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Phylogenetic relationships between cultivated and wild species of the genus *Beta* revealed by DNA "fingerprinting"

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Abstract. Forty-one accessions of the genus *Beta* representing wild and cultivated species of all sections were analyzed by DNA "fingerprinting". Four sugar beet minisatellite DNA probes revealed characteristic banding patterns with Southern-hybridized *Beta* DNA restricted with *Hin*dIII. A total of 111 polymorphic RFLP bands were scored across all accessions. Cluster analysis based on genetic similarity estimates for all 820 combinations of accessions revealed the following results. (1) All accessions could unambiguously be identified by a characteristic RFLP banding pattern. (2) The sugar beet cultivars examined displayed a low level of genetic diversity; they showed high similarity to *B. vulgaris* ssp. *maritima* but low genetic similarity to the other wild species of section I. (3) In most cases, the present taxonomic classification of the genus *Beta* was confirmed. Species of sections II, III, and IV were clearly distinguishable from those of section I except for *B. macrocarpa*, which showed high similarity to wild species of section II. In a second experiment, 108 single-copy RFLP probes from sugar beet were Southern hybridized with *B. procumbens* DNA. A surprisingly low degree of homology (34%) was found. The results are discussed with regard to the taxonomic classification of the genus *Beta*.

Key words: Sugar beet – *Beta* species – DNA "fingerprinting" – Cluster analysis – Evolution

Introduction

Sugar beet (*Beta vulgaris* L.) is the most important crop species of the genus *Beta* (*Chenopodiaceae*), which is classified into four sections: *Beta* (I), *Corollinae* (II), *Nanae* (III), and *Procumbentes* (IV). While sugar beet is closely related to the other cultivated forms, namely fodder beet, leaf beet (Swiss chard), and garden beet, it is also commonly accepted that it is closely related to the wild species *B. vulgaris* ssp. *maritima* (L.) Arcang. The wild beets of section I show a wide geographic distribution extending southward from southwest Norway to the Capverdian Islands and westward presumably from Bangla Desh to the Canary Islands (Frese 1989). Their taxonomic classification, however, is doubtful in some cases, especially if it is mainly based on geographic distribution (e.g., *B. trojana* Pamuk.).

The five species belonging to section II colonize hilly and mountainous regions in Turkey and adjacent countries. *B. corolliflora* Zosimovic ex Buttler, *B. macrorhiza* Steven, and *B. lomatogona* Fisch et Meyer are regarded as basic species that are clearly distinguishable by morphologic characters; *B. intermedia* Bunge and *B. trigyna* Waldstein et Kitabel are considered to be hybrid species (Buttler 1977a). Section III is represented by a single alpine species, *B. nana* Boissier et Heldreich, that grows on the mountain heights of Greece. The three species of section IV form a clearly defined group, and as indicated by their small and remote distribution area, they have most likely reached their final evolutionary state (Frese 1989). These species have received great attention by breeders because they harbor some valuable genes like curly top resistance, nematode resistance, and *Cercospora* resistance (Coons 1954).

The modern sugar beet gene pool is generally regarded to be genetically narrow, mainly for two reasons: All sugar beets presumably descend from a single-source population, the white Silesian beet. In addition, the introduction of cytoplasmic male sterility into elite breeding lines may have further narrowed the genetic variation of the breeding stock. Introgression of wild beet germ plasm

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can broaden the genetic basis and should be extremely valuable for the improvement of certain traits such as disease resistances and cold tolerance (Bosemark 1989). Sugar beet hybridizes readily with the other cultivated forms as well as with wild *B. vulgaris*, however, strong crossing barriers exist between species of section *Beta* and species of sections II, III, and IV (Oldemeyer and Brewbaker 1956; Coons 1975; Abe and Tsuda 1987) mainly due to the presence of nonhomologous chromosomes. Nevertheless, substantial progress has been made in this field during the past decade by introducing genes for nematode resistance from *Procumbentes* species to sugar beet (Jung and Wricke 1987; Heijbroek et al. 1988).

The taxonomic classification of *Beta* species has been based on morphological traits (growth habit, mode of branching, leaf shape, fruit and flower traits, annual habit, leaf color, pollen diameter), reproductive system, and ploidy level. However, these characters did not exhaustively reveal relationships among species (Lange and de Bock 1989). Obviously, there is a need for new types of markers that are suitable for determining genetic similarity with greater precision and for monitoring the introgression of alien germ plasm. RFLP analyses of chloroplast (Fritzsche et al. 1987; Ecke and Michaelis 1990) and mitochondrial DNA (Ecke and Michaelis 1990) were found to be only of limited value because of the low degree of polymorphism, especially in the case of intravarietal comparisons. Isozyme markers have been applied successfully to reveal phylogenetic relationships between *Beta* species (Abe and Tsuda 1987; Wagner et al. 1989) and for varietal discrimination (Nagamine et al. 1989 a).

RFLP markers have been widely accepted for genetic analysis and varietal identification by DNA "fingerprinting". Species relationships and taxonomic studies on the basis of single-copy RFLP markers have been reported for several crop species (Havey and Muehlbauer 1989; Debener et al. 1990; Miller and Tanksley 1990; Song et al. 1990; Kesseli et al. 1991), and recently the first complete linkage map of sugar beet based on RFLP markers was published (Pillen et al. 1992). First results with cDNA (Nagamine et al. 1989 b; Mita et al. 1991) and rDNA markers (Santoni and Bervillé 1992) have also been presented for *Beta* species. A related class of markers hybridizes with repeated DNA elements distributed throughout the genomes of higher plants, yielding complex banding patterns after Southern hybridization with filter-bound, restricted genomic DNA. In general, these markers are classified into three groups: (1) M13 repeat probes, (2) simple repetitive sequences, and (3) minisatellite probes. All of them have been successfully employed for revealing genetic variation in plant nuclear genomes (Rogstad et al. 1988; Nybom et al. 1990; Rogstad et al. 1991) and for phylogenetic studies (Gebhardt et al. 1989).

Our objective was to determine genetic similarities between cultivated and wild *Beta* species on the basis of minisatellite RFLPs. We selected a set of minisatellite and single-copy DNA probes from a shot-gun cloned *B. vulgaris* plasmid library. Here, we report on the application of four minisatellite probes for estimating genetic similarity between 41 accessions of wild and cultivated *Beta* species. Results of cluster and principal coordinate analyses are discussed in relation to the classic taxonomic classification of species from the genus *Beta*.

Materials and methods

Plant materials and DNA extraction

Forty-one accessions representing species from all four sections of the genus *Beta* were examined (Table 1). For the cultivated beets these included 10 sugar beet, 2 fodder beet, 4 Swiss chard, and 3 red garden beet varieties and breeding lines. For the remainder of section I (*Beta*), we included different numbers of accessions from six wild species. Single accessions from three species of section II (*B. macrorhiza, B. corolliflora, B. lomatogona*), one species from section III (*B. nana*), and two species (*B. procumbens* Smith and *B. webbiana* Moquin) from section IV were studied. In addition, one accession of spinach (*Spinacia oleracea* L.) was included as a related species. Accessions characterized by a RNR number were provided by the Dutch German *Beta* program, CGN, Wageningen. *B. nana* was obtained from the genebank Wageningen, the Netherlands.

RFLP analyses

Plants were grown in a greenhouse at 20 °C. Four plants were sampled from each accession, and total DNA was extracted separately as described previously (Jung et al. 1990). Sugar beet DNA was shot-gun cloned into the plasmid vector pBluescribe (Jung et al. 1990; Pillen et al. 1992). Four plasmid probes were used for the Southern hybridizations (Table 2). A total of 108 anonymous sugar beet *Pst*I probes (Pillen et al. 1992) were used in a hybridization experiment with DNA from *B. vulgaris* and *B. procumbens*. Genomic *Beta* DNA was digested with restriction enzyme *Hin*dIII (Boehringer Mannheim) and separated in 0.75% agarose slab gels overnight together with *Hin*dIII-digested λ DNA as fragment length markers. Southern transfer to Biodyne B nylon membranes (Pall) was performed according to the manufacturer's protocol. After electrophoresis in low-melting-point agarose, the plasmid inserts were excised and labelled with [^{32}P]-dCTP by random priming according to Feinberg and Vogelstein (1983). Hybridizations were carried out in 5 × Denhardt's solution, 5 × SSPE, and 0.2% SDS with herring sperm DNA (200 µg/ml). The filters were then washed with 0.5 × SSC, 0.2% SDS at 62 °C. Membranes were rehybridized after stripping with 0.2 *N* NaOH at room temperature.

Statistical analyses

The fingerprint profiles were visually scored by assigning a number to each band. The approximate migration distance was determined by comparison with the flanking lanes of λ markers. Data were binary coded for subsequent numerical analyses using the dBASE IV program package. Presence or absence of a band in a lane was coded by 1 or 0, respectively. Only full intensity bands were scored. Doubtful bands were recorded as missing values. The four individual plants of each accession number were scored independently. Standard line LB was used on each gel for comparison.

Table 1. Geographic location, taxonomic description, and code number of 41 *Beta* accessions used in the DNA fingerprint experiments

Code number	Species	Accession number	Origin	Type, cultivar
Section I:				
Sugar beet				
L15	*B. vulgaris*	RNR 868877	Germany	Maxima[a]
L21	*B. vulgaris*	RNR 880209	Italy	Alba
LB	*B. vulgaris*	B 101-69	Germany	O-type[a]
LC	*B. vulgaris*	C	Germany	O-type[a]
LD	*B. vulgaris*	D 103-54	Germany	O-type[a]
LG	*B. vulgaris*	G	Germany	O-type[a]
LH	*B. vulgaris*	H	Germany	O-type[a]
LI	*B. vulgaris*	I	Germany	O-type[a]
L30	*B. vulgaris*	L30	Germany	Kwmono[b]
L88	*B. vulgaris*	L88	Germany	O-type[a]
Fodder beet				
L69	*B. vulgaris*	L69	Germany	Gelbe Eckendorfer[c]
L70	*B. vulgaris*	L70	Germany	Eckdorot[c]
Swiss chard				
L31	*B. vulgaris*	L31	Germany	?
L32	*B. vulgaris*	L32	Germany	?
L33	*B. vulgaris*	L33	Germany	?
L19	*B. vulgaris*	RNR 870949	Germany	Lukullus
Red garden beet				
L34	*B. vulgaris*	L34	Germany	Nerokugel
L38	*B. vulgaris*	L38	Germany	?
L20	*B. vulgaris*	RNR 870950	France	Rouge Crapaudine
Wild beet				
L18	*B. vulgaris* ssp. *maritima*	RNR 870906	Portugal	–
L22	*B. vulgaris* ssp. *maritima*	RNR 884421	Netherlands	–
L23	*B. vulgaris* ssp. *maritima*	RNR 884421	Netherlands	–
L29	*B. vulgaris* ssp. *maritima*	RNR 892254	Portugal	–
LE	*B. vulgaris* ssp. *maritima*	E 7-4	?	–
LF	*B. vulgaris* ssp. *maritima*	F 80-28	?	–
L28	*B. vulgaris* ssp. *maritima*	RNR 892252	Bretagne	–
L16	*B. vulgaris* ssp. *maritima/adanensis*	RNR 869120	Crete	–
L14	*B. vulgaris* ssp. *adanensis*	RNR 868526	Turkey	–
L17	*B. vulgaris* ssp. *adanensis*	RNR 869830	Turkey	–
L6	*B. vulgaris* ssp. *maritima* var *atriplicifolia*	RNR 891645	Spain	–
L27	*B. macrocarpa*	RNR 892234	Portugal	–
L24	*B. vulgaris* ssp. *orientalis*	RNR 891649	India	–
L25	*B. vulgaris* ssp. *orientalis*	RNR 891649	India	–
L13	*B. patula*	RNR 891652	Madeira	–
Section II:				
L39	*B. lomatogona*	RNR 869013	Turkey	–
L7	*B. corolliflora*	L7		–
L8	*B. macrorhiza*	RNR 869393	Turkey	–
Section III:				
L9	*B. nana*	IDBB 3604	Greece	–
Section IV:				
L10	*B. procumbens*	–		–
L11	*B. webbiana*	–		–
Spinach:				
L12	*S. oleracea*		Germany	–

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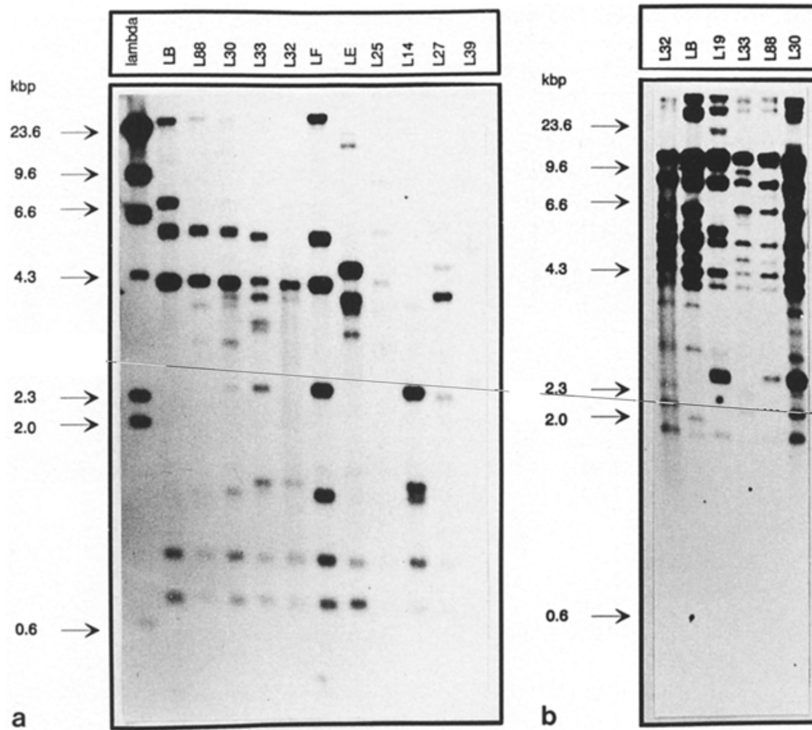

Fig. 1 a, b. Hybridization patterns of two sugar beet minisatellite DNA probes, pCJ943 (**a**) and pKP780 (**b**) *Hin*dIII-digested DNA of several *Beta* species was hybridized with [^{32}P]-labelled probes. Filter exposure: 3 days

Estimates of genetic similarity (GS) were calculated for all 820 pairwise comparisons of accessions according to the method of Nei and Li (1979):

$GS = 2n_{xy}/(n_x + n_y)$.

Here, n_x and n_y are the number of bands in accessions x and y, respectively, and n_{xy} is the number of bands shared by the two accessions. Graphic representations of the associations among the 41 accessions were obtained by standard procedures of numerical taxonomy. Average linkage (UPGMA) cluster analysis and principal coordinate analysis (PCA), according to Gower (1972) were performed with the matrix of RFLP-based GS estimates by using the appropriate procedures of program NTSYS-pc (Rohlf 1989).

Table 2. Characterization of the four DNA probes from *B. vulgaris* used in the DNA fingerprint experiments

Probe designation	Insert length (bp)	Cloning enzyme	Polymorphic bands	Unique bands[a]
pCJ47	1,000	*Hin*dIII	33	2
pCJ57	500	*Hin*dIII	32	4
pKP780	1,500	*Pst*I	25	1
pKP943	1,500	*Pst*I	21	5
Total	–	–	111	12

[a] Visible with only 1 accession

Results

Fingerprint analyses

The four anonymous *B. vulgaris* probes hybridize with repetitive DNA sequences resulting in polymorphic banding patterns. Between 6 (pCJ57) and 19 (pCJ47) bands per lane could clearly be resolved after hybridization with *Hin*dIII-digested DNA (Fig. 1 a). Probe pCJ57 generated conserved bands in the low-molecular-weight range and a number of polymorphic bands in the range between 2 and 14 kb. Among the highly conserved fragments, only 2 *B. vulgaris* ssp. *maritima* accessions displayed polymorphism: a population from West Portugal (RNR 870906) containing male-sterile plants and modified mt-DNA, and RNR 892254, a population of small effective population size from South Portugal (Hall 1989). Probes pCJ47 and pKP780 proved to be the most suitable for revealing polymorphism among cultivated beets (Fig. 1 b). All of the sugar beet cultivars could unambiguously be identified after hybridization with these two probes. The number of polymorphic fragments across all 41 accessions ranged between 21 and 33 (Table 2). Although *Pst*I probes gave less complex banding patterns, one of them (pKP943) yielded the highest frequency of unique bands (24%), which exclusively appeared in one accession. In total, 111 polymorphic fragments were analyzed, 12 of which were unique. For accession numbers RNR 891649 (L24, L25) and RNR 884421 (L22, L23), we found heterogeneity for at least 1 fragment and therefore treated them as different accessions. All accessions could be unambiguously characterized by individual banding patterns except for the two species of section IV.

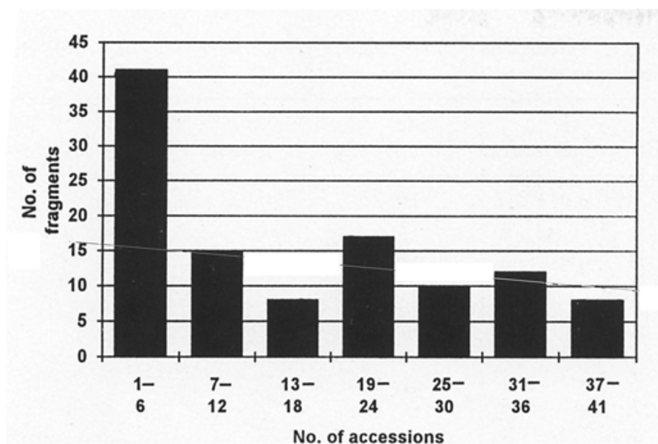

Fig. 2. Histogram of polymorphisms of 111 RFLP fragments detected after Southern hybridization of 41 *Beta* accessions

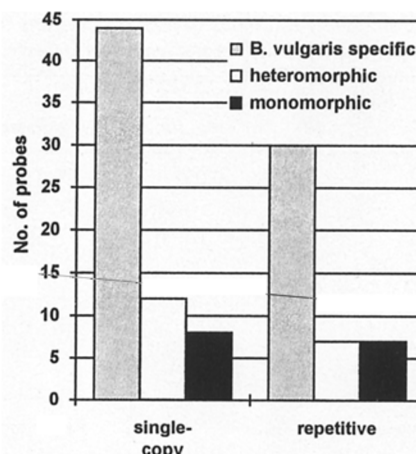

Fig. 4. Histogram of genome specificity of 108 sugar beet DNA probes tested on filter-bound *B. procumbens* DNA

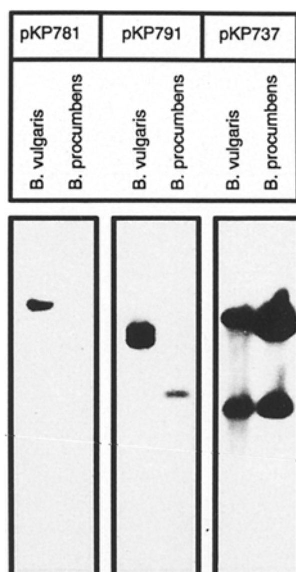

Fig. 3. Homology of randomly cloned sugar beet probes to *B. procumbens* DNA. *Hin*dIII-restricted DNA from *B. vulgaris* and *B. procumbens* was hybridized with three [^{32}P]-labelled single-copy probes. Filter exposure: 5 days

Figure 2 shows the frequency distribution of individual bands across the 41 accessions. A large proportion of fragments (37%) was found in at most 6 accessions. None of the fragments investigated was monomorphic across all accessions.

DNA homology between sugar beet and Procumbentes species

Species of section IV are of particular interest for gene transfer into sugar beet. The cross-hybridization experiment with 108 anonymous sugar beet RFLP probes (Fig. 3) revealed a surprisingly low degree of homology to wild beet DNA. Only 34% of the probes hybridized with wild beet DNA, and 20% yielded RFLPs between sugar beet and *B. procumbens* (Fig. 4).

Cluster analysis, principal coordinate analysis

Associations among all 41 genotypes revealed by cluster analysis based on genetic similarity estimates of all 820 combinations of accessions are presented in Figure 5.

Cultivated beets

The sugar beet varieties and breeding lines included in our assay displayed remarkably high GS estimates (Table 3). Together with the two fodder beet cultivars examined they could be classified into two main clusters except for accessions L15 and LC. The Swiss chard varieties, which displayed low GS estimates in combination with the other cultivated beets, were classified into a different cluster. The red garden beets were highly heterogeneous and did not group in separate clusters. The two fodder beet cultivars were merged into a single cluster.

Wild beets

The *B. vulgaris* ssp. *maritima* populations displayed a broad range of variability; however, they were clearly separated from the other wild taxa of section I, namely *B. adanensis, B. atriplicifolia, B. orientalis, B. macrocarpa*, and *B. patula* (Table 3). The *B. maritima*/*B. adanensis* accession L16 showed low genetic similarity to the other *B. maritima* wild beets investigated. The wild species of sections II, III, and IV showed low GS estimates in combination with species of section I with the exception of *B. macrocarpa*. The lowest GS to other *Beta* species was displayed by the wild species of section IV, which formed a separate cluster with *S. oleracea*. No DNA polymorphism was detectable between species *B. procumbens* and *B. webbiana*.

Table 3. Mean genetic similarity estimates between accessions from cultivated beets (below diagonal) and wild species from section *Beta*. The corresponding standard deviations are shown above the diagonal

B. vulgaris	Sugar beet	ssp. *maritima*	ssp. *adanensis*	ssp. *macrocarpa*	ssp. *orientalis*	ssp. *patula*	ssp. *atriplicifolia*
Sugar beet $n=10$	0.047 0.82	0.071	0.039	0.033	0.052	0.033	0.047
ssp. *maritima* $n=7$	0.75	0.055 0.73	0.054	0.038	0.053	0.016	0.048
ssp. *adanensis* $n=2$	0.67	0.62	– 0.81	0.025	0.054	0.028	0.009
ssp. *macrocarpa* $n=1$	0.60	0.60	0.55	– –	0.037	–	–
ssp. *orientalis* $n=2$	0.70	0.71	0.63	0.55	– 0.83	0.002	0.066
ssp. *patula* $n=1$	0.65	0.65	0.63	0.57	0.59	–	–
ssp. *atriplicifolia* $n=1$	0.63	0.62	0.65	0.60	0.63	0.57	– –

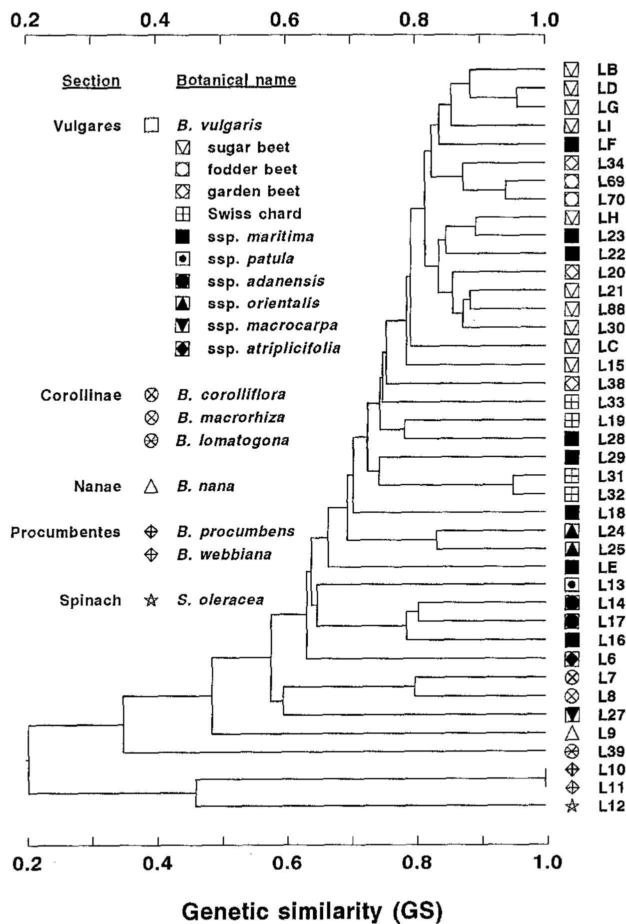

Fig. 5. Dendrogram of 41 *Beta* accessions revealed by UPGMA cluster analysis based on genetic similarity coefficients (GS) calculated from DNA fingerprint data of four minisatellite probes

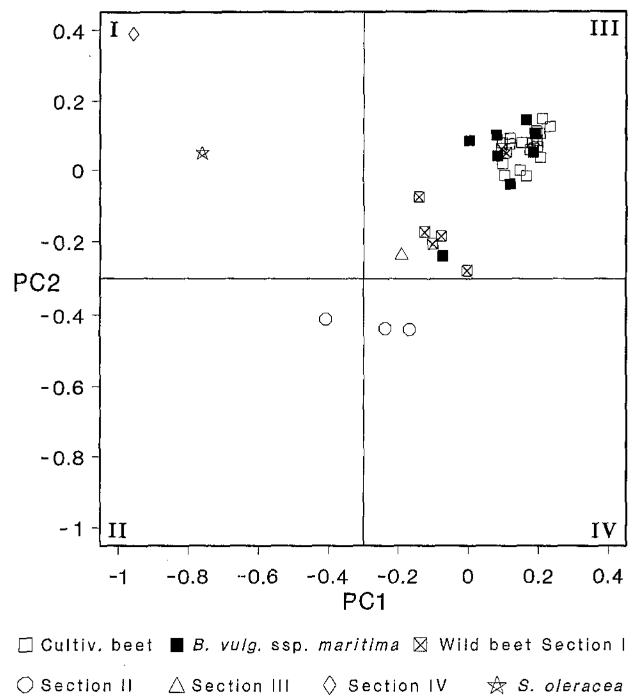

Fig. 6. Associations between 41 *Beta* accessions revealed by principal coordinate analysis (PCA) based on genetic similarity coefficients (GS) calculated from DNA fingerprint data of four minisatellite probes

The low GS estimates between the *B. maritima*/*B. vulgaris* group and the remainder of the *Beta* wild species (Table 3) are demonstrated by the results of the principal coordinate analysis presented in Fig. 6. The first and second principal coordinate (designated as PC1 and PC2)

explained 22.0% and 8.6% of the total variation in the RFLP data, respectively. With a single exception (LE), *B. vulgaris* species were clearly separated from the other species of section I.

Discussion

The proportion of repetitive sequences in the *B. vulgaris* genome is about 64% of the total DNA as revealed by analyzing shot-gun-cloned plasmid libraries (Jung et al. 1990). We have cloned four different repetitive sequences that yielded characteristic banding patterns after Southern hybridization of genomic *Beta* DNA restricted with *Hin*dIII. These probes can be used for varietal discrimination, screening of seed purity, monitoring the introgression of wild beet genes into sugar beet material, characterization of wild *Beta* accessions, and taxonomic studies. We found a substantial genetic variation at the molecular level, particularly among wild species of section I. By comparison, there is a low degree of genetic variation among sugar beet, reflecting the narrow genetic background of this cultivated species.

Earlier phylogenetic studies among *Beta* species and cultivars have been performed with morphologic markers (Buttler 1977b), chloroplast DNA (Fritzsche et al. 1987), mitochondrial DNA (Ecke and Michaelis 1990), and isozyme markers (Abe and Tsuda 1987). These markers were useful for the classification of the genus *Beta*, however, they were of limited value for demonstrating relatedness among *Beta* species within the same section and for varietal discrimination between cultivars (Ecke and Michaelis 1990). Nevertheless, a substantial degree of diversity exists at the level of nuclear DNA, as revealed by cDNA, random genomic DNA probes (Nagamine et al. 1989b, Mita et al. 1991), and rDNA probes. In the latter case, polymorphism between *Beta* species originated from the highly variable intergenic spacer region flanking the genes for the 18S rRNA (Santoni and Bervillé 1992). These results indicated that DNA markers are definitely more suitable for taxonomic studies among *Beta* species than the marker systems previously used.

Genetic similarity among species of section I

Generally, the estimates of genetic similarity based on RFLP patterns obtained with minisatellite DNA probes reflected the established phylogeny of *Beta* species. Cluster analysis and principal coordinate analysis revealed that the genetic similarity between cultivated beet and *B. maritima* accessions, all of which originated from the Atlantic coast, is high. In contrast, low GS was found between sugar beet and leaf beet. Fischer (1989) crossed the fodder beet variety 'Rote Walze' with the leaf beet variety 'Lukullus'. The latter is probably a duplicate of L19 used in our experiment. Since offspring in segregating generations were morphologically similar to sugar beet, Fischer (1989) concluded that sugar beet originated from the cross between a fodder and a leaf beet. The GS estimates presented here contradict this broadly accepted hypothesis.

We found high genetic diversity between *B. macrocarpa* (RNR 892234) and *B. maritima* (RNR 892254), both of which were collected at the same site in South Portugal. The first one was not classified into the cluster of section I but showed a closer similarity to section II species. These findings are in accordance with reports from the literature that suggest that *B. macrocarpa* is likely to be remote from the other species of section I: (1) *B. macrocarpa* × *B.vulgaris* ssp. *vulgaris* species hybrids were partially sterile and chlorotic (Abe and Shimamoto 1989). (2) Crossing barriers are also effective at the natural site (Frese et al. 1990). (3) *B. macrocarpa* displayed many unique alleles and null alleles in isozyme analyses (Abe and Tsuda 1987).

In summary, our results revealed that species of sections II, III, and IV are clearly distinguishable from those of section I, confirming the results of classic taxonomy. Additionally, our findings from principal coordinate analysis support the hypothesis that *B. atriplicifolia* and *B. orientalis* are doubtful taxa (Frese 1989).

Wild species of sections II, III, and IV

Our results demonstrated a close association between the two species of section II, *B. macrorhiza* and *B. corolliflora*, but the third species, *B. lomatogona*, was clearly separated from them. These results are in accordance with previous findings based on morphologic (Buttler 1977b) and rDNA markers (Santoni and Bervillé 1992). In general, genetic similarity indices between the species of sections II, III, and IV were comparatively low.

The wild species of section IV are of particular interest for sugar beet breeders because they carry a number of interesting resistance genes (see above). However, gene transfer by introgression has been hampered by the low viability of species hybrids and the lack of chromosome homology (Savitsky 1975). There are indications that genome-specific satellite DNA is involved in the expression of chromosome homology. Our data suggest that a high degree of heterogeneity exists between the genomes of *B. vulgaris* and *Procumbentes* species. Obviously, genome complexity and DNA content are much higher in the cultivated species. Three lines of evidence support this hypothesis: (1) Our DNA "fingerprint" experiments demonstrated that there is a strikingly low degree of genetic similarity between *B. vulgaris* and *Procumbentes* species. These wild species were classified into one cluster

together with spinach. The degree of DNA homology between both sections was below 34%, even at the level of single-copy sequences. (2) The physical size of the DNA of a *B. procumbens* chromosome carrying the gene for nematode resistance has recently been determined by pulsed field gel electrophoresis. It was in the order of only 25% of the size expected from the nuclear DNA content of the haploid *B. vulgaris* genome (Jung et al. 1992). (3) Minisatellite probes (Schmidt et al. 1991) and genomic and cDNA probes from *B. vulgaris* (Mita et al. 1991) have been described that do not hybridize with *Procumbentes* DNA. These results may shed new light on the relationships between section IV species and the remainder of the *Beta* species. Comparable studies with tomato (*Lycopersicon esculentum*) and potato (*Solanum tuberosum*) have resulted in a high degree of DNA homology. Linkage analyses revealed that these species differ by only five translocations. With few exceptions, all tomato probes hybridized perfectly with potato DNA (Bonierbale et al. 1988). In conclusion, the degree of homology among species of the same genus seems to be much lower for *Beta* species. Scott et al. (1977) suggested that *Procumbentes* species may be treated as a new genus, named *Patellifolia*. In the light of the molecular results presented here, their proposal should be carefully reassessed.

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