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Phylogenetic position of *Raphanus* in relation to *Brassica* species based on 5S rRNA spacer sequence data

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Abstract. Based on RFLP analysis, two evolutionary lineages for *Brassica* diploid species have been proposed. These are (I) the "*nigra*" lineage and (II) the "*rapa/oleracea*" lineage. The phylogenetic relationship of *Raphanus* species to these two lineages is still unclear because chloroplast and mitochondrial DNA genomic restriction site variation suggests that *Raphanus* is more closely related to the *"rapa/oleracea"* lineage, whereas nuclear RFLPs and other lines of evidence suggest that *Raphanus* belongs to the "*nigra*" lineage. Here, we present evidence of the intergenic spacer of nuclear 5S rRNA to support that *Raphanus* is more closely related to the "*nigra*" lineage than to the "*rapa/oleracea*" lineage. Genetic polymorphism within species is also discussed.

Keywords: *Brassica*; Phylogeny; *Raphanus*; 5S rRNA spacer.

Introduction

Brassica is one of the most important plant groups, containing species widely used in our daily life. *Brassica rapa* (n=10), *B. nigra* (n=8), and *B. oleracea* (n=9) are the three basic groups with three amphidiploid species, *B. napus* (n=19), *B. juncea* (n=18) and *B. carinata* (n=17) derived from interspecific hybridization between pairs of these diploid species, *B. rapa* × *B. oleracea, B. rapa* × *B. nigra* and *B. nigra* × *B. oleracea,* respectively (U, 1935). Based on chloroplast DNA (Warwick and Black, 1991), mitochondrial DNA (Palmer and Herbon, 1988), and nuclear DNA variation (Song et al., 1988; 1990) the phylogenetic relationships in *Brassica* and its related genera have been proposed. *Brassica* species can be divided into two evolutionary pathways: the "*nigra*" lineage and the "*rapa/oleracea*" lineage. *Raphanus* is thought to be closely related to the *Brassica* species. However, its relation to either lineage remains unsolved. Based on chloroplast and mitochondrial DNA restriction site variation (Warwick and Black, 1991; Palmer and Herbon, 1988), *Raphanus* was proposed to be more closely related to the "*rapa/oleracea*" lineage, but nuclear RFLPs and other RAPD data have suggested that *Raphanus* is more closely related to the "*nigra*" lineage (Song et al., 1990; Thormann et al., 1994).

5S rRNA genes are arranged in tandom arrays in the nuclear genome with highly conserved regions, separated by the non-transcribed intergenic spacer (IGS) that may vary in length or sequence between and even within species (Gerlach and Dyer, 1980; Long and Dawid, 1980). IGS evolves rapidly and is informative at the level of genus and species, so it has been chosen to study the phylogenetic relationships between and within genera (Appels et al., 1989, 1992; Baum and Appels, 1992; McIntyre et al., 1992; Moran et al., 1992; Playford et al., 1992; Reddy and Appels, 1989; Udovicic et al., 1995). Bhatia et al. (1993) demonstrated great polymorphism in IGS within *B. rapa* and related species. However, the phylogenetic relationship of *Raphanus* to these two lineages has not been studied using IGS sequences. Here we present data from the IGS of nuclear 5S rRNA to support the hypothesis that *Raphanus* is more closely related to the "*nigra*" lineage.

Materials and Methods

Plant Material

A total of twelve accessions of plant materials, which include seven accessions of *B. rapa,* one accession of *B. oleracea*, two accessions of *R. sativus*, one accession of *Rorippa indica* and one accession of *Lepidium virginicum* were used for PCR amplification of 5S rRNA sequences (Table 1). Among them, four accessions were provided by the Asian Vegetable Research and Development Center (AVRDC), and rest of them were collected by the authors. Also, another six 5S rRNA sequences from Genbank, which include sequences from *B. rapa* (X60723), *Eruca sativa* (X63524), *B. nigra* (X65710, X65711), *Sinapis alba* (X56866) and *Arabidopsis athaliana* (M65137) were used in this study (Bhatia et al., 1993; Campell et al., 1992; Capesius, 1991; 1993; Singh et al., 1994).

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Table 1. Different accessions of *Brassica* and its related species used in phylogenetic analysis of 5S rRNA sequence.

Abbr.	Species	Subspecies	Cultivar or accession	Sources
Brc1	*B. rapa*	*chinensis*	20 days Pai-Tai	Sc[a]
Brc2	*B. rapa*	*chinensis*	Chin-Chiang Pai-Tai	Sc[a]
Brc3	*B. rapa*	*chinensis*	Peng-Hop Pai-tai	B00049[b]
Brp1	*B. rapa*	*pekinensis*	Chinese cabbage	Sc[a]
Brp2	*B. rapa*	*pekinensis*	Chinese head cabbage	Sc[a]
Brp3	*B. rapa*	*pekinensis*	Dwarf leaf Chinese cabbage	B00026[b]
Brr1	*B. rapa*	*rapifera*	Goseki	B00475[b]
Brf	*B. rapa*		Field mustard	X60723[c]
Boc	*B. oleracea*	*capitata*		Sc[a]
Rs1	*R. sativus*		Mei Hwa radish	Sc[a]
Rs2	*R. sativus*		Meei-Nong radish	Sc[a]
Es	*E. sativa*			X63524[c]
Bn1	*B. nigra*			X65711[c]
Bn2	*B. nigra*			X65710[c]
Sa	*S. alba*			X56866[c]
Ri	*R. indica*			Sc[a]
At	*A. thalina*			M65137[c]
Lv	*L. virginicum*			Sc[a]

[a] Sc = Collected by the authors.

[b] Accession no. from AVRDC.

[c] Accession no. for 5S rRNA sequence from Genbank.

DNA Isolation and Amplification

Genomic DNA was isolated from 2 g fresh leaves taken from 2–3 plants for each accession grown in the growth chamber according to the method described by Junghans and Metzlaff (1990). This DNA was then used as template for PCR amplification of 5S rRNA repeat based on two primers from *Sinapis alba* (Capesius, 1991). For PCR reaction, DNA was first denatured at 94°C for 4 min prior to the start of each PCR cycle. The complete PCR mixtures in 100 ul contained 100 ng DNA containing 10 mM Tris HCl, pH 8.3, 50 mM KCl, 0.1 mg/ml gelatin, 1.5 mM $MgCl_2$, 0.1 mM dNTP, 0.2 μM primer, and 0.5 unit taq polymerase. Amplification was performed using DNA Thermal Cycler (Perkin Elmer Cetus, Model 2400). DNAs were amplified for 35 cycles of 1 min at 94°C, 45 sec at 55°C, 1 min at 72°C and one final cycle of 5 min at 72°C. The PCR products were then run on 1% agarose gels. Amplified bands representing monomer and dimer of the 5S rRNA genes were cut and purified with JETpure kit (Genomed Inc, NC, USA). These fragments were then ligated with pBluescript II, Sk^- (Stratagene, CA, USA) and transformed into *E. coli* XL-1 cells. The clones were then sequenced with ABI 373 automated sequencer (Applied Biosystem, NJ, USA).

Data Analysis

The IGS sequences from different accessions were aligned using the “MEGALIGN” program of Lasergene system (DNASTAR Inc. Madison, WI, USA) and manual adjustment. The distance between each pair of sequences (OTUs) was then determined by Kimura’s two-parameter method (1980) using the “Mega” program (Kumar et al., 1993). Bootstrap values were also obtained with the same program.

Results and Discussion

The 5S rRNA gene in *Brassica* and related genera is 119 bp long. (Bhatia et al., 1993; Campell et al., 1992; Capesius, 1991; 1993; Singh et al., 1994). A forward primer, 5’-GGATGGGTGACCTCCCGGGAAGTCC-3’ (positions from 81 to 105 of 5S rRNA) and a reverse primer, 5’-CGCTTAACTGCGGAGTTCTGATGGG-3’ (positions from 58 to 34 of 5S rRNA gene) (Capesius, 1991) were used for amplification of 5S rRNA genes. The positive clones containing the regions from position 81 to 119 of 5S rRNA, IGS, and the regions from position 1 to 58 of 5S rRNA were sequenced and aligned with other published DNA sequences in the IGS region (Figure 1). Immediately following the 3’end of 5S RNA, there is a T-rich region of about 20 bp (positions 1 to 31) thought to be required for termination of the 5S rRNA (Hemleben and Werts, 1988). A GC motif with a consensus sequence, (G/T)GGGCGG(G/A)(G/A)(C/T), which may be involved in the regulation of transcription (Bhatia et al., 1993; Hart and Folk, 1982; Sorensen and Frederiksen, 1991) was located in the positions from 159 to 174. A putative TATA box, ATATATA, critical in the initiation of transcription of 5S rRNA genes (Korn, 1982; Selker et al., 1986), is found 5’ to the 5S rRNA from the position 394 to 400. The IGS in the *Brassica* and its related genera ranges from 367 to 399 nucleotides, and the most divergent region is from position 206 to 387. In the polymorphic region, Rs1 and Rs2 of *R. sativus* were found to share the same nucleotide with “Bn1” and “Bn2” of *B. nigra* and “Sa” of *S. alba* in many locations (positions 36–38, 41, 127–128, 168, 170, 172, 215, 235, 249, 251, 258, 275–276, 289–290, 294, 360–363, 365–366, 370), while these two accessions of *R. sativus* were found to share same nucleotides with accessions of *B. rapa* or *B. oleracea* in fewer locations

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Majority  T T T T T A T T T T T T A T T T - - - - - - - - T T T T T T T A - - C A C C T T A A A A C G A G T C
                            10                  20                  30                  40                  50
BRC1.SEQ  T T T T T A T T T T A T A A T - - - - - - - - - T T T T T T C A - - - G C C T - A A A A C G A G T C  37
BRC2.SEQ  T T T T T A T T T T T T A T T T - - - - - - - - T T T T T T A A T G C A T C C T A A G A C G A G T C  42
BRC3.SEQ  T T T T T A T T T T T T A A T T - - - - - - - - T T T T T T T A - - - G C C T - A A A A C G A G T C  38
BRP1.SEQ  T T T T T A T T T T T T T A T T - - - - - - - - T T T T T T T C - - - A C C T T A A A A C G A G T C  39
BRP2.SEQ  T T T T T A T T T T T T A A T T - - - - - - - - T T T T T T T A - - - G C C T - A A A A C G A A T C  38
BRP3.SEQ  T T T T T A T T T T T T A A A A - - - - - - - - T T T T T T T A - - - G C C T - A A A A C G A G T C  38
BRF.SEQ   T T T T T A T T T T T T T A T - - - - - - - - - T T T T T T T A - - - G C C T - A A A A C G A G T C  37
BRR1.SEQ  T T T T T A T T T T T T A A T T - - - - - - - - T T T T T T T A - - - G C C T - A A A A C G A G T C  38
BOC.SEQ   T T T T T A T T T A T T T T T A - - - - - - - - T T T A A T T T A T C G G C C T A G A A C G A G T C  42
ES.SEQ    T T T T T - - - - - - T A T T T - - - - - - - - T T T T T C T G C A C A C C - - A A A A C G T T T C  34
SA.SEQ    T T T T T - - T T T T T T T T T - - - - - - - - T T T T T T T G - - C A A T T T G A A A - A G G C C  37
BN1.SEQ   T T T T T - - T A T T T A T T T - - - - - - - - T T T T T T T G - - C A A T T G G A A A - A C G T C  37
BN2.SEQ   T T T - - - - - - - T T A T T T - - - - - - - - T T T T T T T - - - C A A T T G G A A A - A C G T C  31
RS1.SEQ   T T T T T - - - A T T T T T T T - - - - - - - - T T C T T - - - - - C A T T T T G A A A C G C G T T  34
RS2.SEQ   T T T T T - - T A T A T T T T T - - - - - - - - T T C T T - - - - - C A A T T T G A A A C G G G T C  35
LV.SEQ    T T T T T T T T T C T T C T T T G C T T T T C G T T T T T T T T T T C A A T T G A G A A A A A A C A  50
RI.SEQ    T - T T T A T A T T T T G T T T - - - - - - - - T C T T T T G A A A - - A T T T - - - - - - - G C A  32
AT.SEQ    T C T T T A T A T G T T - T A A - - - - - - - - C C T T T T T T T - - T T T G - - - - - - - - G T T  32

Majority  T A - - A A C T T G A A A A C C T C A T A A C T T T T G A A C C G T G A G G - A A C T A C G T C G C
                            60                  70                  80                  90                 100
BRC1.SEQ  T A - - A A C T T G A A A A C C C C A T A A C T T T T G A A C C G T G A G G - A A A T A T G T C G C  84
BRC2.SEQ  T A - - A A C T T G G A A A C C T C A T A A C T T T T G A A C C G T G A G G - A A A T A C G T C G C  89
BRC3.SEQ  T A - - A A A T T G A A A A C C T C A T A A C T T T T G A A C C G T G A G G - A A C T A C G T C G C  85
BRP1.SEQ  T A - - A A C T T G G A A A C C T C A T A A C T T T T G A A C C G C G A G G - A A A T A T G T C G C  86
BRP2.SEQ  T A - - A A C T T G A A A A C C C C A T A A C T T T T G A A C C G T G A G G - A A C T A C G T C G C  85
BRP3.SEQ  T A - - A A C T T G A A A A C C T C A T A A C T T T T G A A C C G T G A G G - A A C T A C G T C G C  85
BRF.SEQ   T A - - A A C T T G A A A A C C T C A T A A C T T T T G A A C C G T G A G - - A A C T A C G T C G C  83
BRR1.SEQ  T A - - A A C T T G G A A A C C T C A T A A C T T T T G A A C C G T G A G G - A A C T A C G T C G C  85
BOC.SEQ   T G - - A A C T T G G A A A C C T C A T A A C T T T T G A A C C G G G A G G - A A C T A C G T C G C  89
ES.SEQ    T A - - A A C T T A A A A A C T C C A T A A C T T T T G A A C C G T G T G G - A A C T A C G A C G T  81
SA.SEQ    T C - - A A C T T C T A A A C C T T G T A A C T T T T G A A C C G T G A G G - A A C T A C G G C G G  84
BN1.SEQ   T C - - A A C T T C G A A A C C C C A T A A C T T T T G A A C C G T G A G - - A A C T A C G C C G G  83
BN2.SEQ   T C - - A A C T T C G A A A C C C C A T A A C T T T T G A A C C G T G A G - - A A C T A C G C C G G  77
RS1.SEQ   T C - - A T C T T T A A A A C C C C A T A A C T T T T T A A C C G T G A G G - A A A T A C G C C G C  81
RS2.SEQ   A A - - A T C T T G A A A A C C C C A T A A C T T T T G A A C C G T G T G G - A A C T A C G A C G C  82
LV.SEQ    C A G C A T C T T C G T G G C G T C G T A A C T T C T G A A C C G T A A A A - A A C T G C G G G T G  99
RI.SEQ    T C - - G C T T T C G A A A C A T C G T A A C T T T T G A A C C G C G A G G - A A C T A C G G C G G  79
AT.SEQ    A A - - A A C T T T A T G A C T C C A T A A C T T T T A G A C C G T G A G C C A A A C T T G G C A -  80

Majority  - C C A T A G C A C C A T T T C G G A A A G C C C C A G A A A - - - C C A T A A - T G - - G G C G G
                           110                 120                 130                 140                 150
BRC1.SEQ  - C C A T A G C A C C A T T T C G G A A A G C C C C A G A A A - - - C C A T A A - T G - - G G C G G  127
BRC2.SEQ  - C C A T A G C A C C A T T T C G G A A A G C C C C A G A A A - - - C C A T A A - T G - - G G C G G  132
BRC3.SEQ  - C C A T A G C A C C A T T T C G G A A A G C C C C A G A A A - - - C C A T A A - T G - - G G C G G  128
BRP1.SEQ  - C C A T A G C A C C A T T T C G G A A A G C C C C A G A A A - - - C C A T A A - T G - - G G C G G  129
BRP2.SEQ  - C C A T A G C A C C A T T T C G G A A A G C C C C A G A A A - - - C C A T A A - T A - - G G C G G  128
BRP3.SEQ  - C C A T A G C A C C A T T T C G G A A A G C C C C A G A A A - - - C C A T A A - T G - - G G C G G  128
BRF.SEQ   - C C A T A G C A C C A T T T C G G A A A G C C C C A G A A A - - - C C A T A A - T G - - G G C G G  126
BRR1.SEQ  - C A A T A G C A C C A T T T C G G A A A G C C C C A G A A A - - - C C A T A A - T G - - G G C G G  128
BOC.SEQ   - C C A T A G C A C A A T T T C G G A A A G G C C C A G A A A - - - T C A T A A - A G - - G G C G G  132
ES.SEQ    T C C A T A A C A C C A T T T C G G A A A C G C C C C A A A A - - - C C A T A A A T T - - G G C G C  126
SA.SEQ    - C C A T A G C A C C A T T T C G G A A A C G C C C C A A A A - - - C C A T C A - A G - - G C C A G  127
BN1.SEQ   - C C A T A G C A C C A T T T C G G A A A C G C C C C A A A A - - - C C A T C A - A G - - G T C G G  126
BN2.SEQ   - C C A T A G C A C C A T T T C G G A A A C G C C C C A A A A - - - C C A T C A - A G - - G T C G G  120
RS1.SEQ   - C C A C A G C A C C A T T T C G G A A A G C C C C C A A A A - - - C C A T T T - A G - - G T C A G  124
RS2.SEQ   - C C A C A G C A C C A T T T C G G A A A G C C C C C A A A A - - - C C A T C T - A G - - G C C A G  125
LV.SEQ    C C G T C A G C A C C A A T T C G G A A A G C C C C G A G A G A G C T C - C A C A A C T T G G C A G  148
RI.SEQ    - C C A T A A T A C C T T T T C G G A A A G C C C C A G G A A A C G A C T C C G - - - - A G A T G G  124
AT.SEQ    - - T G T G A T A C C T T T T C G G A A A G C C C A A A G A C A G C C C T C C G A C G A A A G A A G  127
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Majority  -TGAATAGGGGG-----CGGCAATTTTTCGGGC-CCAAATTCAGCCGTTT
                   160       170       180       190       200
BRC1.SEQ  -TGAATAGGGGG-----CGC-AATTTTTCGGGC-TCAAATTCAGCCGTTT 169
BRC2.SEQ  -TGAATAGGCGG-----CGCCAATTTTTCGGGC-TCAAATTCAGCCGTTT 175
BRC3.SEQ  -TGAATAGGGGG-----CGC-AATTTTTCGGGC-TCAAATTCAGCCGTTT 170
BRP1.SEQ  -TGAATAGGGGG-----CGC-AATTTTTCGGGC-TCAAATTCAGCCGTTT 171
BRP2.SEQ  -TGAATAGGGGG-----CGC-AATTTTTCCGGC-TCAAATTCAGCCGTTT 170
BRP3.SEQ  -TGAATAGGGGG-----CGC-AATTTTTCGGGC-TCAAATTCAGCCGTTT 170
BRF.SEQ   -GGAATAGGGGG-----CGC-AATTTTTCGGGC-TCAAATTCAGCCGTTT 168
BRR1.SEQ  -TGAATAGGGGG-----CGC-AATTTTTCGGGC-TCAAATTCAGCCTTTT 170
BOC.SEQ   -TGAATAGGCG------TGGAAATTTTTCGGGC-CTAAATTCAGCCGTTT 174
ES.SEQ    CTGATTAAGGCG-----CCGCGATTTTCCGGACGCCAAATTCCGCTGGTT 171
SA.SEQ    -TGAATAGGCC------TGGCGACTTTTCGAGC-CCAAATTCAGCCGTTT 169
BN1.SEQ   -TGAATGGGGG------TGGCCATTTTTCAGGC-CAAAATTCGGCCGTTT 168
BN2.SEQ   -TGAATGGGGG------TGGCCATTTTTCAGGC-CAAAATTCGGCCGTTT 162
RS1.SEQ   -TGAATAGGCC------TGGCCATTTTTCGAGC-CCAAATTCAGCCGTTT 166
RS2.SEQ   -TGAATAGGGC------TGGCGATTTTTCGACC-CCAAATTCTGCCGTTT 167
LV.SEQ    TGTAACAAAGTA-CCACTGGCCCGTTTTCGACCTCCAATTTTGAGTTTTA 197
RI.SEQ    CATCGTCGCCAAACCATCGGCTGATTTTCGGGTCCAAAATTTGACGTTTA 174
AT.SEQ    CAGGACAACTTTTCCATTGACTTTTTGTCGACCCCAAATTTTGACCTTTA 177

Majority  -TG-ACCCTCAAACGGGCTGCGGAAAGTTATGGCCCGTAAAAAAG-ATCG
                   210       220       230       240       250
BRC1.SEQ  -TG-ACCCTCAAACGGGTTGCGGAAAATTATGGCACGTAAAAAAG-ATCG 216
BRC2.SEQ  -TG-ACCCTCAAACGGGCTGCGGAAAGTTTTGGCACGCAAAAAAG-ATCG 222
BRC3.SEQ  -TG-ACCCTCAGACAGGCTGCGAAAAGTTATGGCACGTAAAAAAG-ATCG 217
BRP1.SEQ  -TG-ACCTTCAAACGGGCTGCGGAAAGTTATGGCACGTAAAAAAG-ATCG 218
BRP2.SEQ  -TG-ACCCTCAAACGGGCTGCGGAAAGTTATGGCACGTAAAAAAG-ACCG 217
BRP3.SEQ  -TG-ACCATCAGACGGGCTGCGGAAAGTTATGGCACGTAAAAAAG-ATCG 217
BRF.SEQ   -GG-ACCCTCAAACGGGCTGCGGAAAGTTATGGCACGTAAAAAGA-ATCG 215
BRR1.SEQ  -TG-ACCCTCAAACGGGCTGCGGAAAGTTGTGGCACGTAAAAAAG-ATCG 217
BOC.SEQ   -TG-ACCCTCAAACGGGCTGCAGAAAGGTAAGGGACTTAAAAAAGGATCC 222
ES.SEQ    -TGTACCCTCAAA-AGGCAGTTTAATGTTGAGGACCTAAAATAAAGTCGA 219
SA.SEQ    -TG-AGCCTCAAAAAGGCTGTAAAACATTACGGACCGTAAATTAG-ATGG 216
BN1.SEQ   -TG-AGCCTCAAACAGGCTCTGAAACATTACGGACAGTAAATTAG-ACGC 215
BN2.SEQ   -TG-AGCCTCAAACAGGCTCTGAAACATTACGGACAGTAAATTGT-ATGC 209
RS1.SEQ   -AT-AGCCTCAAACAGGCTGCCCAATGTTACGGGCTGTGAACGAG-ATGC 213
RS2.SEQ   -AG-AGCCTCAAACAGGCTGTCGAATGTTTCGGGCCGTGATTTAG-ATGC 214
LV.SEQ    GAGCCTGTTTTGGCAGTTTCTGTGA--TCCTAACCGAAAATATGGAACAT 245
RI.SEQ    -TGTGGTTTTTGGGAGCCATTTTC-TAATTCTA-CGGCTATAAAGACCTA 221
AT.SEQ    -AGTACTTTTTCGGG--CATTTTCGTGATTTGGGCTATATTACGGACCCA 224

Majority  AAAGCGGATTTTCAGGGTGTTTTTGATGCTTTCTTAACGCCGTTAACCTC
                   260       270       280       290       300
BRC1.SEQ  AAAGCGGATTCTCAAGGTGTTTTTGATGCTTTTTTAACGCCGTTAACCTC 266
BRC2.SEQ  AAAGCGGATTCTCAGGGTGTTTTTGATGCTTTCTGAACGCCATTAACCTC 272
BRC3.SEQ  AAAGCGGATTCTCAAGGTGTTTTTGATGCTTTCTTAACGCCGTTAACCTC 267
BRP1.SEQ  AAAGCAGATTCTCAAGGTGTTTTTGATGCTTTTTTAACGCCGTTAAC-TC 267
BRP2.SEQ  AAAGCGGATTTTCAAGGTGTTTTTGATGCTTTCTTAACGCCGTTAACCTC 267
BRP3.SEQ  AAAGCGGATTTTCAAGGTGTTTTTGATGATTTCTTAACGCCGTTAACCTC 267
BRF.SEQ   AAAGCGGATTCTCAAGGTGTTTTTGATGCTTTCTTAACGCCGTTAACCTC 265
BRR1.SEQ  AAAGCGGATTCTCAAGGTGTTTTTGATGCTTTCTTAACGCCGTTAACCTC 267
BOC.SEQ   CAATCGGATTTTCAGGTTGTTTTTGATGCTTTCTTAACGTCATTAACCTC 272
ES.SEQ    AATCTGGGTTTTCCGTGTGTTATCATTGAATTCT--ACT---TAAATCTC 264
SA.SEQ    GATAAGTTGTTTCTGGGTGTTATT---TTCATTTT--CTACATCAATTTC 261
BN1.SEQ   GATTCAATATTTCTGGGTGTTTTTTTTTTCCTTTT--CTACATCCATTTC 263
BN2.SEQ   GATTCAATATTTCTGGGTGTTTTTTTTTTCCTTTT--CTACATCCATTTC 257
RS1.SEQ   GATCCGCTGTTTCCGGGTGC--TTTT-GCAGTTTA--CTACATCAAATTT 258
RS2.SEQ   GATACGCTGTTTCCGGGTGT--TTTTTGCAGTTTC--CTATATCAATATC 260
LV.SEQ    AACGGACTTTTTTCTCGTGGTTCCGAGGTTTTCGGAG-------AGCCCT 288
RI.SEQ    GAACCTTGTGTTTACGACTGGTTTTCTAATATGCTCAACAACTAAGAATC 271
AT.SEQ    AAATTACTTGTTCAAGCATTGTTTTCGAATTTTTTCATGCATCAAAGCTC 274
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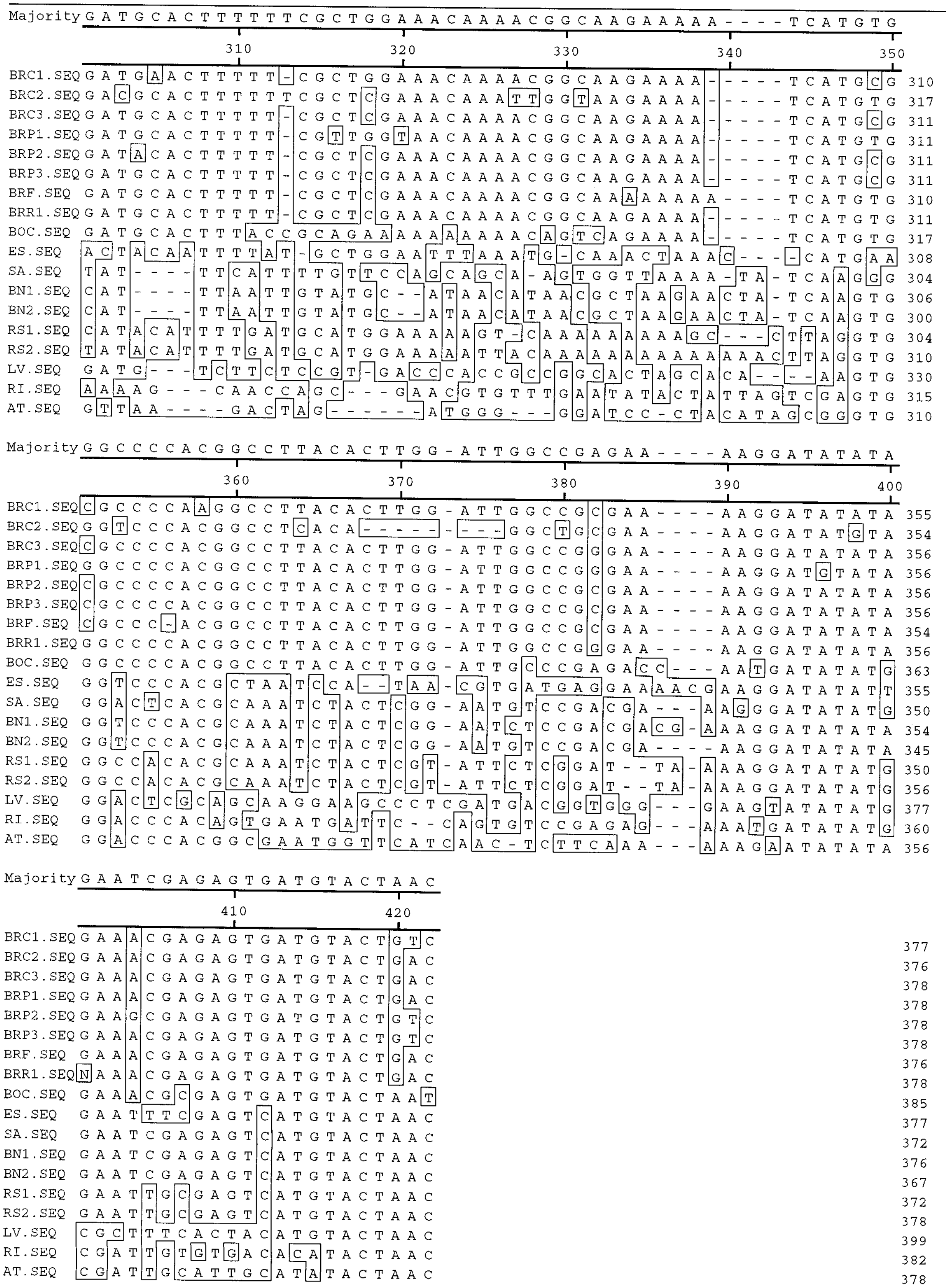

Figure 1. Alignment of IGS sequences begins at the position 1 and ends at the position 422. Different OTUs are indicated in Table 1.

(positions 45–46, 67, 122–123, 157, 227, 328, 332–333, 337, 353, 375). For example, in location 38, all accessions of *B. rapa* and *B. oleracea* have nucleotide "C," while all accessions of *B. nigra, S. alba,* and *R. sativus* have nucleotide "T."

The distances between different OTUs (sequences) were determined at the level of nucleotide substitution using Kimura's two-parameter method (Table 2). The neighbor joining method (Saito and Nei, 1987) was then used to construct a phylogenetic tree among these OTUs and the bootstrap test was performed to determine the bootstrap confidence level (BCL) for each node of the constructed tree (Figure 2). Great polymorphism was observed within *B. rapa*; however, the distances between OTUs within species are always smaller than those between species (Table 2 and Figure 2). Two accessions (Brp2 and Brp3) of ssp. *pekinensis* are clustered with two accessions of ssp. *chinensis* (Brc1 and Brc3) before clustering with Bcf; then

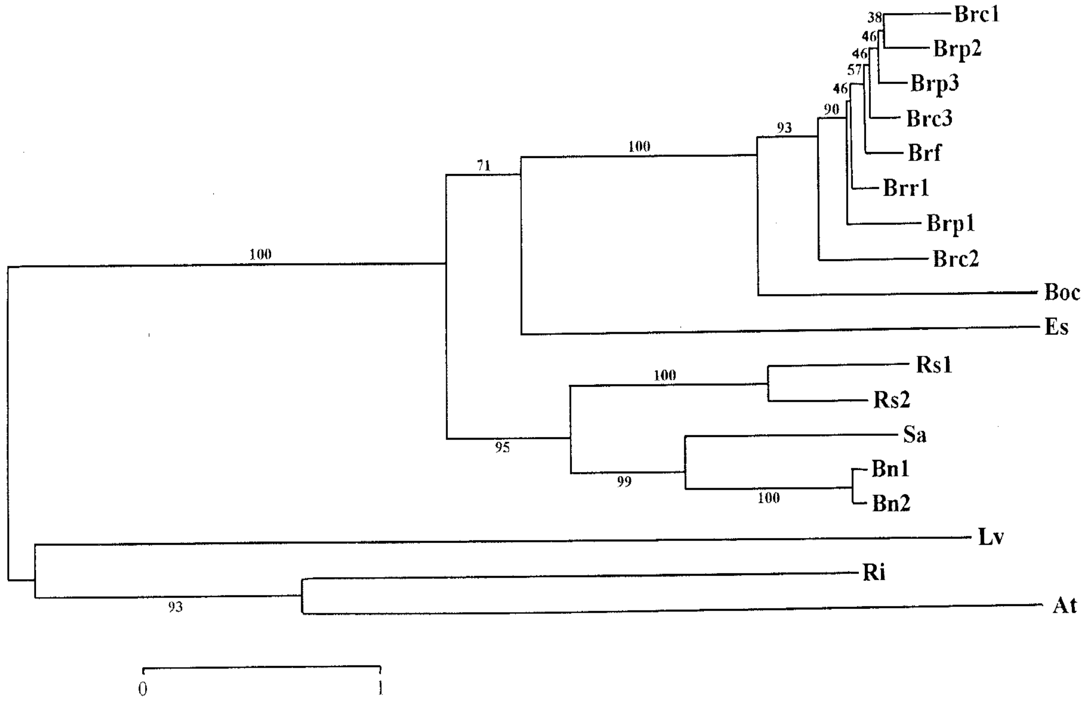

Figure 2. A NJ tree is constructed using the sequence divergences estimated with Kimura's two-parameter method. BCL values are labeled to indicate the percentage of trees that support the node.

Table 2. Number of substitutions per nucleotide site in the intergenic spacer of 5S rRNA genes calculated with Kimura's two-parameter method (Kimura, 1980).

OTUs	Brc1	Brc2	Brc3	Brp1	Brp2	Brp3	Brr1	Brf	Boc	Rs1	Rs2	Es	Bn1	Bn2	Sa	Ri	At	Lv
Brc1																		
Brc2	0.095																	
Brc3	0.046	0.083																
Brp1	0.058	0.089	0.055															
Brp2	0.046	0.095	0.038	0.072														
Brp3	0.043	0.088	0.027	0.063	0.032													
Brr1	0.052	0.068	0.027	0.043	0.043	0.038												
Brf	0.052	0.083	0.032	0.055	0.044	0.035	0.032											
Boc	0.202	0.199	0.181	0.181	0.191	0.177	0.167	0.176										
Rs1	0.409	0.396	0.398	0.390	0.403	0.417	0.403	0.401	0.387									
Rs2	0.391	0.395	0.385	0.381	0.385	0.398	0.380	0.378	0.382	0.103								
Es	0.402	0.379	0.368	0.403	0.359	0.382	0.368	0.386	0.463	0.430	0.364							
Bn1	0.384	0.392	0.369	0.364	0.369	0.387	0.369	0.388	0.389	0.269	0.266	0.418						
Bn2	0.383	0.392	0.368	0.363	0.377	0.386	0.368	0.373	0.385	0.274	0.272	0.415	0.011					
Sa	0.407	0.402	0.378	0.383	0.401	0.396	0.387	0.396	0.403	0.274	0.246	0.428	0.165	0.165				
Ri	0.806	0.728	0.737	0.744	0.759	0.750	0.730	0.768	0.752	0.760	0.731	0.803	0.667	0.683	0.649			
At	0.812	0.796	0.833	0.790	0.804	0.781	0.798	0.794	0.877	0.819	0.798	0.884	0.834	0.841	0.909	0.544		
Lv	0.852	0.771	0.774	0.794	0.793	0.788	0.754	0.773	0.832	0.793	0.746	0.876	0.773	0.767	0.766	0.814	0.742	

they were clustered with Brr1 of ssp. *rapifera* before they met Brp1 of ssp. *pekinensis*. Brc2 of ssp. *chinensis* is located outside these 7 OTUs. Since most values of BCL are low for these accessions within *B. rapa*, the phylogenetic relationship among subspecies is hard to determine on the basis of this DNA sequence. The result may be due to intensive cultivation and occasional outcrossing between different subspecies in *B. rapa*. Nevertheless, 8 OTUs from *B. rapa* were grouped into one composite OTU that is significantly different from the other OTUs in this study. As shown by RFLP data (Song et al., 1988; 1990; Thormann et al., 1994), *B. oleracea* (Boc) is more closely related to *B. rapa* than to *B. nigra*. Two accessions (Bn1 and Bn2) of *B. nigra* were clustered with "Sa" of *Sinapis alba* before they met two accessions (Rs1 and Rs2) of *Raphanus sativus* (Figure 2). The close relation between *S. alba* and *B. nigra* is also reported based on RFLP data (Warwick and Black, 1991). By using *Arabidopsis athaliana, Rorippa indica*, and *Lepidium virginicum* as outgroups, it was clearly shown that *Raphanus sativus* is more closely related to the *B. nigra* lineage than to the *B. rapa/oleracea* lineage. In Table 2, the average distance between *R. sativus* and *B. rapa* is also significantly larger than that between *R. sativus* and *B. nigra* (0.396 ± 0.010 vs. 0.270 ± 0.004). In addition, similar phylogenetic relationships among these OTUs were also observed in the maximum parsimony tree based on the branch and bound search method using the MEGA program (Kumar et al., 1993) (results not shown). Also, data analysis based on the nucleotide sequences of the 18S - 25S spacer region showed a similar result (unpublished data). The discrepancy between nuclear DNA sequence data and chloroplast (or mitochondrial) RFLP data suggests that *R. sativus* may have been derived from hybridization between species belonging to different lineages, as Song et al. (1990) proposed. If this hypothesis is true, two distinct types of IGS must exist in the *Raphanus* with one close to *B. nigra* and the other close to *B. oleracea/rapa* since nuclear DNA sequences are biparentally inherited. However, no such nuclear DNA sequences in *Raphanus* have been found to date.

In conclusion, the IGS of the 5S rRNA gene has been used succesfully to illustrate the phylogenetic relationships among *Brasssica rapa* and its related species. The phylogenetic position of *R. sativus* to the "*nigra*" lineage was demostrated. However, the origin of *R. sativus* may need to be further studied by using more data from chloroplast DNA sequences and other nuclear markers to test the views presented by Palmer and Herbon (1988) and Song et al. (1990). In addition, due to the intensive cultivation or hybridization between cultivars or subspecies, it is hard to determine the phylogenetic relationship among different subspecies within *B. rapa* based on this DNA sequence.

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§Q¥¸ 5S rRNA spacer ‹ª¤s¯‰‡˜ 'MŒ ›a˜ · “«/§¶¡“”¿‰t̂ «Y

•¤‡/[1] ·¿ƒl\“[1] „¥›‰[1] –ifUƒp[2]

[1]//¥¡‹ª¤s |· “««ª¤s'
[2]//¥¡‹ª¤s |¥´“«´ ˙‹ª¤s'

§Q¥¸ RFLP “”/ “R¡AŒ ›a˜ · “«¥i/ ‹ 2 › ”t/˘‡~fi|¡C/@‹O˜ ' ¡§*nigra* ¡¤ “””t/˘‡~fi|¡A¥t/@‹ ¡§*rapa/oleracea* ¡¤ “””t/˘‡~fi|¡Cƒ ' ¯‰‡˜ · “«'M‡o¤ ”t/˘‡~fi|“”ˆ «Y¡A/·ƒ‡¤˙/£†M•¡¡Aƒ]‹ fi ‚›”æ ¯Ø/¸† ‚¢¯Ø“”‹Vƒ ¯Ø“” RFLP “”/ “R¡A¯‰‡˜ 'M *rapa/oleracea* “””t/˘/æ‚ß– “æ¡Aƒ ¥ fi ‹Vƒ ¯Ø“” RFLP /¸¤ ¥ƒ‚Œfi˘¯ª¥ ¡A¯‰‡˜ · “«'M ¡§*nigra* ¡¤ “””t/˘‡~fi|/æ‚ß– “æ¡C 5S rRNA ƒb‹Vƒ ¯Ø/w§e›«´—“”–˘ƒC¡A ƒ § › §Q¥¸ 5S rRNA /§¶¡“”fi »˜§˙ƒC¡A«o¯ª¥ ¯‰‡˜ · “«'M ¡§*nigra* ¡¤ “””t/˘‡~fi|/æ‚ß– “æ¡CŒ ›a˜ · “«‚” /”¿ ¶˙¯ †§'˚¡A/]ƒb¥»/ /”ƒ‡' Q‰ ¡C

ˆ ` ¡G ¿‰t̂ «Y¡F¯‰‡˜ ¡FŒ ›a˜ ¡F 5S rRNA spacer ¡C