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Beta chloroplast genomes: analysis of Fraction I protein and chloroplast DNA variation

Y. Kishima [1], T. Mikami [1], A. Hirai [2], M. Sugiura [3] and T. Kinoshita [1, *]

[1] Plant Breeding Institute, Faculty of Agriculture, Hokkaido University, Sapporo 060, Japan
[2] Graduate Division of Biochemical Regulation, Faculty of Agriculture, Nagoya University, Chikusa, Nagoya 464, Japan
[3] Center for Gene Research, Nagoya University, Chikusa, Nagoya 464, Japan

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Summary. The interrelationships of *Beta* chloroplast genomes have been investigated on the basis of the analysis of Fraction I protein and chloroplast (ct) DNA. Three groups of the chloroplast genomes could be demonstrated by the difference in isoelectric points of the large subunit of Fraction I protein. Restriction enzyme analysis revealed inter- and intra-specific variations among the ctDNAs, which enabled us to detect seven distinct ctDNA types. In *Vulgares* and *Corollinae* species, the observed differences were physically mapped taking advantage of the restriction fragment map available for sugar beet (*B. vulgaris*) ctDNA. The DNA variations were found to result either from gains or losses of restriction sites or from small deletions/insertions, and most of them were located in the large single-copy region of the genome. Moreover, the ctDNAs from *Patellares* species are more diverged from those of other *Beta* taxa. Our results also indicate that there is a close correlation between the chloroplast genome diversity and the accepted taxonomic classification of the species included in this survey.

Key words: *Beta* – Fraction I protein – Chloroplast DNA – Comparative restriction site mapping – Phylogenetic relationship

Introduction

The genus *Beta* is conventionally recognized as comprising four sections, *Vulgares, Corollinae, Nanae* and *Patellares* (Coons 1954, 1975), though the number of species distinguished varies from 10 to 14 depending largely on the classification of the *Vulgares* section (Bosemark 1969).

Analysis of the phylogenetic relationships of the *Beta* species has been attacked through the combined approaches of comparative morphology, cytogenetics and artificial hybridization and genome analysis (Coons 1954, 1975; Bosemark 1969). The patterns of variation reported so far, and the interpretations based upon them, have been considered almost exclusively in terms of nuclear events. We previously described interspecific variability in restriction patterns of chloroplast (ct) DNAs among the six *Beta* species (Mikami et al. 1984b). In addition, recent reports of restriction fragment maps of sugar beet (*B. vulgaris* L.) ctDNA establish a basis for an investigation into the nature of such variations and the locations of these changes on the circular DNA molecule (Brears et al. 1986; Kishima et al. 1986).

We have analyzed restriction endonuclease fragment variation in ctDNAs from sugar beet and its close relatives, to provide new insights upon a phylogenetic outline for the genus. Fraction I protein (Ribulose 1,5-bisphosphate carboxylase/oxygenase, RuBisCO) has also been used as a marker for studying the interrelationship of *Beta* chloroplast genomes.

Materials and methods

Preparation of ctDNA

Sugar beet lines and the accessions of wild species used in this study are listed in Table 1. TK81-MS, NK169-MS and I-12CMS (R) are cytoplasmic male sterile lines with different nuclear genotypes. Their maintainer (Type-0) lines, TK81-0, NK169-0 and I-12-61L possess male fertile (N) cytoplasm. Intact chloroplasts were prepared from green leaves of individual plants as described (Mikami et al. 1984a). The purified chloroplasts were lysed in 2% Sarkosyl and then DNA was fractionated by CsCl density gradient centrifugation.

Restriction endonuclease analysis

Chloroplast DNA was digested with BamHI, EcoRI, HindIII, XhoI, PstI, PvuII and SmaI individually or in combination as

* To whom correspondence should be addressed

Table 1. The relationship of *Beta* chloroplast genomes determined by ctDNA comparison and Fraction I protein analysis

Species	Line or accession	Chromosome no. (2n)	Distribution of ctDNA restriction fragments[a]														Fraction I protein large subunit
			SmaI		PstI		PvuII		XhoI		BamHI		EcoRI		HindIII		
			T	S	T	S	T	S	T	S	T	S	T	S	T	S	
Section *Vulgares*																	
B. vulgaris	TK81-0	18	16	–	12	–	11	–	20	–	26	–	28	–	26	–	I
	TK81-MS	18	16	0	12	0	11	0	20	0	26	0	28	0	25	0	I
	NK169-0	18	16	0	12	0	11	0	20	0	26	0	28	0	26	0	I
	NK169-MS	18	16	0	12	0	11	0	20	0	26	0	28	0	25	0	I
	I-12-61L	18	16	0	12	0	11	0	20	0	26	0	28	0	26	0	I
	I-12CMS (R)	18	16	0	12	0	11	0	20	0	26	0	28	0	25	0	I
B. maritima	SP673000-0	18	16	0	12	0	11	0	20	0	26	0	28	0	26	0	I
	SP733050-01	18	16	0	12	0	11	0	20	0	26	0	28	0	26	0	I
	France	18	16	0	12	0	11	0	20	0	26	0	28	0	26	0	I
	Helgoland	18	16	0	12	0	11	0	20	0	26	0	28	0	26	0	I
	SP581103-0	18	16	1	12	0	12	2	20	0	26	0	28	0	26	0	I
	SP581105-0	18	16	1	12	0	12	2	20	0	26	0	28	0	26	0	I
B. macrocarpa	USDA	18	16	0	12	0	11	0	20	0	26	0	28	0	26	0	I
	Canary Island	36	16	1	12	0	12	2	20	0	26	0	28	0	26	0	I
B. patula	USDA	18	16	0	12	0	11	0	20	0	26	0	28	0	26	0	I
B. atriplicifolia	USDA	18	16	0	12	0	11	0	20	0	26	0	28	0	26	0	I
Section *Corollinae*																	
B. trigyna	WB47	54	14	4	10	4	13	4	19	2	26	3	28	4	27	4	II
B. corolliflora	WB48	36	14	4	10	4	13	4	19	2	26	3	28	4	27	4	II
B. lomatogona	SP743007-0	36	14	4	10	4	13	4	19	2	26	3	27	5	25	2	II
Section *Patellares*																	
B. patellaris	WB10	36	11	8	8	6	10	3	17	9	26	9	26	8	19	4	III
	WB14	36	11	8	8	6	10	3	17	9	26	9	26	8	19	4	III
B. procumbens	327a	18	11	8	8	6	10	3	17	9	26	9	26	8	19	4	III
	SP541205-03	18	11	8	8	6	10	3	17	9	26	9	26	8	19	4	III
B. webbiana	USDA	18	11	8	8	6	10	3	17	9	26	9	26	8	19	4	III

[a] T: Total bands; S: Species-specific bands which are not common with TK81-0

directed by the supplier (Takara Shuzo Co. Ltd). Restriction fragments were separated by electrophoresis in 0.4–1.8% agarose slab gels and visualized after staining with ethidium bromide as described (Sugiura and Kusuda 1979). The EcoRI and HindIII single or double digest fragments of lambda DNA and HaeIII digest fragments of φx174 RF DNA were used as size standards. Some restriction fragments were isolated from the gel according to the protocol of Herrmann et al. (1980) for secondary digestion. The restriction fragment map was constructed using the approach outlined by Seyer et al. (1981).

Isoelectric focusing of Fraction I protein

Fraction I protein was prepared from one gram of freshly harvested leaves according to the protocol of Hirai and Tsunewaki (1981). S-carboxymethylated protein (about 20 μg) was electrophoresed using isoelectric focusing with polyacrylamide gel containing 8 M urea. The methods of electrophoresis and gel staining were the same as those of Kung et al. (1974), except that the concentrations of acrylamide and Ampholine in the gel were changed to 9% and 0.9%, respectively.

Results

Analysis of Fraction I protein

Fraction I protein was isolated from 11 *Beta* species to examine the polypeptide composition by isoelectric focusing in the presence of 8 M urea (Fig. 1). As reported by Chen et al. (1976), the sugar beet protein was shown to be made up of three large subunit polypeptides and a single small subunit polypeptide. The chloroplast-encoded large subunit from Fraction I protein of other *Beta* species also resolved into a cluster of three polypeptides, but there were differences in the isoelectric points of the polypeptides, which could be divided into three groups. One cluster (Type I), which shared common isoelectric points with Fraction I protein of sugar beet, consisted of the large subunits of the proteins from four members of the section *Vulgares;* the proteins from the three *Corollinae* species com-

prised the Type II cluster; and the three *Patellares* species had the Type III cluster of the protein (Table 1).

In all five *Vulgares* species examined, there was an identical single band for the nuclear-encoded small subunit. On the contrary, the proteins from the *Corollinae* and the *Patellares* species contained three distinct small subunits but the difference in their isoelectric focusing patterns between the two sections was apparent (Fig. 1). All accessions were run at least three times: their banding patterns were found to be constant and as described above.

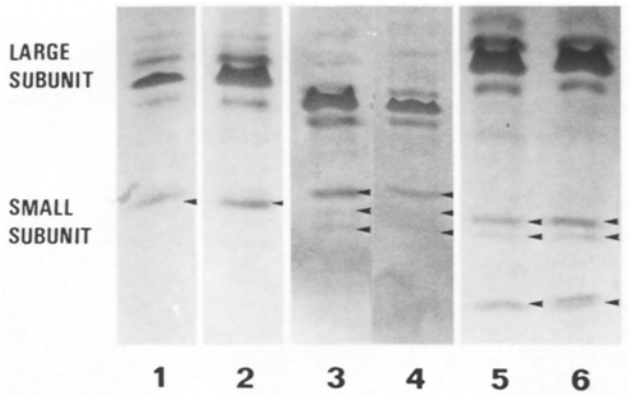

Fig. 1. Isoelectric focusing of Fraction I protein of six *Beta* species. The species are *B. vulgaris* (1), *B. maritima* (2), *B. trigyna* (3), *B. lomatogona* (4), *B. patellaris* (5) and *B. procumbens* (6). The chloroplast-encoded large subunit has three bands; the middle one being the most conspicuous. The small subunit polypeptides are marked with *arrows*

Comparative restriction endonuclease analysis

We have analyzed the ctDNAs of 11 *Beta* species by comparing the banding patterns obtained upon agarose gel electrophoresis of restriction enzyme digests, using SmaI, PstI, PvuII, XhoI, BamHI, EcoRI or HindIII. The first four enzymes were chosen because their cleavage site maps were available for sugar beet ctDNA (Brears et al. 1986; Kishima et al. 1986). In addition, the remaining three enzymes are expected to generate relatively a large number of fragments and therefore have higher resolving power to uncover differences.

Figure 2 illustrates typical SmaI restriction profiles of the ctDNAs, which can be used to sort the *Beta* chloroplast genomes into five distinct groups. All the sugar beet lines invariably gave rise to 16 SmaI fragments. The patterns were indistinguishable. A common restriction pattern was also shared by four of the *B. maritima* (acc. SP673000-0, SP733050-01, France, Helgoland) and one of the *B. macrocarpa* (USDA) accessions, *B. patula* and *B. atriplicifolia*. On the other hand, ctDNAs from two *B. maritima* (SP581103-0,

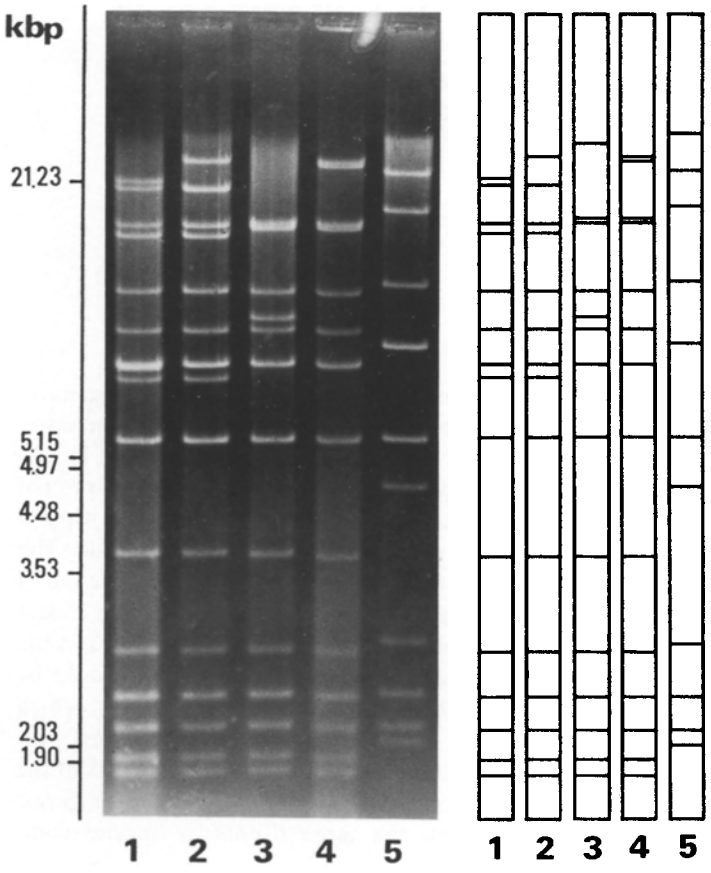

Fig. 2. SmaI restriction patterns of ctDNAs from five *Beta* species. The species are *B. vulgaris* (1), *B. maritima*, acc. SP581103-0 (2), *B. trigyna* (3), *B. lomatogona* (4) and *B. patellaris* (5). The DNA fragments were separated by electrophoresis in 0.8% agarose slab gel

Table 2. Fragment sizes (kb) of *Beta* ctDNAs generated by SmaI

B. vulgaris *B. maritima* (SP673000-0) *B. macrocarpa* (USDA) *B. patula* *B. atriplicifolia*	*B. maritima* (SP581103-0) *B. macrocarpa* (Canary Island)	*B. trigyna* *B. corolliflora*	*B. lomatogona*	*B. patellaris* *B. procumbens* *B. webbiana*
–	–	–	–	51.5
–	–	44.7	–	–
–	28.1	–	28.1	–
–	–	–	25.3	–
–	–	–	–	24.2
21.0	–	–	–	–
18.6	18.6	–	–	–
–	–	–	–	17.0
–	–	15.2	15.2	–
–	–	14.8	14.8	–
14.7	14.7	–	–	–
14.0	14.0	–	–	–
–	–	–	–	9.9
9.8	9.8	9.8	9.8	–
–	–	9.0	–	–
8.4	8.4	8.4	8.4	–
–	–	–	–	7.4 (2×)
7.1 (3×)	7.1 (2×)	7.1 (2×)	7.1 (2×)	–
6.7	6.7	–	–	–
5.4 (2×)	5.4 (2×)	5.4 (2×)	5.4 (2×)	5.4 (2×)
–	–	–	–	4.8
3.7	3.7	3.7	3.7	–
–	–	–	–	2.68
2.64	2.64	2.64	2.64	–
2.33 (2×)	2.33 (2×)	2.33 (2×)	2.33 (2×)	2.33 (2×)
2.10 (2×)	2.10 (2×)	2.10 (2×)	2.10 (2×)	2.10 (2×)
–	–	–	–	1.97 (2×)
1.86 (2×)	1.86 (2×)	1.86 (2×)	1.86 (2×)	–
1.71 (2×)	1.71 (2×)	1.71 (2×)	1.71 (2×)	–
0.75	0.75	–	–	–
Total: 148.39	148.39	149.24	148.94	148.48

There are some slight alterations in SmaI fragment compositions previously determined for the *B. vulgaris* (TK81-0) ctDNA (Kishima et al. 1986): Sma-1 is 21.0 kb and not 21.7 kb; Sma-16 (0.75 kb) was not recognized earlier but is included here. Brears et al. (1986) have mapped the Sma-16 fragment between Sma-1 and Sma-10 while our results indicate that this fragment lies between Sma-4 and Sma-10 (see Fig. 4)

SP581105-0) and a *B. macrocarpa* (Canary Island) accession showed a slight difference: a 28.1 kb fragment is unique and found only in these three accessions in *Vulgares* species (Table 2). The most likely explanation of the observed difference seems to be a single SmaI restriction site mutation within the 28.1 kb fragment, that has produced fragments of 21.0 and 7.1 kb in sugar beet lines.

The SmaI analysis revealed two groups of ctDNAs from the three *Corollinae* species. *B. trigyna* and *B. corolliflora* ctDNAs were characterized by four species-specific bands of 44.7, 15.2, 14.8 and 9.0 kb, whereas the appearance of the four unique bands (28.1, 25.3, 15.2 and 14.8 kb) discriminated *B. lomatogona* from sugar beet, *B. trigyna* or *B. corolliflora* (Table 2). On the basis of the cytogenetic evidence, Zossimovitch (1940) postulated that the hexaploid *B. trigyna* had originated through interspecific hybridization between tetraploid *B. corolliflora* and diploid *B. lomatogona.* If his proposal is correct, our results suggest *B. corolliflora* to serve as the female parent in the original cross. The difference of ctDNA between sugar beet and the three *Patellares* species is remarkable. They differed in 8 out of the 11 distinct SmaI bands (Table 2). This indicates

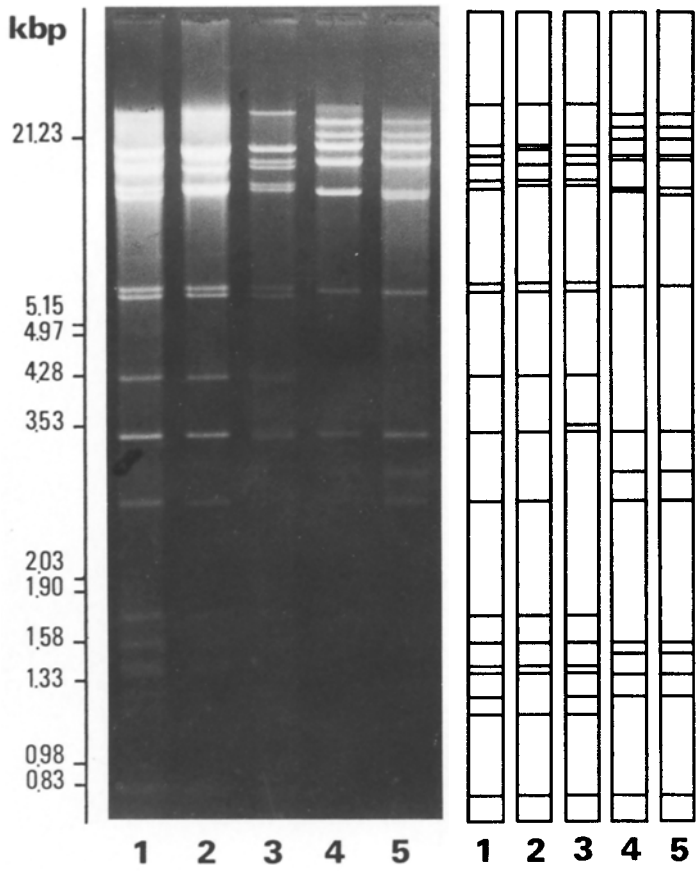

Fig. 3. XhoI restriction patterns of ctDNAs from five *Beta* species. The species are *B. vulgaris* (1), *B. trigyna* (2), *B. lomatogona* (3), *B. patellaris* (4) and *B. procumbens* (5). The DNA fragments were separated by electrophoresis in 0.8% agarose slab gel

that the *Patellares* species are more distantly related to sugar beet than any of the other *Beta* species examined so far.

Similar results were also obtained with ctDNA comparisons using the remaining six enzymes (Table 1). We found previously intraspecific polymorphism for a HindIII restriction site between normal (NK169-0) and male sterile (NK169-MS) cytoplasms in sugar beet (Mikami et al. 1984c). Moreover, XhoI digestion distinguished *B. patellaris* ctDNA from that of *B. procumbens* and *B. webbiana,* though the other enzymes employed here failed to differentiate ctDNAs of these three *Patellares* species from one another (Fig. 3). Thus, the chloroplast genomes of the species analyzed in this survey could be classified into seven groups (Table 1).

Mapping of the differences in ctDNAs from the Vulgares and Corollinae species

Figure 4 shows the serial order of the SmaI, PstI, PvuII and XhoI fragments in the ctDNA from the N cytoplasm of sugar beet. This map is essentially an adaptation of that constructed by Brears et al. (1986) and Kishima et al. (1986).

The similarities in the restriction patterns of ctDNAs from different *Vulgares* and *Corollinae* species indicated that the cleavage site map for the sugar beet chloroplast genome could serve as a reference to characterize and locate the observed differences. In order to substantiate the nature and location of these differences, the analysis of variant fragments was performed in the following manner. After ctDNA was cut with the enzyme that gave the distinctive pattern, the fragments were separated in low melting agarose gels. The variable band was excised and further digested with a second enzyme, followed by the coelectrophoresis of the resulting secondary fragments with primary and double digests of sugar beet and the wild species ctDNAs.

By repeating this experiment with a reverse combination of first and second enzymes, it was possible to trace the origin and conversion of each variable band. Figure 4 summarizes the ctDNA differences among the wild species relative to the map of sugar beet N ctDNA. We detected only two restriction site changes (probably the result of point mutation) in wild *Vulgares* species, while the DNA alterations in *Corollinae* species were shown to result either from gains or losses of restriction sites or from small (ca. 50–500 bp) deletions/insertions. The detailed organization of chloroplast genome in *Patellares* species is currently under investigation.

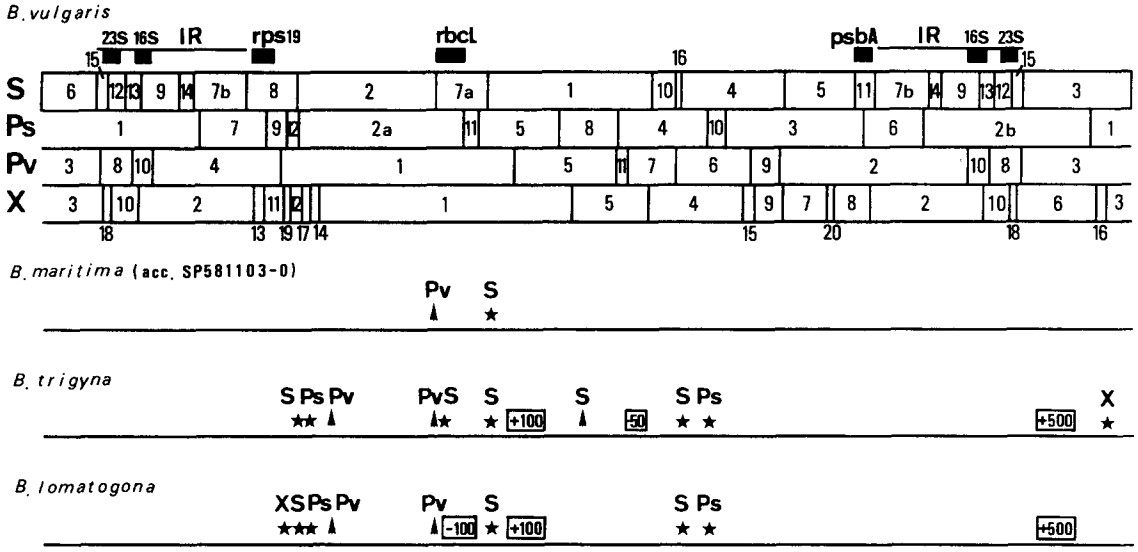

Fig. 4. Location of heterogeneity in the chloroplast genomes of the *Vulgares* and *Corollinae* species. The SmaI (S), PstI (Ps), PvuII (Pv) and XhoI (X) maps of *B. vulgaris* ctDNA (Brears et al. 1986; Kishima et al. 1986) are used as reference and presented in linearized form by cutting the small single-copy region between fragments Sma-3 and Sma-6. The differences for each species or accession are given relative to *B. vulgaris* (TK81-0) ctDNA. Insertions (+) and deletions (–) are *boxed* and their approximate sizes are indicated in bp. Mutations affecting restriction sites are marked by *arrow* (site gains) or *asterisk* (site losses). The extent of the inverted repeat (IR) and the positions of the genes for 23S rRNA, 16S rRNA, RuBisCO large subunit (*rbcL*), chloroplast ribosomal protein S19 (*rps19*) and 32kD protein of the photosystem II reaction center (*psbA*) are also shown

Discussion

Analysis with the combination of seven restriction enzymes revealed inter- and intra-specific variations among the ctDNAs of *Beta* species included in this survey. The degree of variation proved to be sufficient to classify the *Beta* chloroplast genomes into seven different groups. It is also worth noting that there is a close correlation between restriction analysis of ctDNA and isoelectric focusing studies on the chloroplast-encoded large subunit of Fraction I protein. The relationships of *Beta* species expressed in our assay agree well with those inferred from other taxonomic approaches such as comparative morphology and cytogenetics (Coons 1954, 1975; Bosemark 1969).

Previous studies have shown that various types of ctDNA mutations can be distinguished according to a specific phenotype of restriction fragment alterations. These mutations are restriction site changes, deletions/insertions, inversions and transpositions (Gordon et al. 1982; Palmer and Thompson 1982; Tsunewaki and Ogihara 1983; Bowman et al. 1983; Tassopulu and Kung 1984; Salts et al. 1984). In *Nicotiana* chloroplast genomes, for example, the most frequently detected variations are point mutations and deletions/insertions (Tassopulu and Kung 1984; Salts et al. 1984). Furthermore, they are not distributed evenly throughout the genome, but rather are confined in several regions. Our results also demonstrate that the ctDNA changes in *Vulgares* and *Corollinae* species can be attributed to the gains or losses of restriction sites and small deletions/insertions. Additionally, most of the alterations were shown to occur in the large single-copy region of the genome.

In the present study, no attempt has been made to estimate chloroplast genome divergence within *Beta* in greater detail, because we did not consider there to be enough data for this purpose. Our attention is focused on the *Patellares* species, whose ctDNAs are quite divergent from other *Beta* ctDNAs. This finding clearly needs confirmation by the detailed restriction mapping studies. Intensive analysis of the *Patellares* ctDNAs may provide important information about the mutational processes responsible for evolutionary changes in *Beta* chloroplast genome architecture.

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