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Evidence for gene flow via seed dispersal from crop to wild relatives in *Beta vulgaris* (Chenopodiaceae): consequences for the release of genetically modified crop species with weedy lineages

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Gene flow and introgression from cultivated to wild plant populations have important evolutionary and ecological consequences and require detailed investigations for risk assessments of transgene escape into natural ecosystems. Sugar beets (*Beta vulgaris* ssp. *vulgaris*) are of particular concern because: (i) they are cross-compatible with their wild relatives (the sea beet, *B. vulgaris* ssp. *maritima*); (ii) crop-to-wild gene flow is likely to occur via weedy lineages resulting from hybridization events and locally infesting fields. Using a chloroplastic marker and a set of nuclear microsatellite loci, the occurrence of crop-to-wild gene flow was investigated in the French sugar beet production area within a 'contact-zone' in between coastal wild populations and sugar beet fields. The results did not reveal large pollen dispersal from weed to wild beets. However, several pieces of evidence clearly show an escape of weedy lineages from fields via seed flow. Since most studies involving the assessment of transgene escape from crops to wild outcrossing relatives generally focused only on pollen dispersal, this last result was unexpected: it points out the key role of a long-lived seed bank and highlights support for transgene escape via man-mediated long-distance dispersal events.

Keywords: contact zone; chloroplast DNA; crop–wild hybrids; genetically modified organism; microsatellites; risk assessment

1. INTRODUCTION

Over the last decade, the development of transgenic plants has heightened concerns about the potential negative effects of the wide-scale commercial release of genetically engineered crops (Darmency 1994; Raybould & Gray 1994; Gray & Raybould 1998; Hails 2000). Among the commonly listed risks associated with environmental release of genetically engineered cultivars is the hybridization of transgenic plants with wild relatives, and the subsequent introgression of transgenic traits into the gene pool of wild plant populations in natural ecosystems (Ellstrand *et al.* 1999). Furthermore, the risk of a transgenic crop escaping cultivation is likely to be higher in crops where non-transgenic varieties have weedy tendencies (Raybould & Gray 1994; Bartsch *et al.* 1999). Even if gene flow between wild and cultivated relatives results in a transgene escape, its spread will obviously depend on the relative fitness enhancement attributable to the nature of the engineered trait, as well as on other factors such as hybrid fertility, particularly during seedling establishment (Van Raamsdonk & Schouten 1997; Bartsch *et al.* 1999; Hails 2000).

With regard to crop–wild gene flow and its potential consequences, the *Beta vulgaris* complex is of particular interest as crop, wild and weedy forms can be found in parapatry or sympatry in Europe with overlapping flowering periods, are wind-pollinated and all appear to be interfertile (Bartsch *et al.* 1999; Saeglitz *et al.* 2000). In northern France, the sugar beet (*Beta vulgaris* ssp. *vulgaris*) is an extensively cultivated plant. Numerous fields can be found close to the coastline where the common wild form (*B. vulgaris* ssp. *maritima*) also occurs. Conspecific weed beets, which germinate from the seed bank, are also present within the sugar beet fields. Previous studies demonstrated that these weeds result, primarily, from hybridization events between cultivated and wild inland beets within seed production areas for sugar beet (Boudry *et al.* 1993; Bartsch *et al.* 1999; Van Dijk & Desplanque 1999). Various concepts coexist as to which plants are weeds (Baker 1974). In the present study, the term 'weed' is used to define: (i) the in-row and out-row bolters found within crops and resulting from hybridization events; and (ii) beets encountered within man-disturbed habitats in the close vicinity of the agrosystem, such as roadsides, recently built slopes, etc. (Baker 1974; Hornsey & Arnold 1979; Desplanque *et al.* 2002). Sugar beets are biennial, in contrast to weed beets which inherited the possibility of first-year flowering from wild forms and bolt late in the growing season without the requirement of vernalization (dominant B allele; see Boudry *et al.* 1993). Sugar beet seed grown for commercial purposes in northern France contains a fraction of hybrid seeds and the resulting crop–wild F_1 hybrid plants are annuals, bolting, flowering and setting large amounts of seeds. These contaminant hybrids have given rise to weedy lineages; their crucial character 'earliness of flowering' preadapts them for invasive success in cultivated beet

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fields (Van Dijk & Desplanque 1999). Therefore, these characteristics place beets in a high-risk category in terms of the likelihood of transgene escape, since herbicide-resistant transgenic sugar beet lines already exist (Boudry *et al.* 1993; Van Raamsdonk & Schouten 1997; Desplanque *et al.* 2002; Viard *et al.* 2002).

However, at the current time, there is no evidence for gene exchange between weedy lineages and wild coastal relatives in sugar beet production areas. Hybridizations are not necessarily confined to narrow zones of close parapatry, and long-distance pollen dispersal up to hundreds of metres away from the cultivated source could be suspected in this outcrossing species (Lavigne *et al.* 2002). In this context, we attempted to evaluate the genetic features of nearby populations located within the French area of sugar beet production (northern France) by analysing: (i) a typical wild coastal population; (ii) a neighbouring weed population situated within a sugar beet field; and (iii) an intermediate population located between the two latter. Such a linear sampling scheme reflects the geography of a potential contact zone and provides a unique opportunity to evaluate gene exchange from sugar beet crops to coastal wild sea beet populations, and the distance at which pollen and seed dispersal occurs. In this study, the likelihood of weed seed migration was traced back using a maternally inherited chloroplastic marker diagnostic of cultivated lines (Ran & Michaelis 1995). Possible introgression via pollen and/or seed flow was also investigated using nuclear microsatellite markers (Mörchen *et al.* 1996; Viard *et al.* 2002).

2. MATERIAL AND METHODS

(a) *Sampling*

To investigate fine-scale crop–wild gene flow, three locations were sampled: (i) a natural coastal population of wild beets (named COAST); (ii) a weed population (named FIELD) located within a sugar beet field situated 1.5 km apart; and (iii) a contact zone with a possible mixture of wild and weedy beets, located along the river of Wimereux (named RIVER) (figure 1). The present studied area is characterized by the high incidence of sugar beet farming for more than 100 years and is typical of what can be found in northern France where sugar beet crops border the coast. From an intensive field survey, this study site was chosen as representative of a case of close contact between coastal sea beet populations (together with individuals sampled in the river embankments) and sugar beet fields found in close proximity.

The COAST population is a typical sea beet population found along the seashore, with individuals located at the upper level of the high tide. The FIELD population is characteristic of the infestation of sugar beet fields by weed beets. Only weed plants bolting outside the sowing lines ('out-row weeds' following the terminology of Viard *et al.* (2002)) were sampled. Weedy forms found outside the rows of cultivation, were derived from crosses of either physiological bolters or in-row weeds found in the vicinity (Desplanque *et al.* 2002; Viard *et al.* 2002). The RIVER population represents a situation analogous to an estuarial zone, as there is an inland tide stream that can explain the colonization of the river embankments. Although cultivated and wild forms are often difficult to distinguish, the RIVER population was chosen to test for possible introgression because several individual beets displayed conspicuous morphological characters specific to cultivated lines.

Approximately 20% of the individuals belonging to the COAST and FIELD populations were randomly sampled in order to achieve the best representation of the whole population. The sampling of the RIVER population was exhaustive (i.e. all individuals were collected). Altogether, 154 individuals were collected (see figure 1) and fresh leaves were desiccated using silica gels (Prolabo Inc.) prior to DNA extraction.

(b) *Genetic data collection*

Extraction and purification of total DNA was performed using a DNeasy 96 Plant Kit following the standard protocol for isolation of DNA from plant leaf tissue outlined in the DNeasy 96 Plant protocol handbook (Qiagen Inc.).

The maternal cultivated origin was assessed by means of a diagnostic chloroplastic PCR–RFLP marker. Weed beets carry the Owen cytoplasmic male sterility (CMS) (Boudry *et al.* 1993), a trait maternally inherited and characterized by the inability of the plant to produce functional pollen. This CMS is typical of sugar beet cultivars because of its worldwide use in breeding. The Owen CMS cytoplasm is associated with one additional polymorphic *Hin*dIII site on chloroplast (cp) DNA (Ran & Michaelis 1995). Primers used, PCR conditions, and DNA digestion were applied as described by Ran & Michaelis (1995). *Hin*dIII-digested cpDNA products were separated using 0.8% agarose gel electrophoresis and visualized after ethidium bromide staining under UV light.

Individuals were genotyped at six microsatellite loci (CT4, GTT1, GCC1, GAA1, BVM3 and CAA1) according to protocols previously described in Mörchen *et al.* (1996) and Viard *et al.* (2002). One additional unpublished microsatellite locus (CA2) was used with CCTTGCTAGTTGCTGCTGTG and GCATATGTACAAGAGAGCCGTTT as 5′–3′ primer sequences. CA2 is an imperfect and interrupted locus composed of TG repeats, allelic sizes range between 225 and 229 bp, and PCR conditions are identical to those described in Viard *et al.* (2002) except for the annealing temperature (55 °C). Electrophoresis and genotyping were performed on a LI-COR automated DNA sequencer model 4200s (LI-COR Inc., NB, USA).

(c) *Statistical treatments*

Allele frequencies, departures from Hardy–Weinberg equilibrium, linkage disequilibrium, allelic richness following the rarefaction procedure of El Mousadik & Petit (1996), gene diversity, and unbiased intrapopulation fixation index (F_{IS}, a measure of departures from panmixia within populations) were calculated for each population using FSTAT (Goudet 1995). Global differentiation among populations (F_{ST}) was quantified following Weir & Cockerham (1984) and tested for significant departure from zero by randomly permuting multilocus genotypes among samples.

The high number of alleles segregating at hypervariable microsatellite markers is likely to achieve a differentiation of all individuals that are uniquely defined, even with a small number of loci (e.g. Beaumont *et al.* 2001). To depict fine-scaled introgression from crop to wild relatives, and to test individual admixture proportions and the correspondence of genetic clusters with geographically labelled groups, we applied a model-based clustering algorithm introduced by Pritchard *et al.* (2000). Using the software STRUCTURE (Pritchard *et al.* 2000), this Bayesian method enables identification of clusters of genetically similar individuals from multilocus genotypes without prior

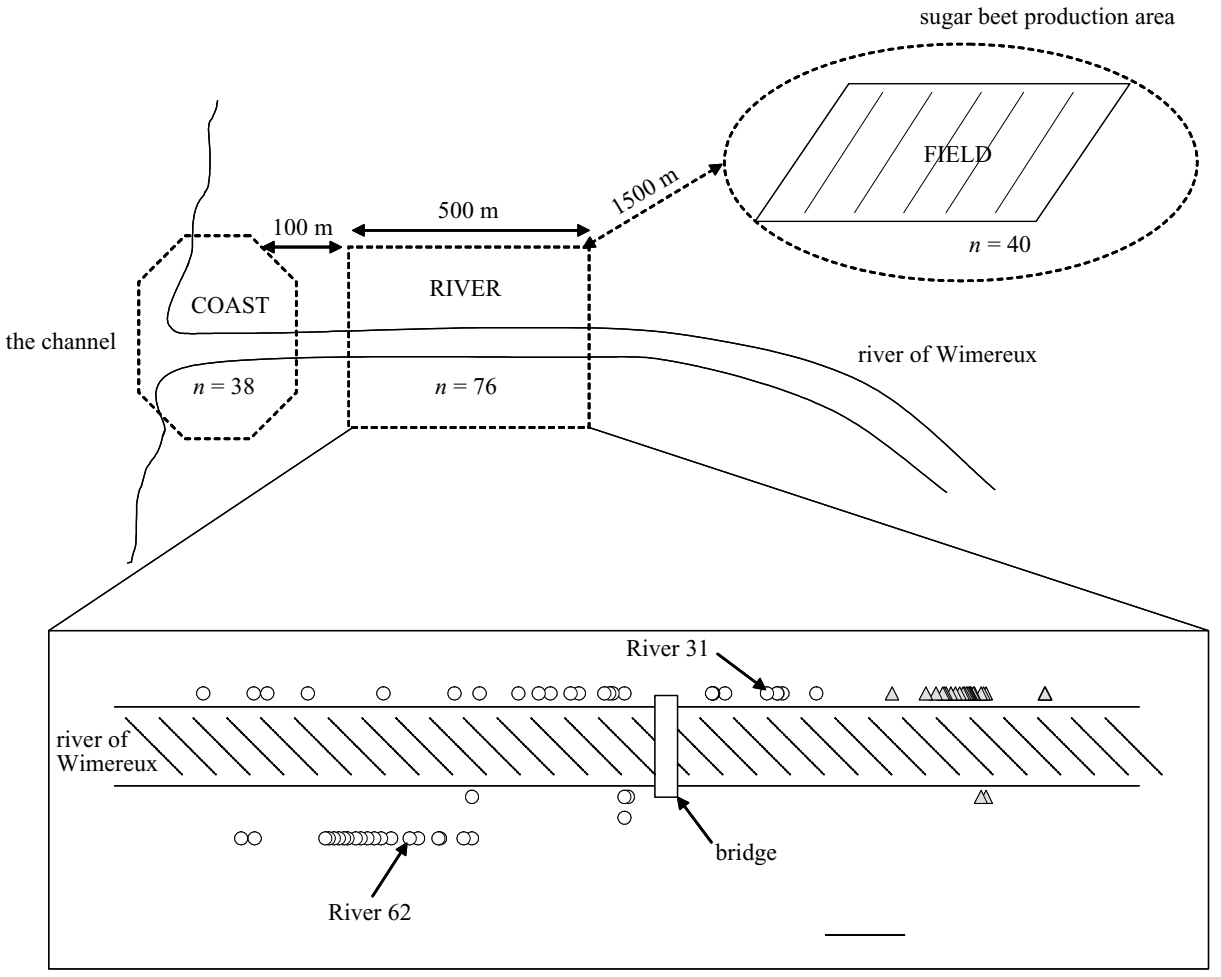

Figure 1. Map of the sampled area. The three *B. vulgaris* populations are located within (COAST and RIVER) and in the vicinity (FIELD) of Wimereux city (northern France, 50°46′ N; 1°37′ W). *n*, sample size. The spatial distribution of the individuals sampled within the RIVER population is also given. Individuals characterized by the Owen cytoplasmic male sterility are indicated with a grey triangle, whereas individuals carrying a non-Owen cytoplasmic type are represented by a white circle. Scale bar, 50 m.

knowledge of their population affiliation. In this approach, it is assumed that there are K populations contributing to the gene pool of the sampled populations. Individuals can have membership in multiple clusters, with membership coefficients summing to unity across clusters. Each run consisted of a burn-in period of 200 000 steps followed by 10^6 MCMC (Monte Carlo Markov chain) replicates, with the number K of specified clusters being from one to six and assuming that allele frequencies are uncorrelated. Repeated runs of STRUCTURE produced identical results to those shown.

3. RESULTS

Chloroplastic DNA markers allowed us to distinguish the cytoplasm associated with Owen's male sterility (found in cultivated sugar beets and their weedy relatives resulting from a crossing with a maternal cultivated plant) from other cytoplasms. All individuals from FIELD and COAST populations were characterized by the OwenCms and non-OwenCms haplotypes, respectively. This demonstrates that extensive seed dispersal does not occur from the fields into the coastal population of the area under study. However, the RIVER population was clearly partitioned into two groups according to the cytoplasm (figure 1). Twenty-five individuals out of 76, spatially clustered in the upstream part of the RIVER population, exhibited the OwenCms haplotype, indicating that one of their maternal ancestors was a cultivated individual. Therefore, in the further nuclear microsatellite analyses the RIVER population was partitioned into two subpopulations according to OwenCms and non-OwenCms lineages.

No linkage disequilibrium occurred for microsatellite loci, except for pair CT4/CAA1 within the FIELD population ($p < 0.05$, after Bonferroni adjustment). Multiple probability tests across all population samples yielded no significant p-adjusted values, showing the independence of each locus. Summary statistics about the genetic diversity within the four populations are given in table 1. Allele frequencies are available upon request. Allelic richness (*Arich.*) ranged from 2 to 16.95 across loci and populations, and amounts of gene diversity (*He*) were relatively high whatever the lineages considered (table 1). In contrast to OwenCms lineages (FIELD and OwenCms RIVER populations), no significant departures from Hardy–Weinberg equilibrium were detected for wild beet populations (COAST and non-OwenCms RIVER), as suggested by F_{IS} values closed to zero (table 1). The

Table 1. Genetic diversity within the FIELD, COAST and RIVER populations.
(The RIVER population was divided into two subpopulations according to the individual chloroplastic haplotypes (OwenCms versus non-OwenCms). $A_{rich.}$, allelic richness estimated following the rarefaction method developed by El Mousadik & Petit (1996); *He*, expected heterozygosity (gene diversity); F_{IS}, intrapopulation fixation index and its associated significance ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ after Bonferroni correction).)

	FIELD ($n = 40$)			RIVER ($n = 76$)						COAST ($n = 38$)		
				OwenCms ($n = 25$)			non-OwenCms ($n = 51$)					
locus	$A_{rich.}$	*He*	F_{IS}	$A_{rich.}$	*He*	F_{IS}	$A_{rich.}$	*He*	F_{IS}	$A_{rich.}$	*He*	F_{IS}
CT4	6.62	0.82	0.33***	6.00	0.79	0.24*	5.34	0.60	−0.07	8.49	0.62	−0.05
GTT1	3.00	0.66	0.28*	3.00	0.40	0.11	3.46	0.41	0.04	3.54	0.40	−0.06
GCC1	2.00	0.49	0.75***	2.00	0.37	−0.07	2.00	0.49	−0.04	2.00	0.50	0.00
GAA1	2.62	0.33	0.09	2.00	0.39	0.085	2.97	0.38	−0.13	3.00	0.45	0.04
BVM3	9.52	0.67	0.52***	10.00	0.76	0.63***	15.39	0.91	0.20***	16.95	0.93	−0.04
CAA1	7.36	0.72	0.24*	9.00	0.74	0.46***	12.73	0.83	0.17**	12.21	0.85	−0.08
CA2	3.00	0.62	0.36*	4.00	0.59	0.46**	2.98	0.45	−0.097	2.99	0.52	0.24
mean	4.87	0.62	0.37***	5.14	0.57	0.33***	6.41	0.58	0.04	7.02	0.61	−0.01

Table 2. Pairwise estimates of genetic differentiation θ (below diagonal) and their associate significance (above diagonal). ($^{**}p < 0.01$; n.s., not significant after Bonferroni correction.)

			RIVER		
		FIELD	OwenCms	non-OwenCms	COAST
FIELD		—	**	**	**
RIVER	OwenCms	0.0536	—	**	**
	non-OwenCms	0.1726	0.2259	—	n.s.
COAST		0.1702	0.2257	−0.0021	—

observed heterozygote deficiencies in weeds (OwenCms lineages) could not be attributed to a particular locus, so that both selective effects and potential presence of null alleles can be neglected. Using permutation procedures implemented in FSTAT, neither $A_{rich.}$, nor *He* showed significant differences between non-OwenCms and OwenCms lineages. F_{IS} values were, however, significantly higher for OwenCms lineages ($p < 0.001$ after 10 000 permutations).

Although there were strong frequency oppositions, we did not find any diagnostic microsatellite alleles to distinguish between cultivated–weed lineages and wild forms of *B. vulgaris* (data not shown). However, even at this microgeographical scale, noticeable significant genetic differentiation ($p < 0.01$) occurred between all pairwise population comparisons, with the clear exception of the COAST population and non-OwenCms individuals of the RIVER population (table 2). Such a lack of differentiation strongly suggests that all non-OwenCms individuals belong to the same breeding group. By assuming uninformative priors on all the K inferred clusters, the Bayesian clustering approach strongly strengthened this hypothesis. Genetic admixture analysis clearly indicated that the posterior probability for the proper number of clusters was maximum for $K = 3$ populations ($\ln P = -2794.7$). By incorporating prior population information to assist the clustering, figure 2 revealed three clusters that: (i) discriminated well COAST/non-OwenCms RIVER beets (i.e. wild lineages) and FIELD/OwenCms

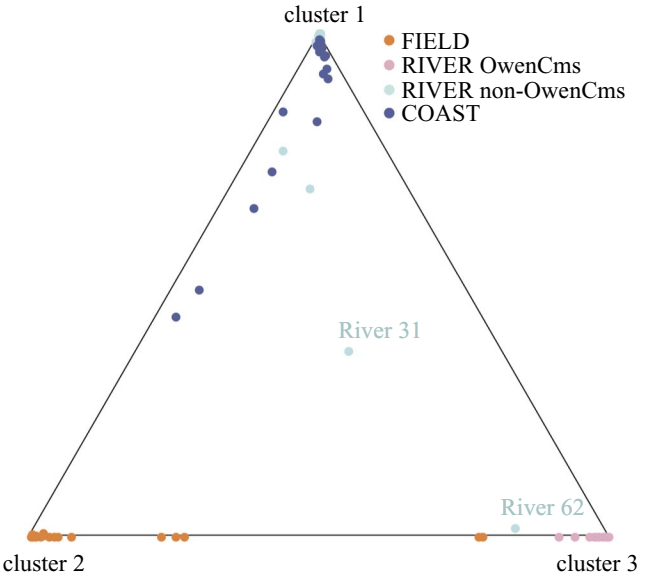

Figure 2. Diagram of three inferred clusters of individuals assuming $K = 3$ populations using STRUCTURE (Pritchard *et al.* 2000). Each point shows the mean ancestry for an individual in the sample. The values of the three coefficients in the ancestry vector q(i) are given by the distances to each of the three sides of the equilateral triangle. After the clustering was performed, the points were labelled according to sampling location and cytoplasmic type, i.e. COAST (dark blue), FIELD (red), RIVER OwenCms (pink) and RIVER non-OwenCms (light blue).

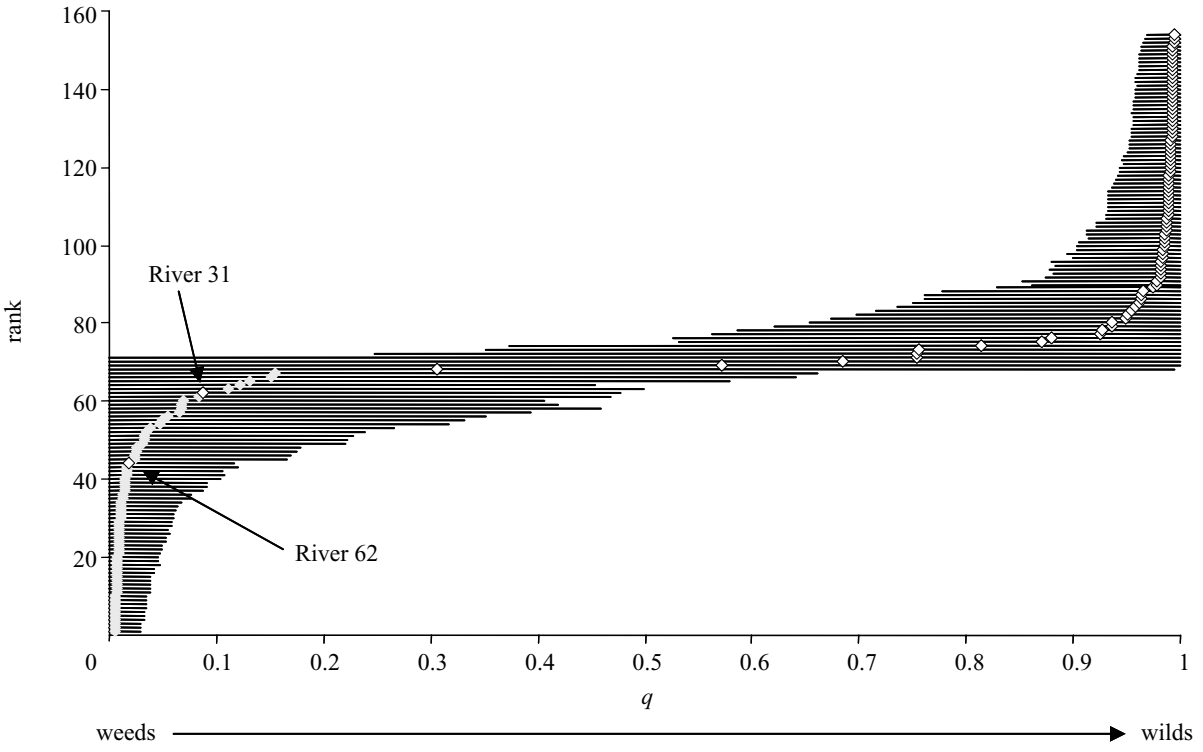

Figure 3. Distribution of the mean individual admixture coefficients q estimated using STRUCTURE (Pritchard *et al.* 2000) without prior population information. In this analysis, K (the number of population contributing to the gene pool of all sampled individuals) is assumed to be 2. Individuals were ranked from lowest to highest q-values and ranks were plotted against q. A q-value of 1 denotes a wild individual, whereas 0 denotes weedy individuals. Also displayed are lines giving the 95% posterior probability intervals of q for each individual. The cytoplasmic status of individuals is represented by a grey diamond for a OwenCms cytoplasm and a white diamond for a non-OwenCms cytoplasm.

RIVER beets (i.e. weedy lineages); and (ii) clustered almost all wild individuals into the same cluster (i.e. cluster 1). Among the 89 individuals carrying the non-OwenCms haplotype, 80 fell into cluster 1, while no OwenCms individuals were assigned into this cluster. Instead, OwenCms individuals split into clusters 2 and 3 in accordance with their spatial location, with, none the less, some intermediate individuals (figure 2). Does the nuclear polymorphism mirror the cytoplasmic partitioning of individuals? By assuming $K = 2$ ($\ln P = -2837.5$), we then reported the means for the individual admixture proportion qi and their 95% probability intervals in figure 3, as in Beaumont *et al.* (2001). It can be seen that there is strong evidence for two distinct groups. These two clusters were perfectly in accordance with the cytoplasmic classification, with only six non-OwenCms (wild) individuals present in an intermediate position ($0.2 < q < 0.8$) with very wide probability limits for q-values. However, within the 'wild part' of the RIVER population (see figure 1), individuals 'River 31' and 'River 62' have a large proportion (0.912 and 0.976, respectively) of their nuclear genome that comes from the weedy lineage although they carry a non-OwenCms cytoplasm. Once again, no OwenCms individuals were assigned into the bulk of wild sea beets (figure 3).

4. DISCUSSION

An increasing number of studies have documented gene exchanges from crops into their wild or weedy relatives, which can result in the long-term establishment of cultivar alleles in wild populations (Darmency 1994; Ellstrand *et al.* 1999). Lavigne *et al.* (2002) predicted the existence of substantial pollen-mediated gene flow from cultivated to wild beets, even in the case where wild beets are supposedly destroyed within 1000 m outside the fields. Nonetheless, the use of nuclear microsatellite markers allowed us to depict a clear genetic cleavage between wild individuals and their weedy relatives. In addition to highlighting such a genetic distinctiveness, admixture analysis also suggested clustering in accordance with the maternal origin and very rare introgression events with only, at best, two individuals (1.29%) resulting from wild–weed hybridization events.

Based on these results, this study surprisingly demonstrates that introgression *via* pollen dispersal into *B. vulgaris* ssp. *maritima* populations occupying their natural habitat may not be the most likely route for transgene spread from agriculture (but see Gray & Raybould 1998). In fact, the occurrence of such gene exchanges may be limited because weedy beet populations usually suffer from a rapid turnover owing to farming practices (crop rotation) and have a limited flowering overlap with wild sea beets. The episodes of close parapatry between cultivated beets and coastal populations are therefore neither continuous in time nor in space so that gene flow from the 'weedy pollen pool' may be spatially and temporally restricted. Weed plant species are indeed often distributed in ephemeral patches (Baker 1974; Hodkinson & Thompson 1997; McCauley 1997) and the location of the 25 weed beets collected along the 'Wimereux' river matched well with recent dyke-building work and soil transport. In fact, 2 years after the first sampling, this population of

weed beets was extinct, because of man-made disturbances (building and cleaning; J.-F. Arnaud and J. Cuguen, personal observation).

In contrast to pollen dispersal, seeds remain in the vicinity of the seed source for the most part, and the long-distance component of seed dispersal distribution is often difficult to document (Levin 1981; Howe & Smallwood 1982; Cain *et al.* 2000; and references therein). Many studies have reported that pollen dispersal is often the major contributor to gene flow in populations of wind-pollinated outcrossing plant species and that striking spatial genetic structuring is mainly the result of restricted seed dispersal (e.g. Hamrick & Nason 1996; McCauley 1997; Latta *et al.* 1998; Laporte *et al.* 2001). Nonetheless, we should bear in mind that dispersal occurs spatially by seed and pollen movement, but also occurs in time via the seed bank (Hodkinson & Thompson 1997; Raybould 1999). In the present study, several lines of evidence show that seed migration can occur from fields to wild populations, through weedy lineages via human activities such as soil transport. Individuals resulting from the emergence of a weed seed bank (generally found within sugar beet fields) were found only a few metres apart from typical sea beet individuals. Since most studies involving the assessment of transgene escape from crops to wild relatives generally focus only on pollen dispersal (reviewed in Lavigne *et al.* 2002) this last result was unexpected. It is interesting with regard to the processes associated with transgene escape in *B. vulgaris* (Gray & Raybould 1998), as well as other key aspects of the biology of plants, such as invasiveness or the evolution of metapopulation dynamics (Cain *et al.* 2000). Because beets have a long-lived seed bank and can shed thousands of seeds, this means that weeds are likely to reappear each spring despite stochastic disturbances (Desplanque *et al.* 2002). Together with stochastic founder effects, occasional long-distance seed dispersal, without regard to the extent of geographical separation, also means that gene flow would not necessarily decline regularly as an exponential function of distance from the genetically engineered crop (Raybould & Gray 1994; Van Raamsdonk & Schouten 1997).

Evaluation of the relative contribution of pollen and seed movement using maternally (chloroplastic DNA) or biparentally (microsatellites) inherited markers is also especially interesting in weedy plant species (McCauley 1997). In human-dispersed species, such as weedy forms of *B. vulgaris*, many colonization events may result from long-distance dispersal of a relatively few seeds into ephemeral vacant habitats, a situation in which extinction–recolonization models of genetic differentiation would be more adequate than classical models of gene flow (Hamrick & Nason 1996; McCauley 1997). Indeed, weed beets are adapted to man-disturbed habitats (e.g. very high seed output in favourable environmental circumstances) and may display life histories equivalent to the demography assumed in the so-called metapopulation model (Hamrick & Nason 1996; Hanski 1999). Therefore, seed movement should be relatively common in the sugar beet production areas and may involve multiple colonization events through either the seed bank and/or human-mediated long-range dispersal. As (i) the weed population located along the river embankments is clearly differentiated from the neighbouring FIELD population; and (ii) substantial departures from Hardy–Weinberg equilibrium are found within both weed populations ($F_{IS} > 0$; see table 1), these findings highlight the occurrence of stochastic colonization events involving a mixture of genetically distinct individuals coming from several sources (Wahlund effect). Accordingly, an additional subclustering analysis using STRUCTURE showed that weedy individuals sampled within the sugar beet crop split into seven distinct clusters with the highest probability (results not shown). Hence, the evolution of *B. vulgaris* weediness is likely to result from multiple exports of genetically differentiated crop–wild hybrids from the seed production areas, followed by secondary hybridization events (see also Viard *et al.* 2002).

In conclusion, although pollen usually represents a significant vector for the spread of genetically modified traits, the present results suggest: (i) that seed flow may have a deeper and longer impact in connecting wild and crop relatives within the complex *Beta*; and (ii) point out the key role of a long-lived seed bank, a factor often neglected.

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