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Mitochondrial gene variation and phylogenetic relationships in the genus *Beta*

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Abstract Restriction fragment length polymorphisms (RFLPs) for three mitochondrial genes, *coxI*, *coxII* and *atpA*, were used to determine mitochondrial (mt) DNA diversity in 21 accessions of the genus *Beta* representing wild and cultivated species. On the basis of distribution of the RFLP patterns these *Beta* genotypes were assigned into six distinct chondriome groups. A high degree of heterogeneity was found to exist between the mitochondrial genomes of the sugarbeet cultivar and the wild species of *Procumbentes* section. The polymorphic fragments from wild *Beta* species were cloned and subjected to fine mapping. We found that most of the RFLPs are due to sequence rearrangements rather than point mutations. Our data also suggest that the close linkage between *coxII* and *coxI* is taxonomically localized to an evolutionary lineage that led to *Vulgares* and *Corollinae* species but not to *Procumbentes* species. This linkage is most likely to have arisen via the mutation(s) that inserted the DNA segment containing *coxI* downstream of *coxII* in the common ancestor of *Vulgares* and *Corollinae* species. The results are discussed with regard to the taxonomic and phylogenetic relationships of the *Beta* species.

Key words Sugarbeet · *Beta* species · Mitochondrial gene · Taxonomy · Phylogeny

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Introduction

The genus *Beta* has been subdivided into four sections: *Vulgares* (syn. *Beta*), *Corollinae*, *Nanae* and *Procumbentes* (syn. *Patellares*) (Coons 1954). All of the selected beet cultivars fall into *B. vulgaris*, which is included in the section *Vulgares* (Ford-Lloyd and Williams 1975). This section also contains a wide range of more primitive beet crops and wild forms. The *Corollinae* and *Procumbentes* sections exclusively comprise wild species that are found along the islands and the Atlantic coasts of Africa, and in southeast Europe. The section *Nanae* is represented by a single alpine species, *B. nana*, which occurs on the mountain heights of Greece. Cultivated beets and their wild relatives have been subjected to morphological, cytogenetic, crossability and isozyme studies (Coons 1954,1975; Bosemark 1969; Oléo et al. 1986), and all have contributed in complementary ways to our current understanding of the genus.

Analysis of DNA variation has proved to be useful in elucidating the taxonomic and phylogenetic relationships between related taxa. Putative phylogenies can be routed bi-parentally by examining nuclear DNA sequences or, in organisms where male transmission of organelles does not occur, through the maternal lineage, by using cytoplasmic DNA markers. In *Beta* species, cDNA, rDNA and minisatellite DNA markers have been employed for revealing genetic variation in their nuclear genomes and for evolutionary studies (Nagamine et al. 1989; Mita et al. 1991; Santoni and Bervillé 1992; Jung et al. 1993). Chloroplast DNA (cpDNA) also enables phylogenetic relationships between the *Beta* species and the sections to be established (Mikami et al. 1984a; Kishima et al. 1987; Fritzsche et al. 1987). Mitochondrial DNA (mtDNA) analyses in beets have been carried out predominantly on male-sterile and male-fertile cultivars, with the aim of understanding the molecular mechanism(s) of cytoplasmic male sterility (Powling 1982; Mikami et al. 1984b, 1985; Boutin et al. 1987; Halldén et al. 1988; Mann et al. 1989; Senda et al. 1991, 1993). Little is known of the amount and distribution of

mtDNA diversity in the genus *Beta*. We describe herein the classification of mitochondrial genomes of *Beta* species on the basis of a probe hybridization analysis and clone mapping study. An attempt has also been made to relate our data to the existing taxonomic classifications.

Materials and methods

DNA preparation

The *Beta* species used in this study are listed in Table 1. MtDNA was isolated from either taproots (*B. vulgaris* sugarbeet) or green leaves (wild species) of field-grown plants as described (Mikami et al. 1984b; Hanson et al. 1986). Total cellular DNA was extracted from leaves using a CTAB-based DNA extraction method (Ishikawa et al. 1992).

Southern blot analysis

DNA was digested with restriction enzymes under the conditions specified by the enzyme supplier. Standard techniques were applied in the construction of Southern blots and their hybridization to [^{32}P]-labelled probes (Senda et al. 1991). The following cloned sugarbeet mitochondrial genes were used as probes: cytochrome *c* oxidase subunit I (*coxI*) (Senda et al. 1991), cytochrome *c* oxidase subunit II (*coxII*) 5′ exon and 3′ exon (Senda et al. 1991), and F_1-ATPase alpha subunit (*atpA*) (Senda et al. 1993).

DNA cloning

*Bam*HI or *Hin*dIII digests of mtDNAs from wild species were ligated into the plasmid vector pUC119 and transformed into *E. coli* strain JM109 (Senda et al. 1991). The sugarbeet mitochondrial gene probes were used to screen the recombinant plasmid library.

Results

RFLP analyses

The sugarbeet probes were used to hybridize Southern blots of *Bam*HI, *Eco*RI or *Hin*dIII-digested total DNAs from *B. vulgaris* sugarbeet and its wild relatives. Figure 1A shows the four restriction fragment length polymor-

Table 1 Restriction fragment length polymorphisms in mtDNAs of *Beta* species. Mitochondrial gene probes used were *coxI* (1600-bp *Eco*RI fragment; probe 3 in Fig. 2), *coxII* 5′ exon (800-bp *Xho*I-*Bam*HI fragment; probe 1 in Fig. 2), *coxII* 3′ exon (*Sal*I-*Hin*dIII 400-bp fragment; probe 2 in Fig. 2) and *atpA* (700-bp *Eco*RI-*Bam*HI fragment; probe 4 in Fig. 3). Sizes of hybridizing fragments are indicated in kb

Taxon	Cultivar/ accession	Filter-bound DNA hybridized with											
		coxI			*coxII* 5′ exon			*coxII* 3′ exon			*atpA*		
		*Bam*HI	*Eco*RI	*Hin*dIII	*Bam*HI	*Eco*RI	*Hin*dIII	*Bam*HI	*Eco*RI	*Hin*dIII	*Bam*HI	*Eco*RI	*Hin*dIII
Section *Vulgares*													
B. vulgaris	TK81-0	2.5	1.6	7.2	1.6	1.5	5.2	1.8	2.1	5.2	3.1	1.8	3.7
B. vulgaris	SP581103-0	2.5	1.6	7.2	1.6	1.5	5.2	1.8	2.1	5.2	3.1	1.8	3.7
ssp. *maritima*	SP673000-0	2.5	1.6	7.2	1.6	1.5	5.2	1.8	2.1	5.2	3.1	1.8	3.7
	WB37	2.5	1.6	7.2	1.6	1.5	5.2	1.8	2.1	5.2	3.1	1.8	3.7
	WB35b	2.5	1.6	7.2	1.6	1.5	5.2	1.8	2.1	5.2	3.1	1.8	3.7
	Guilianova	2.5	1.6	7.2	1.6	1.5	5.2	1.8	2.1	5.2	3.1	1.8	3.7
B. vulgaris ssp. *maritima* var *atriplicifolia*	USDA	2.5	1.6	7.2	1.6	1.5	5.2	1.8	2.1	5.2	3.1	1.8	3.7
B. vulgaris ssp. *adanensis*	Egypt	2.5	1.6	7.2	1.6	1.5	5.2	1.8	2.1	5.2	3.1	1.8	3.7
B. macrocarpa	Canary Island	2.5	1.6	7.2	1.6	1.5	5.2	1.8	2.1	5.2	3.1	1.8	3.7
B. patula	USDA	2.5	1.6	7.2	1.6	1.5	5.2	1.8	2.1	5.2	3.1	5.4,1.8	5.4,3.7
Section *Corollinae*													
B. trigyna	SP753012-0	4.4	1.6	7.2	4.8	3.2	3.2	4.4	2.0	3.2	3.1	1.8	3.7
	WB47	4.4	1.6	7.2	4.8	3.2	3.2	4.4	2.0	3.2	3.1	1.8	3.7
B. lomatogona	SP743007-0	4.4	1.6	7.2	4.8	3.2	3.2	4.4	2.0	3.2	3.1	1.8	3.7
	SP583041-0	4.4	1.6	7.2	4.8	3.2	3.2	4.4	2.0	3.2	3.1	1.8	3.7
B. corolliflora	WB48	4.4	1.6	7.2	4.8	3.2	3.2	4.4	2.0	3.2	3.1	1.8	3.7
Section *Procumbentes*													
B. patellaris	WB14	3.9	1.6	7.2	4.2	3.0	3.2	3.7	1.2	3.2	9.0	6.0	5.8
	WB29	3.9	1.6	7.2	4.2	3.0	3.2	3.7	1.2	3.2	9.0	6.0	5.8
B. patellaris ssp. *campanulata*	–	2.8	1.6	7.2	5.4	2.1	6.5	3.7	1.2	6.5	9.0	6.0,2.2	5.8,3.8
B. procumbens	327a	2.8	1.6	7.2	5.4	2.1	6.5	3.7	1.2	6.5	9.0	6.0	5.8
	SP541205-03	2.8	1.6	7.2	5.4	2.1	6.5	3.7	1.2	6.5	9.0	6.0	5.8
B. webbiana	WB11	2.8	1.6	7.2	5.4	2.1	6.5	3.7	1.2	6.5	9.0	6.0	5.8

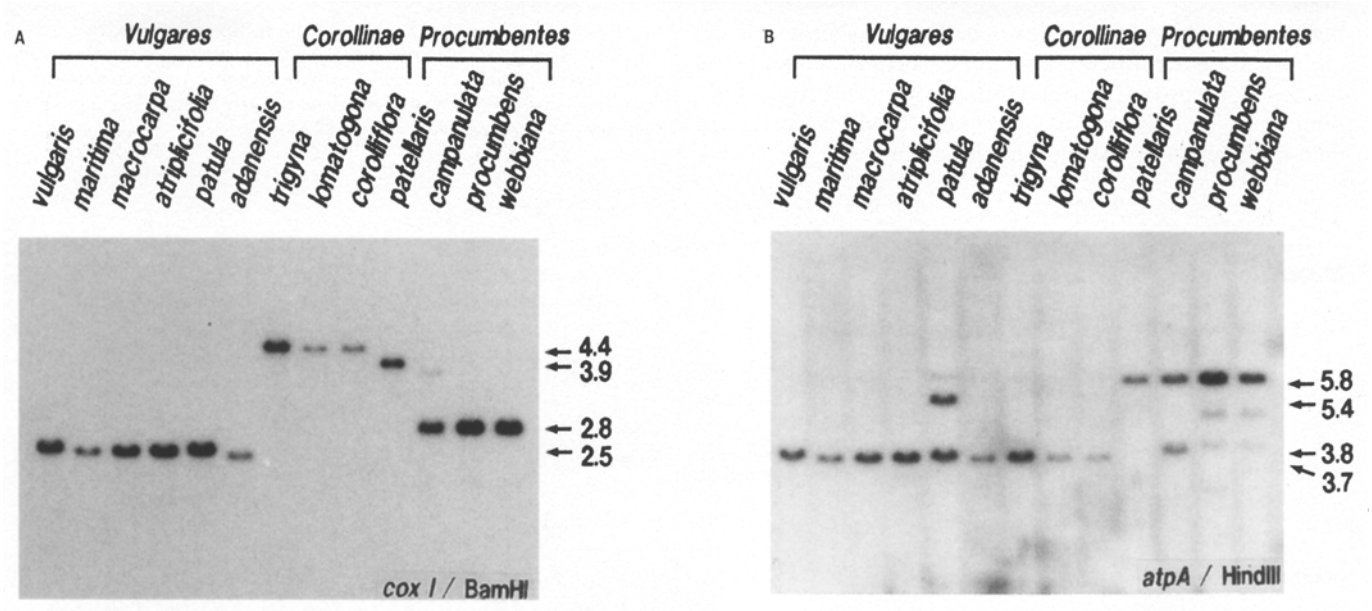

Fig. 1 A,B Southern blot hybridization of total DNAs from *Beta* species. DNA sources are: *B. vulgaris* ('TK81-0'), *B. vulgaris* ssp. *maritima* (SP581103-0), *B. macrocarpa* (Canary Island), *B. vulgaris* ssp. *maritima* var '*atriplicifolia*' (USDA), *B. patula* (USDA), *B. vulgaris* ssp. *adanensis* (Egypt), *B. trigyna* (SP753012-0), *B. lomatogona* (SP743007-0), *B. corolliflora* (WB48), *B. patellaris* (WB29), *B. patellaris* ssp. *campanulata, B. procumbens* (SP541205-03) and *B. webbiana* (WB11). DNA was digested with *Bam*HI (**panel A**) or *Hin*dIII (**panel B**) and electrophoresed on 0.8% agarose gel. The gel was blotted onto a nylon membrane filter and hybridized with the radiolabelled *coxI* (**panel A**) and *atpA* (**panel B**) probes. Sizes of the hybridizing fragments are indicated in kb

phisms (RFLPs) with the *coxI* probe. This probe hybridized with a 2.5-kb *Bam*HI restriction fragment in sugarbeet (cv 'TK81-0'). The 2.5-kb fragment was shared by the wild beet genotypes of the *Vulgares* section examined, while the 3 *Corollinae* species contained *coxI* sequences on a unique 4.4-kb *Bam*HI fragment. Hybridization of the *Bam*HI Southern blot with the *coxI* probe separated the accessions of the *Procumbentes* section into two groups: two accessions (WB14 and WB29) of *B. patellaris* displayed a polymorphic fragment of 3.9 kb that replaced a fragment of 2.8 kb found in all other *Procumbentes* accessions surveyed.

Additional polymorphisms were noted with other probe/restriction enzyme combinations. For instance, the combination of *atpA*/*Hin*dIII enabled us to distinguish *B. patula* from the rest of the *Vulgares* species: *B. patula* had an extra 5.4-kb *Hin*dIII fragment (Fig. 1B). As seen in Fig. 1B, the *Procumbentes* species possessed a 5.8-kb *Hin*dIII fragment in common when hybridized to the *atpA* probe, but in *B. patellaris* ssp. *campanulata* that probe hybridized to an additional 3.8-kb fragment. The distribution of RFLP patterns thus allowed us to assign these *Beta* genotypes into six mitochondrial groups (Table 1).

Clone mapping

In order to reveal the nature of the mutations giving rise to the RFLPs, the polymorphic fragments from wild *Beta* species were cloned into the plasmid pUC119 and subjected to fine mapping.

coxI *and* coxII

We previously sequenced the *coxI* and *coxII* genes from sugarbeet mitochondria and showed that these two genes are organized in a tandem array within 5.3 kb (Senda et al. 1991). The sugarbeet *coxI* and *coxII* probes were used to screen the *Bam*HI or *Hin*dIII library of mtDNAs from *B. trigyna* acc. SP753012-0, *B. patellaris* acc. WB29 and *B. webbiana* acc. WB11. The cloned fragments were then mapped with seven restriction enzymes, and the resulting maps were compared with the map of the sugarbeet counterpart (Fig. 2).

Our comparison indicated that the overall organization of the *coxII-coxI* gene cluster is conserved between sugarbeet and *B. trigyna*. Restriction site changes are confined to two areas: the 5′ flank of the *coxII* gene and the *coxII/coxI* intergenic spacer. Southern blot analysis showed significant homology between the *coxII/coxI* intergenic spacer of sugarbeet and that of *B. trigyna* (data not shown). On the other hand, the sequences 5′ to the *coxII* genes from the both species failed to hybridize with each other (data not shown), suggesting that the DNA sequence upstream of the gene is rearranged. An observation we next want to describe is that *B. patellaris* and *B. webbiana* lack the close linkage of *coxII* and *coxI* (Fig. 2). In addition, the *coxII* genes in *B. patellaris* and *B. webbiana* could be readily discriminated both from each other and from the sugarbeet gene by the divergence in their 5′ flanking sequences. This is also the case in the *B. patellaris* and *B. webbiana coxI* loci in which novel DNA sequences were found in their 5′ flanks (Fig. 2).

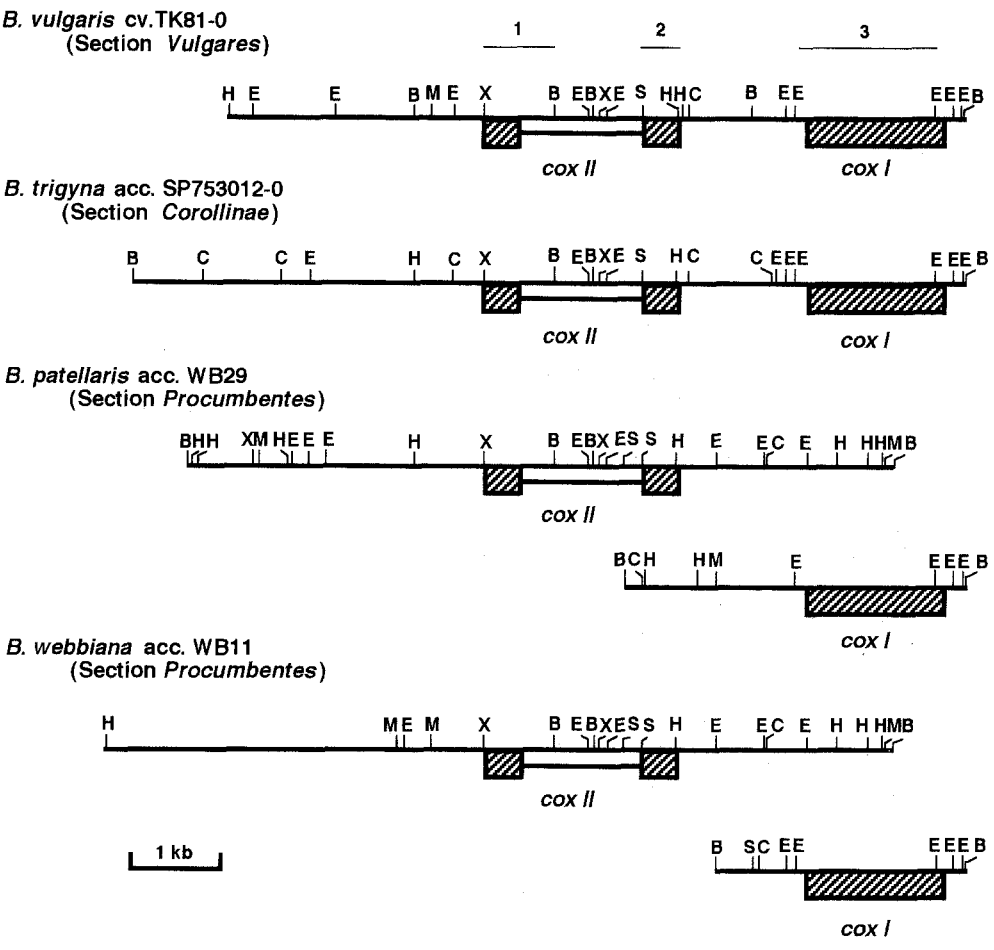

Fig. 2 Restriction maps of the *coxI*- and *coxII*-containing regions from the mitochondria of *B. vulgaris* sugarbeet and three wild *Beta* species. The sugarbeet mapping data are taken from Senda et al. (1991). The *hatched box* represents the exons, while the *bar* shows the *coxII* intron. Restriction sites are designated as follows: *B Bam*HI, *C Sac* I, *E Eco*RI, *H Hin*dIII, *M Sma*I, *S Sal*I, *X Xho*I. The location and extent of the three probe DNAs (probes 1, 2 and 3) used for Southern blot analysis is also indicated

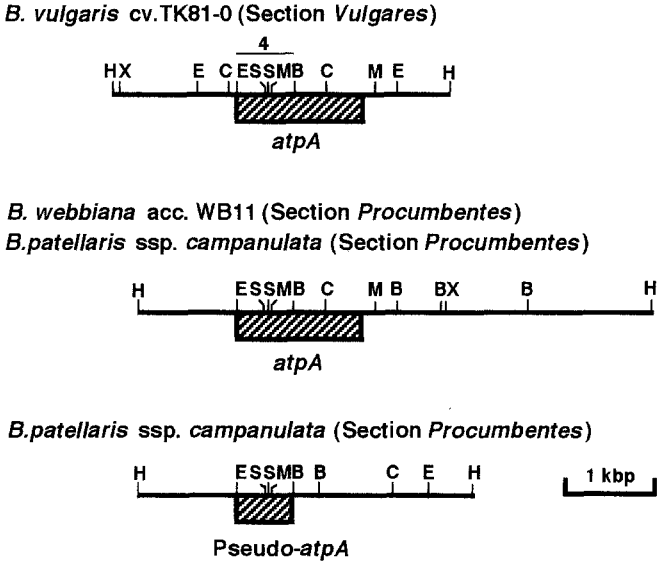

Fig. 3 Restriction maps of the *atpA*-containing regions from the mitochondria of *B. vulgaris* sugarbeet and two wild *Beta* species. The sugarbeet mapping data are taken from Senda et al. (1993). The *hatched box* represents the coding sequence and its homology in the pseudocopy. Restriction sites are designated as follows: *B Bam*HI, *C Sac*I, *E Eco*RI, *H Hin*dIII, *M Sma*I, *S Sal*I, *X Xho*I. The location and extent of the probe DNA (probe 4) used for Southern blot analysis is also indicated

atpA

Fig. 3 illustrates the restriction maps of the *atpA*-containing clones. In *B. patula* the number of seeds available was limited, thus making it difficult to prepare sufficient amounts of mtDNA for clone mapping. The pattern of restriction fragments indicates the nucleotide sequence conservation within the *atpA* reading frames carried by the 3.7-kb *Hin*dIII fragments from sugarbeet and by the 5.8-kb *Hin*dIII fragments from *B. patellaris* ssp. *campanulata* and *B. webbiana*. All of the restriction site polymorphisms were found in the 5′ and 3′ flanking regions of these genes, in comparison with the sugarbeet counterpart.

An additional 3.8-kb *Hin*dIII fragment was also isolated from a clone library of *B. patellaris* ssp. *campanulata* mtDNA. As shown in Fig. 3, the two *atpA* copies (the 5.8-kb and 3.8-kb fragments) of *B. patellaris* ssp. *campanulata* appear to have the 5′ flanking and the N-terminal coding sequences in common, but to diverge from each other after the gene-internal *Bam*HI site. This result led us to suppose that the 3.8-kb clone may harbour a truncated copy of the *atpA* gene.

Discussion

The large size, complexity and rearrangements of the plant mitochondrial genomes create highly variable restriction patterns that limit interpretation of experimental data (Lonsdale et al. 1984). In this study, those limitations were overcome by comparing the variability of restriction fragments identified by three mitochondrial gene probes (*coxI*, *coxII* and *atpA*) hybridized to Southern blots of DNAs from *Beta* species.

Our analysis led to five observations. (1) We detected enough mtDNA sequence diversity within the *Beta* genotypes examined to delineate six groups. (2) Identical RFLP profiles were observed in the *Vulgares* species, except for *B. patula*, which could be differentiated from the other members of the *Vulgares* section when the DNAs were probed with *atpA*. In this section, therefore, the remarkable morphological variability (Ford-Lloyd 1986) is not accompanied by a similar chondriome variability. (3) Kishima et al. (1987) and Fritzsche et al. (1987) previously reported that the *Corollinae* species can be separated into two groups on the basis of cpDNA RFLPs. This contrasts with the situation found in the present study where no mtDNA variation was noted amongst the 3 species belonging to the *Corollinae* section. (4) Our analysis failed to reveal mtDNA heterogeneity between *B. procumbens* and *B. webbiana*, an observation that supports the hypothesis that these 2 *Procumbentes* species are two extremes of a single ecospecies (Curtis 1968; Wagner et al. 1989). The mitochondrial genomes of *B. patellaris* ssp. *campanulata* and two *B. patellaris* accessions (WB14 and WB29) could be discriminated from each other, and from *B. procumbens* and *B. webbiana* mtDNAs, and as a result, there are three mtDNA haplotypes amongst the *Procumbentes* species. Note also that a high degree of heterogeneity exists between the mitochondrial genomes of sugarbeet and *Procumbentes* species. (5) The clone mapping study indicated that most of the fragment polymorphisms detected are due to sequence rearrangements.

The *Procumbentes* species have received great attention by sugarbeet breeders because they harbour the genes for resistance to the beet cyst nematode *Heterodera schachtii* (Reamon-Ramos and Wricke 1992). Also of economic importance are their monogermity and resistance to *Cercospora* leaf spot and curly top virus (Coons 1954). *B. patellaris* is known to occur in the western and outer Mediterranean region and on the Cape Verde, Canary, Salvage and Madeira Islands, whereas *B. procumbens* and *B. webbiana* are restricted to the Canary Islands (Coons 1954). The characterization of monosomic additions in *B. vulgaris* from the *Procumbentes* species led Reamon-Ramos and Wricke (1992) to suppose that earlier in evolution the 3 *Procumbentes* species had the same basic complement but that *B. patellaris* had undergone further polyploidization. The evolutionary relationships within *Procumbentes* section, however, remain to be fully elucidated. In this respect *B. patellaris* ssp. *campanulata* is particulary interesting in that it shares the sequence arrangements involving the *coxI* and *coxII* loci with *B. procumbens* and *B. webbiana* but not those with the two *B. patellaris* accessions.

As pointed out above, the mitochondrial genomes of the *Vulgares* and *Corollinae* species differ from those of the *Procumbentes* species in having the *coxI* locus approximately 1.5 kb downstream of the *coxII* locus. This raises the question of whether or not the close linkage of *coxII* and *coxI* resulted from the insertional mutation(s) in the lineage that gave rise to *Vulgares* and *Corollinae* species but not to *Procumbentes* species. Interestingly, the *coxI* locus was found to be absent in the vicinity of *coxII* in the mitochondrial genome of spinach (*Spinacia oleracea* L.), which is a member of the *Chenopodiaceae*, as is sugarbeet (Stern and Palmer 1986): spinach thus represents an evolutionary outgroup to *Beta* species. The results suggest that the common ancestor of *Vulgares* and *Corollinae* species underwent sequence rearrangements that placed the *coxI*-containing segment downstream of *coxII*.

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