbreds. As a matter of fact, the high value of the fixation index (G_{ST}=0.315) is consistent with a DNA polymorphism rate more pronounced among entries within type than between types. This result suggests that in each radicchio type, populations produced by breeders through controlled intercrossing (VR and CF) or repeated selfing (TVP, TVT and CH) conserved their gene pools well separated over the years, as confirmed by the rather low estimates of gene flow (Nm = 1.09). On the basis of the reproductive system of radicchio, two factors can be taken into account: i) the incompatibility system that limits both selfing and intercrossing between plants with an identical phenotype at the multiallelic S-locus, thus allowing a certain amount of heterozygosity to be maintained even in inbred populations; ii) the selection criteria of mother plants to be used for seed production applied each year by each farmer most likely allowed to limit the genetic contamination between types and to preserve the phenotypic identity of each type.

Molecular characterization and breeding of radicchio

Selections C_0, C_1 and C_2 of the two experimental selections of "Rosso di Verona" scored 14 polymorphic marker loci. The genetic similarity over the three generations improved only with the first cycle of selection, increasing in VR-OP from 0.651 to 0.683 and in VR-OM from 0.629 to 0.690. The second cycle of selection was instead shown to be ineffective since the coefficient of similarity reduces in both VR-OP and VR-OM (0.667 and 0.671, respectively). Despite the reduction in similarity, it is interesting to note that the coefficient of variability decreases from 25-26% to around 19-20% over the three generations, thereby demonstrating an increase in genetic uniformity within populations after both cycles of selection (Fig. 2). Analysis of molecular fingerprints demonstrated the presence of some individuals with rare marker alleles that could be classified as molecular offtypes. The molecular information was used to carry out a negative selection of all offtypes within each population to maintain the most similar and distinguishable individuals. Molecular markers will be be used to support field selection programs aimed to reach a degree of genetic uniformity that would probably not be achievable with the simple phenotypic evaluation made by farmers.

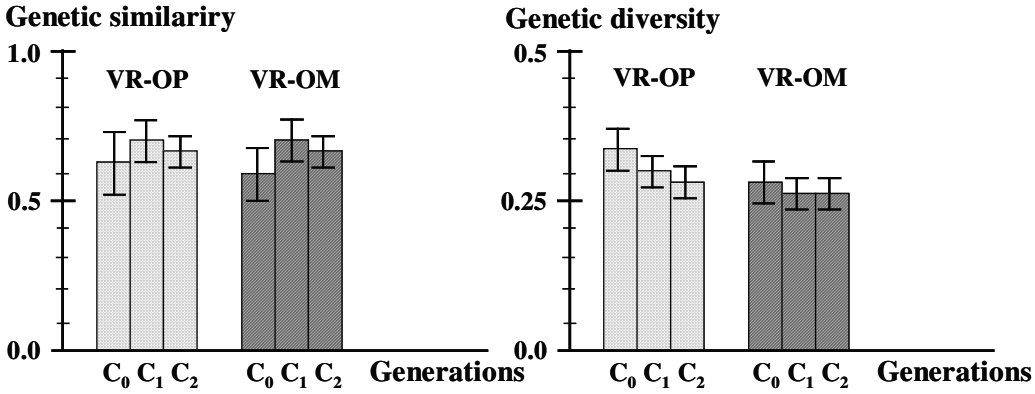

Figure 2. Histograms of mean genetic similarity and genetic diversity of selections C_0, C_1 and C_2 of "Rosso di Verona" along with variability coefficients for each generation.

The total genetic diversity of the six selections analyzed was 0.363. The C_0 generation registered values of genetic diversity of 0.330 for VR-OP and 0.306 for VR-OM while C_1 and C_2 generations showed comparable values that were lower than those of C_0 (0.320, 0.313 for C_1 and C_2 of VR-OP, 0.275 and 0.277 for C_1 and C_2 of VR-OM). On the whole, 85% of the total genetic variation was within generations and that between generations within selection was less than 15%. This means that two cycles of selection for earliness did not determine a clear molecular differentiation, at least on the basis of the exploited marker data set.

The genetic differentiation between experimental selections and varieties of "Variegato di Castelfranco" most commonly used by farmers was assessed by AFLP markers. A total of 126 markers were obtained, 31 (24.6%) of which were polymorphic.

Fingerprint analysis revealed that only one marker was always present in both local and commercial varieties and always absent in the experimental selections, which in turn scored four additional markers that never appeared in the other materials. The dendrogram showed four major nodes (Fig. 3). The first clustered samples of the four experimental populations CF-Q1 and CF-Q2, and displayed a mean similarity of 0.915, ranging from 0.907 to 0.956. The local varieties Rol1 and Rol2 formed a major sub-group with a mean similarity of 0.761, while the third major sub-group with a mean similarity of 0.842 contained the other two local varieties analyzed, M1 and M2. The two commercial varieties Badia and Corma formed the

fourth sub-group of the dendrogram, with a mean similarity of 0.934. These two lines registered the highest degree of uniformity and showed the highest degree of genetic differentiation compared to the experimental selections of "Variegato di Castelfranco".

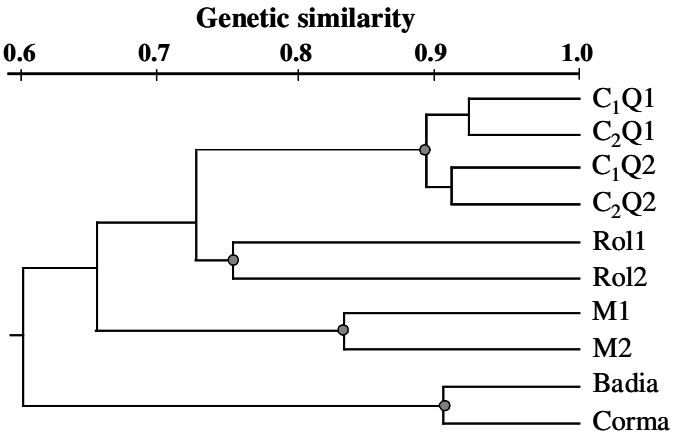

Figure 3. Dendrogram of the experimental selections (C_1Q1, C_2Q1, C_1Q2, C_2Q2) and commercial varieties (M1, M2, Rol1, Rol2, Badia and Corma) of "Variegato di Castelfranco" based on the mean genetic similarity estimates from AFLP data.

The breeding programs at present under way by local breeders and regional seed institutions are aimed: i) to isolate, within the best local selections, individuals amenable to be used as parents for the constitution of synthetic varieties and, although not easily feasible; ii) to select inbred lines suitable for the production of commercial F_1 hybrids. These breeding procedures could be greatly helped by the use of molecular markers that allow to discard molecular off-types, to better exploit parental genetic polymorphisms for synthetics and to identify the most genetically distant inbreds as parental lines for hybrids.

All things considered, the set up of a molecular reference system seems to be feasible for the precise identification of the single types of radicchio and suitable for the evaluation of the extent of natural hybridization that can occur between different types. The molecular characterization of radicchio can form the basis for an additional criterion of selection of phenotypically homogeneous genotypes to be used in breeding synthetic varieties and commercial hybrids. Moreover, the possibility of identifying the types of radicchio in commercial use in Veneto through their molecular characterization can be an essential element for certifying typical local products and in the near future could represent a basic requisite for their use in a serious and consumer-oriented production and marketing context.

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