
Fast and Simple Monitoring of Introgressive Gene Flow from Wild Beet into Sugarbeet

C. Stark^{1,2}, S. Liepelt¹, M. Dieckvoss³,
D. Bartsch⁴, B. Ziegenhagen¹ and A. Ulrich³

¹Philipps-University Marburg, Faculty of Biology, Conservation Biology, Karl-von-Frisch-Strasse 8, D-35032 Marburg, Germany; ²Current address: Helmholtz Centre for Environmental Research – UFZ, Department of Community Ecology, Theodor-Lieser-Str. 4, D-06120 Halle, Germany; ³Leibniz-Centre for Agricultural Landscape Research, Institute of Landscape Matter Dynamics, Eberswalder Str. 84, D-15374 Müncheberg, Germany; ⁴Federal Office of Consumer Protection and Food Safety (BVL), Taubenstrasse 42/ 43, D-13533 Berlin, Germany.
Corresponding author: C. Stark (christiane.stark@ufz.de)

ABSTRACT

Weed beet, so-called bolters, can arise either from self dedomestication of sugarbeet or from hybridisation with wild sea beet relatives. Previous investigations indicated that weed beet may evolve from hybridisation between wild and cultivated beet when they occur in close proximity. Beet seed can persist several years in soil allowing the formation of a seed bank that subsequently releases weed beet again. Microsatellite markers were used to trace hybridisation events within weed beet populations from 12 fields in Brandenburg, Germany. Seeds derived from bolter offspring were analyzed at three microsatellite loci and population allelic patterns were compared with sugarbeet varieties and wild beet as reference genotypes. The results demonstrate past hybridisation and introgression of wild beet alleles, even in the seeds of the bolters. The use of only three highly polymorphic microsatellites provides a simple and fast method to monitor individuals for feral or wild beet characters that may cause adverse effects if recombination with GM beets were to occur.

Additional key words: *Beta vulgaris*, biosafety, hybridisation, microsatellites

The cultivated beet, *Beta vulgaris* L. ssp. *vulgaris*, is a biennial crop. Wild relatives of sugarbeet, *B. vulgaris* ssp. *maritima*, are typically found on the coastlines of South- and Western-Europe and sometimes inland, for example France (Hegi, 1979; Desplanque et al., 1999). Most of the populations of *B. vulgaris* ssp. *maritima* are annuals (Hansen et al., 2001; Hautekèete et al., 2002).

Feral plants are derived from partially or fully dedomesticated crop plants, dedomestication in terms of a return of a previously domesticated species to a feral form by back mutations. Sugarbeet (*B. vulgaris* ssp. *vulgaris*) is not fully domesticated; they often have a high frequency of feral traits like bolting (Gressel, 2005). These bolting beet ('bolters') are unwanted in commercial production and thus are considered as weeds. Sugarbeet gone feral in a sugarbeet field will not necessarily be identical to the wild type of the species, the sea beet (*B. vulgaris* ssp. *maritima*). Previous investigations showed that gene flow between cultivated and wild beets is probable where they occur in close proximity (Bartsch & Schmidt, 1997; Bartsch et al., 1999). Mörchen et al. (1996) and Viard et al. (2002) developed microsatellite markers to investigate hybridisation on a local scale, especially to explore the influence of hybridisation on the genetic diversity of beet in commercial fields. Subsequently, these markers were also utilized to analyze seed and pollen flow among wild and weed beet populations on a regional scale (Viard et al., 2004). However, a very important role in gene flow is denoted to seeds: spatially via seed dispersal through human activities and in time via seed banks (Arnaud et al., 2003). As they can persist 3 to 7 years in soil (Gunn, 1982 cited in Longden, 1993; Desplanque et al., 2002) we examined DNA-microsatellite markers to determine if they are adequate to trace back former hybridisation even in the seeds of bolters. It is desirable to identify alleles or genotypes diagnostic for certain varieties of sugarbeet or characteristic for certain provenances of wild beet. Hence we studied whether the three beet groups 'wild', 'weed' and 'cultivar' can be distinguished by frequency based genetic differentiation.

Knowledge about the genetic origin of bolters and their ability to produce offspring is important, especially for weed management and monitoring practices. There is still a great need to harmonize monitoring systems by the development of appropriate methods to evaluate the environmental impact of introgressed transgenes in case of genetically modified sugarbeet (Bartsch et al., 1996; Saeglitz et al., 2000; Bartsch et al., 2003). Thus the development of a simple detection system is a first step to assess the necessity for control measures such as mechanical bolter eradication in production fields or containment strategies during sugarbeet seed production.

MATERIAL AND METHODS

Plant Material

Bolters were sampled in Brandenburg, (north-eastern) Germany. In each of the three cultivation regions, Havelland, Uckermark and Oderbruch, four randomly chosen sugarbeet fields were sampled (Table 1). At each field site, seeds of 20 bolters were collected, dried and planted in a green house. Analyzing seedlings derived from bolters assured that only fertile bolters were analyzed as contributing to the accumulation of a seed bank.

As references, seeds of each variety cultivated on the investigated fields were obtained from seed companies. Additionally seeds from six wild beet regions in Europe were included in this study (Table 2).

Microsatellite-Analysis

For DNA extraction, 50-70 mg fresh plant material from leaves was used. The extraction followed the method described by Dumolin et al. (1995), with the modification of using cetyltrimethylammonium bromide (CTAB) instead of alkyltrimethylammonium bromide (ATMAB). For the analysis of genetic differentiation, the microsatellite loci Bmv3 (Mörchen et al., 1996), CAA1 and CT4 (Viard et al., 2002), that were shown to be polymorphic between closely related individuals, were chosen. The PCR-amplification of the three microsatellite loci fol-

Table 1. Detailed information about the analyzed bolter populations of three sugar beet cultivation regions in Brandenburg (Germany).

Region	Pop. Identity	Cultivated varieties	Sample size
Havelland	Bolters H1	Helix-Sorella-Tomba	
		Wiebke-Ricarda-Mosaik	12
	Bolters H2	Katinka-Ricarda-Mosaik	12
	Bolters H3	Wiebke-Paloma-Katinka	10
Uckermark	Bolters H4	Katinka	10
	Bolters U1	Cortina	6
	Bolters U2	Kristall	15
	Bolters U3	Kristall-Manhattan	12
Oderbruch	Bolters U4	Kassandra	18
	Bolters O1	Achat	11
	Bolters O2	Helix	14
	Bolters O3	Mosaik	14
	Bolters O4	Mosaik	11

Table 2. Detailed information about the analyzed references.**a) Varieties**

Variety	Ref. Identity	Seed company	Sample size
Ricarda	Ref V 1	KWS	3
Paloma	Ref V 2	KWS	3
Kassandra	Ref V 3	KWS	3
Helix	Ref V 4	Strube-Dieckmann	3
Sorella	Ref V 5	Syngenta Seeds	3
Mosaik	Ref V 6	Strube-Dieckmann	3
Achat	Ref V 7	Strube-Dieckmann	3
Wiebke	Ref V 8	KWS	3
Katinka	Ref V 9	KWS	3
Tomba	Ref V 10	Syngenta Seeds	3
Manhattan	Ref V 11	Danisco Seed	3
Kristall	Ref V 12	Danisco Seed	3
Cortina	Ref V 13	Danisco Seed	3

b) Wild beet

Region	Ref. Identity	Country	Sample size
Helgoland	Ref W 1	Germany	3
Fehmarn	Ref W 2	Germany	3
San Michele	Ref W 3	Italy	3
Murano	Ref W 4	Italy	3
Grado	Ref W 5	Italy	3
Bocasette	Ref W 6	Italy	3

lowed the protocol of Viard et al. (2002). For each PCR-reaction, 20-30 ng DNA were used. The resulting PCR fragments were separated along with the internal size standard GeneScan 500 Rox (Applied Biosystems) on an ABI 310 DNA sequencer (Applied Biosystems). For detection of the fragments the forward primer of each primer pair was fluorescence-labeled with 6-Fam or Joe (MWG Biotech). Sizing of the fragments was done with the GeneScan Analytical Software v.3.1.2 (Applied Biosystems). To assess the genetic diversity we used the computer software GenAlEx V5 (Genetic Analysis in Excel) (Peakall and Smouse, 2001) for calculating the average number of alleles per locus (allelic diversity, A_{div}), the actual level of heterozygosity (observed heterozygosity, H_O), the heterozygosity expected for a random mating population (expected heterozygosity, H_e), and the number of alleles that if equally frequent would result in the observed homozygosity (effective number of alleles n_e) (Frankham et al., 2002). Some of the varieties

were triploid, so the peaks were visually scored. For triploid varieties with only two alleles, the relative highest peak was assumed to appear twice. Effective number of alleles and expected heterozygosity were not calculated for varieties as Mendelian-based inheritance cannot be assumed. The cluster analysis was carried out using the genetic distances of Nei (Nei, 1978) and the UPGMA-method (Sneath and Sokal, 1973) with the software POPGENE vers. 1.31 (Population Genetic Analysis Software) (Yeh et al., 1999).

RESULTS AND CONCLUSIONS

In order to identify foreign genotypes or alleles in seedlings derived from the bolters, reference genotypes of cultivated and wild beets were screened for typical alleles or genotypes, respectively.

Based on the genotyping of the 13 varieties included in this study, it was not possible to identify diagnostic alleles that would characterize each variety. All of the 19 alleles found were identified in at least two varieties. The varieties are dominated by 3-4 abundant alleles per locus that occurred with frequencies up to 0.9. In contrast to the cultivated beet, several alleles in the wild beet references were unique to specific regions. Altogether 57% of all alleles obtained for the wild beet were restricted to particular regions. This suggests that there are indeed alleles characteristic for certain regions, but due to the restricted sample size and the postulated high genetic diversity of wild beet (Desplanque, 1999), it needs to be verified by analyzing more individuals. The observed genetic diversity between the three investigated beet groups revealed considerable genetic differences (Table 3). For the varieties we ascertained a low allelic diversity but a high observed heterozygosity. These results confirm the observations of de Riek et al. (2001) who showed that most of the genetic diversity of sugarbeet varieties can be found within and not between varieties. The high degree of heterozygosity is due to the fact that the analyzed varieties are exclusively hybrid-varieties. The wild beet however, had a large number of effective alleles and, considering the small number of

Table 3. Results of the genetic comparison of the three analyzed beet types wild beets, bolters and varieties.

	Wild beet	Bolters	Varieties
Number of samples, N	18	145	99
Allelic diversity, A_{div}	10	17.7	6.3
Number of effective alleles, n_e / Locus	6.9	4.0	-
Observed heterozygosity, H_0 / Locus	0.6	0.5	0.7
Expected heterozygosity, H_e / Locus	0.8	0.7	-

individuals sampled, a high allelic diversity, which was also found previously in other wild beet provenances (Desplanque et al., 1999). The bolters shared the high allelic diversity of the wild beet but the low number of effective alleles of the varieties, indicating an intermediate genetic structure, at least at the microsatellite loci analyzed. The high allelic diversity strongly suggests a wild beet introgression into the bolters and consequently their progeny. Furthermore the seedlings comprised 93% of all 57 alleles found over all three loci and groups of beets. The three dominating alleles for the cultivated beet were also the most abundant alleles in the bolters, but they occurred with lower frequencies. In the genotypes of the bolters, 64% of the alleles were foreign alleles that were not detected in the varieties, whereas 41% were also identified for the wild beets. Altogether we found such foreign genotypes in half of all analyzed bolters. This supports the hypothesis of wild beet genetic introgression. The cluster analysis, based on Nei's genetic distances, grouped all bolter populations

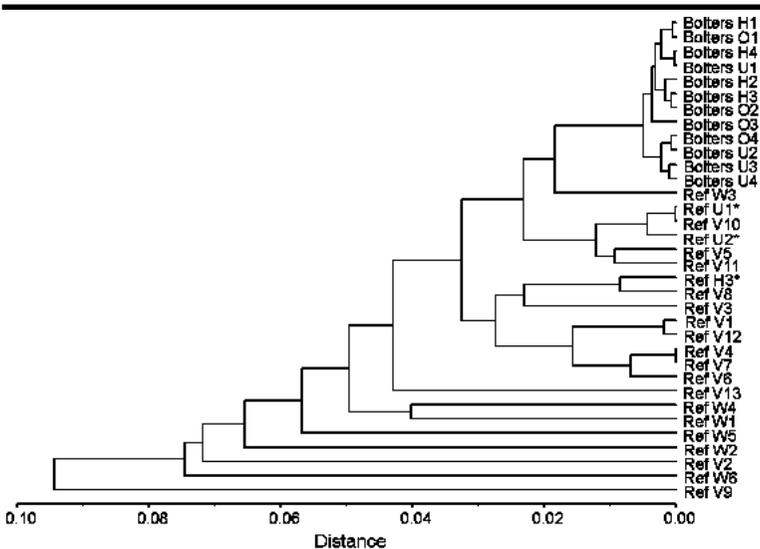


Fig. 1. Dendrogramm (UPGMA-method) of the three analyzed beet types based on the genetic distances of Nei (1978). The bolters from the three different regions Havelland (H1-H4), Uckermark (U1- U4) and Oderbruch (O1- O4) are clearly separated from the references of the varieties (Ref V 1- Ref V 13) and of the wild beets (Ref W 1- Ref W 6).

*-marked references come from plant material sampled directly on the respective field sites. For further details see Tables 1 and 2 in 'Materials

in a single cluster, clearly separated from the references (Figure 1). This indicates that the bolters are not identical to the references, neither the varieties nor the wild beet and again confirms the genetic differences. The fact that the cluster of the bolters is arranged next to a reference of a wild beet provenance suggests a possible origin of introgressive gene flow.

We identified genetic introgression in about half of the bolters, representing at least the second generation after hybridisation. This may underestimate an even higher percentage occurring in the bolters as F₁ hybrids. Our results suggest that bolting has been induced by genetic introgression from wild beet; otherwise, the bolters would not have been separated from the varieties. Hence, we were able to detect the footprints of previous hybridisation between wild beet and cultivars in the seeds of bolters.

Our results demonstrate that bolters reproduce in commercial beet fields in Germany as we analyzed seedlings derived from bolters. Moreover it is possible to detect the traces of introgression from wild beets in the progeny of bolters. Considering the large number of varieties represented in this study, it should be easy to conduct similar analyses with different varieties and in different beet production areas. The possibility of detecting foreign genotypes together with the high reproducibility of the microsatellite-method facilitates the establishment of a simple monitoring system capable of detecting foreign genotypes in varieties. Thus it is possible to screen bolting individuals or even complete seed banks for possible wild beet introgression. This will be extremely useful in risk assessment for estimating the potential accumulation of non-desired genotypes, i.e. weed beet and genetically modified beet, in the seed reservoirs of commercial fields.

For technical adjustment and scientific exchange we are willing to provide size standards for the three microsatellites we used together with the list of allele sizes obtained.

ACKNOWLEDGEMENT

We thank Danisco Seed, KWS, Strube-Dieckmann and Syngenta Seeds for providing seed of their sugarbeet varieties. We thank Sigune Weinert, Ilona Bartelt and Christina Mengel for valuable advice in the laboratory. This study was carried out with the financial support of the Ministry of Science, Research and Culture of Brandenburg (Az: 24#2598-09/399;2003).

LITERATURE CITED

- Arnaud, J.-F., F. Viard, M. Delescluse, and J. Cuguen. 2003. 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. Proc. R. Soc. Lond. B 1524: 1565-1571.
- Bartsch, D., M. Schmidt, M. Pohl-Orf, C. Haag, and I. Schuphan. 1996. Competitiveness of transgenic sugar beet resistant to beet necrotic yellow vein virus and potential impact on wild beet populations. Mol. Ecol. 5: 199-205.
- Bartsch, D., and M. Schmidt. 1997. Influence of sugar beet breeding on populations of *Beta vulgaris* ssp. *maritima* in Italy. J. Veg. Sci. 8: 81-84.
- Bartsch, D., M. Lehnen, J. Clegg, M. Pohl-Orf, I. Schuphan, and N.C. Ellstrand. 1999. Impact of gene flow from cultivated beet on genetic diversity of wild sea beet populations. Mol. Ecol. 8: 1733-1741.
- Bartsch, D., J. Cuguen, E. Biancardi, and J. Sweet. 2003. Environmental implications of gene flow from sugar beet to wild beet- current status and future research needs. Environ. Biosafety Res. 2: 105-115.
- de Riek, J., E. Calsyn, I. Everaert, E. van Bockstaele, and M. de Loose. 2001: AFLP based alternatives for the assessment of distinctness, uniformity and stability of sugar beet varieties. Theor. Appl. Genet. 103: 1254- 1265.
- Desplanque, B., P. Boudry, K. Broomberg, P. Saumitou-Laprade, J. Cuguen, and H. van Dijk. 1999. Genetic diversity and gene flow between wild, cultivated and weedy forms of *Beta vulgaris* L. (Chenopodiaceae), assessed by RFLP and microsatellite markers. Theor. Appl. Genet. 98: 1194- 1201.
- Desplanque, B., N. Hautekète, and H. van Dijk. 2002. Transgenic weed beets: possible, probable, avoidable? J. Appl. Ecol. 39: 561-571.

- Dumolin, S., B. Demesure, and R.J. Petit. 1995. Inheritance of chloroplast and mitochondrial genomes in pedunculate oak investigated with an efficient PCR method. *Theor. Appl. Genet.* 91: 1253-1256.
- Frankham, R., J.D. Ballou, and D.A. Briscoe. 2002. *Introduction to Conservation Genetics*. Cambridge University Press, Cambridge, UK. 617 pp.
- Gressel, J. 2005. Introduction- the challenges of ferality. Pages 1- 7. In J. Gressel (ed.). *Crop Ferality & Volunteerism*. CRC Press, Inc., Boca Raton, FL. 422 pp.
- Gunn, J.S. 1982. Population dynamics of weed beet. *Proceedings 1982: British Crop Protection Conference – Weeds*, 61-66.
- Hansen, M., T. Kraft, S. Ganestam, T. Säll, and N.-O. Nilsson. 2001. Linkage disequilibrium mapping of the bolting gene in sea beet using AFLP markers. *Genet. Res.* 77: 61-66.
- Hautekèete, N.-C., Y. Piquot, and H. van Dijk. 2002. Life span in *Beta vulgaris* ssp. *maritima*: the effects of age at first reproduction and disturbance. *J. Ecol.* 90: 508-516.
- Hegi, G. 1979. *Illustrierte Flora von Mitteleuropa III/2*, 2nd edn. Paul Parey, Berlin und Hamburg.
- Longden, P.C. 1993. Weed beet: a review. *Aspects Appl. Biol.* 35: 185-194.
- Mörchen, M., J. Cuguen, G. Michaelis, C. Hänni, and P. Saumitru-Laprade. 1996. Abundance and length polymorphism of microsatellite repeats in *Beta vulgaris* L. *Theor. Appl. Genet.* 92: 326- 333.
- Nei, M. 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* 89: 583-590.
- Peakall, R., and P.E. Smouse. 2001. *GenAlEx V5: Genetic Analysis in Excel*. Population genetic software for teaching and research.

Australian National University, Canberra, Australia. <http://anu.edu.au/BoZo/GenAIEx>

- Saeglitz, C., M. Pohl, and D. Bartsch. 2000. Monitoring gene flow from transgenic sugar beet using cytoplasmic male-sterile bait plants. *Mol. Ecol.* 9: 2035-2040.
- Sneath, P.H.A., and R.R. Sokal. 1973. Numerical taxonomy; the principle and practice of numerical classification. W.H. Freeman, New York.
- Viard, F., J. Bernard, and B. Desplanque. 2002. Crop-weed interactions in the *Beta vulgaris* complex at a local scale: allelic diversity and gene flow within sugar beet fields. *Theor. Appl. Genet.* 104: 688-697.
- Viard, F., J.-F. Arnaud, M. Delescluse, and J. Cuguen. 2004. Tracing back seed and pollen flow within the crop-wild *Beta vulgaris* complex: genetic distinctiveness vs. hot spots of hybridisation over a regional scale. *Mol. Ecol.* 13: 1357-1364.
- Yeh, F.C., R.-C. Yang, T.B.J. Boyle, Z.-H. Yeh, and J.X. Mao. 1997. POPGENE, the user-friendly shareware for population genetic analysis. Molecular Biology and Biotechnology Centre, University of Alberta, Canada. <http://www.ualberta.ca/~fyeh/>