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Distinction between cultivated and wild chicory gene pools using AFLP markers

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Abstract The cultivation area of industrial chicory, *Cichorium intybus* L. cv Sativum, coincides with the natural distribution area of its wild relative, *C. intybus* L., which could lead to gene flow between wild and cultivated types. The genetic diversity within and between the two types has therefore been studied using AFLP genotyping of samples from 12 wild populations collected in Belgium and ten commercial varieties. The genotyping of 233 individuals allowed the identification of 254 AFLP markers. Similar levels of genetic diversity were observed within wild populations and cultivated varieties, suggesting the absence of any strong bottleneck in the history of the cultivated types. The phylogenetic analysis pointed to a monophyletic origin of cultivated varieties as compared to the local wild populations studied, hence the two types of chicory form two separate gene pools. The genotyping of some individuals sampled in ruderal sites clearly showed that they belong to the cultivated gene pool, which suggests the existence of feral or weedy types. The low differentiation observed among wild populations indicates that gene flow might be important in this species.

Keywords *Cichorium intybus* · Chicory · AFLP · GMO · Genetic structure

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Introduction

Among chicory varieties, *Cichorium intybus* L. cv Sativum is bred and cultivated for its roots that are still used as a coffee substitute but more and more for its storage polyfructan, inulin. Inulin is a food ingredient that can be converted into fructose syrup upon chemical or enzymatic hydrolysis. While chicory can be found in most parts of Europe, industrial chicory cultivation is mainly restricted to Belgium, the north-eastern part of France and the Netherlands. Genetic engineering has been applied to industrial chicory but is still confined to laboratories and tightly controlled experimental plots. However, wild populations of *C. intybus* do occur in the cultivation area, which raises the question of possible gene flow from upcoming genetically modified varieties to wild relatives. Being a cross-pollinated species, exchanges between wild and cultivated types are expected, but cultivation practices may largely limit gene flow: as a biennial plant harvested on the first year, gene transfer can only happen during seed production (generally carefully controlled by seed breeders in southern France, more than 1,000 km away) or through weedy individuals. However, chicory is very sensitive to spring vernalization and early sown fields are thus more or less prone to bolting on the first year, depending on the varieties used: recent varieties have been improved for that trait. Hence, opportunities for crop to wild gene flow do exist. The situation in chicory resembles to some extent the pattern of co-occurrence of wild and cultivated types of sugar beet, *Beta vulgaris* L., in Western Europe (Bartsch et al. 1999). In beet, evidence for wild-to-crop and crop-to-wild gene flow has been suggested from analyses of samples from crop and wild and weedy forms with nuclear genetic markers (Bartsch et

al. 1999; Desplanque et al. 1999). These studies pointed out the necessity of considering the role of weedy forms of sugar beets in the context of risk assessment of transgenic beets. Although genetic polymorphism in cultivated chicory has been studied using different markers (allozyme markers, Baes and Van Cutsem 1992, 1993; RAPD markers, Bellamy et al. 1996; Koch and Jung 1997; AFLP markers, Kiers et al. 2000), no comparative study between wild and cultivated chicory populations has been carried out.

The aim of the present study is to contribute to the knowledge of the genetic structure of the different chicory populations encountered in Belgium, the central area of cultivation of industrial chicory, and to evaluate whether genetic markers specific to cultivated and wild types can be identified. The AFLP genotyping technique was chosen because of its high multiplex ratio (number of polymorphic markers generated in a single PCR experiment), high reproducibility (Jones et al. 1998, Pejic et al. 1998), and because of the lack of publicly available codominant markers (nuclear RFLP, SSR or SNP) in this species. AFLP markers were therefore used to assess: (1) the genetic structure within and among varieties in the industrial chicory type; (2) the population genetic structure of *C. intybus* in the wild in Belgium; and (3) the genetic variation between cultivated and wild varieties. Such a comparison between wild and cultivated types of chicory has never been reported and will help understanding the current status of this recently bred crop as well as contributing to the risk assessment of transgenic varieties.

Materials and methods

Plant material

Seeds from ten commercial varieties of industrial chicory were sown in a greenhouse at 20–25 °C and 16 h-day/8 h-night regime (Table 1). The leaves from 132 plants were sampled 4 to 8 weeks later. We also sampled leaves from 100 individuals of wild chicory belonging to 12 populations from Belgium (Table 1). These wild populations are mainly located in the area of the River Meuse. Two "wild" samples (Rixensart and Evere) were found very close to the Brussels region, where chicory has been historically grown as a coffee substitute.

DNA extraction

Fresh young leaves (no more than 100 mg) were finely ground in liquid nitrogen and genomic DNA was extracted according to Murray and Thompson (1980), with the following modification: a step of PEG purification was carried out before the two last ethanol rinses, in order to eliminate remaining polysaccharides [200 μl of 20% (w/v) PEG 8000/1.2 M of NaCl were added to the DNA and the solution was incubated on ice for 20 min].

AFLP protocol

AFLP was performed using the commercial kits from Applied Biosystems for fluorescent fragment reaction. Genomic DNA was restricted with *Eco*RI and *Mse*I before adaptator ligation. The preselective (*Eco*RI primer E-A and *Mse*I primer M-C) and selective amplifications with a single primer combination (E-AAG and M-CTA primers) were then carried out. The E-AAG primer was linked to a green fluorescent label at the 5′ end. The PCR products were run on a 4.25% w/v polyacrylamide gel on an ABI prism 377 DNA Sequencer. An internal lane-size marker (Genescan Rox-500; 35–500 bp) was present in each sample.

Band scoring and data analysis

The Genescan 2.1 software was used to obtain the detection time, signal peak height, surface and size of each amplification fragment. Sizing of the fragments was performed by interpolation of the internal lane standard. The raw data were analysed with the Perkin-Elmer Genotyper 2.0 analysis software which created tables with fragment sizes for each genotyped individual. The table was then converted into a matrix where fragments were scored as either present (1) or absent (0). Using the software AFLP-SURV v1.0 (Vekemans et al. 2002, available at http://www.ulb.ac.be/sciences/lagev), estimates of pairwise relatedness coefficients (r) were calculated between individuals according to Lynch and Milligan (1994). A principal co-ordinate (PCO) analysis was performed based on this matrix using NTSys (Rohlf 2000) and the first two axes were plotted graphically. Pairwise genetic distances (Reynolds et al. 1983) were calculated between samples. A neighbour-joining tree was computed, based on these distance measurements using the procedure NEIGHBOUR from the PHYLIP software package (Felsenstein 1993). A thousand bootstraps were performed over AFLP loci using AFLP-SURV and PHYLIP. Statistics of genetic variation within and between samples were computed using the software AFLP-SURV v1.0. Because AFLP markers were scored as dominant, allelic frequencies at AFLP loci were calculated from the observed frequencies of fragments, using the Bayesian approach proposed by Zhivotovsky (1999) for diploid species and assuming Hardy-Weinberg equilibrium. This is justified by the outcrossing breeding-system of chicory which is self-incompatible. A non-uniform prior distribution of allelic frequencies was assumed with its parameters derived from the observed distribution of fragment

Table 1 Origin of cultivated and wild samples of chicory

Cultivated chicory			Wild chicory		
Variety	Seed company	Sample size	Population	Location	Sample size
Bergues	Desprez	14	Beauraing	55°40′N 2°57′E	3
Dageraad	DVP	20	Bure	55°38′N 3°14′E	6
Eva	DVP	11	Chimay	55°36′N 2°13′E	12
Hera	DVP	11	Froid-Lieu	55°39′N 3°01′E	8
Katrien	DVP	14	Han	55°48′N 3°08′E	4
Madona	Warcoing	12	Longueville	55°59′N 3°28′E	9
Oesia	Nestlé	9	Mazée	55°40′N 2°37′E	5
Orchies	Desprez	14	Prayon	56°12′N 3°43′E	11
Régalo	SES	12	Treignes	55°40′N 2°34′E	10
Tilda	DVP	15	Vaulx	55°37′N 2°15′E	18
			Rixensart	56°22′N 2°25′E	11
			Evere	56°31′N 2°18′E	3

frequencies among loci (Zhivotovsky 1999). This procedure has been shown to produce almost unbiased estimates of allelic frequencies in dominant markers (Krauss 2000). These allelic frequencies were used as input for the analysis of genetic diversity within and between samples following the method described in Lynch and Milligan (1994). Within samples we report values of the number and proportion of polymorphic loci at the 5% level (loci with the frequency of the marker allele comprised between 0.05 and 0.95), and of the gene diversity H_j which is an estimate of the average heterozygosity expected under Hardy-Weinberg equilibrium. For each type of chicory we report the overall gene diversity (H_T), the average gene diversity within populations or varieties (H_S), the differentiation among populations or varieties (D_{ST}) and the proportion of genetic differentiation (G_{ST}). The significance of the genetic differentiation between samples or between types was tested by comparison of the observed G_{ST} with a distribution of G_{ST} under the hypothesis of no genetic structure, obtained by means of 1,000 random permutations of individuals among groups.

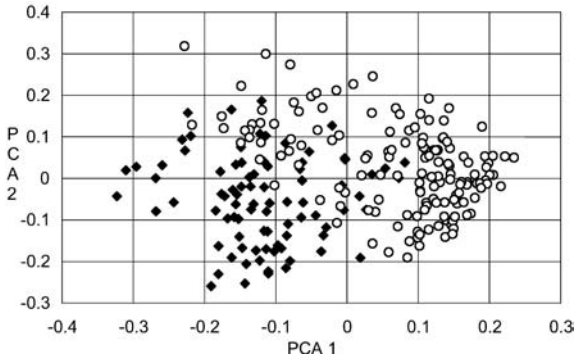

Fig. 1 Plot of sampled individuals of cultivated (*empty circles*) and wild (*filled diamonds*) chicory against the first two principal coordinates obtained by PCO on the matrix of pairwise relatedness coefficients between individuals

Results

Genetic differentiation between cultivated and wild chicory

The principal coordinate analysis (PCO) showed that wild and cultivated types of chicory formed two distinct clouds of points with some overlap (Fig. 1). Comparable sizes of the two clouds suggest that the levels of genetic variation in the two types are similar. Genetic differentiation between samples of cultivated and wild chicory can be visualised on a neighbour-joining tree obtained from a matrix of pairwise genetic distances between varieties and/or populations (Fig. 2). Two main clusters can be recognised, with a bootstrap support of 69%. The first cluster comprises all natural populations except those from Evere and Rixensart, whereas the second cluster contains all commercial varieties together with these two populations.

In order to test the hypothesis of differentiated gene pools, we computed the proportion of genetic variation expressed between, as opposed to within the two types, after exclusion of the populations from Evere and Rixensart. We found that only 3.8% of total genetic variation can be explained by the differentiation between the two gene pools (G_{st} = 0.038, see Table 4). However, this value of G_{st} was significantly higher than values obtained after random permutations of individuals between the two types ($P < 0.001$). Hence, in Belgium, the cultivated and wild gene pools of chicory are significantly genetically differentiated. Although we did not find AFLP markers strictly associated with one of the two types, we identified ten markers showing substantial differences in the frequency of occurrence between the cultivated and wild gene pools (Table 2). Among these, nine AFLP markers were indicators of the cultivated type whereas only one (A231) was an indicator of the wild gene pool. An extensive screening of many more AFLP markers would clearly lead to the detection of more specific markers.

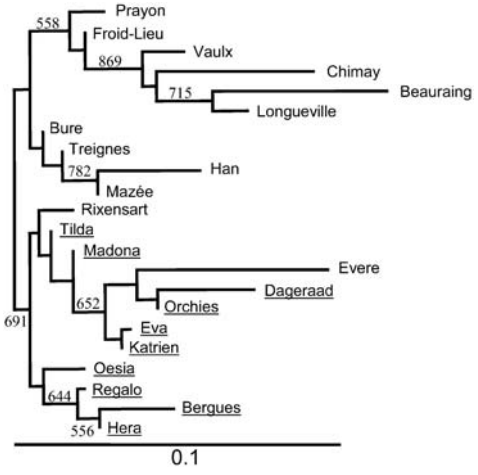

Fig. 2 Cluster analysis of samples from cultivated and wild chicory using neighbour-joining from a matrix of pairwise genetic distances. Names of samples from commercial varieties of chicory are *underlined*. The number of bootstraps that support a given internal branch is written above the branch when this number is higher than 500

Genetic variation within commercial varieties and natural populations of chicory

Statistics of genetic variation within commercial varieties and within natural populations of chicory are given in Table 3. For the wild type, we discarded populations with a sample size lower than eight. The selected primer combination amplified, on average, about 42 AFLP fragments for each individual plant. The number of polymorphic loci within samples varied between 75 to 149 over a total of 254 AFLP loci. The average proportion of polymorphic loci was very similar in the two chicory types: 45.4% in cultivated samples and 47.1% in wild samples. The average gene diversity within samples was also similar in the two types: H_j = 0.153 ± 0.017 in samples of cultivated chicory and 0.137 ± 0.014 in samples of wild chicory.

Table 2 Frequencies of occurrence of the ten AFLP markers (obtained with the single primer combination *Eco*RI-AAG/*Mse*I-CTA) that discriminate best between the cultivated and wild types of chicory. For each AFLP marker, its molecular size in bp is given

	Sample frequency of each discriminant AFLP marker									
AFLP fragment	A195	A200	A127	A136	A188	A229	A253	A231	A223	A213
Molecular weight	339	350	230	244	329	404	448	406	394	374
Cultivated type										
Bergues	0.86	0.64	0.93	0.64	0.71	0.14	0.50	0.07	0.36	0.14
Dageraad	0.65	0.80	0.65	0.75	0.35	0.60	1.00	0.00	0.60	0.65
Eva	0.36	0.45	0.64	0.73	0.18	0.82	0.82	0.00	0.36	0.73
Hera	0.45	0.73	0.73	0.64	0.73	0.36	0.27	0.00	0.45	0.00
Katrien	0.50	0.43	0.64	0.64	0.71	0.86	1.00	0.00	0.29	0.71
Madona	0.75	0.67	0.58	0.58	0.75	0.33	0.50	0.00	0.50	0.33
Orchies	1.00	0.50	0.86	0.64	0.50	0.50	0.86	0.00	0.36	0.57
Oesia	0.56	0.22	0.56	0.67	0.56	0.56	0.67	0.00	0.56	0.11
Regalo	0.58	0.75	0.50	0.58	0.92	0.00	0.08	0.00	0.17	0.08
Tilda	0.47	0.67	0.27	0.47	0.33	0.67	0.67	0.00	0.53	0.27
Overall	0.63	0.61	0.64	0.64	0.56	0.49	0.67	0.01	0.42	0.39
Wild type										
Chimay	0.00	0.00	0.08	0.00	0.08	0.00	0.00	0.92	0.08	0.08
Froid-Lieu	0.25	0.00	0.00	0.13	0.13	0.00	0.00	0.25	0.00	0.25
Longueville	0.22	0.00	0.00	0.22	0.00	0.00	0.56	0.33	0.00	0.11
Prayon	0.00	0.09	0.18	0.27	0.18	0.09	0.55	0.36	0.00	0.00
Treignes	0.10	0.30	0.10	0.20	0.00	0.10	0.50	0.00	0.20	0.20
Vaulx	0.00	0.00	0.11	0.17	0.11	0.00	0.00	0.61	0.00	0.00
Overall	0.06	0.06	0.14	0.15	0.08	0.05	0.23	0.41	0.03	0.07
Weedy type										
Evere	0.67	0.67	0.67	0.67	0.67	0.00	0.67	0.00	0.00	0.67
Rixensart	0.91	0.55	0.45	0.73	0.64	0.55	0.73	0.09	0.09	0.45

Table 3 Genetic variation within commercial varieties of industrial chicory and natural chicory populations sampled in Belgium

Type	Mean number of fragments per plant	Number of polymorphic loci	Proportion of polymorphic loci (in %)	H_j[a]	S.E. (H_j)[b]
Commercial varieties					
Bergues	40.6	91	35.8	0.132	0.010
Dageraad	50.4	123	48.4	0.163	0.011
Eva	47.8	136	53.5	0.165	0.011
Hera	43.5	140	55.1	0.153	0.010
Katrien	47.9	121	47.6	0.164	0.010
Madona	43.7	99	39.0	0.154	0.010
Orchies	49.5	120	47.2	0.170	0.010
Oesia	31.4	101	39.8	0.115	0.009
Regalo	44.3	103	40.6	0.157	0.010
Tilda	42.3	118	46.5	0.154	0.009
Mean	44.1	115.2	45.4	0.153	
S.D.[c]	5.5	16.2	6.4	0.017	
Natural populations					
Chimay	36	75	29.5	0.119	0.010
Froid-Lieu	32.9	114	44.9	0.122	0.009
Longue-ville	44.6	112	44.1	0.148	0.011
Prayon	39.6	147	57.9	0.143	0.009
Treignes	37.1	149	58.7	0.139	0.008
Vaulx	44.7	121	47.6	0.152	0.010
Mean	40.3	120	47.1	0.137	
S.D.[c]	5.3	27.2	10.7	0.014	

[a] Gene diversity
[b] Standard error of gene diversity over individuals and loci

Table 4 Comparisons of the genetic structure within the cultivated and wild gene pools of chicory

Sampling groups	Overall gene diversity H_T	Average gene diversity within sampling group H_S	Differentiation among sampling groups D_{ST}	Proportion of genetic differentiation $G_{ST} = D_{ST}/H_T$
Commercial varieties	0.158	0.153	0.006	0.037
Natural populations	0.145	0.137	0.008	0.056
Cultivated vs wild gene pools	0.148	0.142	0.006	0.038

Genetic structure in the cultivated and wild gene pools

The partitioning of genetic diversity within and between sampling groups (i.e. varieties for cultivated chicory, and populations for wild chicory) is described in Table 4. As noted previously, the amount of genetic variation within varieties or populations was similar in both gene pools, with slightly higher values in the cultivated type. However, average gene differentiation (D_{ST}) among natural populations was slightly higher than differentiation among commercial varieties of industrial chicory. Hence, 5.6% (G_{ST} = 0.056) of genetic variation in wild chicory occurs among populations rather than within, whereas the figure is only of 3.7% for cultivated chicory. In other words, commercial varieties are more closely related to each other than are natural populations from Belgium.

Discussion

The AFLP technique can be used to estimate genetic variation of cultivated and related wild species as shown in this study on industrial and wild chicory: the level of genetic variation has been found to be high in cultivated industrial chicory and similar to that found in wild populations. This suggests that no major event of bottleneck occurred in the history of the cultivated varieties of industrial chicory, thus indicating a low breeding pressure. This result is in agreement with studies by Baes and Van Cutsem (1993) using allozymes, and Koch and Jung (1997) using RAPD and AFLP markers, which reported high levels of genetic variation in cultivated industrial chicory. The situation seems to be different in commercial witloof chicory, *C. intybus* L. cv Foliosum, where a low level of genetic variation was found (Bellamy et al. 1996) as a consequence of a more-intensive breeding activity. Indeed witloof chicory commercial varieties are produced through hybrid breeding, whereas most varieties of industrial chicory are synthetic populations, i.e. they are produced by open-pollination of a restricted number of genotypes (usually between 6 and 12 elite plants) selected for their general aptitude to combination and the agronomic performances of their offspring. Kiers et al. (2000) compared genetic variation within the two cultivated species of *Cichorium*, i.e. *C. intybus* (chicory) and *Cichorium endivia* (endive), using AFLP markers. They found substantially higher levels of polymorphism within chicory than within endive, although they did not distinguish between wild and cultivated accessions. The higher genetic diversity of *C. intybus* can be explained by its mating system, characterised by a self-incompatibility system, whereas *C. endivia* is self-compatible. The situation in sugar beet is similar to that of witloof chicory, with much lower diversity within the cultivated type than within wild populations, as a consequence of hybrid breeding programmes (Desplanque et al. 1999). This suggests that it will be more difficult to find genetic markers associated to the cultivated type in industrial chicory as compared to witloof chicory or to sugar beet. In the latter, allozyme markers provided evidence for introgression of cultivated genes in sea beet populations near the Italian cultivated beet-seed production region (Bartsch et al. 1999). Moreover, the occurrence of wild-to-crop gene flow in the regions of multiplication of commercial beet seeds has been demonstrated through molecular analyses of weed beets sampled within sugar beet fields (Desplanque et al. 1999).

The comparison of genetic variation within and between populations indicates that both wild and cultivated populations are not much differentiated, as expected for a largely allogamous species, but the gene pool of cultivated individuals can nevertheless be distinguished from the gene pool of wild individuals. The inclusion of samples of natural populations from more-diverse areas would undoubtedly increase the value of G_{ST} for wild chicory, and hence would reinforce the difference in genetic structure between the cultivated and wild gene pools. When compared to values of genetic differentiation in other plant species, the value observed in the wild gene pool of chicory lies within the range of variation typical of an outcrossing species with animal pollination (G_{ST} = 0.20 ± 0.19; Hamrick and Godt 1989), whereas values for autogamous species are much higher (G_{ST} = 0.51 ± 0.31; Hamrick and Godt 1989). Such a low genetic differentiation among populations indicates that gene flow through pollen or seed dispersal may be substantial within wild populations of chicory in Belgium.

The present data allow no conclusion about the frequency of gene exchange between cultivated and wild gene pools. However two populations from the survey of wild chicory were found to be genetically similar to cultivated varieties. These two populations (Evere and Rixensart) are the only ones from the region of Brussels. Preliminary morphological observations of these individuals suggest that they are feral or weedy types of chicory (unpublished). Wild, cultivated and putatively feral types should be grown under controlled conditions in order to assess their comparative phenotypes. If confirmed, this

would indicate that the cultivated gene-pool occurs outside cultivated fields.

In conclusion, our results seem to indicate that cultivated varieties of industrial chicory and natural populations of *C. intybus* from Belgium form two distinct gene-pools, with the existence of individuals in the wild which seem to belong to the cultivated pool or which represent intermediate genotypes. However, no AFLP marker could be found strictly linked to either the cultivated or the wild gene pool, which is the normal consequence of a recent domestication process of the crop.

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