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Comparative restriction endonuclease analysis and molecular cloning of plastid DNAs from wild species and cultivated varieties of the genus *Beta* (L.)

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Summary. A phyletic tree of the genus *Beta* has been constructed based on EcoRI and PstI plastid DNA restriction patterns of eight species from three sections of the genus. In contrast to the remarkable morphological variability of the varieties of *B. vulgaris* the restriction patterns of the plastid DNA of this species were found to be almost identical. The comparison of plastic DNAs of *B. vulgaris crassa* fertile and sterile lines with 13 different restriction enzymes revealed only a single fragment polymorphism in the HindIII patterns. Hybridization analyses in the plastidal rDNA region revealed an interesting loss of an EcoRI restriction site in all cultivated *B. vulgaris* varieties in contrast to wild species. The results of the construction of clone banks for SalI and BamHI fragments of plastid DNA from fertile *B. vulgaris crassa* are reported and difficulties in the cloning of specific fragments are discussed.

Key words: *Beta* – Plastid DNA – Restriction enzyme analysis – Cytoplasmic male sterility

Introduction

Restriction enzyme analysis of plastid DNA has become a widely used approach for studying the phylogenetic relationship among plant genera and species (review Palmer 1985a, b). Plant plastid DNA molecules range between 120 kbp and 217 kbp and the resulting restriction patterns consist of distinct bands which can be used as molecular markers for characterizing a specific cytoplasm. Phyletic trees for plant genera have been established from restriction fragment polymorphisms in plastid DNA patterns (Bowman et al. 1983; Palmer and Zamir 1982).

In the genus *Beta,* 13 species have been described, some of great economic importance. Most of the *Beta* hybrid varieties in worldwide use have been produced with the aid of cytoplasmic male sterile (cms) lines. Most molecular analyses of cms lines suggest an involvement of mitochondria in the inheritance of cms, but diversities in restriction patterns have been described in mitochondrial and plastid DNAs of fertile and sterile lines (Mikami et al. 1984a, b, c, 1985; Samoylov et al. 1986). A physical map of the plastid DNA of fertile sugar beet (*Beta vulgaris altissima*) was published recently by Brears et al. (1986) and Kishima et al. (1986). The organization of plastid DNA is similar to that of many other higher plants. The plastid DNA molecule has an inverted repeat of approximately 25 kbp separated by unique DNA sequences of approximately 15 kbp and 82 kbp. So far, the genes *rrs, psbA* and *rbcL* have been localized on the physical map.

In this paper we report the results of restriction enzyme analyses of the plastid DNAs of different species within the genus *Beta.* Using a heterologous plastidal rDNA probe for hybridization, the plastid DNAs of three wild species can be distinguished from that of *B. vulgaris* lines. Similarly to the findings of Mikami et al. (1984b) for the plastid DNAs of fertile and sterile lines of *B. vulgaris altissima,* we can distinguish the HindIII patterns of plastid DNAs from fertile and sterile lines of *B. vulgaris crassa.* A phyletic tree for the analysed species is presented and the construction of clone banks of plastid DNA fragments of *B. vulgaris crassa* is reported.

Materials and methods

Beta species and varieties

All species and varieties were provided by the Institute of Beta Research, Klein Wanzleben, GDR. The following species and varieties were analysed:

Section *Beta*	*B. macrocarpa, B. orientalis, B. vulgaris* (*altissima* N, *crassa* N and cms, *altissima* × *conditiva, altissima* × *flavescens*);

section *Corollinae*	*B. lomatogona, B. trigyna;*
section *Procumbentes*	*B. patellaris, B. procumbens, B. webbiana.*

Isolation of plastid DNA

Plastid DNA was isolated following a method of Palmer (1982) which was adapted to our laboratory conditions by F. Baldauf. The plastids were purified in sucrose step gradients (60% and 30% sucrose). After lysis of plastids with 2% sarcosyle, the lysate was centrifuged in a discontinuous CsCl-ethidiumbromide-gradient for 16 h at 25,000 rpm (Herrmann et al. 1975). The DNA containing band was collected and centrifuged for 40 h at 40,000 rpm.

Restriction enzyme analysis

Up to 5 µg of plastid DNA were digested with various restriction enzymes following manufacturers' recommendations. DNA fragments were separated on 0.7% to 1.2% horizontal agarose gels. The restriction enzyme analysis of plastid DNA in organello followed the procedure published by Atchison et al. (1976) with some modifications (Metzlaff et al. 1981).

Molecular cloning of plastid DNA fragments

For this 0.5 µg vector plasmid pBR 322 and 4 µg plastid DNA were digested with BamHI and SalI, respectively, and ligated with 1 unit of T4-DNA-ligase for 2 h at 14 °C. The ligation mixture was used directly for transformation of *E. coli* DH1 cells.

Hybridization

Plasmids were labelled by nick translation (Maniatis et al. 1975) with ^{32}P- or ^{35}S-alphadATP (Amersham). The two recombinant plasmids used as probes contain the 16S rDNA and the 23S, 4.5S and 5S rDNA of spinach plastid DNA. These plasmids were constructed by cloning BamHI pt DNA fragments into pBR 322 (Barbier 1980). They were kindly provided by Professor R. Mache, University of Grenoble, France. The transfer of DNA fragments to nitrocellulose filters was carried out according to Southern (1975). Prehybridizations were performed at 65 °C. After hybridization, filters were washed twice for 15 min at 60 °C in 2×SSC, 0.1% SDS. Filters were used for hybridization up to 4 times after extensive washing for 30 min in 2×SSC, 0.1 *M* NaOH, for 5 times in 2×SSC and once in distilled water.

Results

Restriction enzyme analyses and hybridizations

On the basis of preliminary studies by Friedrich (1983) we have analysed restriction fragment polymorphism in PstI and EcoRI patterns of plastid DNAs and have established a phyletic tree for the genus *Beta* (Fig. 1). There is remarkable interspecific plastome variation between the species of different sections. The patterns of species from the sections *Corollinae* and *Beta* show a higher degree of homology to each other than to those of the section *Procumbentes*. We were also able to differentiate with the two restriction enzymes between species within the sections *Corollinae* and *Procumbentes*. The

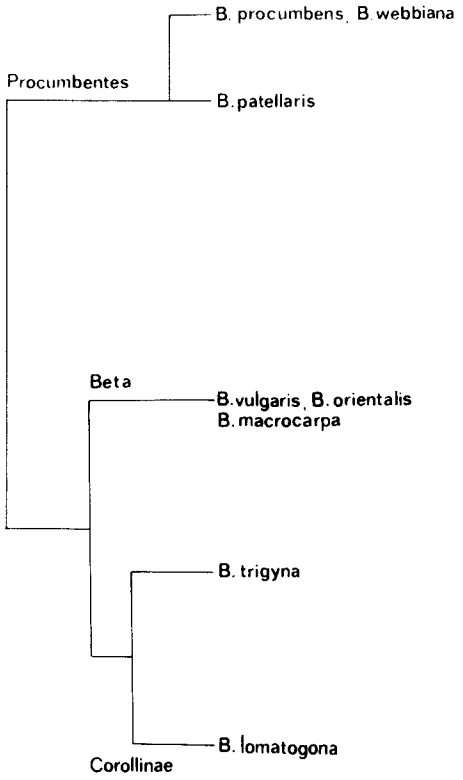

Fig. 1. Genetic tree of the genus *Beta* L. based on polymorphisms in PstI and EcoRI restriction endonuclease patterns of plastidal DNAs

differences in the restriction patterns of *B. trigyna* and *B. lomatogona* are particularly distinct. Therefore we agree with the differentiation of the section *Corollinae* into the subsections *Lomatogonae* and *Trigynae* which was suggested by several botanists and breeders (Barocka 1966). However, within the section *Beta* the EcoRI and PstI restriction patterns were identical. In this section, the strong morphological variability is not accompanied by a similar plastome variability.

In Fig. 2, the hybridization patterns of spinach rDNA probes to EcoRI patterns of plastid DNAs from various species and varieties of the genus *Beta* are shown. In the hybridization patterns of 16S rDNA (A, Fig. 2) these taxons show a unique hybridization band of 3.7 kbp. However, the wild species differ from the cultivated *B. vulgaris* lines in the hybridization patterns with the 23S- 4.5S-5S rDNA probe (B, Fig. 2). In these patterns three bands of 4.75 kbp, 1.26 kbp and 0.78 kbp can be observed in all three wild species, but the cultivated lines of *B. vulgaris* hybridize only with two bands of 4.75 kbp and 1.88 kbp. We interpret this finding as due to a small deletion in the plastomes of cultivated *B. vulgaris* varieties. It includes an EcoRI restriction site present in the plastomes of wild species.

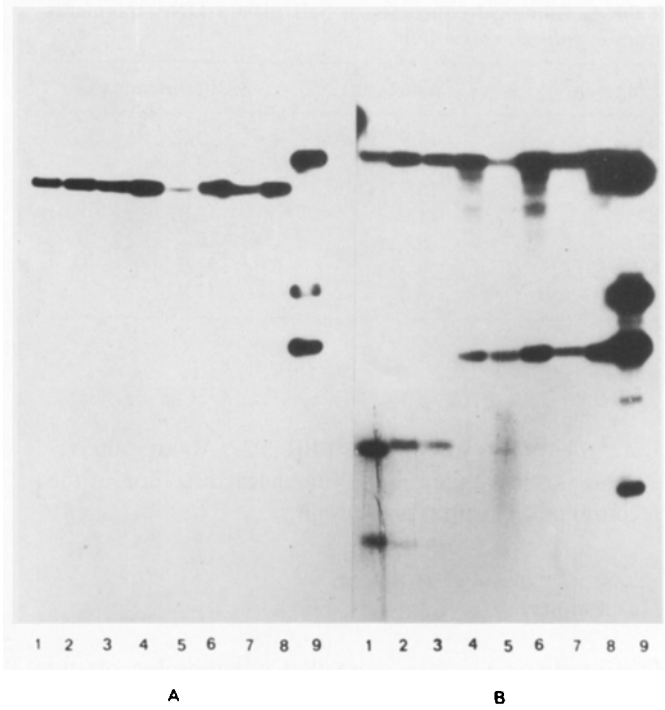

Fig. 2 A, B. Hybridization of spinach rDNA probes to EcoRI restriction endonuclease patterns of plastid DNAs from *Beta.* **A** 16S rDNA probe; **B** 23S-4.5S-5S rDNA probe. *1: B. trigyna; 2: B. patellaris; 3: B. procumbens; 4: B. vulgaris altissima × crassa; 5: B. vulgaris altissima × flavescens; 6: B. vulgaris altissima; 7: B. vulgaris crassa* (N); *8: B. vulgaris crassa* (cms); *9:* BglI digested pBR 322

Comparison of plastid DNAs from B. vulgaris crassa lines with normal and male sterile cytoplasms

Diversities in the plastid DNA restriction patterns of sugar beets (*B. vulgaris altissima*) with normal and sterile cytoplasms have already been reported by Mikami et al. (1984 a, b). Our aim was to check whether there are also differences between the plastid DNA restriction patterns from *B. vulgaris crassa* lines with normal and sterile cytoplasms. We compared the restriction patterns generated by 13 restriction enzymes – AluI, BamHI, BglII, BspI, ClaI, EcoRI, EcoRV, HaeIII, HincII, HindIII, HinfI, MspI and XbaI. Most of these patterns are shown in Fig. 3. Nearly all patterns are identical; only in the HindIII patterns we could detect a fragment polymorphism (Fig. 4): a 5.7 kbp HindIII fragment, present in the plastid DNA of the fertile line, is absent in the sterile line. Instead of this fragment two smaller fragments of 3.0 and 2.7 kbp occur in the plastid DNA of the sterile line.

These findings demonstrate that the plastid DNAs of fertile and sterile lines from *B. vulgaris crassa* are homologous. However, the additional HindIII site in the plastid DNA of the sterile line is a valuable molecular marker for distinguishing the fertile and sterile cytoplasms.

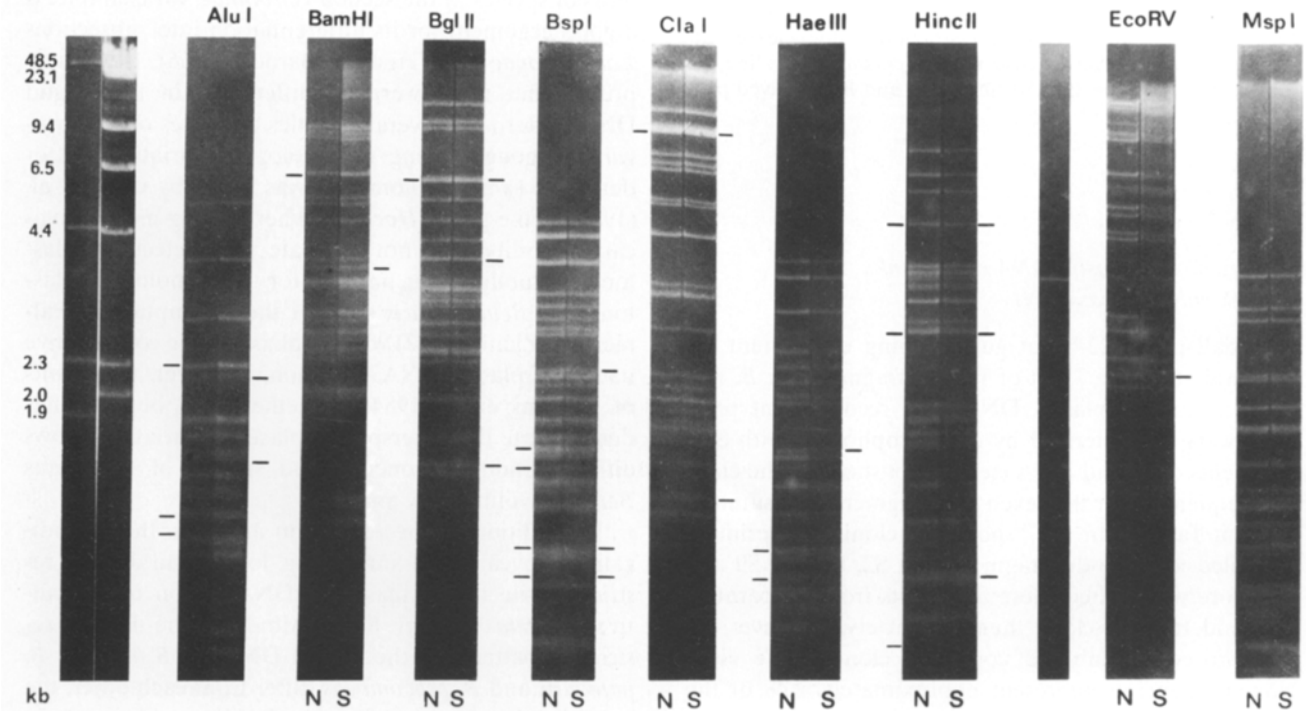

Fig. 3. Restriction endonuclease patterns of plastid DNA from *Beta vulgaris crassa:* N: fertile line; S: cms line; *bars on left,* hybridization with the spinach 23S-4.5S-5S rDNA probe; *bars on right,* hybridization with the spinach 16S rDNA probe

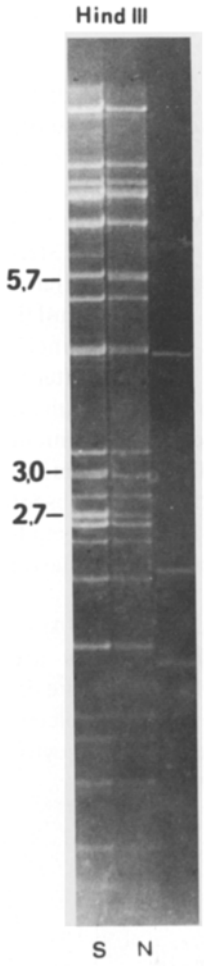

Fig. 4. Comparison of HindIII restriction endonuclease patterns of plastid DNA from *Beta vulgaris crassa:* S: cms line; N: fertile line; *right lane,* BamHI linearized and BglI cleaved pBR 322

Construction of plastid DNA clone banks from B. vulgaris crassa (N)

In a SalI-pBR 322-"shot gun"-cloning experiment we were able to clone 7 out of 11 SalI fragments of *B. vulgaris crassa* (N) plastid DNA. The recombinant plasmids were characterized by coelectrophoresis with SalI fragments of plastid DNA (results not shown). The cloning frequencies for the seven SalI fragments are summarized in Table 1. In this "shot gun"-cloning experiment we failed to clone the fragments S1, S2, S6 and S9 and therefore we isolated these fragments from preparative gels and tried to clone them separately. However, by this procedure only S2 could be cloned. The eight cloned fragments represent approximately 73% of the total plastid DNA. In a second "shot gun"-cloning experiment we cloned smaller BamHI fragments ranging from 14.6 to 0.5 kbp into pBR 322. About 300 recombinants were identified. The characterization of the recombinant plasmids is in progress.

Table 1. Cloning frequencies of SalI plastid DNA fragments from *B. vulgaris crassa* (N)

Fragment	Size (kbp)	Frequency (%)
S3	18.9	3.8
S4	17.2	3.8
S5	16.7	1.3
S7	11.9	12.8
S8	9.8	16.7
S10	6.3	32.0
S11	4.2	17.9

Discussion

Our results show once more that polymorphic plastid DNA restriction patterns are a powerful tool for elucidating the evolutionary relationships within plant genera. The phylogenetic tree established for the genus *Beta* correlates well with analyses based on taxonomic morphological data (Buttler 1977). Therefore, the plastid DNA analyses can provide useful additional data for the verification of hypotheses on the evolution of species. The distinct variability of the restriction patterns of species in the section *Corollinae*, for example, is a good argument for its differentiation into subsections *Lomatogonae* and *Trigynae* (Barocka 1966). It was surprising that there were no differences in the plastid DNA patterns of several varieties and lines of *Beta vulgaris,* although strong morphological variation is evident. The same phenomenon was found by Clegg et al. (1984) in the genus *Hordeum* where strong morphological variability does not correlate with detectable plastome variability. Our findings for the homologous plastomes in *Beta vulgaris* support the assumption of Palmer and Zamir (1982) who emphasized the conservative nature of plastid DNA evolution. However, the results of Mikami et al. (1984c) together with our findings demonstrate that interspecific plastome variation allows differentiation of noncultivated species of the genus *Beta* for evolutionary analyses.

In addition to the restriction analyses, the hybridizations revealed an interesting loss of an EcoRI restriction site in the plastidal rDNA region of the cultivated *Beta vulgaris* lines. Although the EcoRI restriction patterns of the plastid DNAs of *B. trigyna, B. patellaris* and *B. procumbens* differ from each other, the hybridization patterns for the rDNA are identical. In contrast all cultivated *B. vulgaris* lines share a loss of an

EcoRI site which is included in a small deletion of about 160 bp. It will be interesting to find out where this deletion is located on the plastid DNA of *B. vulgaris* and whether the two wild species of the same section *Beta, B. macrocarpa* and *B. orientalis,* also carry this deletion.

The results of the comparison of plastid DNAs of *B. vulgaris crassa* fertile and sterile lines with 13 different restriction enzymes are in agreement with Powling and Ellis (1983) and Mikami et al. (1984a, b, 1985) for *B. vulgaris altissima.* The plastid DNAs of fertile and sterile lines are almost identical in both varieties. In accordance with the results of Mikami et al. (1984b) for *B. vulgaris altissima,* we observed only one polymorphism in the HindIII patterns of *B. vulgaris crassa* lines. The correlation of these two findings is interesting as the sources of the Japanese *B. vulgaris altissima* line and our German *B. vulgaris crassa* line are certainly different.

We do not wish to suggest that the observed HindIII polymorphism is causally connected with cms. In *Beta,* the mitochondrial DNA variability between fertile and sterile lines is much stronger than that of plastid DNA (Powling and Ellis 1983; Mikami et al. 1984b, 1985). This was also observed in other plant species, for example in maize (Pring and Levings 1978) and sorghum (Conde et al. 1982; Pring et al. 1982). However, the HindIII polymorphism correlates with the distribution of the male sterile cytoplasms in *Beta vulgaris altissima* and *crassa* and is therefore a useful molecular marker for distinguishing the fertile and sterile lines.

For future molecular analyses of the plastid DNA of *Beta vulgaris crassa* we have begun to construct a clone bank of the plastid DNA of the fertile line. Up to now we have failed to clone three SalI fragments (S1, S6 and S9). By cloning the large fragment S2 (20.4 kbp) after fragment isolation we showed that it is possible to clone such large fragments using the vector pBR 322. It is still unclear why we failed to clone the smaller fragments S6 (12.1 kbp) and S9 (9.6 kbp). We suppose that these two fragments code for gene products which may cause a selective disadvantage for the bacterial host cells. Similar difficulties were reported by Zhu et al. (1982) for a 4.8 kbp fragment of *Nicotiana otophora* plastid DNA, by Palmer and Thompson (1981) for a 12.2 kbp fragment of *Pisum sativum* plastid DNA and by de Heij et al. (1983) for two fragments (10.0 and 1.0 kbp) of *Spirodela oligorrhiza* plastid DNA.

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