Heredity **80** (1998) 624–632 *Received 11 July 1997*

Genetic relationships within the genus *Beta* determined using both PCR-based marker and DNA sequencing techniques

YULONG SHEN, BRIAN V. FORD-LLOYD & H. JOHN NEWBURY*
School of Biological Sciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

The sequences of ITS1 of the internal transcribed spacer regions of nuclear ribosomal DNA from 11 species or subspecies in four sections of the genus *Beta* were compared. Phylogeny of these wild beet taxa was inferred from the sequence data using phenetic and phylogenetic analyses. Multiple accessions from the same 11 taxa were subjected to random amplified polymorphic DNA (RAPD) analysis, and the data were analysed phenetically. With both molecular techniques and each analysis, three distinctive groups were formed: species from section *Beta* formed one group; species from section *Procumbentes* formed a very distinct group; and species from both section *Nanae* and section *Corollinae* clustered together forming the third group, which is closer to *Beta* than to *Procumbentes.* The RAPD data revealed within-section interspecies relationships that are consistent with those reported previously; this was not always the case using the single-locus sequence data.

Keywords: *Beta*, internal transcribed spacer (ITS), phylogeny, rDNA, RAPD, wild beets.

Introduction

The genus *Beta* is divided into four sections: *Beta*, *Corollinae*, *Nanae* and *Procumbentes*. Section *Beta* includes the crop species *B. vulgaris* which contains sugar beet, fodder beet and chards. The systematics within this economically important genus have been subject to disagreement (Kishima *et al*., 1987; Santoni & Berville, 1992), but molecular data may provide a solution to the taxonomic problems, as well as information about the possible evolutionary relationships of sections and species within the genus. DNA sequence data are increasingly providing valuable information for evolutionary studies (Olmstead & Palmer, 1994) and, in plants, chloroplast genes and the 18S–5.8S–25S ribosomal DNA have been the main sequences used for such studies. The 18S–5.8S–25S rDNA is attractive for phylogeny reconstruction because of high copy number, universality and diverse rate of evolution within and among component subunits and spacers (Baldwin, 1994). Although the regions of rDNA that encode the mature rRNAs are useful for deep phylogeny inference within angiosperms as a whole, the two internal transcribed spacers (ITS1 and ITS2; Fig. 1) of nuclear rDNA have evolved more rapidly than the coding regions that flank them and are suitable for comparison of closely related taxa. For example, phylogenetic analysis of ITS sequences from several genera in the subtribe Masinae of the Compositae (Baldwin, 1992) has yielded results highly concordant with the phylogeny of species based on chloroplast DNA restriction site mutations. The ITS sequences have also revealed phylogeny in the genus *Calycadenia* which has close agreement with that based on the interpretation of cytological and morphological data (Baldwin, 1992). In sugar beet, the 18S–5.8S–25S rDNA repeats have been mapped onto chromosomes using fluorescent *in situ* hybridization (Schmidt *et al*., 1994). Restriction fragment length polymorphism (RFLP) analysis of *Beta* species has been carried out using rDNA probes, and variation in restriction sites was detected within the nontranscribed intergenic spacer (IGS) region but not in the transcribed ITS region (Santoni & Berville, 1992).

As a contrasting technique, RAPD (Williams *et al*., 1990) has been widely used to reveal genetic variation in crops. The technique has been used successfully for revealing polymorphism within species (Demeke & Adams, 1994). For the study of genetic relationships above the species level, the use of RAPD has been criticized for revealing unreliable phylogenies because of possible lack of homology of

*Correspondence. E-mail: h.j.newbury@bham.ac.uk

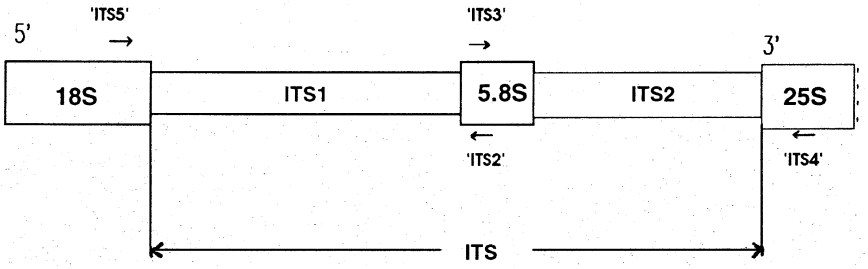

Fig. 1 Diagram of the organization of the ITS region of the 18S–5.8S–25S nuclear rDNA repeat. Arrows indicate approximate positions of primers for sequencing. Primer names follow White *et al*. (1990). The nontranscribed intergenic spacer (IGS) between 25S and 18S is not shown.

co-migrating bands (Brummer *et al*., 1995). However, several studies have used RAPD successfully to reveal relationships at the section level or above in several genera, including *Oryza* (Martin *et al*., 1997), *Medicago* (Brummer *et al*., 1995) and *Rosa* (Millan *et al*., 1996); in each case, the RAPD-derived phylogenies have been found to be in good agreement with those produced using other methods. In beets, RAPD analysis has so far been used in the taxonomic characterization of species/subspecies of wild annual beets within the section *Beta* (Shen *et al*., 1996) and five species within section *Corollinae* (Reamon-Buttner *et al*., 1996).

Here, we present results using ITS1 sequence data to illustrate the phylogenetic relationships of species in the genus *Beta.* We also show that similar genetic relationships are revealed using RAPD when species from each of the sections of the genus are analysed, confirming the reliability of RAPD for studying variation between species and sections of a genus.

Materials and methods

ITS (ITS1–5.8S–ITS2) amplification and sequencing

Thirteen accessions from the University of Birmingham Beet Germplasm Collection, representing members of all four sections, were used for ITS1 sequencing (Table 1). In addition, the ITS1 region of *Chenopodium album* was sequenced to provide an outgroup for the phylogenetic analysis. Leaf tissues from greenhouse-grown beet plants and a *C. album* plant growing naturally were used for DNA extraction according to the method of Sabir *et al*. (1992). Between two and 10 plants were sampled for each accession used in DNA sequencing. The yield of DNA was estimated by electrophoresis on an agarose gel (0.7%) along with λ phage DNA standards. PCRs were performed in 25 μL volumes containing 1.0 ng of genomic DNA, 200 μM of each dATP, dCTP, dGTP and dTTP, 2 μM of each primer, 1.0 U of *Taq* polymerase, 1 × ammonium incubation buffer and 2.5 mM magnesium chloride. The primers were ITS5 (5′-GGAAG-TAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTATATGATATGC-3′) (White *et al*., 1990; Fig. 1). Amplifications were performed in a thermocycler (Hybaid-Omnigene) programmed as follows: one cycle at 95°C for 2 min; two cycles of 30 s at 95°C, 1 min at 57°C and 2 min at 72°C; two cycles of 30 s at 95°C, 1 min at 55°C and 2 min at 72°C; 31 cycles of 30 s at 94°C, 1 min at 55°C and 2 min at 72°C; and finally 72°C for 5 min. In order to separate the amplified product from the residual primers, 10–12 reaction mixtures from one accession were pooled and subjected to electrophoresis in a 1.2% (w/v) low-melting-point agarose gel using TAE buffer (Sambrook *et al*., 1989). The desired DNA fragment was cut out of the gel and recovered using a Geneclean II Kit (Bio 101, Vista, CA, USA) according to the manufacturer's instructions. The DNA was used for sequencing using an Applied Biosystem 373A Automatic DNA Sequencer. Only the ITS1 region (White *et al*., 1990; Fig. 1) was sequenced, using ITS5 as a forward primer and ITS2 (ITS2: 5′-GCTGCGTTCTTCATCGATGC-3′; White *et al*., 1990; Fig. 1) as reverse.

Alignment of ITS1 sequences and phylogenetic reconstruction

The sequences of ITS1 obtained using the two different primers (ITS5 and ITS2) for each of the 13 samples of beet plus *C. album* were compared and checked. The sequences were aligned by eye using the LINE-UP program of the GCG sequence analysis software package (GCG, 1995). Divergence between ITS1 sequences in pairwise comparisons was calculated using the Kimura two-parameter method (Swofford & Olsen, 1990). In this method, different rates of transversions and transitions are taken into account and the gaps are not scored. The calculation was carried out using the DISTANCES program in the GCG software package (GCG, 1995). Phylogenetic trees were generated using the distances with the

GROWTREE program and using both UPGMA and neighbour-joining options.

RAPD analysis

DNA was extracted from 25 accessions representing all the taxa in the Birmingham *Beta* collection (Table 1) as described above. PCR amplification and electrophoresis were carried out in duplicate following the method used for rice (Virk *et al*., 1995). The primers used were OPG-12, OPH-16, OPK-10, OPM-17 and OPM-18 (Operon Technology). Except for accessions from section *Beta*, in which DNA samples used for amplification were pooled, all the other DNA samples were from single plants. The DNA amplification reactions were performed in a volume of 25 μL containing approximately 1 ng of genomic DNA, 200 μM of each dATP, dCPT, dGTP and dTTP, 0.4 μM primer, 1.0 U of *Taq* polymerase, 1 × ammonium incubation buffer and 2.5 mM magnesium chloride. The amplification was performed in a thermocycler (Hybaid-Omnigene) programmed as follows: one cycle of 95°C for 2 min; two cycles of 30 s at 95°C, 1 min at 37°C and 2 min at 72°C; two cycles of 30 s at 95°C, 1 min at 36°C and 2 min at 72°C; 41 cycles of 30 s at 94°C, 1 min at 36°C and 2 min at 72°C; and finally 72°C for 5 min. Aliquots of 16 μL of amplified products were loaded onto 1.4% (w/v) agarose gels for electrophoresis in 0.5 × TBE buffer and run at 200 V for about 2 h. Gels were stained with ethidium bromide and photographed under UV light using the IS-500 Digital Imaging System (Alpha Innotech Corporation). Only strong bands that were observed in both duplicate

Table 1 *Beta* material used

Accession	Species/subspecies	Abbreviation	Section	No. of plants used for RAPD	Country of origin*
NA	*Chenopodium album*	ALB†	NA	1	UK
B0205	*lomatogona*	LOM	*Corollinae*	2	Turkey
B0213	*lomatogona*	LOM†	*Corollinae*	2	Turkey
B0234	*lomatogona*	LOM	*Corollinae*	3	Turkey
B0221	*macrorhiza*	MCR	*Corollinae*	1	Turkey
B0397	*macrorhiza*	MCR†	*Corollinae*	2	Czech.
B0224	*trigyna*	TRI	*Corollinae*	1	Turkey
B0349	*trigyna*	TRI	*Corollinae*	1	
B0367	*trigyna*	TRI†	*Corollinae*	2	
B0368	*corolliflora*	COR	*Corollinae*	2	
B0403	*corolliflora*	COR	*Corollinae*	2	Armenia
B0537	*corolliflora*	COR†	*Corollinae*	2	
B0317	*maritima*	MAR	*Beta*	10‡	Greece
B0731	*maritima*	MAR	*Beta*	10‡	Algeria
B0334	*maritima*	MAR†	*Beta*	1	Greece
B0424	*adanensis*	ADA	*Beta*	10‡	Greece
B0423	*adanensis*	ADA†	*Beta*	1	Greece
B0588	*macrocarpa*	MCC†	*Beta*	4‡	Canary Is.
B0051	*vulgaris* spinach beet	VU1†	*Beta*	1	
B0079	*vulgaris* sugar beet	VU2†	*Beta*	1	
B0534	*patellaris*	PAT	*Procumbentes*	1	Canary Is.
B0555	*patellaris*	PAT	*Procumbentes*	2	Canary Is.
B1108	*patellaris*	PAT	*Procumbentes*	3	
B0576	*procumbens*	PRO(PR1)†	*Procumbentes*	5	Canary Is.
B0535	*procumbens*	PRO(PR2)†	*Procumbentes*	3	Canary Is.
B0536	*webbiana*	WEB	*Procumbentes*	3	
B0566	*webbiana*	WEB†	*Procumbentes*	3	Canary Is.
FD19	*nana*	NAN†	*Nanae*	1	
FD24	*nana*	NAN	*Nanae*	2	
FD25	*nana*	NAN	*Nanae*	1	

*Where information available.
†Accessions used for RAPD and sequencing.
‡Accessions for which pooled samples were used.

amplifications were scored. RAPD bands showing variation across the 48 samples were used as polymorphic markers and were scored as present (1) or absent (0) for each accession. The data were then subjected to analysis using Jaccard's similarity coefficient, and a dendrogram was generated using UPGMA cluster analysis (NTSYS-pc: Rohlf, 1992).

Results

ITS fragments were efficiently amplified for all the 13 beet accessions and *C. album*. A band of the expected size (740 bp) was obtained, although some accessions also yielded a faint band of about 480 bp, probably because of weak, non-specific primer binding within the ITS region during PCR. The sequences of ITS1 fragments were aligned and, by comparison of the sequence data with published sequences in other crops (Yokota *et al*., 1989), the boundaries of ITS1 were determined. Because of insertions or deletions, four gaps of between one and four bases were included in the alignment (Fig. 2). Species in section *Procumbentes* have 238–239 bp sequences, in section *Beta* 241 bp and in sections *Nanae* and *Corollinae* 243–244 base sequences, whereas ITS1 in *C. album* is 221 bp long. Omitting base insertions and deletions leaves 211 bases, of which 86 (41%) are variable across the 14 sequences.

Pairwise comparisons of transitions and transversions of the ITS1 regions and the Kimura two-parameter distances were calculated. The distances among pairwise comparisons ranged from 0 to 48.85. The distances between species within sections were usually less (0–2.53) than those between sections, except for those between species in sections *Nanae* and *Corollinae*.

The phylogenetic relationships among species were the same in the trees generated using the distance data and both UPGMA and neighbour-joining methods. To illustrate the distances between accessions, a phylogenetic tree was drawn using the data from the neighbour-joining analysis (Fig. 3). Three groups were formed: species from section *Procumbentes* formed a distinct group, species from section *Beta* formed another and species from sections *Nanae* and section *Corollinae* formed the third group (closer to *Beta* than to *Procumbentes*). In section *Beta*, two accessions from *B. vulgaris* and one accession from *B. maritima* did not show any variation in the ITS1 region and grouped as one. *Beta macrocarpa* is more distantly related to *B. vulgaris* than is *B. adanensis.* In section *Procumbentes*, two accessions from *B. procumbens* were separated; one of them had the same sequence as *B. webbiana* and the other differed by six bases. In section *Corollinae*, *B. macrorhiza* and *B. trigyna* did not show sequence variation in the ITS1 region and clustered together. *Beta nana,* the single species in section *Nanae*, was grouped with species from section *Corollinae*; *B. nana* was closer to *B. trigyna* and *B. macrorhiza* than to *B. corolliflora* or *B. lomatogona.*

Forty-eight DNA samples derived from 25 accessions were analysed using RAPD (see Table 1). The primers used were selections of those that had been used in a previous study (Shen *et al*., 1996), and all five primers produced reliable and reproducible banding patterns. Accessions from the four sections gave rise to characteristic RAPD profiles, which were so obviously different as to allow identification at the section level by eye. An example of the profiles is shown in Fig. 4. For numerical analysis, 31 polymorphic bands were scored, and the data were analysed using the simple matching coefficient and UPGMA clustering (NTSYS-pc: Rohlf, 1992) to produce a dendrogram (Fig. 5). All accessions from section *Procumbentes* formed one group (A) well separated from the others. Within this group, plants from each accession were generally clustered together. However, accessions putatively belonging to the same species were not always grouped together. The remainder of the accessions formed another main group consisting of three subgroups: accessions from section *Beta* formed one group (B), although the four accessions (B0317, B0424, B0588 and B0731) were well separated; accessions from section *Nanae* showed no polymorphism and formed a tight group (C), which is closer to section *Corollinae* than section *Beta*. Within section *Corollinae* (Fig. 5), accessions from *B. lomatogona* clustered together and formed a distinctive group (E). Accessions from the other three species grouped together (D) but, although plants from the same accessions did cluster together, the subgrouping did not correlate well with the putative species identification.

Discussion

ITS1 base sequence and RAPD banding data have been obtained for 11 species or subspecies of *Beta* and for *Chenopodium album*. Both the phylogeny obtained using the ITS1 sequence data and the genetic relationships revealed using RAPD are in general agreement with the relationships defined using other methods; these include relationships revealed from taxonomically more restricted analyses of RFLP studies of chloroplast (Kishima *et al*., 1987, 1995), mitochondrial (Senda *et al*., 1995)

```
           1                                                    50
      NAN  TCGAAACCTG CAAAGCAGA. GCAACCAGCG AACATGTTTT ACATCCGTGG
      TRI  TCGAAACCTG CAAAGCAGA. GCAACCAGCG AACATGTTTT ACATCCGTGG
      LOM  TCGAAACCTG CAAAGCAGA. GCAACCAGCG AACATGTTTT ACATCCGTGG
      COR  TCGAAACCTG CAAAGCAGA. GCAACCAGCG AACATGTTTA ACATCCGTGG
      MCR  TCGAAACCTG CAAAGCAGA. GCAACCAGCG AACATGTTTT ACATCCGTGG
      WEB  TCGAAACCTG CAAAGCAGA. GCAACCAGCG AACTTGTTTA TTACCCGTGG
      RP2  TCGAAACCTG CAAAGCAGA. GCAACCAGCG AACTTGTTTA TTACCCGTGG
      PR1  TCGAAACCTG CAAAGCAGA. GCAACCAGCG AACTTGTTTA TTACCCGTGG
      VU1  TCGAATC.TG CAAAGCAGA. GCAACCAGCG AACATGTTTT ACATCTGTGG
      VU2  TCGAATC.TG CAAAGCAGA. GCAACCAGCG AACATGTTTT ACATCTGTGG
      ADA  TCGAATC.TG CAAAGCAGA. GCAACCAGCG AACATGTTTT ACATCTGTGG
      MCC  TCGAATC.TG CAAAGCAGA. GCAACCAGCG AACATGTTTT ACATCTGTGG
      MAR  TCGAATC.TG CAAAGCAGA. GCAACCAGCG AACATGTTTT ACATCTGTGG
      ALB  TCGAAACCTG CCCAGCAGAA GCGACCAGAG AACATGTTTA TCAT......
CONSENSUS  TCGAAaCcTG CaaAGCAGA. GCaACCAGcG AACaTGTTTt acAtccgtgg

           51                                                   100
      NAN  GACGGGGGTG TTGGTATGAT GCTTTAGCTT GTGCCAGCCC CTCCCCC.CA
      TRI  GACGGGGGTG TTGGTATGAT GCTTCAGCTT GTGCCAGCCC CTCCCCC.CA
      LOM  GACGGGGGTG TTGGTACGAT GCTTTAGCTT GTGCCAGCCC CTCCCCC.CA
      COR  GACGGGGGTG TTGGTATGAT GCTTTAGCTT GTGCCAGCCC CTCCCCC.CA
      MCR  GACGGGGGTG TTGGTATGAT GCTTCAGCTT GTGCCAGCCC CTCCCCC.CA
      WEB  GATAGGAGTG CTTGCATGCA CCTTTGGTTC ATGCAAGTCC TTATCCTGCA
      RP2  GATAGGAGTG CTTGCATGCA CCTTTGGTTC ATGCAAGTCC TTATCCTGCA
      PR1  GATGGGAGTG CTTGCATGCA CCTGTGGTTC ATGCAAGCCC TTATCCTGCA
      VU1  GAAGGGGGTG CTGGCACGAT GCTTTGGGTT GTGCCAGCCC CTCCCC..CA
      VU2  GAAGGGGGTG CTGGCACGAT GCTTTGGGTT GTGCCAGCCC CTCCCC..CA
      ADA  GAAGGGGGTG CTGGGACGAT GCTTTGGGTT GTGCCAGCCC CTCCCC..CA
      MCC  GACGGGGGTG TTGGCACGAT TCTTTAGGTT GTGCCAGCCC CTCCCC..CA
      MAR  GAAGGGGGTG CTGGCACGAT GCTTTGGGTT GTGCCAGCCC CTCCCC..CA
      ALB  GAACGGGGTC GGGGTGAAAG CCCCTTCCTC AAGCCGGGGA ATCGCTCCGC
CONSENSUS  GAcgGGgGTg ctgGcatgat gCtttggcTt gtGCcaGccc cTccCcc.ca

           101                                                  150
      NAN  TGTCGCGGGG CACTCCTACT TGG.TGTGCT CCCCGGC.GA AAAAAACAAA
      TRI  TGTCGCGGGG CACTCCTACT TGG.TGTGCT CCCCGGC.GA AAAAAACAAA
      LOM  TGTCGCGGGG CACTCCTACT TGG.TGTGCT CCCCGGCAAA AAAAAACAAA
      COR  TGTCGCGGGG CACTCCTACT TGG.TGTGCT CCCCGGC.GA AAAAAACAAA
      MCR  TGTCGCGGGG CACTCCTACT TGG.TGTGCT CCCCGGC.GA AAAAAACAAA
      WEB  T..CGAGGGG CTCTCCTCTA TGGGAGTGCT TCTCGGC... AAATAACAAA
      RP2  T..CGAGGGG CTCTCCTCTA TGGGAGTGCT TCTCGGC... AAATAACAAA
      PR1  T..CGAGGGG CTCTCCTCTT TGGGAGTGCT TCTCGGC... AAATAACAAA
      VU1  TGTCGCGGGG CACTCCTACT TGG.TGTGCT CCCCGGC.GA AAAAAACAAA
      VU2  TGTCGCGGGG CACTCCTACT TGG.TGTGCT CCCCGGC.GA AAAAAACAAA
      ADA  TGTCGCGGGG CACTCCTACT TGG.TGTGCT CCCCGGC.GA AAAAAACAAA
      MCC  TGTCGCGGGG CACTCCTACT TGG.TGTGCT CCCCGGC.GA AAAAAACAAA
      MAR  TGTCGCGGGG CACTCCTACT TGG.TGTGCT CCCCGGC.GA AAAAAACAAA
      ALB  CTTGGCGGGG C......... .....GTCCT TCCCGGC..A CAATAACGAA
CONSENSUS  tgtcGcGGGG Cactcctact tgg.tGTgCT cCcCGGC.ga aAAaAACaAA

           151                                                  200
      NAN  CCCCGGCGCT TACTGCGCCA AGGAACATGA AAA.GGAGTG TGCCTGTCCC
      TRI  CCCCGGCGCT TACTGCGCCA AGGAACATGA AAA.GGAGTG TGCCTGTCCC
      LOM  CCCCGGCGCT TACTGCGCCA AGGAACATGA AAA.GGAGTG TGCCTGTCCC
      COR  CCCCGGCGCT TACTGCGCCA AGGAACATGA AAA.GGAGTG TGCCGGTCCC
      MCR  CCCCGGCGCT TACTGCGCCA AGGAACATGA AAA.GGAGTG TGCCTGTCCC
      WEB  CCCCGGCGCG GTCTGCGCCA AGGAACAAAA AA.TGGATTG TGCCTATT.T
      RP2  CCCCGGCGCG GTCTGCGCCA AGGAACAAAA AA.TGGATTG TGCCTATT.T
      PR1  CCCCGGCGCG GTCTGCGCCA AGGAACAAAA AAATGGATTG TGCCTATT.T
      VU1  CCACGGCGCT TACTGCGCCA AGGAACATGA AAA.GGAGTG TGCCTGTCCT
      VU2  CCACGGCGCT TACTGCGCCA AGGAACATGA AAA.GGAGTG TGCCTGTCCT
      ADA  CCACGGCGCT TACTGCGCCA AGGAACATGA AAA.GGAGTG TGCCTGTCCT
      MCC  CCACGGCGCT TACTGCGCCA AGGAACATGA AAA.GGAGTG TGCCTGTCCT
      MAR  CCACGGCGCT TACTGCGCCA AGGAACATGA AAA.GGAGTG TGCCTGTCCT
      ALB  CCCCGGCGCG GTCTGCGCCA AGGAACATGA ATACAAGTGT GCCCC.TCCG
CONSENSUS  CCcCGGCGCt taCTGCGCCA AGGAACAtgA Aaa.ggagtg tgCCtgTcct

           201                                                  248
      NAN  GTGCATCGGT TGGCCGGTGT GGGGACGTGG CACCCAGTAT TAAGAGAT
      TRI  GTGCATCGGT TTGCCGGTGT GGGGACGTGG CACCCAGTAT TAAGAGAT
      LOM  GTGCATCGGT TTGCCGGTGT GGGGACGTGG CACCCATTAT TAAGAGAT
      COR  GTGCATCGGT TTGCCGGTGT GGGGACGTGG CACCCATTAT TAAGAGAT
      MCR  GTGCATCGGT TTGCCGGTGT GGGGACGTGG CACCCAGTAT TAAGAGAT
      WEB  GTGC.TCGGT TTACCGAAGC AGGGATGTGG CACTCGATAT TA.TATAT
      RP2  GTGC.TCGGT TTACCGAAGC AGGGATGTGG CACTCGATAT TA.TATAT
      PR1  GTGC.TCGGT TTACCGAAGC AGGGATGTGG CACTCGGTAT TA.TATAT
      VU1  ATGCATCGGT TTGCCGGTGC GGGGATGTGG CACCCAGTAT TAAGAGAT
      VU2  ATGCATCGGT TTGCCGGTGC GGGGATGTGG CACCCAGTAT TAAGAGAT
      ADA  ATGCATCGGT TTGCCGGTGC GGGGATGTGG CACCCAGTAT TAAGAGAT
      MCC  GTGCATCGGT TTGCCGGTGC GGGGATGTGG CACCCAGTAT TAAGAGAT
      MAR  ATGCATCGGT TTGCCGGTGC GGGGATGTGG CACCCAGTAT TAAGAGAT
      ALB  CAG..TCGGT TCGCCGGCCG TGGAA.GTGG CACCAAGTCG TA.TATAA
CONSENSUS  gtGcaTCGGT TtgCCGgtgc gGGgAtGTGG CACccagTat TAagAgAt
```

Fig. 2 Aligned DNA sequences of the ITS1 region of the 18S–5.8S–26S nuclear ribosomal DNA from 13 beet accessions in the genus *Beta* and an outgroup species (*Chenopodium album*). Accessions abbreviations are indicated on the left and the gap positions are indicated as dots.

and total genomic DNA (Jung *et al*., 1993) and the distribution of satellite DNA families (Schmidt *et al*., 1991; Schmidt & Heslop-Harrison, 1993). There is also general agreement with the classifications produced by Santoni & Berville (1992) using variation in four restriction enzyme sites in the IGS region of rDNA in nine *Beta* species; no differences in restriction sites for the enzymes *Eco*RI, *Bam*HI, *Hin*dIII or *Sac*I were found in the ITS1 region of these species, and this is in agreement with our data.

A number of key points emerge from our results with respect to *Beta* taxonomy and phylogeny. For example, it is clear from both ITS1 sequence data and RAPD banding profiles that there is a considerable divergence of section *Procumbentes* from the other three sections; this agrees with results from other molecular studies (Kishima *et al*., 1987; Mita *et al*., 1991; Santoni & Berville, 1992; Senda *et al*., 1995). With regard to section *Nanae*, represented by the single species *B. nana*, the RAPD banding patterns suggest a lack of genetic variation within this species and that it is closely related to species of section *Corollinae.* This relationship has also been demonstrated in studies using RFLPs of minisatellite DNA (Jung *et al*., 1993) and hybridization of satellite DNA family probes cloned from species *B. corolliflora* and *B. trigyna* (Schmidt & Heslop-Harrison, 1993). In the studies of Jung *et al*. (1993), *Nanae* was grouped within section *Corollinae*, which is also the case for our ITS1 results, whereas our RAPD data and the study by Schmidt & Heslop-Harrison (1993) showed *Nanae* to be separated from, but closely allied to, section *Corollinae*. In either case, the conclusion must be that sections *Nanae* and *Corollinae* are closely related phylogenetically.

Section *Procumbentes* includes three species, *B. patellaris*, *B. procumbens* and *B. webbiana*. *Beta patel-*

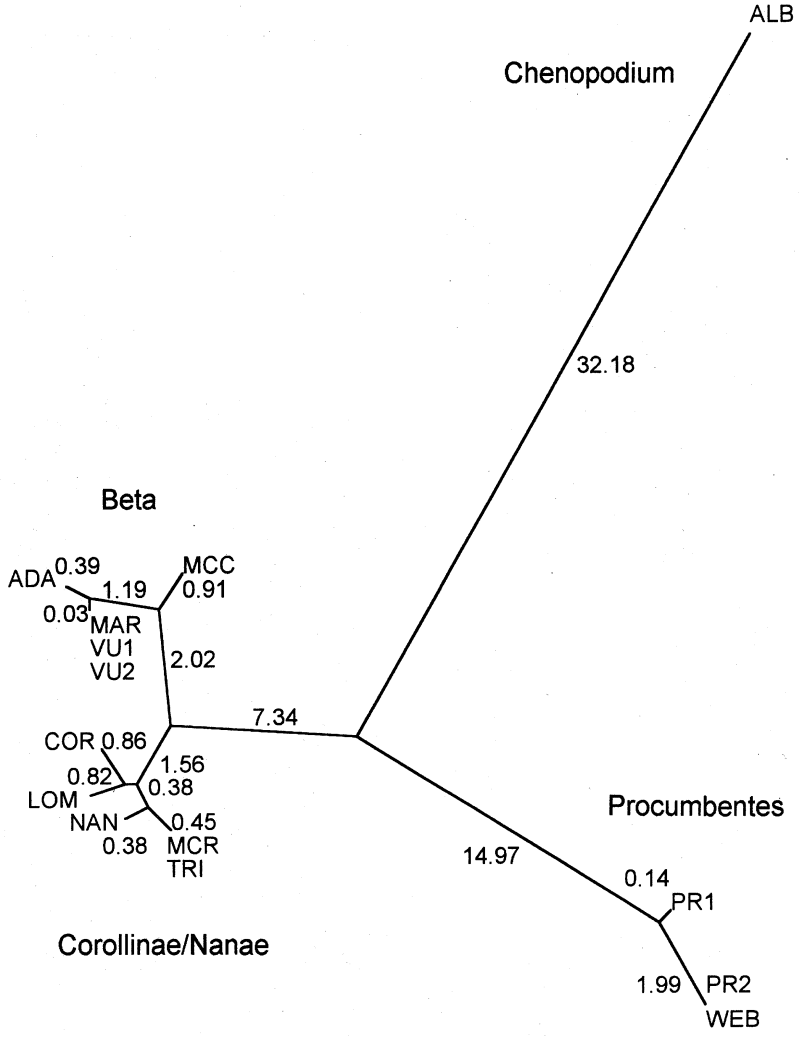

Fig. 3 Phylogenetic tree based upon data using the neighbour-joining method with the Kimura two-parameter distances displayed.

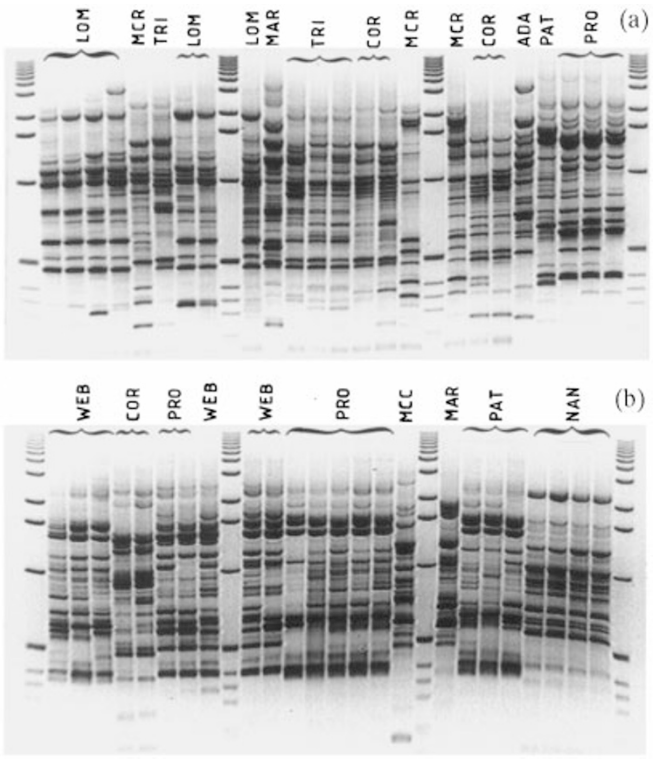

Fig. 4 RAPD profiles showing variation in band patterns between samples. The amplification was carried out using primer OPM17. M indicates molecular size markers. Lanes 1–24 (from left to right): (a) B0205–1, B0205–2, B0213–1, B0213–2, B0221–3, B0224–2, B0234–1, B0234–2, B0234–3, B0317, B0349–1, B0367–1, B0367–3, B0368–1, B0368–2, B0397–1, B0397–2, B0403–1, B0403–2, B0424, B0534–1, B0535–1, B0535–2 and B0535–3; (b) B0536–1, B0536–2, B0536–3, B0537–1, B537–2, B0555–1, B0555–2, B0566–1, B0566–2, B0566–3, B0576–1, B0576–2, B0576–3, B0576–4, B0576–5, B0588, B0731, B1108–1, B1108–2, B1108–3, FD19–1, FD24–1, FD24–2, FD25–1 (numbers after the hyphens are plant numbers within accessions). Species are identified using the abbreviations included in Table 1.

laris has been regarded as an allotetraploid (Abe *et al*., 1987; Senda *et al*., 1995), with *B. procumbens* and *B. webbiana* as diploids. Molecular studies have recently suggested that the last two are extreme forms of the same species (Wagner *et al*., 1989; Mita *et al*., 1991; Jung *et al*., 1993; Kishima *et al*., 1995), a situation originally proposed by Curtis (1968) based on morphological characteristics. Our ITS1 sequence data suggest that one accession (B0535), designated by the original collector as *B. procumbens*, may actually be *B. webbiana*. The RAPD results show that considerable variation exists within this section. The subgroupings do not correlate with the original classification into species, which might result from natural gene exchange and could support the single-species hypothesis, and an autopolyploid origin for *B. patellaris* (Santoni & Berville, 1992). Section *Procumbentes* is, however, the most genetically distinct of all sections within the genus, and this is supported by both RAPD and sequence data. Phylogenetic analysis of the sequence data, with *C. album* included as an outgroup to root the tree, indicates that section *Procumbentes* diverged from the other forms of beet at a relatively early stage in the evolution of the genus, a conclusion that is also supported by Santoni & Berville (1992).

As for section *Corollinae*, accessions of *B. lomatogona* formed a distinctive group using RAPD data, but much variation was observed in the group composed of the other three species. This may, in part, result from difficulty with taxonomic identification within this section. It seems just as likely, however, that it is because *B. macrorhiza*, *B. corolliflora* and *B. trigyna* are closely related phylogenetically. Reamon-Buttner *et al*. (1996) indicated that these three species are distinct from *B. lomatogona* and that *B. macrorhiza* is ancestral to both *B. corolliflora* and *B. trigyna*, which have evolved through various hybridizations. If this is the case, then one might expect a lack of discrimination between these species in our RAPD analysis. Santoni & Berville (1992) also separated *B. lomatogona* from *B. macrorhiza*, *B. trigyna* and *B. corolliflora* using variation in restriction sites within the IGS region of rDNA.

The sequence data provide direct information about mutations in DNA that accompany divergence of species. In further work, we have developed protocols that exploit these ITS1 sequence differences to define specific primers, which allow the identification of *Beta* taxa using single locus PCR (data not shown). However, in the context of genetic relationships, it is important to remember that the sequence data reflect variation at only a single locus (ITS1), whereas the RAPD analysis used data from 31 loci. A potential problem with the RAPD data is some co-migrating bands, which may not be allelic.

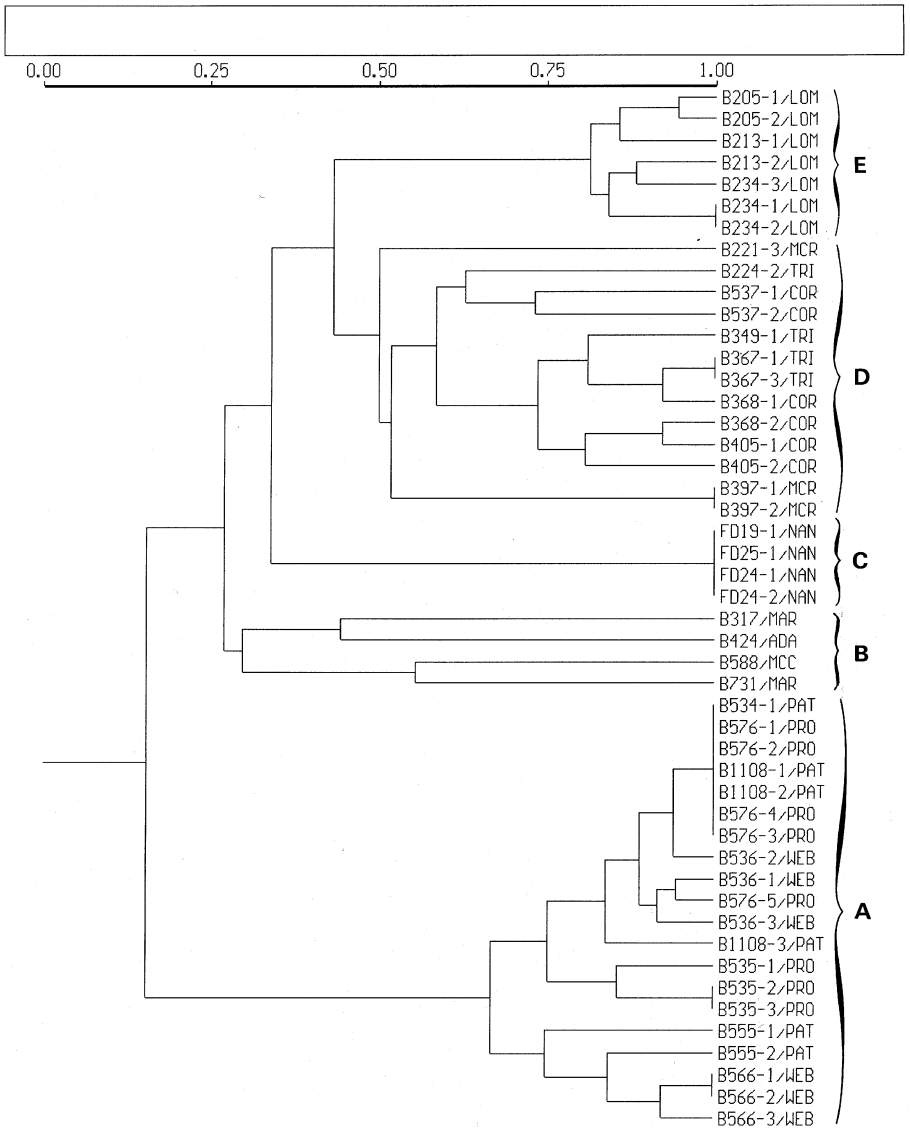

Fig. 5 Dendrogram of genetic similarity (Jaccard's) and the UPGMA cluster method using the 48 wild beet samples and 31 RAPD markers. Numbers after the hyphens are individual plant numbers; species abbreviations are also included.

The results obtained here indicate that the patterns of interspecific relationships revealed by the RAPD data are broadly similar to those revealed using sequence data, although there are some differences. For instance, the plants of *B. macrorhiza* and *B. trigyna* possessed identical ITS1 sequences and were closely linked to *B. nana*; however, all three were clearly separated using RAPD data. This suggests that the RAPD data may provide a more accurate picture of relationships at the species level. This is supported by the concordance of these results with those of Reamon-Buttner *et al*. (1996) even to the extent that two forms of *B. macrorhiza* may exist; the two accessions used here appear to be very distinct. Overall, the results demonstrate the value of using more than one molecular technique for the analysis of genetic relationships.

References

ABE, J., NAKASHIMA, H. AND TSUDA, C. 1987. Isozyme variation and species relationships in the genus Beta. *Mem. Fac. Agr. Hokkaido Univ.*, **15**.

BALDWIN, B. G. 1992. Phylogenetic utility of the internal transcribed spacer of nuclear ribosomal DNA in plants: an example from Compositae. *Mol. Phylogenet. Evol.*, **1**, 3–16.

BALDWIN, B. G. 1994. Molecular phylogenetics of *Calycadenia* (Compositae) based on ITS sequences of nuclear ribosomal DNA: chromosomal and morphological evolution re-examined. *Am. J. Bot.*, **81**, 254.

BRUMMER, E. C., BOUTON, J. H. AND KOCHERT, G. 1995. Analysis of annual *Medicago* species using RAPD markers. *Genome*, **38**, 362–367.

CURTIS, G. J. 1968. Observations of fruit shape and other characters in the species of the section *Patellares*, genus *Beta*. *Euphytica*, **17**, 485–491.

DEMEKE, T. AND ADAMS, R. P. 1994. The use of PCR-RAPD analysis in plant taxonomy and evolution. In: Griffin, H. G. and Griffin, A. M. (eds) *PCR Technology: Current Innovations*, pp. 179–191. CRC Press, Boca Raton, FL.

GCG. 1995. *GCG Program Manual Version 8.1 (VMS)*, Vol. 2. Genetics Computer Group Inc., Madison, WI.

JUNG, C., PILLEN, K., FRESE, L., FAHR, S. AND MELCHINGER, A. E. 1993. Phylogenetic relationships between cultivated and wild species in the genus *Beta* revealed by DNA 'fingerprinting'. *Theor. Appl. Genet.*, **86**, 449–457.

KISHIMA, Y., MIKAMI, T., HIRAI, A., SUGIURA, M. AND KINOSHITA, T. 1987. *Beta* chloroplast genomes: analysis of fraction I protein and chloroplast DNA variation. *Theor. Appl. Genet.*, **73**, 330–336.

KISHIMA, Y., YANAI, Y., KINOSHITA, T. AND MIKAMI, T. 1995. Physical mapping of differences between the chloroplast DNAs of *Beta vulgaris* and *B. webbiana. Plant Breed.*, **114**, 361–362.

MARTIN, C., JULIANO, A., NEWBURY, H. J., LU, B.-R., JACKSON, M. T. AND FORD-LLOYD, B. V. 1997. The use of RAPD markers to facilitate the identification of *Oryza* species within a germplasm collection. *Genet. Res. Crop Evol.*, **44**, 175–183.

MILLAN, T., OSUNA, F., COBOS, S. AND TORRES, A. M. 1996. Using RAPDs to study phylogenetic relationships in *Rosa*. *Theor. Appl. Genet.*, **92**, 273–277.

MITA, G., DANI, M., CASCIARI, P., PASQUALI, A., SELVA, E., MINGANTI, C. AND PICCARDI, P. 1991. Assessment of the degree of genetic variation in beet based on RFLP analysis and the taxonomy of *Beta. Euphytica*, **55**, 1–6.

OLMSTEAD, R. G. AND PALMER, J. D. 1994. Chloroplast DNA systematics: a review of methods and data analysis. *Am. J. Bot.*, **81**, 1205–1224.

REAMON-BUTTNER, S. M., WRICKE, G. AND FRESE, L. 1996. Interspecific relationship and genetic diversity in wild beets in section Corollinae genus *Beta*: isozyme and RAPD analyses. *Genet. Res. Crop Evol.*, **43**, 261–274.

ROHLF, F. J. 1992. *NTSYS-pc*, *Numerical Taxonomy and Multivariate Analysis System*. Exeter Software, New York.

SABIR, A., NEWBURY, H. J., TODD, G., CATTY, J. P. AND FORD-LLOYD, B. V. 1992. Determination of genetic stability using isozymes and RFLPs in beet plants regenerated *in vitro*. *Theor. Appl. Genet.*, **84**, 113–117.

SAMBROOK, K., FRITSCH, J. F. AND MANIATIS, T. 1989. *Molecular Cloning: a Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

SANTONI, S. AND BERVILLE, A. 1992. Characterisation of the nuclear ribosomal DNA units and phylogeny of *Beta* L. wild forms and cultivated beets. *Theor. Appl. Genet.*, **83**, 533–542.

SCHMIDT, T. AND HESLOP-HARRISON, J. S. 1993. Variability and evolution of highly repeated DNA sequences in the genus *Beta*. *Genome*, **36**, 1074–1079.

SCHMIDT, T., JUNG, C. AND METZLAFF, M. 1991. Distribution and evolution of two satellite DNAs in the genus *Beta*. *Theor. Appl. Genet.*, **82**, 793–799.

SCHMIDT, T., SCHWARZACHER, T. AND HESLOP-HARRISON, J. S. 1994. Physical mapping of rRNA genes by fluorescent in-situ hybridization and structural analysis of 5S rRNA genes and intergenic spacer sequences in sugar beet (*Beta vulgaris*). *Theor. Appl. Genet.*, **88**, 629–636.

SENDA, M., ONODERA, Y., KINOSHITA, T. AND MIKAMI, T. 1995. Mitochondrial gene variation and phylogenetic relationships in the genus *Beta*. *Theor. Appl. Genet.*, **90**, 914–919.

SHEN, Y., NEWBURY, H. J. AND FORD-LLOYD, B. V. 1996. The taxonomic characterisation of annual *Beta* germplasm in a genetic resources collection using molecular markers. *Euphytica*, **91**, 205–212.

SWOFFORD, D. L. AND OLSEN, G. J. 1990. Phylogeny reconstruction, In: Hillis, D. M. and Moritz, C. (eds) *Molecular Systematics*, pp. 411–501. Sinauer Associates, Sunderland, MA.

VIRK, P. S., FORD-LLOYD, B. V., JACKSON, M. T. AND NEWBURY, H. J. 1995. Use of RAPD for the study of diversity within plant germplasm collections. *Heredity*, **74**, 170–179.

WAGNER, H., GIMBEL, E. M. AND WRICKE, G. 1989. Are *Beta procumbens* Chr. Sm. and *Beta webbiana* Moq. different species? *Plant Breed.*, **102**, 17–21.

WHITE, T. J., BRUNS, T., LEE, S. AND TAYLOR, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innes, M., Gelfand, D., Sninsky, J. and White, T. (eds) *PCR Protocols: a Guide to Methods and Applications*, pp. 315–322. Academic Press, San Diego, CA.

WILLIAMS, J. G. K., KUBILEK, A. R., LIVAK, K. J., RAFALSKI, J. A. AND TINGEY, S. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucl. Acids Res.*, **18**, 6531–6535.

YOKOTA, Y., KAWATA, T., KATO, A. AND TANIFUJI, S. 1989. Nucleotide sequences of the 5.8S rRNA gene and internal transcribed spacer regions in carrot and broad bean ribosomal DNA. *J. Mol. Evol.*, **29**, 294–301.