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Crop-weed interactions in the *Beta vulgaris* complex at a local scale: allelic diversity and gene flow within sugar beet fields

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Abstract Crop-wild hybrids and weed beets are the main source of agronomic concern for sugar beet production all over Europe. In order to understand the dynamics of crop-wild interactions and the evolution of weediness in *Beta vulgaris*, we investigated genetic features of bolting individuals occurring at a local scale, i.e. within two sugar beet fields of the French northern area of sugar beet production. By analysing ploidy level, mitochondrial DNA and microsatellite polymorphism, the genetic diversity and the genetic relationships among three different classes of individuals (variety, in-row and out-row weed-beets) from a given field were examined. Such genetic analyses provide a unique opportunity to obtain evidence for the weeds origin and the evolutionary hypotheses previously stated. All the individuals shared in common the *Svulg* mitochondrial haplotype, and thus a common maternal origin. Conversely, the large genetic diversity at microsatellite loci highlighted the large diversity of the pollinator plants (cultivated and wild plants) during the-seed production process, as well as during the further evolution of weed beets in the sugar production area.

Keywords *Beta vulgaris* · Crop-wild hybrids · Weed beets · Bolting · Ploidy · Mitochondrial DNA · Microsatellites

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Introduction

Determining the level of gene flow and gene introgression between crops and their wild relatives is critical for the improvement of domesticated plants as well as for sustainable management of genetic resources in the wild (Bartsch et al. 1999; Ellstrand et al. 1999; Jarvis and Hodgkin 1999). Considerable attention has also been focused on weeds as a potential way for the escape of transgenes from crops to wild populations (Dale 1994; Ellstrand et al. 1999). In this context, the *Beta vulgaris* (L.) complex is of particular interest as crop, wild and weedy forms may all be found in Europe.

In the western European countries, two sub-species of *B. vulgaris* (L.) can be recognised: *B. vulgaris* ssp. *maritima* Arcang, the typical wild sub-species, usually found along the coastlines but also reported in inland habitats especially in the Western Mediterranean regions (e.g. in the south-west of France, see Boudry et al. 1993; Desplanque et al. 1999), and *B. v.* ssp. *vulgaris*, the cultivated beet. Sugar beet is a biennial crop selected and harvested for the sucrose in its root. Hybrid varieties are sown in spring and harvested in autumn to prevent vernalization effects that would induce bolting and concomitant storage exhaustion in the next year. Nevertheless, a very particular situation can be observed in sugar beet fields, as bolting-individuals occur during the cultivation period. Three kinds of bolting beets are found within a crop. Two (from the seeds sown) emerge within the sowing line: cultivated individuals ('variety bolters') characterized by a low vernalization requirement, and 'in-row weeds' (i.e. F_1 crop-wild hybrids) which have been thought to originate from contamination of the individuals producing crop seeds by the pollen of wild individuals during seed multiplication (Santoni and Bervillé 1992b; Boudry et al. 1993); they both carry the dominant *B* allele which cancels any cold requirement (Boudry et al. 1994; Abe et al. 1997). The third class of bolters comes up outside the sowing line ('out-row weeds'). These out-row weed-beets (i.e. back-crossed crop-wild hybrids) originate from a seed bank and are likely to

evolve from crosses between the different flowering individuals within, or in the vicinity of, the sugar-beet fields (e.g. variety bolters, crop-wild hybrids, and locally wild sea beets in coastal sugar-production areas; Van Dijk and Desplanque 1999). This so-called 'weed-beet problem' is of major agronomic concern since the 1970s in Europe (Longden 1989, 1993), as the weed-beets cannot be chemically weeded and compete with the crop. The presence of weed-beets also has implications for GMO's use and management (Desplanque 1999; Ellstrand et al. 1999). The introduction of transgenic herbicide-tolerant sugar beets may bring an alternative to the manual removal of weed-beets. However, the possibilities of escape of transgenes from the crop to the surrounding wild populations of sea beets, as well as the risk for transgenic weed-beets to arise, has to be evaluated and controlled (Desplanque 1999; Saeglitz et al. 2000).

In order to understand the dynamics of crop-wild interactions and the evolution of weediness within the *B. vulgaris* complex, the main goal of this study was to investigate some genetic features of bolting individuals occurring at a local scale, i.e. within sugar beet fields of the French northern area of sugar beet production. Numerous studies have been devoted to assess the genetic diversity among sugar beet breeding lines and germplasm collections (see for example Kraft et al. 1997; McGrath et al. 1999, and references therein). But as far as the authors are aware, there have been no published studies to analyse the genetic diversity and distance between the weeds and varieties over a local scale (i.e. within a given crop field). Indirect evidences for the hybrid origin of the weeds were obtained through the analyses of the presence of the *B* allele which is known to be present from the wild beets in the seed production area and absent in the wild populations in the sugar beet production area (Santoni and Bervillé 1992b; Boudry et al. 1993). Besides, using a neighbor-joining tree constructed with several French wild, weed and sugar beet accessions, Desplanque et al. (1999) showed that weed-beets were halfway between sugar beet varieties and wild beets from the seed production area. However, more direct (i.e. genetic) evidence about the hybrid origin of weeds, as well as questions about the genetic diversity and relationships among varieties, in-row and out-row weeds still await local-scale studies.

Such studies would be of interest not only to obtain evidence for the hypotheses previously proposed for the appearance of weeds (Boudry et al. 1993; Desplanque et al. 1999) but also to gain insight on the evolution of the weediness in the seed-production area. According to the studies mentioned above, some expectations can be formulated: (1) a large genetic diversity is expected in varieties of sugar beet, as they have been maintained as open-pollinated populations for a long time (McGrath et al. 1999), (2) a larger diversity of in-row weeds is expected when compared with varieties, as in-row weeds are likely to originate from the hybridisation between two genetically differentiated groups (namely cultivated seed bearers and wild beets, Desplanque et al. 1999), (3) the in-row weeds are supposed to share with the variety a common cytoplasm, i.e. the Owen cytoplasmic male-sterility (Owen CMS) type used in breeding programs (Mackay et al. 1999); (4) an increase in genetic diversity is expected in out-row weeds when compared to either in-row weeds and varieties, because out-row weeds originate from the seed bank and are the result of the crossing of all the flowering plants in the vicinity (in-row weeds, wild, surrounding weeds); the out-row weeds might integrate the recurrent contributions of plants from different geographical areas and from different generations.

We chose to investigate those expectations by analysing the genetic diversity displayed by the different classes of beets (varieties, in-row and out-row weeds) from two sugar beet fields located in the French sugar-production area. We traced back the maternal genome with maternally inherited mitochondrial markers. We also investigated the nuclear genetic diversity of these three groups by using co-dominant and polymorphic microsatellite markers, five out of the six loci used being specifically characterized for the present study.

Materials and methods

Plant materials

In France, beet seed production mainly takes place in the south-western regions (Gers, Lot et Garonne) whereas cultivation occurs for a large part in the northern regions. Two sugar beet fields, separated by about 5 km, namely 'Sainghin' (about 1 hectare) and '4 Cantons' (about 2.5 hectares), were sampled in the sugar production area, and were located near the University Campus of Lille 1. These two fields were chosen because: (1) by surveying them over the crop season, we were sure that no agricultural practices to remove the weeds were carried out so that sampling of the individuals was not biased, and (2) the infestation level was not so high that it prevented a clear recognition of bolter individuals within and outside the sowing line. The size and abundance of the weeds was larger in 4 Cantons. Leaves from 48 and 94 individuals in Sainghin and 4 Cantons, respectively, were collected during the summer of 1999. Three classes of individuals were taken into consideration during the sampling: cultivated individuals, bolting plants within the sowing line and bolters outside the sowing line (i.e. out-row weeds).

A small fragment of each fresh leaf was used to estimate the ploidy level. The other part was frozen prior to DNA extraction, carried out using a slightly modified procedure described in Dellaporta et al. (1983).

Ploidy level analyses

During the seed-production processes, diploid or triploid varieties are obtained through the pollination of a diploid maternal male-sterile line by pollen from a diploid or a tetraploid paternal line respectively. When triploid seeds are sown in sugar beet field production (and this was the case for the two sampled fields), the variety bolters are triploid but in-row weeds coming from an accidental hybridization of the maternal line with pollen from surrounding wild populations (Boudry et al. 1993) are diploid. The ploidy level is thus an efficient diagnostic feature for bolters within the sowing line to classify the individuals into two groups, namely variety bolters and in-row weeds. The ploidy level was estimated in all the sampled individuals by counting the number of chloroplasts in the stoma guard cells after $AgNO_3$ staining (Brown

et al. 1991; Jahier 1992). The range of values per stoma indicated for discriminating diploids and triploids are 12–18 and 19–25 respectively (Brown et al. 1991; Jahier 1992). This quick and reliable procedure is an efficient alternative to flow cytometry measures. Ten stomata per individual sampled within the sowing lines of the two fields were analysed.

mtDNA polymorphism

The mitochondrial DNA polymorphism was investigated using Southern-RFLP. For each individual sample, about 5 µg of DNA was digested with *Eco*RI (2 U/µg). DNA fragments were separated on 0.7% agarose gels in TAE buffer (1×), and blotted onto a Nylon membrane (Allefs et al. 1990). Three mitochondrial probes were used: ATP6, the ATPase subunit 6 from maize; pBV4, a non-coding mitochondrial sequence from sugar beet; and NvulgN2, a 12.5-kb fragment isolated from *EcoRI* digestion of the mtDNA of beets. Probes were labelled, hybridised on total DNA, and detected as described in Cuguen et al. (1994). The analysis of the *Eco*RI restriction fragments leads to the determination of mitotypes (i.e. mitochondrial haplotypes) by combining the information from the three probes. The mitotypes were named using the same nomenclature as in Cuguen et al. (1994) and Desplanque et al. (2000).

Microsatellite-marker isolation and genotyping

Nuclear DNA polymorphism was investigated by using microsatellite loci. One microsatellite locus isolated from a previous genomic library, namely *Bvm3* (Mörchen et al. 1996), together with five microsatellite loci isolated from a new genomic library, were used.

For the characterisation of the new microsatellite markers, the following protocol was employed: nuclear DNA was extracted following Pillen et al. (1992) from two individuals of the wild species *B. vulgaris* subsp. *maritima*. One individual was derived from a crossing experiment between two French Mediterranean individuals and the other one was sampled on the French Atlantic coast. Two aliquots of DNA were digested with *Sau*IIIA and *Asc*I respectively and electrophoresed in a 0.8% agarose gel. Fragments in the size range of 500–1500 bp were recovered as described by Mörchen et al. (1996) and ligated into the *Bam*H1- or *Eco*RI-cut de-phosphorylated pUC19 cloning vector. After transformation into SURE competent cells (Stratagene), 20736 clones were transferred onto Nylon membrane with a Biomek 1000 robot (Beckman) and hybridized with 11 ^{32}P-labelled oligonucleotide probes [i.e. $(GT)_8$, $(CT)_8$, $(TAA)_6(TA)$, $(AAC)_5$, $(TAC)_5$, $(CAC)_5$, $(GCC)_5$, $(TCC)_5$, $(TCT)_6$, $(GATA)_4$, $(GGAT)_4$] as described in Epplen (1992). Selected positive clones were sequenced on a *LI-COR* automated DNA sequencer 4000L. Primer pairs were designed using the software PRIMER3 (Rozen and Skaletski 1998).

The microsatellite genotyping was carried out for each locus as follows: the PCR amplifications were performed using a Perkin-Elmer Gene-Amp system 9600 thermocycler, with the following method: 3 min denaturing at 95°C, followed by 25 to 35 cycles (depending on the locus; see Table 2) of 45 s denaturing at 94°C, 45 s annealing at 54°C and 45 s extension at 72°C, with a final extension step of 72°C for 10 min. The reaction solution (20 µl) contained 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 0.75 pmol of forward and reverse primers, 0.5 pmol of M13-labelled primer, 1 U of *Taq* polymerase (Amplitaq, Perkin-Elmer), and 20 ng of DNA. A M13-tail was added to the forward primers (Gibco BRL) to use a M13-labelled primer on a *LI-COR* automated DNA sequencer 4200 (Oetting et al. 1995); 15 µl of stop solution (Fuschine-based) were added prior to the loading of 1 µl on a 7% denaturing polyacrylamide gel.

Data analyses

Standard population-genetic parameters were estimated to analyze the genetic diversity within each group of individuals (i.e. for varieties, in-row and out-row weeds) as well as the genetic structure among these groups within and among the two fields. For the diploid samples (in-row and out-row weeds), the allelic frequencies, the number of alleles (N_{all}), the observed heterozygosity (H_O), and the gene diversity [H_E, according to Nei (1978)] were estimated for each microsatellite locus and across loci by using the software Genetix 4.0 (Belkir et al. 2000). The *f*-statistic which measures the deviation from Hardy Weinberg expectations at the within-population level [i.e. the F_{IS} estimate according to Weir and Cockerham (1984)] were estimated using the software FSTAT 2.9.1 (Goudet 1995). Tests for deviations from Hardy Weinberg expectations at each locus and for genotypic linkage disequilibria among loci were computed within each population with the GENEPOP v. 3.2 software (Raymond and Rousset 1995b). For triploid samples (i.e. cultivated plants and variety bolters), both the likely departure from Hardy Weinberg expectation and the impossibility to score microsatellite genotypes prevent an accurate estimation of the allelic frequencies. The mean number of alleles (N_{all}) across loci and the presence/absence of each allele at each locus were recorded.

The genetic differentiation among groups was analysed by testing for allelic differentiation with exact tests, and computing the $\hat{\theta}$ estimate of F_{ST} according to Weir and Cockerham (1984) by using GENEPOP v.3.2 (Raymond and Rousset 1995a, b). Nei's genetic distances between the samples were calculated and used to draw a neighbor-joining tree by using the PHYLIP V.3.52 package (Felsenstein 1993).

Results

Ploidy level

The mean, the standard deviation and the range of the number of chloroplasts, as well as the sampling size for each group of individuals, are given in Table 1. The three kinds of beets (i.e. variety, in-row and out-row bolters) sampled finally split up unambiguously into four categories, from which two were triploid (variety and variety bolters) and two diploid (in-row and out-row weeds). All the out-row weeds were found to be diploid. The classification was unambiguous except for one bolting individual sampled in one row in the field of Sainghin (this individual was removed from further analyses). The four groups were significantly differentiated according to the number of chloroplasts in Sainghain [non-parametric Kruskall-Wallis test, chi-square (df=3)=33.4, $P<10^{-4}$] and in 4 Cantons [non-parametric Kruskall-Wallis test, chi-square (df=3)=76.5, $P<10^{-4}$]. The status and the characteristics of each of the four categories are summarised in Table 1.

We found variety bolters as well as in-row weeds in both fields. From the 24 and 48 bolted individuals sampled within the sowing line, five and 14 appeared to be variety bolters in Sainghin and 4 Cantons respectively. The percentage of variety bolters among the bolters (25% and 29.2% in Sainghin and 4 Cantons respectively) was similar in the two fields.

Mitochondrial haplotypes

All the 142 individuals exhibited the same mitochondrial haplotype, namely *Svulg* according to the nomenclature

Table 1 Summary of the characteristics of the four categories of individuals sampled in a field. *Location* stands for the location of the individual within or outside the sowing line (i.e. in or out of the row). The ploidy level (*Ploidy*) is given together with the mean number of chloroplasts per stoma (*Cp*; standard deviation in parentheses; range underlined) over all individuals for each class. N is the sample size of each class of individuals used for chloroplast counts and molecular analyses. Var., wild and ? stands for 'Variety', 'wild' and 'unknown' maternal or paternal origin

Name used	Definition/genetic description	Location	Bolters	Maternal	Paternal	Ploidy	Sainghin		4 Cantons	
							Cp	N	Cp	N
Variety	Variety	Within	No	Var.	Var.	2n=3*x*	23.78 (0.89) 22.3–25.2	12	23.65 (0.81) 22.3–24.8	24
	Variety bolters	Within	Yes	Var.	Var.	2n=3x	19.41 (2.39) 18.4–24.0	5	20.73 (1.65) 19.3–24.8	14
In-row weeds	F_1 crop-wild hybrids	Within	Yes	Var.	Wild	2n=2*x*	14.69 (0.54) 13.5–15.5	18	15.60 (0.90) 13.3–16.8	33
Out-row weeds	Backcross crop-wild hybrids	Outside	Yes	Var.	?	2n=2*x*	15.13 (1.27) 12.9–17.6	12	14.51 (0.67) 13.5–15.4	23
Total								48		94

Table 2 Microsatellite loci analysed. "Size" is the expected size in base pairs according to the length of the sequenced insert. "Cycles" indicates the number of PCR cycles used. "N_{all} (SR)" stands for the total number (and size range in base pairs) of alleles observed over the whole study

Locus	Forward and reverse primers (5′–3′)	Core sequence	Size (bp)	Cycles	N_{all} (SR)
CAA1	TCCTATCTCCTCACCACAAC TCAAATGTAAGAAACCTTGTT	$(CAT)_4(CAA)_{14}$	164	35	13 (145–183)
CT4	TACCCCTTCAGCATCATCC CTGCGCGAATTTTGTCTAGT	$(CT)_{11}$	159	25	14 (148–161)
GCC1	TAGACCAAAACCAGAGCAGC TGCTCTCATTTCGTATGCAC	$(GGC)_6$	125	30	4 (97–125)
GAA1	TGGATGTTGTACTAAAGCCTCA TCCTACCAAAATGCTGCTTC	$CAA(GAA)_3T(GAAAGAA)_2(GA)_2$	187	35	2 (184–187)
GTT1	CAAAAGCTCCCTAGGCTT ACTAGCTCGCAGAGTAATCG	$(C)_5AAC(CCA)_5$	120	30	4 (117–126)
Bvm3	ACCAAATGACTTCCCTCTTCTT ATGGTGGTCAACAATGGGAT	$(CT)_{15}CC(T)_{10}$	112	25	19 (98–122)

by Cuguen et al. (1994) and Desplanque et al. (2000), whatever group they belonged to.

Microsatellite isolation and nuclear genetic diversity

From more than 20000 clones screened with 11 probes, a total of 582 positive clones were obtained of which 228 gave a strong hybridisation signal. Out of these 228 clones, 72, 6, 40, 15, 2, 23, 9, 4, 56, 1 and 1 were revealed with the probes $(GT)_8$, $(CT)_8$, $(TAA)_6(TA)$, $(AAC)_5$, $(TAC)_5$, $(CAC)_5$, $(GCC)_5$, $(TCC)_5$, $(TCT)_6$, $(GATA)_4$, $(GGAT)_4$ respectively.

Out of 23 selected and sequenced clones, 11 primer pairs were defined and five microsatellite loci (see Table 2) were selected for their polymorphism, clear amplification pattern and Mendelian segregation (tested in controlled crosses).

The six loci used were polymorphic among the 142 individuals analysed. A large difference of allele numbers was observed across loci. Three loci, GCC1, GTT1 and GAA1 were only slightly polymorphic with two to four alleles. Three loci, CAA1, CT4 and Bvm3, were highly polymorphic (13 to 19 alleles) given the small spatial scale of the study. Only four out of 54 triploid individuals (variety and variety bolters) exhibited three alleles at one locus. One individual from Sainghin showed a triallelic genotype at the locus *Bvm3*. Three individuals from 4 Cantons exhibited three alleles at the CAA1 locus.

The allelic distribution for each group of individuals, the allele frequencies for in-row and out-row weeds, together with the diversity parameters, are displayed in Table 3. A large number of alleles was observed across the six loci used with up to 43 alleles in the out-row weeds sample from 4 Cantons. The mean number of al-

Table 3 Allelic distribution and diversity. The alleles are named according to the length of the amplified fragment (in base pairs). For triploids, '+' indicates the presence of the allele, otherwise (for diploids) the frequency is given. H_o, H_e, F_{is}, N_{ind}, U_{all}, N_{all} stand for the observed heterozygosity, the gene diversity, the estimator of the parameter F_{is} of Weir and Cockerham (1984), the mean number of individuals, the total and mean (per locus) number of alleles, respectively. Standard deviations are in parenthesis. * and *** indicate that the F_{is} -estimate is significant at the 5% (*) and 0.1% (***) level respectively, when tested against the alternate hypothesis of a heterozygote deficiency (one-tailed test). N.B.: 'Variety' groups both bolters ('Bolt') and non-bolters ('Non bolt')

Locus		Sainghin				4 Cantons			
		Variety		In-row weeds	Out-row weeds	Variety		In-row weeds	Out-row weeds
		Non-bolt.	Bolt.			Non-bolt.	Bolt.		
GCC1	97	+	+	0.42	0.92	+	+	0.29	0.33
	100	+	+	0.58	0.08	+	+	0.71	0.61
	106								0.04
	125								0.02
GTT1	117	+	+	0.14	0.33	+	+	0.18	0.15
	120		+	0.25	0.13	+	+	0.12	0.07
	123	+	+	0.61	0.54	+	+	0.67	0.72
	126							0.03	0.07
CAA1	145								0.02
	146			0.06					
	147			0.15		+	+	0.08	0.11
	151								0.02
	152		+	0.03	0.09				
	155	+	+	0.38	0.59	+	+	0.36	0.45
	156							0.02	
	158							0.03	
	162								0.05
	165								0.02
	175	+	+	0.35	0.41	+	+	0.52	0.23
	178					+	+		
	183			0.03					
GAA1	184			0.12	0.05	+		0.03	0.11
	187	+	+	0.88	0.95	+		0.97	0.89
CT4	148			0.06				0.02	
	149							0.03	0.04
	150			0.06				0.05	0.04
	151					+	+	0.12	0.04
	152	+	+	0.50	0.42	+	+	0.18	0.28
	153					+	+	0.09	0.07
	154	+	+	0.03	0.08	+	+	0.02	0.04
	155					+	+	0.05	0.07
	156	+	+	0.33	0.50	+	+	0.17	0.17
	157					+	+	0.24	0.17
	158			0.03		+	+	0.03	0.02
	159					+			
	160							0.02	0.04
	161					+			
Bvm3	98				0.03				
	100							0.05	0.02
	101								0.04
	102			0.06	0.04			0.03	0.04
	103								0.04
	104								0.04
	110					+		0.02	0.13
	111	+	+	0.08	0.08	+	+	0.17	0.24
	112	+	+	0.47	0.04	+	+	0.27	0.17
	113	+	+	0.14	0.29	+	+	0.08	0.02
	114	+	+	0.03	0.29	+	+	0.03	0.04
	115				0.04	+			
	116	+			0.04	+		0.03	
	117					+	+		
	118					+		0.03	
	119	+			0.17	+	+	0.21	0.13
	120			0.08				0.03	0.02
	121			0.06					0.02
	122			0.06				0.06	0.02
H_o		/		0.56 (0.18)	0.45 (0.28)	/		0.46 (0.22)	0.55 (0.26)

Table 3 (continued)

Locus	Sainghin				4 Cantons			
	Variety		In-row weeds	Out-row weeds	Variety		In-row weeds	Out-row weeds
	Non-bolt.	Bolt.			Non-bolt	Bolt.		
H_e	/		0.57 (0.20)	0.46 (0.28)	/		0.55 (0.30)	0.61 (0.26)
F_{is}	/		0.02	0.02	/		0.17***	0.11*
N_{ind}	16.83		17.00	11.67	34.67		32.67	22.67
U_{all}.	18		27	21	31		37	43
N_{all}	3.00 (1.67)		4.67 (2.80)	3.33 (2.33)	5.17 (3.82)		6.13 (4.66)	7.17 (4.66)

Table 4 Estimators of the F_{ST}-values. All the values are significant (exact test of differentiation at the 5% level)

F_{ST}-values	Sainghin in-row weeds	Sainghin out-row weeds	4 Cantons in-row weeds	4 Cantons out-row weeds
Sainghin in-row weeds	–			
Sainghin out-row weeds	0.110	–		
4 Cantons in-row weeds	0.038	0.151	–	
4 Cantons out-row weeds	0.027	0.120	0.010	–

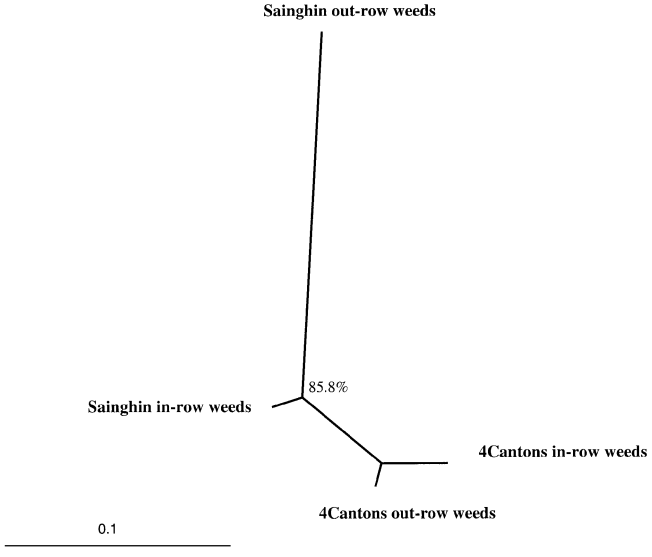

Fig. 1 Genetic relationships between in-row and out-row weeds of the two sampled fields. A Nei's genetic distance matrix between the different samples was used to draw an unrooted dendrogram by using the neighbor-joining method

leles per locus ranged from 3 to 7.2 among the samples. The lowest and highest values for the total, as well as the mean number of alleles, were observed in the variety and in the weed samples respectively. The mean number of alleles was not correlated with sampling size, as a non-significant (Pearson product-moment correlation analysis, n=3, r=0.36, P=0.78) positive correlation was observed in Sainghin and a non-significant (n=3, r=0.61, P=0.23) negative correlation was observed in 4 Cantons. The variety in Sainghin exhibited considerably less alleles than the variety sampled in 4 Cantons. A substantially larger mean number of alleles per locus was observed in the in-row and out-row weed samples of both fields.

However, no diagnostic allele could be observed, and there was a strong overlap in allele presence between both fields. The finding that the groups share a large number of alleles at almost the same frequency explains the low, although significant, F_{ST} values estimated (Table 4). Despite this overlap in allele presence, a closer genetic relationships between in-row and out-row weeds from a given field were observed by comparison with a given group of individuals across fields (e.g. in-row weeds from the two fields). This is illustrated by a neighbor-joining tree in Fig. 1.

Discussion

An interesting feature of the *B. vulgaris* ssp. complex is that at a very local spatial scale, i.e. within a crop field, three closely related forms coexist: variety, in-row weeds (i.e. crop-wild hybrids) and out-row weeds (i.e. backcrossed crop-wild hybrids). Table 1 summarises some of their characteristics. The joint analysis of ploidy level, as well as cytoplasmic and nuclear markers among 150 individuals from two sugar beet fields was particularly helpful: (1) to evaluate the genetic basis of the crop variety sown, (2) to examine the ploidy and genetic status of in-row weeds and thus obtain direct evidence for a previous scenario resulting from studies on a macro-geographic scale (Desplanque et al. 1999) and a phenotypic

assay on plants sampled in the seed production area (Boudry et al. 1993), and (3) to investigate the maternal and paternal origins of weeds with respect to the two other classes of individuals.

Microsatellite loci are well-suited markers for local-scale genetic analyses in both cultivated and wild beets (see for example germplasm or population genetic analyses in beets in Raybould et al. 1998; Desplanque et al. 1999; Mücher et al. 2000; Rae et al. 2000; Laporte et al. 2001). The mutation rates of the microsatellite loci are large enough to lead to a substantial polymorphism within a complex of highly related forms of the same species. The five loci isolated for the present study exhibited similar features to those derived by Morchen et al. (1996) and Rae et al. (2000) despite the fact that the latter used an enriched bank. Among the most-significant common properties, we noticed that: (1) most of the loci isolated were compound or imperfect repeats with a sometimes complex structure [e.g. Table 1 of the present study and Table 3 in Rae et al. (2000)], and (2) short SSRs (e.g. locus GCC1 in Table 1) exhibited polymorphism at the within-field and within-variety level.

Considering the genetic basis of the cultivars analysed in the present study, three points have to be examined. First, as expected from the history of the sugar beet breeding programs, the genetic diversity, in terms of the mean number of alleles detected, in the hybrid cultivars from the two fields was large. For example, up to ten alleles were observed among 36 cultivated individuals at the locus CT4 in 4 Cantons. In the present study, hybrid varieties [i.e. resulting from genetically different maternal and paternal breeding lines (Kraft et al. 1997)] were analysed. The observed allelic diversity cannot be directly compared with previous studies on gene diversity in cultivated beets since only breeding lines were investigated in those studies (e.g. Kraft et al. 1997; Hansen et al. 1999; Rae et al. 2000). Secondly, the varieties of both fields appeared to be far from homogeneous. Over the six loci used, a total of 18 and 31 alleles were found in Sainghin and 4 Cantons respectively, although only 17 and 37 individuals were analyzed in the two fields (Table 3). A similar pattern was reported by Van Den Broecke et al. (1998) who examined 15 sugar beet varieties with AFLPs and found that up to 90% of the total genetic variance investigated was at the within-variety level. A large variation between years for a given variety was also noticed and explained by the lack of production of homozygous lines in beets (Kraft et al. 1997). Finally, although the varieties here analysed were triploid, their triploid nature did not appear at the nuclear loci used except for a few individuals (4 out of 54 variety individuals from the two fields). These few cases of triallelic genotypes were observed at only two loci, namely Bvm3 and CAA1, two out of the three most-polymorphic loci. Such a result is not completely unexpected as the tetraploid cultivated pollen donors used for the seed production are mainly ‘doubled diploids’ and are therefore expected to transmit two copies of the same allele (Desplanque 1999 and references therein).

As previously observed in other fields of the production area in the north of France (Desplanque 1999, unpublished data), diploid bolters within the sowing line, i.e. in-row weeds, were easily found in the two fields, although at a low rate largely beyond the maximum value of one-per-thousand allowed by law. The ploidy analysis was the only efficient way to discriminate between in-row weeds and variety bolters as: (1) both are situated in the sowing line, (2) they share a common domestication syndrome that prevents a distinction between them within the field or by experimental essay on the basis of simple morphological characters (Desplanque, 1999; Ellstrand 2000), and (3) the nuclear genetic analysis showed no clear diagnostic allele between variety bolters and in-row weeds (Table 3), so that a classification of bolters would not be possible *a posteriori* with the microsatellite loci. However, a qualitative analysis of the allele distribution clearly demonstrated that triploid bolters, i.e. ‘variety bolters’, were correctly classified as variety individuals. An almost exact match of the alleles between both types of individuals was observed. When combining the alleles observed across the six loci used, only four alleles out of 18 and six alleles out of 31 were not common to both the ‘non-bolting variety’ and to the ‘variety-bolters’ in Sainghin and 4 Cantons respectively. The few exceptions observed are likely to be due to a limited sampling size.

The use of the ploidy level as a diagnostic feature is possible only for triploid varieties. An alternative to ploidy analysis would be to check for the presence of the *B* allele in the plants bolting within the sowing line. In-row weeds are indeed genetically characterised by the presence of the dominant allele *B* of the bolting gene, which allows bolting without vernalization and confers a selective advantage (Ellstrand 2000). The *B* allele is lacking in sugar beet so that its presence, assessed through controlled experiments in the greenhouse (Boudry et al. 1993), could be connected with the contamination of the seed bearers by pollen from surrounding *B*-carrying wild populations. A future approach of great interest would be to screen for the presence of the *B* allele by using a specific molecular marker. Unfortunately, such a marker is not available so far and the alternative method to detect the *B* allele requires long-term greenhouse experiments.

Surprisingly, a similar level of the frequency of in-row weeds (75% and 71% of the bolters sampled in the sowing line in Sainghin and 4 Cantons respectively) was observed in both fields. This rate could be interpreted with caution as only two fields were examined. However, similar observations were made by Desplanque (1999, unpublished data) who analyzed the ploidy level of 250 bolting individuals sampled from ten fields. In all the fields, in-row weeds were always more common (73% to 85%) than variety bolters. These results may suggest a constancy of the contamination rate by pollen from the wild populations surrounding the seed production areas, despite intensive care for locating and destroying those populations.

The joint use of mitochondrial and nuclear markers also gave insight into the maternal origin of the three class of individuals (variety, in-row and out-row weeds) as well as the genetic diversity of the respective pollen clouds.

First, our study revealed a total uniformity of the mitochondrial haplotypes, as all the 150 individuals were characterised by the haplotype *Svulg*. For the varieties and the in-row weeds, this finding gives evidence for the hypothesis proposed by Boudry et al. (1993). *Svulg* is the single mitochondrial haplotype present in all sugar beet varieties for more than 30 years and is associated with Owen cytoplasmic male-sterility (CMS) (Senda et al. 1998). In-row weeds are expected to possess this haplotype which is transmitted by their maternal parent, the cultivated seed bearer (Mackay et al. 1999). More interesting is the occurrence of this haplotype in the weed beets of the two study fields. This demonstrated a common maternal origin with cultivated individuals and that these are likely to be derived from in-row weeds. Our results are to some extent analogous to those by Mücher et al. (2000). They showed that weed beets from the German sugar beet-production area are likely to originate from 'primary' weed beets observed in the Italian Po-valley seed-production area. These results, combined with those from the present study, ascertain the scenario proposed by Boudry et al. (1993) for the appearance of weeds, as well as the hypothesis of Mücher et al (2000) that seed-production areas are "hybridization hotspots."

Conversely to the cytoplasmic maternally inherited markers, the genetic diversity observed with the biparentally inherited microsatellite loci increased from cultivated to in-row weeds and from in-row to out-row weeds. In the field of 4 Cantons, the mean number of alleles per locus equalled to 5.17, 6.13 and 7.17 in cultivars, in-row and out-row weeds, respectively, despite a smaller sampling size for the latter. The same holds for Sainghin when taking into account sampling-size bias. This is in agreement with previous studies using different molecular markers (allozymes, RFLPs, RAPDs, AFLPs) in beets (e.g. Bartsch and Ellstrand 1999; McGrath et al. 1999).

Significant heterozygote deficiencies were observed in the 4 Cantons field in both in-row and out-row weed beets (Table 3). Such deviations from Hardy-Weinberg expectations may be due to two main factors: (1) the presence of a low frequency of self-compatibility alleles which are commonly used in breeding programs (Mackay et al. 1999), and (2) a Walhund effect due to the mixing of spatially or temporally genetically differentiated sub-populations. With highly polymorphic loci it is difficult to discriminate between both hypotheses (inbreeding vs the Walhund effect). However, several points make the second hypothesis far from being unlikely. First, in the in-row and out-row weed samples of 4 Cantons different loci showed heterozygote deficiencies, in each case with only two out of the six: CT4 and Bvm3 in in-row weeds and GCC1 and Bvm3 in the out-row weeds. It is thus unlikely that these deficiencies arose from inbreeding (Hedrick 2000). Moreover, the in-row and out-row weeds from Sainghin did not show the same pattern. 4 Cantons is a larger field (2.5 ha) than Sainghin (1 ha) so that it was sampled over a larger area and heterogeneous sub-samples may have been collected. The frequency of weeds within 4 Cantons was also larger than in Sainghin. This may be related to a longer history of contamination by weeds in this field, which increased the seed bank, so that populations may have been lumped over time with temporal variation in allelic frequency. Only two fields were analysed in the present study so that this hypothesis can not be confirmed. A comparative analysis of a large number of fields with contrasted history (e.g. various dates since the first sugar beet cultivation) should be carried out to test for the relationships between the gene diversity of out-row weeds and the history of contamination by in-row weeds. However, for the two fields, the occurrence of heterozygote deficiencies was a common pattern observed. It highlights the large polymorphism introduced by in-row weeds and reinforces the importance of a detailed investigation of the seed bank. Given the large gene diversity of in-row and out-row weeds, and the recurrent and still-continuing introduction of in-row weeds in sugar beet fields (ascertained through direct counting; Desplanque 1999; unpublished data), the hypothesis that the out-row weeds inside one field all originate from a unique or only a few bolting plants within the sowing line is unlikely. The evolution of weediness is thus related to multiple recurrent introductions and hybridization events. Ellstrand (2000) recently underlined the role of these complex hybridization events as a source for evolutionary novelty and the subsequent invasive success of weed beets.

The significant genetic differentiation between in-row and out-row weeds also supports the hypothesis of the recurrent introduction of in-row weeds and illustrates the large diversity of the wild paternal pool in the seed-production area. In-row and out-row weeds were significantly genetically differentiated in both fields, although a lack of diagnostic alleles at the microsatellite loci was noted to differentiate these three groups. A more clear-cut pattern of differentiation between variety and in-row could have been expected as they differ by their paternal parents (i.e. pollinator plants and wild individuals respectively). Although significant, the F_{st}-estimates were small (especially when comparing in-row and out-row weeds from 4 Cantons; see Table 4). This result may have several explanations: (1) the wild parents of the in-row weeds may contribute 'domestic' alleles as they can be introgressed by the sugar beet lines multiplied in the vicinity: both seeds and pollen can escape from multiplication fields in the seed-production area (Van Dijk and Desplanque 1999), (2) the weak genetic distance between crop and wild forms in beets may be directly related to the history of the breeding in sugar beets as they were only recently domesticated from wild sea beet and were regularly hybridised with wild beets by the latter (Bosemark 1989; Santoni and Bervillé 1992a; McGrath et al. 1999). Theoretically, weeds may also derive from

pollinations by natural populations of *B. vulgaris maritima* inhabiting the coastline in northern France. However, given that those populations are at least at 60 km from our fields and given the local pollen cloud density, it is largely unlikely that wild pollen can genetically contribute to the weeds studied there. A different pattern could be observed if such analyses were extended to sugar beet fields located near the coastlines. Here, the measured differentiation of out-row weeds from in-row weeds should rather depend on the history of farmer practices (manual removing of bolters or not), of the different varieties grown in a given field, and to the recurrence of infestation by in-row weeds.

In conclusion, by analysing the nuclear and cytoplasmic diversity at the level of a sugar beet field, we demonstrated: (1) the occurrence of the *Svulg* haplotype in all the individuals studied, (2) the increase of gene diversity from variety to in-row weeds and to in-row to out-row weeds, and (3) the genetic differentiation between in-row and out-row weeds. Thus, this study, although restricted to two fields, definitely confirms the hypotheses of the recurrent introduction of in-row weeds (Desplanque 1999) and of the "accidental pollinations of seed-production plants by wild-beets as the origin of weed beet populations in the sugar beet production area" (Boudry et al. 1993). Further studies focusing on the seed bank are now needed to investigate more precisely the origin of the large genetic diversity of weed-beets, which is likely to have been underestimated. It would also be of great interest to compare our data with data from fields located in the vicinity of wild coastal populations and to compare the gene diversity of weed-beets with wild populations. Such investigations would be of valuable interest for GMOs management policies (Desplanque et al. 1999; Saeglitz et al. 2000).

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