

Genomic DNA fingerprints as a tool for identifying cultivated types of radicchio (*Cichorium intybus* L.) from Veneto, Italy

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Abstract

Red or variegated chicory (*Cichorium intybus* L., $2n = 2x = 18$) 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 polymerase chain reaction (PCR)-derived markers. The experimental material was represented by two outbred populations (one of 'Variegato di Castelfranco' (CF) and one of 'Rosso di Verona' (VR)) and by eight inbred lines (three of early 'Rosso di Treviso' (TVP), three of late 'Rosso di Treviso' (TVT) and two of 'Rosso di Chioggia' (CH)). A total of 96 individual plant DNAs and 16 bulked DNAs of six plants each were assayed. The different types were well distinguished from one another if analysed by means of bulks using amplified fragment length polymorphism markers at the population level, while they were not if analysed at the individual level using random amplified polymorphic DNA, inter-simple sequence repeat and Arbitrarily Primed (AP)-PCR markers. The genetic variation was shown to be much higher within types 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 well-separated gene pools over the years. The setting up of a molecular reference system seems to be feasible and suitable both for the precise identification of the single types of radicchio and for the evaluation of the extent of natural hybridization that can occur between different types.

Key words: *Cichorium intybus* — molecular markers — genetic diversity

The genus *Cichorium* (Asteraceae) is made up of two cultivated species, *C. intybus* L. (chicory) and *C. endivia* L. (endive), and one (*C. spinosum* L.) to two (*C. spinosum* L. and *C. pumilum* Jacq.) to four (*C. spinosum* L., *C. pumilum* Jacq., *C. calvum* Sch. Bip. and *C. bottae* A. Deflers) wild species, on the basis of different classifications (Tutin et al. 1976, Pignatti 1982, Kiers et al. 2000).

Cichorium endivia is an autogamous annual species, known only in its cultivated form as a leafy vegetable. *Cichorium intybus* reproduces mainly by cross-fertilization, but up to 20% of selfing is possible (Bellamy et al. 1995). Any classification within the species *C. intybus* is based mainly on the use of the different types. A first distinction between 'root' chicory (*C. intybus* L. var. *sativum* Bishoff) and 'leaf' chicory (*C. intybus* L. var. *foliosum* (Hegi) Bishoff) is made. Among the different types of leafy vegetables, the 'Red or variegated chicory' native to, and very extensively grown in north-eastern Italy, and

called 'Radicchio' (Paulet 1985, Baes and Cutsem 1993, Bellamy et al. 1996, Koch and Jung 1997, Kiers et al. 2000; van Stallen et al. 2000, 2001), is acquiring more and more commercial interest. All the red types of radicchio now being cultivated derive from red-leafed individuals belonging to the botanical variety *foliosum*, while the types with spotted or variegated leaves originated from spontaneous or controlled crosses between these individuals and plants of the species *C. endivia* L. var. *latifolium* Hegi, commonly known as broad-leaved endive (Bianchedi 1961, Paulet 1985). Radicchio is an allogamous plant, with entomophilous pollination, characterized by sporophytic incompatibility (Varotto et al. 1995). Selfing is also limited by an unfavourable flower morphology and a strong gametophytic competition between self and cross-pollination (Bellamy et al. 1995).

Currently, the main types of radicchio cultivated in Veneto 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. Although a clear-cut morphological differentiation among the five types does exist, their genetic identification is becoming increasingly important. Materials grown are usually represented by local populations known to possess a high variation and adaptation to the natural and anthropological environment where they have originated and are still cultivated (Soattin et al. 2001). These populations are maintained by farmers through phenotypical selection according to their own criteria and sometimes by exploiting to controlled hybridizations among different types in order to obtain recombinant genotypes showing superior agronomic and commercial traits.

The breeding programmes at present under way by local breeders and regional seed institutions aim (1) to isolate, within the best local selections, individuals amenable for use as parents for the constitution of synthetic varieties and, although not easily feasible (2) to select inbred lines suitable for the production of commercial F₁ hybrids. These breeding procedures could be greatly helped by the use of molecular markers that allow the discarding of molecular off-types, to better exploit parental genetic polymorphisms for synthetics and to identify the most genetically distant inbreds as parental lines for hybrids.

A few genetic studies using molecular markers have been carried out on *Cichorium* spp. mainly to characterize commercial varieties and experimental materials (Bellamy et al. 1995, Koch and Jung 1997, van Stallen et al. 2000), to evaluate the

genetic homogeneity and purity, respectively, of inbreds and hybrids (Bellamy et al. 1996), and to investigate phylogenetic relationships between cultivars and cultivar groups of *C. intybus* and other species, both cultivated and wild, belonging to the same genus (Vermeulen et al. 1994, Kiers et al. 2000; van Stallen et al. 2001). Amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) markers were also used to construct the only genetic map of *C. intybus* reported in the literature (De Simone et al. 1997).

The molecular investigations reported in this paper aimed to evaluate the genetic 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. This information, 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 geographical indication (IGP) mark.

Materials and Methods

Plant materials: The plant materials of *C. intybus* L. were provided by Veneto Agricoltura, Centro Sperimentale Ortofloricolo 'Po di Tramontana' at Rosolina (Rovigo, Italy). They were developed from a large number of local farmers' populations initially selected on the basis of their morphological and phenological traits.

In this study, an experimental population of 'Variegato di Castel-franco' (CF-C₂) and one of 'Rosso di Verona' (VR-C₂), both obtained after two selection cycles for earliness and morphological uniformity, were analysed along with three groups of experimental lines, one of early 'Rosso di Treviso' (TVP-S₄), one of late 'Rosso di Treviso' (TVT-S₄) and one of 'Rosso di Chioggia' (CH-S₅). Each of the experimental populations CF-C₂ and VR-C₂ was represented by 24 plants taken at random from a population obtained by intercrossing in isolation previously phenotypically selected plants. Each of the two groups of experimental lines TVP-S₄ and TVT-S₄ 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-S₅ was formed by 12 plants from two inbreds (six plants each) obtained after five generations of selfing.

It is worth mentioning that the experimental materials (both outbred populations and inbred lines) used in this work were derived from autochthonous populations and maintained over generations by phenotypical selection; they can thus be considered as belonging to gene pools representative of the five different types of radicchio cultivated in the Veneto region.

Genomic DNA isolation: Total genomic DNA was isolated from 0.5 g of leaf tissue according to the protocol described by Barcaccia and Rosellini (1996). The DNA pellet was washed twice with 70% ethanol, dried and redissolved in 100 µl of TE buffer (10 mM Tris-HCl, 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0). The concentration of DNA samples was determined by optical density readings at 260 nm (1 optical density (OD) = 50 µg/ml) and their purity calculated by the OD₂₆₀/OD₂₈₀ ratio and by the OD₂₁₀-OD₃₁₀ pattern (Sambrook et al. 1989). An aliquot of genomic DNA was also assayed by electrophoresis on 1% agarose gels.

Molecular markers: The molecular analysis was performed on a single-plant DNA basis (96 samples) with RAPD (primers OP-A1 and OP-C7), inter-simple sequence repeat (I-SSR) (primer I-33) and AP-PCR (primer M13) markers. Moreover, a bulk-plant DNA analysis was performed by randomly grouping six by six the plants within each type, for a total of 16 samples distributed as follows: four for CF-C₂ and VR-C₂, three for TVP-S₄ and TVT-S₄, and two for CH-S₅ (one sample for each inbred line). AFLP markers were adopted using three different *EcoRI* + 3/*MseI* + 3 primer combinations.

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 MgCl₂) and were performed in a 9700 Thermal Cycler (Perkin Elmer, Norwalk, CT, USA).

RAPD markers: PCR parameters adopted for visualizing RAPD markers were those described by Barcaccia et al. (1997). The sequences (5' to 3') of the oligonucleotide primers used are as follows: OP-A1 = CAGGCCCTTC and OP-C7 = GTCCCGACGA.

I-SSR markers: Inter-microsatellite markers were assayed using a short primer anchored at the 3' terminus of the AGC repeat by one nucleotide residue (I-33 = AGCAGCAGCAGCT). The PCR protocol used for detecting ISSR polymorphisms was that reported by Barcaccia et al. (2003).

AP-PCR markers: AP-PCR experiments were carried out using the universal primer M13 = TTATGAAACGACGGCCAGT. Amplification of these markers was based on the PCR conditions optimized by Soattin et al. (2001).

RAPD, I-SSR and AP-PCR amplification products were separated by electrophoresis in 2% agarose gels run with 1× TBE buffer (45 mM Tris-HCl, 45 mM orthoboric acid and 1 mM EDTA) at 150 V for 3 h. Photographs of the polymerized genomic fragments were taken after staining of the agarose gels with ethidium bromide.

AFLP markers: Restriction-ligation, preamplification and hot-PCR experiments were performed according to the AFLP protocol reported by Barcaccia et al. (1998). The analysis of DNA fingerprints was based on the amplification of *EcoRI/MseI* genomic restriction fragments with three different primer combinations having three selective bases (E + CAC/M + ATC, E + CCA/M + AGG, and E + CAC/M + AAG).

The PCR products were analysed by 6% denaturing polyacrylamide gel (acrylamide/bis 19 : 1 solution) electrophoresis run with 1× TBE buffer (45 mM Tris-HCl, 45 mM orthoboric acid and 1 mM EDTA) using a sequencing cell apparatus. Gels were blotted on Whatman 3 MM paper, dried at 75°C for 1 h and visualized by autoradiogram after 12 h exposure at -80°C using intensifying screens.

Data analysis: DNA markers were scored as present (1) or absent (0) over all single or bulked plant DNA samples. Data were recorded as a binary matrix by assigning the molecular weight to each monomorphic and polymorphic marker identified by comparing sample lanes with known DNA ladders.

Dice's (1945) genetic similarity estimates between single or bulked plant DNA samples were calculated in all possible pairwise molecular fingerprint comparisons. The 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 within-type mean genetic similarity estimate was calculated taking into account all plants belonging to that type. The ordination analysis was performed according to the unweighted pair-group arithmetic average method (UPGMA) clustering algorithm (Sneath and Sokal 1973). Centroids of all 96 single-DNA plants were bidimensionally plotted according to the principal coordinates extracted from the double-centred genetic similarity matrix. The dendrogram of the 16 bulked DNA samples was constructed from the symmetrical genetic similarity matrix. Relationships among the five types, as estimated on the basis of single and bulked DNAs were visualized by comparing mean genetic similarity matrices.

All calculations and analyses were conducted using the appropriate routines of the software NTSYS version 1.80 (Rohlf 1993).

Additional statistics were computed to estimate the degrees of polymorphism within types and the genetic distances between types. The average marker allele frequency (p_i) for each primer and over all primers was calculated for each single type and over all types taking into account the genotypic nature of plant materials.

Genetic diversity (H) and populations differentiation (D_{ST}) statistics of Nei (1973) were used to summarize the data for molecular markers. The fixation index (G_{ST}) was computed as proportion of genetic diversity expressed between types. From this, the levels of gene flow (Nm) between types were derived and interpreted according to McDermott and McDonald (1993). Nei's (1978) genetic distance values between types were calculated in all possible pair-wise comparisons using marker allele frequencies over all marker loci.

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

Results

Analysis of individual DNAs

The RAPD, AP-PCR and I-SSR markers used in the single-plant DNA analysis allowed reproducible and informative polymorphisms to be obtained in the five types of radicchio (Fig. 1). Selected primers generated a total of 34 markers, 27 of which were polymorphic (79.4%). The I-33 primer gave the highest number of polymorphisms (13).

Of the 27 polymorphic marker loci identified, four were observed only in one type, one in two types, four and five were

always absent from one or two types, respectively. Theoretically, the presence or absence of marker loci specific to one or two radicchio types, should allow discrimination from the others. This was, however, not possible because of the low frequency of their respective specific marker alleles. For example, in the case of the five loci present only in one or two types, the frequency of the individuals showing the marker allele was, on average, only 38.2% for each type (minimum value 4.2%, maximum value 50%).

The cluster analysis based on single plant DNA polymorphisms was thus unable to separate the 96 samples in distinct subgroups corresponding to the five types. A number of individuals of a given type were clustered within subgroups of different types, thus showing substantial genomic similarities among plants belonging to different types. As a consequence, although some of the marker alleles screened were type-specific, none of them allowed the individuals of the different types to be pooled in distinct subgroups. Individuals of a given type could never be identified by type-specific haplotypes. The best results were obtained with marker alleles I-33/1, I-33/5 and M13/5 which re-classified correctly 14 of the 18 TVT plants and with marker alleles OP-C7/3 and I-33/6, which distinguished 13 of the 24 VR plants. No distinguishing haplotypes were found for CF, VR and CH types.

Similar results were obtained with the principal coordinate analysis applied to plot centroids of the 96 plants. According to the first two coordinates, which accounted for 45.3% of the total genetic variance, a number of individuals of each type overlap the distribution of individuals of other types. However, most of the TVP and TVT individuals, as well as the two CH lines, were discriminated by the first coordinate, while TVT and TVP lines as well as most of the individuals of CF and VR populations were separated by the second coordinate (Fig. 2).

These results were in agreement with the amount and partition of genetic diversity. The total Nei's genetic diversity (H_T) was 0.217, while that calculated for single types (H_S) was, on average, 0.149. The average value of genetic diversity between types (D_{ST}) was 0.068. The fixation index ($G_{ST} = 0.315$) showed that as much as 70% of the total genetic variability observed can be attributed to within-type differences and around 30% is due to differences among the

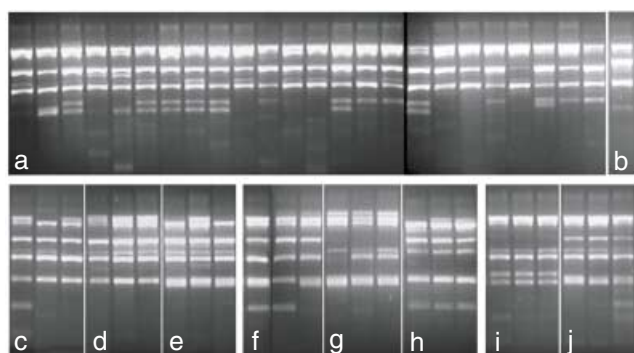


Fig. 1: Examples of inter-simple sequence repeats (I-SSRs) markers generated by primer I-33 on the basis of a single DNA sample analysis: a. 24 plants of the 'Variegato di Castelfranco' (CF-C₂) selection; b. one representative plant of 'Rosso di Verona' (VR-C₂) selection; c-e. three inbred lines of early 'Rosso di Treviso' (TVP-S₄); f-h. Three inbred lines of late 'Rosso di Treviso' (TVT-S₄); i,j. two inbred lines of 'Rosso di Chioggia' (CH-S₃). (Three plants for each line)

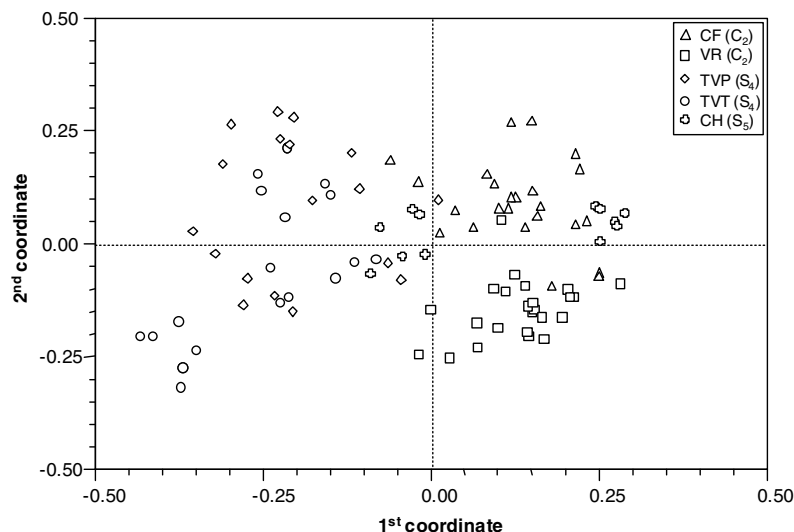


Fig. 2: Centroids of the 96 individuals belonging to the five radicchio types (CF, 'Variegato di Castelfranco'; VR, 'Rosso di Verona'; TVP, early 'Rosso di Treviso'; TVT, late 'Rosso di Treviso'; CH, 'Rosso di Chioggia') constructed by the first two coordinates according to random amplified polymorphic DNA, AP-polymerase chain reaction, and inter-simple sequence repeat markers and based on the Dice's similarity coefficients. The first two coordinates accounted for 45.3% of the total genetic variance

Table 1: Matrix of genetic similarity (GS) coefficients (below diagonal) and genetic distance (GD) estimates (above diagonal) calculated on the basis of random amplified polymorphic DNA, AP-polymerase chain reaction and inter-simple sequence repeat markers

	CF	VR	CH	TVP	TVT	Mean GD
CF		0.049	0.071	0.095	0.137	0.088
VR	0.848		0.084	0.133	0.137	0.101
CH	0.841	0.841		0.148	0.151	0.114
TVP	0.801	0.778	0.802		0.056	0.108
TVT	0.761	0.768	0.766	0.809		0.120
Mean GS	0.813	0.809	0.813	0.798	0.776	

five types. The estimated gene flow was rather low ($Nm = 1.09$).

The average Dice's genetic similarities among the five types of radicchio were also calculated (Table 1). 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). These data are in agreement with those obtained by the calculation of the Nei's genetic distances between the different types of red chicory (Table 1). It was confirmed that those genetically closer are VR and CF (0.049) and TVP and TVT (0.056), while TVT was, on average, the most distant from the other types (0.120). On the basis of both genetic similarities and genetic distances, CH was found to be much closer to CF and VR than to TVP and TVT.

Analysis of bulked DNAs

The AFLP analysis performed on bulk-plant DNAs yielded a total of 197 amplification products, 118 (59.9%) of which were polymorphic. In particular, the primer combination *Eco* + C-CA/*Mse* + AGG produced 60 markers, 44 of which (73.3%) were polymorphic.

Fingerprint analysis enabled the identification of specific markers for each of the types. Three type-specific markers were found for TVP and TVT that allowed their discrimination from the other types. The two 'Treviso' types are distinguished from one another thanks to six markers present only in TVP and to one present only in TVT. VR and CF, share five markers that never appeared in the banding patterns of the other types and are differentiated from one another by seven markers present only in CF and six present only in VR. CH displays six specific markers that clearly distinguish it from the other materials evaluated. Considering the markers lacking in the bulks of a type, six marker alleles are always absent from TVP. These loci therefore allow each of the two 'Treviso' types to be identified, while other two markers, always absent from both types, allow them to be distinguished from the others where they are always present. The absence of five marker alleles differentiates VR. The fact that 11 markers absent from CH are always found in the other types makes this type the most easily identifiable one.

Cluster analysis was performed using Dice's coefficient matrix of similarity calculated from the monomorphic and polymorphic marker data set. The dendrogram in Fig. 3 singles out main subgroups corresponding to the five types, each with a characteristic degree of genetic uniformity. Results clearly indicate that the genetic similarity among samples within a given type is higher in populations obtained after two

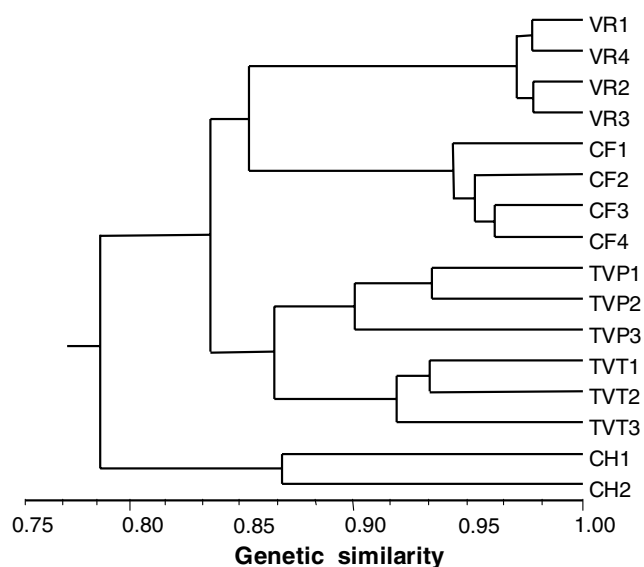


Fig. 3: The UPGMA dendrogram of the 16 bulked DNA samples obtained from the genetic similarity matrix based on AFLP markers (VR1-4, bulks of 'Rosso di Verona'; CF1-4, bulks of 'Variegato di Castelfranco'; TVP1-3, inbred lines of early 'Rosso di Treviso'; TVT1-3, inbred lines of late 'Rosso di Treviso'; CH1-2, inbred lines of 'Rosso di Chioggia')

cycles of intercrossing (VR and CF) than in the groups of inbred lines developed from the same material through four or five cycles of selfing (TVP, TVT and CH). The average genetic similarities calculated within each type were: 0.974 for VR, 0.950 for CF, 0.912 for TVP, 0.924 for TVT and 0.869 for CH. Lines of CH were the most differentiated from the other plant materials. The average value of genetic similarity calculated between CH and all the other types was, in fact, the lowest (0.789). The value of similarity registered between TVP and TVT was by far the highest among the different types (0.865). This means that these two are genetically the most similar types.

An evaluation of the efficiency of DNA sampling procedures (bulked and single plant analysis) and marker systems in detecting the genetic relationships among the five types of radicchio has been attempted by comparing centroids calculated for the five types on the basis of the two approaches. As shown in Fig. 4, the classification based on the mean genetic similarity matrices from single and bulked plant DNA polymorphisms gave a comparable bidimensional plotting result.

Discussion

The molecular investigations of the materials chosen as representative of the five types of radicchio currently grown in Veneto has allowed the evaluation of the degree of genetic differentiation reached through phenotypic selection and controlled matings.

The sampling procedure and marker system used are efficient in discriminating the plant materials. The different types (VR, CF, TVP, TVT and CH) are well distinguished from one another if they are analysed by means of bulks using AFLP markers at the population level, but they are not if analysed at the individual level using RAPD, I-SSR and AP-PCR markers. These marker systems were useful for tracing the diversity between types and for estimating the gene

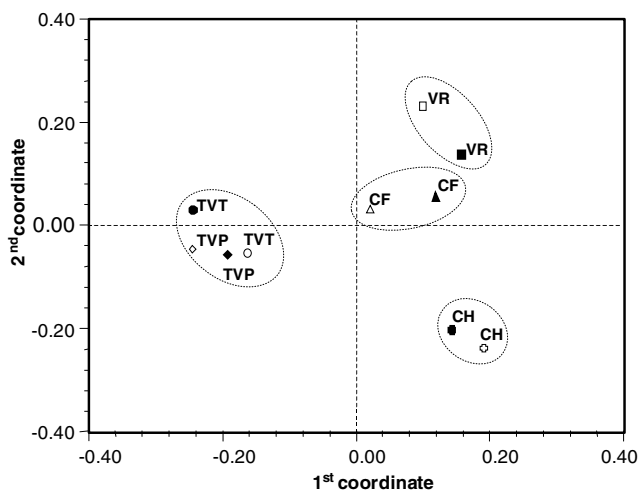


Fig. 4: Centroids of the five radicchio types (CF, 'Variiegato di Castelfranco'; VR, 'Rosso di Verona'; TVP, early 'Rosso di Treviso'; TVT, late 'Rosso di Treviso'; CH, 'Rosso di Chioggia') detected on the basis of two different approaches applied: individual plant DNA analysis with random amplified polymorphic DNA, AP-polymerase chain reaction, and inter-simple sequence repeat markers (white symbols) vs. bulked plant DNA analysis with amplified fragment length polymorphism markers (black symbols). The first two coordinates accounted for 75.9% (individuals) and 64.1% (bulks) of the total genetic variance

flow. Levels of gene flow are low and tend to exclude an exchange of alleles between individuals belonging to different types. This reflects the fact that the Veneto region areas of production of each of the five radicchio types are fairly well isolated from one another.

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 performed in isolation among selected plants, it is likely that their gene pools did not change over the mere 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 thus to the formation of genetically differentiated inbreds, and this seems particularly true for CH. 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 well-separated gene pools over the years, as confirmed by the rather low estimates of gene flow. On the basis of the reproductive system of radicchio, such a finding may be explained taking into account two factors: first, in this species, inbreeding is hampered by a sporophytic incompatibility mechanism that limits both selfing and intercrossing between plants with an identical phenotype at the multi-allelic S-locus, thus allowing a certain amount of heterozygosity to be maintained even in inbred populations; second, the selection criteria of mother plants to be used for seed production applied each year by each farmer probably allowed contamination between types to be

limited, preserving the phenotypic identity of each type. Moreover, seed multiplication carried out over the years by each farmer according to their own criteria produced a clear genetic differentiation between types within the species.

The classes of molecular markers adopted in this study deserve additional discussion. The key of the success of multilocus PCR-based markers has to be found in their high multiplex ratio. As a result of their own genetic nature, RAPD, AP-PCR, I-SSR and AFLP systems detect simultaneously many loci randomly distributed within the genome. Moreover, compared with codominant markers, these systems allow a more precise estimate of marker allele frequencies at single loci and a faster estimate of population polymorphisms over several loci, e.g. they require a lower number of sampled plants per population to investigate presence vs. absence marker alleles and a much lower number of experiments to investigate a given number of marker loci.

The results obtained confirm once again the great versatility, reliability and precision of the techniques based on molecular markers, which can be used to aid the classical evaluation of the differentiation between selections/lines based on the observation of morphological characteristics. 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 the radicchios can form the basis for an additional criterion of selection of phenotypically homogeneous genotypes to be used in breeding synthetic varieties and commercial hybrids.

The possibility of identifying the types of radicchio in commercial use in Veneto through their molecular characterization could be an essential element for certifying typical local products and could represent a basic requisite for their use in a serious, transparent and consumer-orientated production and marketing context.

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