Theor Appl Genet (1999) 98: 1194–1201

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Genetic diversity and gene flow between wild, cultivated and weedy forms of *Beta vulgaris* L. (Chenopodiaceae), assessed by RFLP and microsatellite markers

Received: 8 June 1998 / Accepted: 8 October 1998

Abstract Beets belonging to the species *Beta vulgaris* L. can be found in crop, wild and weedy forms, all of which are interfertile. We studied the intra-specific genetic relationships of about 300 individuals from 54 populations of various French geographic origins using nuclear molecular markers (five single-copy RFLP loci and one microsatellite locus). The patterns of diversity were congruent for both types of markers. Genetic diversity in wild beets appeared to be high, both in term of allele number and observed heterozygosity, whereas the narrowness of the cultivated-beet gene pool was confirmed. Genetic distances between all forms showed that weed beets in northern France are intermediates between sugar beet and inland wild beets in south-western France. This analysis allowed us to infer the paternal origin of weed beets and furthermore, is in agreement with a previous study which focused on their maternal origin: weed beet infesting sugar-beet fields originated from accidental and recurrent hybridization between cultivated lines and ruderal inland wild beets during the production of commercial seeds in south-western France. Inland wild beets are genetically close to Mediterranean coastal wild beets, but differ from other coastal forms (from Biscay, Brittany and northern France). The study of gene flow in the beet complex contributes to the risk assessment of transgenic beets.

Key words RFLPs · Microsatellite · Gene flow · Genus *Beta* · Transgenic sugar beet

Communicated by G. Wenzel

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Introduction

Many domestic species have wild relatives, the geographical distribution of which sometimes overlaps with parts of their own distribution area (see Van Raamsdonk and Van Der Maesen 1996 for a crop-weed complex definition). Crop-to-wild hybridizations are well described for many genera such as *Brassica* (Lefol et al. 1991; Kerlan et al. 1992; Darmency 1994; Baranger et al. 1996; Mikkelsen et al. 1996), *Helianthus* (Cronn et al. 1997), *Lactuca* (De Vries 1997), *Pennisetum* (Till-Bottraud et al. 1992), *Raphanus* (Klinger et al. 1992), *Sorghum* (Arriola and Ellstrand 1996) and *Zea* (Doebley 1990). The interest for studies on gene flow in crop-weed complexes has recently increased with the possibility of producing transgenic crops (Rogers and Parkes 1995; Snow and Palma 1997). The dispersion of a transgene can be assessed via modelling and computer simulation (Lavigne et al. 1996; Timmons et al. 1996), field experiments (see Conner and Dale 1996 for an example), or by in situ analysis of gene flow between crops and wild relatives using genetic markers (Raybould and Gray 1993; Gliddon 1994; Linder and Schmitt 1994; Luby and McNicol 1995; reviewed in Kareiva et al. 1996).

In this context, the beet complex is of particular interest as crop, wild and weedy forms can all be found in Europe. Moreover, these taxa are all interfertile and can easily be found in sympatry (Santoni and Bervillé 1992 a; Boudry et al. 1993). Consequently, the probability for hybridization to occur in *Beta* sp. is expected to be high (Kapteijns 1993; Raybould and Gray 1993).

Herbicide-resistant transgenic sugar beet lines already exist (Dhalluin et al. 1992; Tsaftaris 1996) and hybrids between transgenic sugar beet and wild relatives have been obtained experimentally (Bartsch and Pohl-Orf 1996; Fredshavn and Poulsen 1996).

Interspecific relationships within the genus *Beta* have been described in detail by Letschert (1993) and by Jung et al. (1993). Several interfertile forms coexist within the species *Beta vulgaris* (De Bock 1986; Ford-Lloyd 1986; Ford-Lloyd and Hawkes 1986). According to their habitats, four groups can be defined: (1) sea beets [*B. vulgaris* subsp. *maritima* (L.) Arcangeli] mostly found along the western European coasts; (2) cultivated beets (*B. vulgaris* subsp. *vulgaris* L.), i.e. sugar beet, but also privately grown table beet (red beet) or leaf beet (Swiss chard) and fodder beet for cattle; (3) weed beets, which infest sugar-beet fields in northern France and have led to severe agronomic problems since the seventies (Horsney and Arnold 1979); (4) inland beets, which are typical of man-disturbed habitats and qualify as ruderal beets, are particularly common in the seed-production area of sugar beet in southern France. The genetic origin of weed beets and south-western inland beets is still poorly known. Inland wild forms in the seed-production area may originate from either natural non-coastal forms of *B. vulgaris* subsp. *maritima* (De Bock 1986) or from feral forms (for a review see Bartsch et al. 1993).

Weed beets are believed to result from accidental pollination between cultivated lines and ruderal pollen donors in the seed-production area of sugar beet (Santoni and Bervillé 1992 b). Their maternal cultivated origin was suggested by the analysis of cytoplasmic RFLP markers (Boudry et al. 1993): weed beets carry the typical cultivated mitochondrial haplotype associated with Owen cytoplasmic male sterility (CMS), while this character is never found among wild populations in France (Cuguen et al. 1994). Paternal origin was only postulated on the basis of vernalization requirement. Variation for this trait is determined by the polymorphism of a major gene (Munerati 1931): the dominant B allele enables bolting within the year the plant was sown without any vernalization and is suspected to cause the invasive character of weed beet. The B allele is highly selected in weeds because of the fast crop turnover. While this allele is absent in the cultivated beets, it is extremely frequent among both the ruderal populations in the seed-production area (Boudry et al. 1993) and the natural coastal populations of Biscay and the Mediterranean (Van Dijk et al. 1997). In addition, because the penetrance of the B allele is incomplete, the efficiency of its detection is reduced (Owen 1954; Boudry et al. 1994 a). Therefore, neutral genetic markers are needed to clearly identify the paternal origin of weed beet.

The present study addresses three major issues. By using co-dominant markers, we analysed the genetic diversity within and between the different groups referred to above in order to: (1) genetically characterize the groups defined on a habitat basis and, more specifically, (2) determine the paternal origin of the weeds and, consequently, (3) discuss the risks associated with the use of transgenic sugar-beet cultivars.

Materials and methods

Collection of plant material

Previous analyses suggested that weed beets in northern France could result from hybridization events between both ruderal and cultivated beets within the seed-production area (Boudry et al. 1993). Weed beets are easily found in sugar-beet crops from northern France. In this area, most of the crop seeds sown [between 70 and 90%, M. Monjarret (GNIS), personal communication] are produced in south-western France, where ruderal beets are also present. Therefore we chose to sample mainly ruderal beets from this geographic area.

We analysed a total of about 300 individuals (see Table 1) from 82 populations separately (mostly between two and five individuals per population but, exceptionally, higher or lower numbers). The populations (Fig. 1) fell into the following categories: (1) cultivated beets for which the seeds were multiplied in most cases in the south-western seed production area: 28 cultivars of sugar beet and seven breeder's lines; two cultivars of fodder beet, one leaf beet and one table beet; (2) rural inland beets from the same south-western seed-production area; (3) weed beets from sugar-beet fields, and (4) wild coastal beets, which were mainly found along the French coast (from the Netherlands to the Mediterranean) and classified according to their geographical origin (Channel, Brittany, Biscay and Mediterranean). All non-cultivated accessions were sown in a greenhouse from seeds sampled in natural populations.

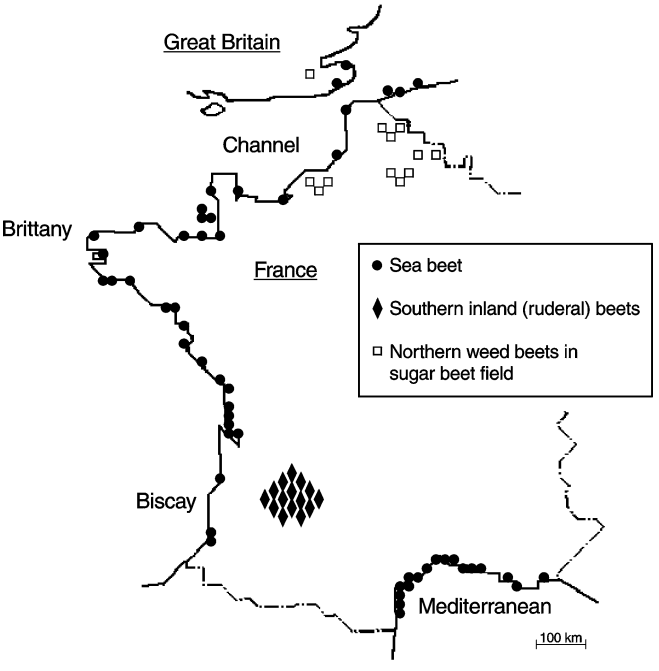

Fig. 1 Location of the beet populations studied

DNA extraction

Total genomic DNA was extracted from 2 g of young leaves with a modified Dellaporta protocol as described in Saumitou-Laprade et al. (1991).

Nuclear RFLPs

A preliminary test was conducted on 12 individuals with 42 enzyme/probe combinations (four restriction enzymes: *Eco*RI, *Eco*RV, *Hin*dIII, *Xba*I and 18 RFLP probes). This is led us to select five combinations (three enzymes and five probes) according to the following criteria: unambiguous reading and strictly single-copy markers. The selected probes (see Table 1) were derived from four of the nine linkage groups in the beet genome (Pillen et al. 1992). For each individual, 5 µg of total DNA were digested with *Eco*RI (2 U/µg), *Eco*RV (4 U/µg) and *Hin*dIII (8 U/µg). Restriction DNA fragments were separated by electrophoresis for 16 h at 30 V on an 0.8% agarose gel using TAE buffer (40 mM Tris-acetate, 20 mM Na acetate, 2 mM EDTA, 18 mM NaCl at pH 8). The DNA was transferred onto a Nylon membrane (Biodyne A from Pall) using the vacuum-blot system of Pharmacia. After transfer, DNA was UV cross-linked (1.2 J/cm^2) to the Nylon support.

Labelling of 10 ng of plasmid DNA containing a sugar-beet probe was performed with PCR using the "Dig labelling Mix" (Boehringer Mannheim) and 1 unit of *Taq* Standard (Perkin Elmer). Half of the resulting labelled probe was hybridized overnight at 68°C. Membranes were then washed and hybridization signals detected according to the chemiluminescence method of Allefs (1990).

Microsatellite locus

A polymerase chain reaction with primers for the *Beta* microsatellite Bvm3 locus (Mörchen et al. 1996) was carried out in a Perkin Elmer GeneAmp PCR System 9600, using 0.75 units of Perkin Elmer standard DNA *Taq* polymerase and about 50 ng of total DNA in a final volume of 15 µl. Further conditions were 1.5 mM $MgCl_2$, 50 µM of each nucleotide, and 0.2 µM of each primer (purchased at MWG Biotech). One of the primers was labeled with the infra-red fluorescent dye IRD41. Due to the sensitivity of the detection system, only 19 cycles at an annealing temperature of 60°C were necessary. After the addition of half a volume of loading buffer, 0.5–1 µl of each reaction was loaded onto a 25-cm denaturing 'LongRanger' gel (polyacrylamide derivative, FMC Bioproducts). Electrophoresis and detection were performed on an LI-COR automated sequencer model 4000L (LI-COR Inc., Nebraska, USA). Fragment size (between 96 and 128 nucleotides) was first determined by a pUC 19 sequence, and then by a mixture of alleles of known nucleotide numbers.

Data analysis

The number of alleles, as well as the observed and expected heterozygosity (H_o and H_e, respectively), were calculated using GENETIX V. 3.0 software (Belkhir et al. 1996). A Mantel-test was also performed using this software. *F*-statistics were estimated using GENEPOP V. 3.0 (Raymond and Rousset 1995), following Weir and Cockerham (1984). By partitioning the variance of allele frequencies between populations, among individuals within a population, and within individuals, this estimation procedure allows the analysis of the heterozygote deficiency within populations (F_{is} estimates) and the genetic differentiation among populations (F_{st} estimates) (see Hartl and Clark 1989). Exact tests for gene differentiation and genotypic linkage-disequilibrium, as well as for other tests of independence, were performed using GENEPOP V. 3.0. The genetic distance between groups was calculated using Reynolds' distance (Reynolds et al. 1983). The loci were all pooled together except for the microsatellite locus, and two genetic distance matrices were calculated from the allelic frequencies: one for the RFLP loci and one for the microsatellite locus. These two matrices were then compared in order to check the congruence between the two different markers employed. Since the two matrices appeared to be highly positively correlated (Mantel test significance probability level = 0.002 with 1000 re-samplings) we combined them. A resulting unrooted tree was performed using the Neighbour-Joining method. These analyses were carried out with the PHYLIP V. 3.52 package (Felsenstein 1993).

Results

Levels of polymorphism within groups

All six loci turned out to be highly polymorphic (Table 1): 26 alleles were identified for the microsatellite locus while there was a mean number of 10.2 (SD = 0.2) for the five RFLP loci. When analysing each locus separately, every group showed a similar number of alleles, except for the cultivated beet group which exhibited a smaller value (this applied also for Biscay beets, although the difference was not as pronounced). Values for the observed and expected heterozygosity, as well as the fixation index F_{is}, are provided in Table 1. The H_o values appear to be high and more or less equivalent for all the groups. The F_{is} estimates were tested by an exact test based upon deviations from Hardy-Weinberg expectations: except for the cultivated beets, all the deviations were significant for both RFLP and microsatellite loci.

Relationships among groups

When all groups were pooled, no linkage disequilibrium was found for each pair of loci over all groups (exact test, data not shown). The overall F_{st} value is significant ($P < 0.01$) and is estimated as 0.10 (SE = 0.02, based on a jacknife procedure performed with GENETIX). Table 2 displays the matrix of Weir and Cockerham F_{st} estimates when groups were compared two by two (loci were not considered individually here). An exact test on allelic differentiation (allelic frequency distribution) was performed for each locus and each pair of populations: the only non-significant case concerns the comparison of Biscay versus Channel and Brittany. All the other values attributed to group couples showed a significant allelic differentiation for five out of six loci (data not shown).

Reynolds' genetic distances are given in Table 2, and Fig. 2 shows their graphic representation using a Neighbour-Joining method. Weed beets clearly appeared to be intermediate between cultivated and ruderal beets from south-western France. The latter are closer to Mediterranean beets than to the beets of Biscay. Mediterranean beets are isolated from the other

Table 1 Levels of polymorphism within groups for the five RFLP loci and the microsatellite locus. H_o: observed heterozygosities; H_e: expected heterozygosities

Groups	Number of populations	Number of individuals	Number of alleles						
			RFLP loci (probe/restriction enzyme)[a]						Microsat. locus
			pk495/ *Eco*RI	pk753/ *Eco*RV	pk815/ *Hin*dIII	pk851/ *Eco*RV	pk967/ *Eco*RV	Unweighted mean (and SD) over RFLP loci	BVM3[b]
Cultivated beets	39 var.	39	6	6	2	4	3	4.2(1.8)	9
Southern inland (ruderal) beets	15	82	10	10	10	9	8	9.4(0.9)	16
Northern weed beets	12	71	10	7	7	9	6	7.8(1.6)	13
Mediterranean sea beets	18	47	8	9	7	8	9	8.2(0.8)	19
Sea beets of Biscay	10	24	7	6	6	7	6	6.4(0.5)	10
Sea beets of Channel and Brittany	27	54	9	9	4	10	8	8.0(2.3)	18
Over all groups of beets	82 (without cultivated)	319	10	11	10	10	10	10.2	26

$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$
[a] Nomenclature according to Pillen et al. 1992
[b] Nomenclature according to Mörchen et al. 1996

coastal beets of the same group (Biscay, Brittany and Channel). The relative position of each group to the other was tested by bootstrapping (10 000 re-samplings): the only value below 98% concerned the south-western ruderal beets, which seems to be closer to the coastal beets of Biscay than to Mediterranean beets in 20% of the trees (data not shown).

Discussion

Genetic diversity

Nuclear RFLPs are commonly used for gene-flow analysis within a given species, as in the case of *Hordeum* (Zhang et al. 1993) and *Triticum* (Le Corre and Bernard 1995). They are also appropriate for studies of crop-weed complexes, as was the case for *Brassica* (Crouch et al. 1995), *Hevea* (Besse et al. 1993), *Sorghum* (Aldrich and Doebley 1992), *Aegilops* (Sasanuma et al. 1996), etc. Currently, the use of microsatellites is rapidly expanding and is no longer exceptional for plants. For a partial review see Morgante and Olivieri (1993), as well as examples in Awadalla and Rietland (1997), Streiff et al. (1998); and for *Beta* see Mörchen et al. (1996), Raybould et al. (1998). The number of alleles is usually high: in the present study, all five RFLPs, chosen for their high polymorphism, exhibited about ten alleles whereas the microsatellite locus displayed 26. This microsatellite locus demonstrated patterns that were identical to those obtained with the five RFLP loci. This finding is based on only one locus but is nevertheless compatible with the results of recently published studies on RFLPs versus microsatellites: see for example *Oryza* (Wu and Tanksley 1993), *Solanum* (Provan et al. 1996), and *Pisum* (Lu et al. 1996). The physical independence of 4 out of 5 RFLP markers, as well as the congruence between the six loci analysed, strongly suggest that the use of additional markers would not significantly increase the reliability of the population genetic estimates (see the review by Bossart and Pashley Prowell 1998 for a more detailed discussion).

A high degree of polymorphism was observed within all the groups, except for cultivated beets. In terms of genetic resources, this suggests that wild *B. vulgaris* presents a significant genetic diversity. The small number of alleles found in the cultivated group confirmed the narrowness of the cultivated gene pool in *B. vulgaris* (Jung et al. 1993). Indeed, in most species genetic diversity is greater among wild populations than among crop populations in terms of numbers of alleles (see Saghai Maroof et al. 1994 for an example with microsatellite data). The high values of the F_{is} estimator may be due to a Wahlund effect, since different populations were pooled in each group. Conversely, we did not observe any deviation from Hardy-Weinberg expectation and the F_{is} was low for the cultivated beets, which is an additional argument for the genetic homogeneity of cultivated beets.

In addition, our study showed that the different groups we defined according to habitat and

Table 1 Continued

H_o (SD)		H_e (SD)		Weir and Cockerham F_{is} estimator (SD)	
RFLPs	BVM3	RFLPs	BVM3	RFLPs	BVM3
0.538 (0.255)	0.657	0.480 (0.250)	0.719	-0.069^{ns} (0.170)	0.100^{ns}
0.634 (0.094)	0.766	0.724 (0.091)	0.867	0.165*** (0.094)	0.122*
0.497 (0.153)	0.609	0.632 (0.153)	0.823	0.261*** (0.111)	0.267***
0.655 (0.083)	0.659	0.772 (0.044)	0.900	0.163*** (0.099)	0.278***
0.508 (0.211)	0.609	0.644 (0.180)	0.867	0.251** (0.164)	0.318**
0.556 (0.244)	0.796	0.609 (0.265)	0.862	0.222*** (0.155)	0.085*
		0.725	0.894		

geographical characteristics have a genetic basis: coastal wild beets are genetically distinct from the other groups and, within the coastal beets, one can easily distinguish between Mediterranean and Atlantic (Biscay) or Northern coastal types. The only non-significant genetic differentiation concerns the comparison of Channel and Biscay populations. Along these coasts, populations of sea beets are continuously distributed and gene flow is likely to take place. The lack of significant genetic differentiation between these areas can also be due to the limited power of the present study, as only 78 individuals were sampled.

Origin of weed beets

Our study demonstrated the intermediate position of weed beets between the cultivated and south-western ruderal inland gene pools of beets. Weed beets clearly appeared to be produced by accidental hybridization of cultivated lines and ruderal-beet pollen donors in the seed-production area. This result confirms the previous study (Boudry et al. 1993) which identified their maternal origin. Indeed, the genetic distances used here are based on nuclear markers with a biparental transmission. This, in turn, makes it possible to infer the paternal contribution, which had been previously determined on the sole basis of the transmission of the bolting gene's B allele. Another interesting result that emerges from the present study is the high genetic diversity of weed beets despite their recent evolutionary history. Their high nuclear genetic diversity contrasts with the previously found uniformity of mtDNA (Boudry et al. 1993). This suggests that: (1) pollen flow from inland to cultivated beets is likely to be both frequent and recurrent, and (2) the transportation of crop-wild hybrids from the seed-production area to the sugar-beet fields in Northern France is also likely to be a recurrent phenomenon rather than the result of a single introgressive event followed by local expansion in sugar-beet fields.

By using greenhouse studies on life-history traits, Bartsch and Schmidt (1997) suggested that a similar scenario occurs in northern Italy, the other important european seed-production area. Introgression between cultivated and wild beets could therefore be a general trend but one which has to be ascertained more accurately by fine-scale genetic analyses in crop-wild sympatric areas.

Origin of ruderal beets

The origin of south-western rural inland beets is less obvious. Their genetic diversity appeared to be high, since one can find as many alleles in this group as among coastal forms. In addition, their closest relatives appeared to be the coastal beets, in particular those of the Mediterranean, but not the Atlantic beets despite the latter's closer geographic proximity. Genetic proximity is corroborated by the fact that many ruderal populations occur all along a geographic

Table 2 Relationships among groups: Reynolds' genetic distance matrix (below diagonal) and F_{st} estimator values over six loci (above diagonal)

Groups of beets	Cultivated beets	Southern inland (ruderal) beets	Northern Weed beet	Mediterranean sea beets	Sea beets of Biscay	Sea beets of Channel and Brittany
Cultivated beets	0	0.115	0.043	0.174	0.186	0.199
Southern inland (ruderal) beets	0.115	0	0.042	0.022	0.079	0.112
Weed beet	0.052	0.049	0	0.088	0.128	0.157
Mediterranean sea beets	0.176	0.034	0.099	0	0.067	0.102
Sea beets of Biscay	0.186	0.093	0.142	0.087	0	0.002
Sea beets of Channel and Brittany	0.204	0.120	0.166	0.113	0.020	0

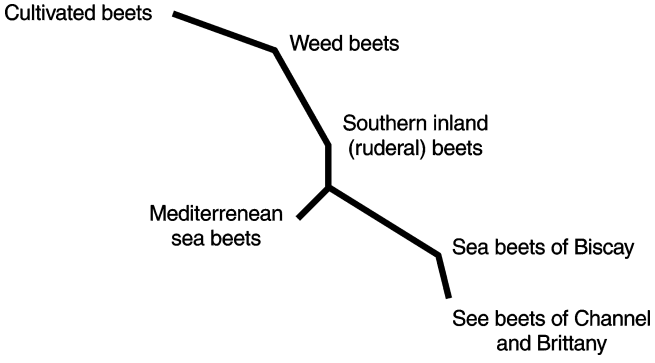

Fig. 2 Unrooted dendrogram inferred from Reynolds' genetic distance matrix between the different forms of beet, based on six nuclear loci (Neighbor-Joining method)

continuum, linking up the south-western seed-production area to the Mediterranean coastlines, while no populations were observed when moving towards the Atlantic coasts (data not shown). Although these ruderal populations seem to be of wild origin, their position in the tree (Fig. 2) indicated an introgression with cultivated forms. Further studies are needed to clarify this point.

Gene flow between beet populations and risk assessment

Crops which are cultivated annually for their seeds often have companion weed races (Harlan 1992), which, among other characteristics, shatter their seeds more readily. Crop-weed gene exchange is generally easy, especially among allogamous species. Sugar beet belongs to that category of species that is cultivated for its vegetative biomass only. Weed beets can only persist in annual cultures if they complete their reproduction before crop harvesting. For beets, crop-weed gene exchange is far more difficult. It is only possible when some crop individuals reproduce early, although this can be prevented by selection (Longden et al. 1975). The fact, however, that beets are grown from seed requires large-scale seed production. Gene exchange is in fact restricted to crop-wild hybridization during seed production, the wild (ruderal) forms being, however, already rather 'weedy'. Beets are in a special situation because of the presence of a dominant gene in ruderal beets which has a very strong effect on early flowering (Boudry et al. 1994 b). This leads directly to weedy forms in the sugar-beet fields.

Multiple interactions seem to occur between the different forms of beet. On the one hand, weed beets in the sugar-beet fields of northern France are sometimes in parapatry with coastal beets. But, up to now, there is no evidence for gene exchange between them, as shown by their F_{st} value which is among the highest (Table 2). On the other hand, there are frequent gene exchanges in the seed-production area. These hybridization events need to be examined in greater detail because in one direction, from ruderal to cultivated beets, weed beets are generated: if a transgene is introduced via the maternal route then transgenic weeds will be produced. In the other direction, from cultivated lines to ruderal beets, hybridization events could cause the transgene to escape to the wild if it is introduced by the paternal route. The other seed-production areas in Europe are obviously submitted to the same risks because of the presence of coastal wild beets near the cultivated lines, especially in England and Northern Italy (Bartsch et al. 1996). Thus, evaluating every possible gene flow is a pre-requisite for the risk assessment of transgenic beets.

Acknowledgements We thank R. Dron for technical assistance, M. Mörchen for help on microsatellite data, as well as Dr. F. Viard and Pr. Ph. Vernet. The authors are grateful to Pr. Ch. Jung (Institute of Crop Science and Plant Breeding, Kiel, Germany) and Dr. G. Steinrücken (A. Dieckmann Company) for providing the RFLP probes. This work was funded by the French Ministry of Environment (grant No. 94153), the Région Nord Pas-De-Calais and FEDER. B. Desplanque was supported by a CNRS fellowship.

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