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Phylogenetic analysis of *Brassiceae* based on the nucleotide sequences of the *S*-locus related gene, *SLR1*

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Abstract Nucleotide sequences of orthologs of the *S*-locus related gene, *SLR1*, in 20 species of Brassicaceae were determined and compared with the previously reported *SLR1* sequences of six species. Identities of deduced amino-acid sequences with *Brassica oleracea SLR1* ranged from 66.0% to 97.6%, and those with *B. oleracea SRK* and *SLR2* were less than 62% and 55%, respectively. In multiple alignment of deduced amino-acid sequences, the 180–190th amino-acid residues from the initial methionine were highly variable, this variable region corresponding to hypervariable region I of SLG and SRK. A phylogenetic tree based on the deduced amino-acid sequences showed a close relationship of *SLR1* orthologs of species in the *Brassicinae* and *Raphaninae*. *Brassica nigra SLR1* was found to belong to the same clade as *Sinapis arvensis* and *Diplotaxis siifolia*, while the sequences of the other *Brassica* species belonged to another clade together with *B. oleracea* and *Brassica rapa*. The phylogenetic tree was similar to previously reported trees constructed using the data of electrophoretic band patterns of chloroplast DNA, though minor differences were found. Based on synonymous substitution rates in *SLR1*, the diversification time of *SLR1* orthologs between species in the *Brassicinae* was estimated. The evolution and function of *SLR1* and the phylogenetic relationship of *Brassiceae* plants are discussed.

Keywords *S*-multigene family · Pollen-stigma interaction · Phylogenetic relationship · Molecular evolution · Brassicaceae

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Introduction

The recent development of molecular techniques has enabled us to analyze the phylogenetic relationships of various living organisms using the DNA sequence data of some genes. The estimation of the phylogenetic relationships of species based on DNA sequence analysis is more objective than that obtained by traditional methods based on morphology. Comparison of nucleotide sequences of the orthologs from various species is also useful for studying gene function and evolution.

The Brassicaceae includes various economically important crops, such as vegetables, oil crops and the mustard family, as well as the model plant for molecular genetic study, *Arabidopsis thaliana*. Most of the economically important crops in the Brassicaceae belong to the tribe *Brassiceae*. In *Brassiceae* crops, because of their economical importance, the large variation of their chromosome numbers, and the ease of interspecific hybridization, the cytological relationships of the species have been intensively studied (Mizushima 1980). Phylogenetic analyses based on morphology (Schulz 1936; Gomez-Campo 1980, 1999; Takahata and Hinata 1980) and restriction fragment length polymorphism of nuclear, chloroplast and mitochondrial DNA (Erickson et al. 1983; Yanagino et al. 1987; Song et al. 1988, 1990; Warwick and Black 1991; Pradham et al. 1992) have also been carried out. Although nucleotide sequence analyses for the phylogenetic study of the Brassicaceae on a higher taxonomic level have been carried out (Galloway et al. 1998; Koch et al. 2001), there are only a few phylogenetic studies of the *Brassiceae* using nucleotide sequence data (Yang et al. 1999; Wroblewski et al. 2000), and the number of analyzed species in the *Brassiceae* is small.

The *S*-locus related gene, *SLR1*, has been isolated as a homologue of the *S*-locus glycoprotein gene (*SLG*) from *Brassica oleracea* (Lalonde et al. 1989; Trick and Flavell 1989). *SLR1* and *SLG* are members of the *S* multigene family including *SRK*, which is the determinant of self-recognition specificity of the stigma in self-incompatibility. Despite its structural similarity to *SRK*, *SLR1* is not

linked to the *S* locus (Lalonde et al. 1989), suggesting no function of *SLR1* in self-recognition specificity of the self-incompatibility response. The *SLR1* protein has been reported to be responsible for pollen-stigma adhesion (Luu et al. 1999). An ortholog of *SLR1* has been isolated from *A. thaliana, Ats1* (Dwyer et al. 1992), suggesting a broad distribution of *SLR1* orthologs in self-incompatible and self-compatible Brassicaceae plants. In contrast to the high intraspecific sequence variation of *SLG* and *SRK*, the sequence variation of *SLR1* is low in a species (Hinata et al. 1995). Sakamoto et al. (1988) suggested that *SLR1* is a suitable gene for the study of the phylogenetic relationship of Brassicaceae species.

In the present study, we determined the nucleotide sequences of *SLR1* orthologs in 20 species of the Brassicaceae and compared them to elucidate the variation and evolution of *SLR1* genes and the phylogenetic relationships of *Brassiceae* species.

Materials and methods

Twenty four species in the Brassicaceae (see Table 1), which are maintained in the Tohoku University Brassica Seed Bank (http://www.agri.tohoku.ac.jp/pbreed/Seed_Stock_DB/SeedStocktop.html), were used as plant material. Genomic DNA was isolated from a single seed according to Sakamoto et al. (2000). *SLR1* orthologs were amplified by the polymerase chain reaction (PCR) using the primer pair of PS-24 (5′-ATGAGAGGTGTAATACCAAAC-3′) and PS-25 (5′-GAGATAAAGATCTTGACCTC-3′) (Sakamoto et al. 1998). Amplified DNA was cloned into a pCRII vector using a TA Cloning Kit (Invitrogen).

Nucleotide sequences of the cloned PCR products were determined by a DNA sequencer (CEQ2000, Beckman Coulter). Three independent clones obtained from different seeds of the same batch were sequenced to avoid errors that may have occurred during the PCR process. The data were joined to make up the sequence of the *SLR1* coding region using the computer software program Sequencher (Hitachi Software Engineering). The primer sequence of PS-24 starts at the translation initiation codon of *SLR1*, and that of PS-25 corresponds to nucleotides 1,283 to 1,302 in the *SLR1* sequence from *B. oleracea S*[29] (Trick and Fravell 1989). The primer sequences were removed from the determined sequence and deposited in DDBJ (Table 1). A phylogenetic tree was constructed with the neighbor joining method (Saitou and Nei 1987) after removal of insertions and deletions (indels) from multiple alignments, and bootstrap probabilities of 1,000 trials were calculated. Synonymous substitutions were calculated by the method of Satta et al. (1993). The sequence data of *SLR1* orthologs in *Hirshfeldia incana* and *Brassica insularis* are available (Luu et al. 2001), but they were not included in this analysis, because these sequences are approximately 180 bp shorter than our sequence data.

Results

Amplification and sequencing of *SLR1*

In 20 out of the 24 species, DNA fragments of approximately 1.3 kb (1,237 bp–1,276 bp without the primer sequences) were amplified (Table 1). The deduced amino-acid sequences in all the species had the 12 cysteine residues and the N-terminal hydrophobic signal peptides conserved in *SLR1*. Identities of the deduced amino-acid sequences with reported *S*-related sequences, i.e. *SLR1* (Trick and Flavell 1989), *SLR2* (Boyes et al. 1991), *SLR3* (Cock et al. 1995), *SLG-6* (Nasrallah et al. 1987) and *SRK-6* (Stein et al. 1991) in *B. oleracea*, *AtS1* in *A. thaliana* (Dwyer et al. 1992) and *SRK-a* in *Arabidopsis lyrata* (Kusaba et al. 2001), are shown in Table 2. Their sequence identity with *B. oleracea SLR1* ranged from

Table 1 List of the species used in this study and length and accession number of the nucleotide sequence of *SLR1* orthologs

Species name	Code number in seed bank	Length of SLR1 sequence (bp)	Accession number of the sequence in DDBJ
Alyssum psilocarpum	ALS-PSI-1		
Brassica amplexicaulis	Am-1	1,258	AB075407
Brassica barrelieri	Ba-103	1,258	AB075408
Brassica deflexa	Df-1	1,276	AB075409
Brassica erucastrum	Ec-101	1,261	AB075410
Brassica maurorum	Ma-1	1,267	AB075411
Brassica nigra	Ni-141	1,258	AB075412
Brassica oxyrrhina	Ox-101	1,258	AB075413
Brassica tournefortii	T-162	1,267	AB075414
Brassica villosa	Vill-1	1,261	AB075415
Crambe kralikii	CRM-KRA-1	1,237	AB075416
Diplotaxis erucoides	DIP-ERU-9	1,261	AB075417
Diplotaxis siifolia	DIP-SII-3	1,261	AB075418
Eruca sativa	ERU-SAT-12	1,258	AB075419
Eruca vesicaria	ERU-VES-3	1,258	AB075420
Erucastrum abyssinicum	EST-ABY-1	1,261	AB075421
Erucastrum gallicum	EST-GAL-1	1,258	AB075422
Hirschfeldia incana	Ad-120		
Ionopsis uticularioides	ION-UTI-1		
Lepidium sativum	LEP-SAT-1		
Lunaria annua	LUN-ANN-1	1,246	AB075423
Moricandia arvensis	MOR-ARV-14	1,249	AB075424
Sinapis alba	SIN-ALB-25	1,261	AB075425
Sinapis arvensis	SIN-ARV-13	1,261	AB075426
Sinapis arvensis	SIN-ARV-20[a]	1,261	AB075427

[a] Code No. SIN-ARV-20 has been originally identified as *Brassica cheiranthos*.

Table 2 Identity of the deduced amino-acid sequences of the *SLR1* orthologs in Brassicaceae species with reported *S*-related sequences

Species name	*B. oleracea*					*Arabidopsis*	
	SLR1	*SLR2*	*SLR3*	*SLG-6*	*SRK-6*	*AtS1*	*SRK-a*
Brassica amplexicaulis	76.7%	53.4%	50.9%	57.8%	56.6%	63.0%	54.4%
Brassica barrelieri	84.8	52.9	48.3	57.2	56.4	61.9	53.6
Brassica deflexa	86.1	50.5	48.8	55.8	55.6	61.1	53.9
Brassica erucastrum	85.9	51.9	51.6	57.0	55.6	60.4	54.0
Brassica maurorum	86.3	52.0	46.2	57.8	57.1	62.3	54.0
Brassica nigra	81.7	52.8	51.5	59.1	58.3	63.0	54.7
Brassica oxyrrhina	85.2	53.6	49.4	57.5	57.0	63.4	55.8
Brassica tournefortii	86.6	52.2	52.5	56.8	55.2	62.3	53.1
Brassica villosa	97.6	51.9	49.6	58.3	58.0	63.4	54.0
Crambe kralikii	80.2	53.1	49.8	59.9	59.5	63.5	56.5
Diplotaxis erucoides	85.9	50.7	47.9	56.0	54.0	59.4	50.9
Diplotaxis siifolia	82.9	50.1	48.6	57.7	57.3	60.3	53.2
Eruca sativa	77.4	53.4	55.3	57.2	57.5	61.4	53.4
Eruca vesicaria	79.4	54.2	55.3	58.0	58.5	62.7	53.9
Erucastrum abyssinicum	86.0	50.1	49.2	56.4	56.0	60.7	55.7
Erucastrum gallicum	86.4	51.4	50.0	58.0	57.5	61.2	55.3
Lunaria annua	66.0	55.0	54.8	61.4	61.2	70.6	57.2
Moricandia arvensis	78.7	54.2	49.8	59.1	59.2	64.0	55.0
Sinapis alba	85.0	51.7	48.1	59.6	57.7	63.0	55.6
Sinapis arvensis-13	80.0	51.2	51.3	58.6	58.1	62.6	53.4
Sinapis arvensis-20	81.0	51.9	51.6	59.1	58.9	62.9	54.8

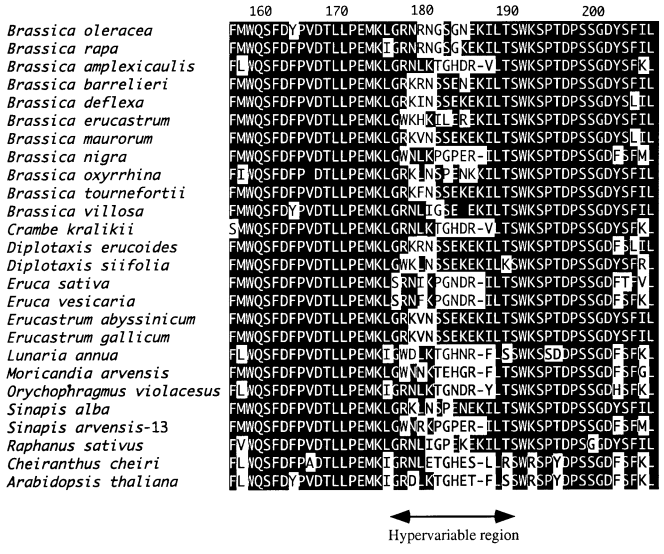

Fig. 1 Alignment of deduced amino-acid sequences in the variable region of SLR1. The most frequent amino-acid residues in the columns are indicated by *black boxes*

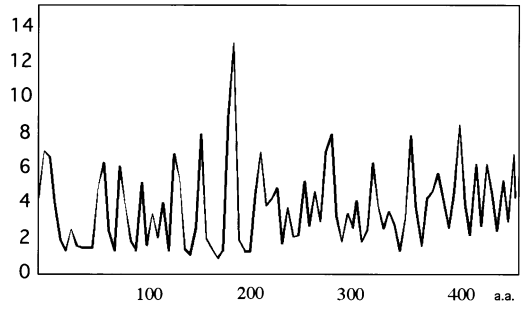

Fig. 2 Linear distribution of amino-acid variation in SLR1. The *numbers* on the left are values of the number of different amino-acid residues divided by the rate of the most frequent amino-acid residue in each column of the multiple alignment of SLR1 orthologs

66.0 to 97.6% in the deduced amino-acid sequences (77.1–99.0% in the nucleotide sequences). The sequence of *Brassica villosa* showed the highest similarity with *B. oleracea SLR1*, while that of *Lunaria annua* showed the lowest. In comparison with *SLG-6* and *SRK-6* in *B. oleracea*, the deduced amino-acid had less than 62% identity. Their amino-acid sequence identities with *SLR2*, which is linked to *SLR1* and highly homologous to class II *SLG*, and those with *SLR3* were less than 55%. *AtS1* had more than 60% sequence identities with these sequences except that of *Diplotaxis erucoides* (59.4%), and *SRK-a* in *A. lyrata* had less than 60% sequence identities. These results suggest that the PCR products were of the *SLR1* orthologs.

In multiple alignment of the SLR1 sequences of the 20 species and the reported SLR1 sequences of six species, the 180–190th amino-acid residues from the initial methionine were highly variable (Fig. 1). The number of different amino-acid residues in each column of the multiple alignment was divided by a percentage of the most frequent amino-acid residue in the column according to Wu and Kabat (1970). The values from the N-terminal amino-acid to the C-terminal amino-acid are shown in Fig. 2. The values in the 180–190th amino-acid residues were 13.9, while the average in the other region was 3.9.

In the pairwise comparison of *SLR1* orthologs, combinations having more than 90% amino-acid sequence identity were seven in number: *B. oleracea–Brassica rapa* (91.8%) *B. villosa–B. rapa* (91.9%), *Eruca sativa–Eruca vesicaria* (94.5%), *Erucastrum abyssini-*

cum–Erucastrum gallicum (96.7%), *Brassica erucastrum–D. erucoides* (94.0%), *Brassica barrelieri–Brassica oxyrrhina* (90.5% and *B. oleracea–B. villosa* (97.6%). Although the *SLR1* orthologs of the species belonging to the same genus generally have high sequence similarity, *SLR1* of *Brassica amplexicaulis* has less than 80% identity with *B. oleracea SLR1*, suggesting that this species is distantly related to *B. oleracea*. In *Diplotaxis* and *Sinapis* as well, there were species having the *SLR1* orthologs which are not so similar to each other in a genus. The *SLR1* orthologs of *L. annua, Cheiranthus cheiri, A. thaliana*, and *Orychophragmus violaceus* showed less than 70% amino-acid sequence identity with those of the other species. These species were considered to have diverged from the other species in the Brassicaceae at an earlier point in phylogenetic development.

Phylogenetic tree

A phylogenetic tree was constructed using the deduced amino-acid sequences of 26 *SLR1* orthologs (Fig. 3). *B. oleracea SLR2* was adopted as the outgroup. *C. cheiri* and *A. thaliana* formed a pair independent of the major clade including *Brassica* species with a high bootstrap probability (100%). In the major clade, *L. annua, O. violaceus*, and *Moricandia arvensis* were grouped separate from the other species with a bootstrap probability of 100%. *Diplotaxis siifolia, Sinapis arvensis*, and *Brassica nigra* formed a cluster in the remaining species. The combinations having more than 90% amino-acid sequence identity, *E. gallicum* and *E. abyssinicum, B. erucastrum* and *D. erucoides, B. oleracea* and *B. villosa*, and *E. sativa* and *E. vesicaria*, formed pairs with a bootstrap probability of 100%.

The chromosome number of each species is shown in the phylogenetic tree (Fig. 3). There was no relationship between the chromosome number and the position of the species in the phylogenetic tree. Despite the close relationship between *B. oleracea* and *B. rapa*, between *E. abysimicum* and *E. gallicum*, and between *B. barrelieri* and *B. oxyrrhina*, the chromosome numbers of each pair were different, suggesting recent diversification of the chromosome number.

Estimation of the divergence time of the *SLR1* gene

Synonymous and nonsynonymous substitutions per site were calculated for each combination of *SLR1* orthologs. The lowest value of the synonymous substitutions per site was observed between *B. oleracea* and *B. villosa* (0.008). The time of divergence of the *SLR1* gene was estimated using the synonymous substitution rate of *SLR1*. The divergence time of *Brassica* and *Arabidopsis* has been inferred to be 20 million years (Koch et al. 2001). The synonymous substitutions per site of the *SLR1* gene between *B. oleracea* and *A. thaliana* were calculated to be 0.512. Therefore, the divergence time of

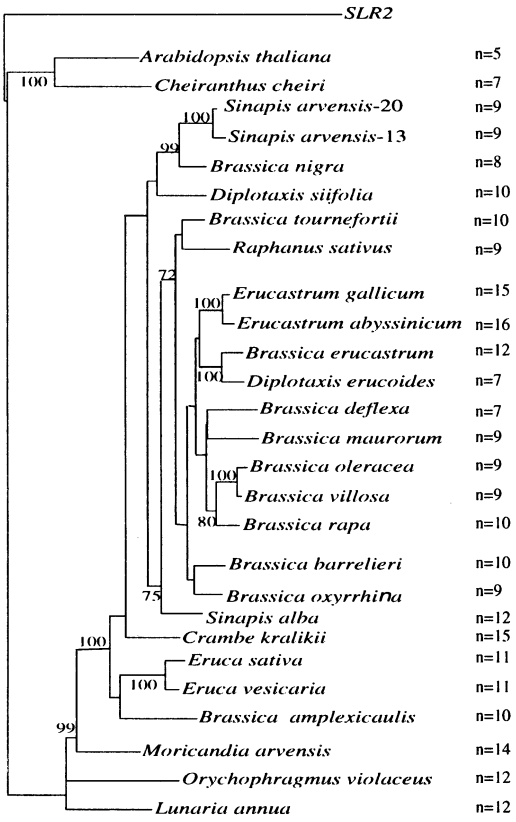

Fig. 3 Phylogenetic tree of deduced amino-acid sequences of *SLR1* orthologs in Brassicaceae species constructed by the neighbor-joining method. *B. oleracea SLR2* was used as the outgroup. The *numbers* besides the branches are the bootstrap probability (%) with 1,000 repeats. Chromosome numbers are shown on the right

SLR1 between *B. oleracea* and *B. villosa* was estimated to be 0.31 million years, and that of the *SLR1* alleles from SIN-ARV-13 and SIN-ARV-20 in *S. arvensis* was estimated to be 1.37 million years. Divergence times of the *SLR1* orthologs between *B. oleracea* and *B. rapa*, between *E. abyssinicum* and *E. gallicum*, and between *S. arvensis* and *B. nigra*, were estimated to be 3.75, 2.11 and 2.81 million years ago, respectively. These results suggest that isolation of populations causing the speciation of *Brassicinae* species occurred 2 to 4 million years ago.

Discussion

Variation and evolution of *SLR1*

The sequences determined in this study were similar to *Brassica SLR1* and *Arabidopsis Ats1*, and not to the other genes in the *S* multigene family. For the amplification of DNA, the sequences unique to *SLR1* were used as specific primers (Sakamoto et al. 1998). In the phylogenetic analysis, these sequences formed a clade independent from the *SLG* and *SRK* clade (data not shown) as

shown by Sakamoto et al. (1998), although some *SLR1* sequences reported by Luu et al. (2001) were intermingled with *SLG* alleles in a phylogenetic tree (Luu et al. 2001). Since *SLR1* is a single-copy gene in the monogenomic Brassicaceae species so far examined (Lalonde et al. 1989; Trick and Flavell 1989; Watanabe et al. 1992; Luu et al. 2001), the sequences highly similar to *Brassica SLR1* are considered to be the *SLR1* orthologs.

In about 420 amino-acid residues in SLR1, highly variable regions were found, while there were conserved amino-acids in all the 26 species. The region from 180 a.a. to 190 a.a. had an especially high variation. This region corresponds to the hypervariable region I of SLG and SRK, which have three hypervariable regions (Kusaba et al. 1997; Nishio and Kusaba 2000). The other regions were similar between different species, and the 12 cysteine residues, which are present in the proteins encoded by the *S*-multigene family, were completely conserved, suggesting that these *SLR1* orthologs have a similar structural configuration and probably a common function in the Brassicaceae species.

The possible function of SLR1 in pollen-stigma adhesion (Luu et al. 1999), and the presence of the hypervariable region having species-specific sequences, suggest the possibility that this region is responsible for species-specific adhesion. The *SLR1*-binding protein, SLR1-BP, has been identified in the pollen coat protein (Takayama et al. 2000). If pollen-stigma adhesion is a prerequisite for pollen tube penetration into the stigma, SLR1 may be one of the determinants of interspecific incompatibility. However, it has been reported that transgenic plants with an antisense *SLR1* construct exhibited no abnormality in pollen tube behavior after self- and cross-pollination (Franklin et al. 1996). Further examination is necessary to elucidate the participation of SLR1 in interspecific incompatibility.

SLR1 is a member of the *S* multigene family. Although *SRK* and *SLG* are present in only self-incompatible species, *SLR1* has been found in most of the Brassicaceae species, suggesting that *SLR1* has a more fundamental function in pollen stigma interaction than *SLG* and *SRK*. *SLR1* and *SRK*/*SLG* are considered to have originated from an ancestral gene by duplication. The ancestral gene is inferred to have a function similar to that of *SLR1* because of its possible fundamental function. Luu et al. (1999, 2001) proposed that *SLG* and *SRK* may have been derived from the duplication of *SLR1* and translocation into the *S* locus.

The length of the sequences varied from 1,237 bp in *Crambe kralikii* to 1,276 bp in *Brassica deflexa*. All the three species having the *SLR1* sequences remarkably shorter than average, i.e., *C. kralikii, L. annua*, and *M. arvensis*, were distantly related in the phylogenetic tree, suggesting a possible relationship between the length of the gene and the phylogeny of Brassicaceae species. However, the positions of the deletions were different between these three species. In three species having *SLR1* genes longer than average, *B. deflexa, Brassica tournefortii* and *Brassica maurorum*, the positions of insertions were independent between these sequences. These results suggest that the insertion and the deletion in these sequences occurred recently.

Phylogenetic relationship of Brassicaceae species

SLR1 alleles from the different lines in *S. arvensis* showed 98.3% amino-acid sequence identity. Nucleotide sequence identity between *SLR1* clones obtained from *B. rapa* was more than 95% (Watanabe et al. 1992). In the comparison of *SLR1* sequences of 26 species, seven combinations, namely, *B. oleracea–Brassica virosa, E. abyssinicum–E. gallicum, E. sativa–E. vesicaria, B. erucastrum–D. erucoides, B. rapa–B. virosa, B. oleracea–B. rapa*, and *B. barrelieri–B. oxyrrhina*, showed more than 90% amino-acid identity. *B. virosa*, *SLR1* of which has 97.6% identity with that of *B. oleracea*, has been assigned to the *B. oleracea* section (Raimond and Mazzola 1997). The *B. virosa* sequence was also highly homologous (97.6% amino-acid sequence identity) to *B. insularis SLR1* (Luu et al. 2001), which has been assigned to the same section as *B. oleracea* (Gomez-Campo 1999). We found the same sequence as *B. virosa SLR1* in an *SLR1* clone obtained from a broccoli cultivar (Sato and Nishio, unpublished), suggesting that the *SLR1* sequence obtained in *B. virosa* is an intraspecific variant in *B. oleracea*. *E. sativa*, *SLR1* of which has 94.5% identity with that of *E. vesicaria*, has been classified as a subspecies of *E. vesicaria* (Gomez-Campo 1999). Therefore, a high similarity between *B. erucastrum* and *D. erucoides*, 94.0%, suggests the necessity of a re-examination of their species names.

According to a classification of Brassicaceae species based on plant morphology (Schulz 1936), 23 species excluding *A. thaliana, C. cheiri*, and *L. annua* belong to the tribe *Brassiceae*. The *Brassiceae* is divided into three subtribes, namely, the *Brassicinae, Raphaninae* and *Morcandiinae*. *Moricandia* and *Orychophragmus* belong to the *Morcandiinae*, *Raphanus* and *Crambe* to the *Raphaninae*, and *Brassica, Diplotaxis, Eruca*, *Erucastrum*, and *Sinapis* to the *Brassicinae*. *M. arvensis* was not included in the clade composed of the species in the *Brassicinae* and the *Raphaninae*, and *O. violaceus* was more distantly related to these species. Gomez-Campo (1980) excluded *O. violaceus* from the tribe *Brassiceae*, and the present result supports his classification. *SLR1* of *Raphanus sativus* and *C. kralikii*, both of which belong to the *Raphaninae* (79.7% amino-acid identity), are not so similar, but *SLR1* of *R. sativus* is similar to those of *B. tournefoltii* (88.4%) and *B. oleracea* (86.0%), suggesting that *Raphaninae* species are not monophyletic.

In the three species of U's triangle (U 1934), *B. rapa* and *B. oleracea* including *B. virosa* formed one clade, but *B. nigra* was distantly related to these species in the phylogenetic relationship of *SLR1*. RFLP analysis of nuclear DNA by Song et al. (1988) has suggested that *B. nigra* originated from one evolutionary pathway together with *S. arvensis*, whereas *B. rapa* and *B. oleracea* came from another pathway. Phylogenetic analysis using

RFLP data of chloroplast DNA by Warwick and Black (1991) has indicated a clear division of the subtribe *Brassicinae* into two ancient evolutionary linages, the Nigra lineage and the Rapa/Oleracea lineage. Based on RFLP data of the chloroplast and mitochondrial DNA, Pradham et al. (1992) classified *Brassica* coenospecies into two lineages, the *Brassica* lineage corresponding to the Rapa/Oleracea lineage and the *Sinapis* lineage corresponding to the Nigra lineage, and placed *B. nigra* in the *Sinapis* lineage. The present study on *B. nigra* supported these previous phylogenetic studies of *Brassica* coenospecies. However, the phylogenetic tree of *Brassicinae* species did not completely correspond to that composed of the two lineages of the previous studies. *B. maurorum* and *B. tournefortii* were placed in the clade of *B. oleracea* and *B. rapa*. The differences of the phylogenetic tree are considered to be due to the difference of the data used in the analyses, the sequence data and band patterns, a nuclear gene and chloroplast DNA. Since interspecific hybridization between the species in the different lineages has occurred in the evolution of *Brassica juncea* and *Brassica carinata*, i.e. between *B. nigra* and *B. rapa* and between *B. nigra* and *B. oleracea*, introgression of nuclear genes or chloroplast and mitochondrial DNA might have also occurred in the evolution of monogenomic species in the *Brassicinae*. Studies of more genes in nuclei, chloroplasts and mitochondria are needed for a comprehensive understanding of the phylogenetic relationships of Brassicaceae species.

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