Genetic diversity of cultivated and wild radish and phylogenetic relationships among *Raphanus* and *Brassica* species revealed by the analysis of *trnK/matK* sequence

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Nucleotide sequence variations of the *trnK/matK* region were investigated in cultivated and wild radish species and related *Brassica* species. Two insertions/deletions and 9 substitutions were detected among the *Raphanus* accessions. The value of the nucleotide diversity (π) was found to be higher in cultivated radish (0.00184) than in the wild species (0.00134). Based on the nucleotide diversity, the phylogenetic relationships of *Raphanus* and its related species were inferred by constructing a Neighbor-Joining tree. *Raphanus* species and *Brassica barraelieri* formed a sister clade located between the Rapa/Oleracea group and the Nigra group of *Brassica*. These results were in complete agreement with those obtained by Warwick and Black. The placement of *Raphanus* species at this position showed the existence of a paraphyletic relationship among the *Brassica* species. Each of the three varieties of cultivated radish, *R. sativus* var. *sativus* (European small radish), var. *hortensis* (East Asian big radish) and var. *niger* (black radish), belonged to a different cluster of the phylogenetic tree, suggesting the existence of independent multiple origins of these varieties. Based on the phylogenetic tree, problems related to the identification of the wild ancestral species of cultivated radish and original birthplaces of cultivated radish varieties were discussed.

Key Words: *Raphanus*, *Brassica*, *trnK/matK*, nucleotide diversity, phylogenetic relationships, origin of cultivated radish.

Introduction

Raphanus ($2n=18$), the radish genus includes one cultivated species and several wild species. Cultivated radish has been classified into many varieties based on the morphology of its edible root and different uses (Kitamura 1958) as follows: *R. sativus* var. *sativus* L. (syn. var. *radicula* Pers.) (European small radish), var. *hortensis* Becker (East Asian big long radish), var. *niger* Kerner (black Spanish radish), var. *chinensis* Gallizioli (Chinese oil radish) and var. *caudatus* Hooker & Anderson (tail-podded radish or rat-tail radish). Among the wild species, *R. raphanistrum* L., *R. landra* DC. and *R. maritimus* Smith are distributed in the coastal areas of the Mediterranean Sea. Both *R. raphanistrum* and *R. landra* were once considered to be candidates for the wild ancestor of cultivated radish. They cannot be discriminated from each other morphologically at the species level, and some authors considered that *R. landra* was a subspecies of *R. raphanistrum* (Munoz and Bermejo 1978). On the other hand, *R. sativus* var. *raphanistroides* Makino, another

wild *Raphanus* species, is distributed in the Far East, mainly in the coastal areas of Japan, Korea and China (Makino 1961). However, most of the radish scientists were not convinced that the East Asian wild species was an endemic true wild species. Since this wild species is morphologically similar to cultivated radish in East Asia, it was assumed to be an escaped form of cultivated radish, and it was classified as *R. sativus* var. *hortensis* f. *raphanistroides* Kitamura (Kitamura 1958). However, analysis of mitochondrial DNA (Yamagishi and Terachi 2001, Yamagishi 2004) did not confirm this assumption.

The phylogenetic relationships among cultivated radish and its wild relatives have been studied using molecular markers. For example, Lewis-Jones *et al.* (1982) who studied allozyme variations failed to detect any critical differentiation among the wild species and concluded that *R. raphanistrum* and *R. landra* could not be identified as distinct species. However, differentiation between wild radish species from the Mediterranean area and the Far East was difficult because no sample of East Asian wild radish was included in their study. A RAPD survey on *Raphanus* (Yamagishi *et al.* 1998) suggested that East Asian wild radish was more closely related to cultivated radish in East Asia than to the wild radish in Europe, *R. raphanistrum*. As for the

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wild ancestor of cultivated radish, Lewis-Jonas *et al.* (1982) suggested that a variant of the raphanistrum-landra complex from Italy or the East Mediterranean area might be a candidate for the wild ancestor. Recently, Yamagishi and Terachi (2003) have analyzed mtDNA variation in *Raphanus*. They showed that haplotypes of cultivated radish were derived from multiple sources of wild radish and concluded that cultivated radish displayed multiple origins. A similar conclusion was drawn from the studies on mtDNA conducted by Yamagishi (2004) and on chloroplast DNA by Yamane *et al.* (2005). However, it remains to be determined which wild species provided haplotype genomes to cultivated radish.

Brassica is the genetically closest genus to *Raphanus*, and cultivated forms of this genus are important vegetables, such as cabbage, mustard and Chinese cabbage. The basic diploid species include *B. oleracea* L. (n=9), *B. nigra* (L.) Koch (n=8) and *B. rapa* L. (syn. *B. campestris* (L.) Clapman, n=10), and the allotetraploid species, *B. napus* L. (n=19), *B. juncea* (L.) Czern (n=18) and *B. carinata* Braun (n=17) were derived by interspecific hybridization among these basic groups (U 1935). The interspecific relationships of *Brassica* species including *Raphanus* have been studied based on chloroplast DNA diversity (Warwick and Black 1991, 1993, 1997 and Warwick *et al.* 1992). The results suggested that *Brassica* could be divided into two lineages (Rapa/Oleracea lineage and Nigra lineage), and that the *Raphanus* species were located in the Rapa/Oleracea lineage. On the other hand, the results from nuclear DNA analysis indicated that *Raphanus* was more closely related to the Nigra lineage (RFLP: Song *et al.* 1990, 5S rRNA sequence: Yang *et al.* 1998). When using mtDNA, two contradictory conclusions have been obtained. Palmer and Herbon (1988) suggested that *Raphanus* was located in the Rapa/Oleracea lineage, whereas Pradhan *et al.* (1992) indicated that *Raphanus* was closer to *B. nigra*. As early as in 1990, Song *et al.* (1990) suggested that *R. sativus* might have been derived from species that belonged to two different lineages by introgression or hybridization. However, they could not identify the ancestral wild species because no wild species was utilized in their study.

Chloroplast DNA, which is characterized by a maternal inheritance, the absence of recombination and a slow evolutionary rate of base substitutions, has been used to analyze all the types of plant species. One of the chloroplast genes, *matK* exhibits a higher nucleotide substitution rate than other chloroplast genes (Olmstead and Palmer 1994), and its substitution rate is 3 times higher than that of the *rbcL* gene (Johnson and Soltis 1995). It is considered to be a useful gene to resolve phylogenetic and evolutionary problems in a wide range of taxonomic levels, especially at the closely related taxonomic levels, such as inter- and intra- species (Steele and Vilgalys 1994, Johnson and Soltis 1994, 1995, Soltis *et al.* 1996, Ohsako and Ohnishi 2000, 2001, Yamane *et al.* 2003).

The present study was undertaken to achieve two objectives as follows: 1) to clarify the genetic relationships

among wild radish species, including the Far Eastern wild species *R. sativus* var. *raphanistroides*, which has been scarcely investigated simultaneously with European wild species; 2) to identify the wild ancestral species of cultivated radish. To achieve these objectives, we analyzed 9 accessions of three wild species and 9 accessions of different cultivated varieties of *R. sativus*. Since it had been postulated that a wild *Brassica* species was involved in the origin of cultivated radish, we also included several *Brassica* species in the present investigation. This is the first study in which wild and cultivated radish and related *Brassica* species was analyzed to elucidate the origin of cultivated radish.

Materials and Methods

Plant materials

In the present study, 18 accessions of *Raphanus* were used. They consisted of 9 cultivated radish accessions (*R. sativus*), 2 accessions of East Asian wild radish, 3 of *R. raphanistrum*, and 4 of *R. landra*. Seven accessions of *Brassica* (*B. barrelieri*, *B. rapa* (syn. *B. campestris*), *B. oleracea*, *B. nigra*, *B. juncea*, *B. napus* and *B. carinata*) and one accession of *Sinapis alba* were also used to analyze the phylogenetic relationships of *Raphanus* and *Brassica* (Table 1). The *Isatis tinctoria* L. species was used as an out-group in our study, because *I. tinctoria* formed a sister group with the group that included *Brassica* in the phylogenetic tree of Beilstein *et al.* (2006). All the *R. landra*, *Brassica*, *S. alba* and *I. tinctoria* samples were lines stored at the gene bank of the Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany (Table 1). The other accessions were collected by one of us (O. Ohnishi) during expeditions conducted from 1990 to 2005 (Table 1). The samples of wild radish populations, consisting of one hundred to one thousand siliques, were originally collected from natural populations. For the cultivated species, mass seed samples were bought at local markets.

Polymerase chain reaction and sequencing

The plants were grown in a greenhouse and total DNA was isolated from young leaf tissues of an arbitrarily chosen individual for each accession, according to the plant DNAzol Reagent protocol (Gibco BRL, Grand Island, NY, USA).

Using the two universal primers trnK/3914F and trnK/2R (Johnson and Soltis 1994), the *trnK/matK* region was amplified for all the plants described above. PCR amplifications were performed in a final reaction solution of 50 µl containing 50 ng DNA template, 10 mM Tris-HCl (pH 8.3), 20 mM KCl, 1.5 mM MgCl₂, 100 mM each of dATP, dCTP, dGTP, dTTP, 0.2 mM of each primer and 2.5 units of Taq DNA polymerase (Takara Shuzo Co. Ltd., Otsu, Japan). The thermal cycling protocol consisted of 25 cycles of denaturation at 94°C for 45 sec, annealing at 50°C for 1 min, extension at 72°C for 3 min, and a final extension at 72°C for 5 min.

The DNA sequence was determined using an Applied Biosystems 3730xl DNA Analyzer (Hitachi Instruments

Table 1. Plant materials, length of *trnK/matK* sequences and accession numbers of these accessions in EMBL/GenBank/DDBJ

Accession No.	Species and Variety	Country	Locality, Cultivar or Strain	Length of sequence (bp)	Accession No. (EMBL/GenBank/DDBJ)
<i>Raphanus</i>					
C2	<i>R. sativus</i> var. <i>hortensis</i>	China	Jintiaohong	2211	AB354253
J3	<i>R. sativus</i> var. <i>hortensis</i>	Japan	Sakurajima	2211	AB354252
R4	<i>R. sativus</i> var. <i>hortensis</i>	Uzbekistan	Samarkand	2211	AB354254
C13	<i>R. sativus</i> var. <i>sativus</i>	China	Hongyingtao	2209	AB354260
I1	<i>R. sativus</i> var. <i>sativus</i>	Italy	Round red outdoor	2209	AB354257
S2	<i>R. sativus</i> var. <i>sativus</i>	Slovenia	National	2209	AB354258
T6	<i>R. sativus</i> var. <i>sativus</i>	Turkey	Local Diyarbakir	2209	AB354259
F1	<i>R. sativus</i> var. <i>niger</i>	France	Noir Gros long	2209	AB354255
I0330	<i>R. sativus</i> var. <i>caudatus</i>	India	Rat-tail radish Mandi	2209	AB354256
J9	<i>R. sativus</i> var. <i>raphanistroides</i>	Japan	Higashihama	2211	AB354261
J13	<i>R. sativus</i> var. <i>raphanistroides</i>	Japan	Chitose	2209	AB354262
I8	<i>R. raphanistrum</i>	Italy	Rome	2202	AB354269
R1	<i>R. raphanistrum</i>	Russia	Orel	2209	AB354263
P1	<i>R. raphanistrum</i>	Poland	Pulawy	2209	AB354264
RA230/80	<i>R. landra</i>	Russia	—	2211	AB354265
RA410/95	<i>R. landra</i>	France	—	2209	AB354266
RA401/98	<i>R. landra</i>	Italy	—	2209	AB354268
RA411/94	<i>R. landra</i>	Italy	—	2209	AB354267
<i>Brassica</i>					
BRA1200/96	<i>B. barrelieri</i>	—	—	2198	AB354270
CR1021/95	<i>B. rapa</i> (syn. <i>B. campestris</i>)	—	—	2205	AB354276
BRA530/95	<i>B. oleracea</i>	England	—	2208	AB354271
CR1203/97	<i>B. nigra</i>	—	—	2205	AB354272
CR76/90	<i>B. juncea</i>	—	—	2205	AB354274
CR162/95	<i>B. napus</i>	—	—	2203	AB354273
BRA927/00	<i>B. carinata</i>	Ethiopia	—	2205	AB354275
<i>Sinapis</i>					
CR2165/97	<i>S. alba</i>	—	—	2205	AB354277
<i>Isatis</i>					
ISA 23/03	<i>I. tinctoria</i>	Tajikistan	—	2203	AB354278

—: unknown

Service, Tokyo, Japan). The sequencing primers were as follows: matK1460L/F (5'-ATT-TCC-CTT-TTT-AGA-AGA-CA), 1480matK/R (5'-GTC-GAT-CGT-AAA-TGC-GAA-GA), matK615L/F (5'-CAT-TCT-GGC-AAC-AAA-GGA-TA), matK209L/R (5'-CAC-AAC-AAA-GAC-CAA-GTA-TA), matK144L/F (5'-AAA-AAG-GTT-GCG-CTC-TGG-TT), trnK677L/R (5'-CAT-AGT-GCG-ATA-CAG-TCA-AA) and trnK3'/1946 (5'-TAG-CAG-TCT-ACC-TGT-TTA-TG). In addition to the accessions mentioned above, the sequence data of 11 lines of *Brassica*, which were obtained from the GenBank of DDBJ (sequence identification numbers are shown in Table 2 and Fig. 1) were also analyzed. The following species were included: *B. rapa*, *B. oleracea* and wild related species, such as *B. insularis* Moris, *B. cretica* Lam., *B. hilarionis* Post., *B. incana* Ten., *B. montana* Pourret, *B. rupestris* Rafin, *B. villosa* Biv., *B. macrocarpa* Guss. (see Table 2). All these wild species are known to be distributed in Europe and on the Mediterranean coast (Harberd 1972), where wild radish is growing naturally.

Table 2. Accessions with sequence data originally provided by EMBL/GenBank/DDBJ

Species	Country	Accession No. (EMBL/GenBank/DDBJ)
<i>Brassica cretica</i>	Greece	AY541611
<i>B. hilarionis</i>	Turkey	AY541612
<i>B. incana</i>	Italy	AY541613
<i>B. insularis</i>	Italy	AY541614
<i>B. macrocarpa</i>	Italy	AY541615
<i>B. montana</i>	Spain	AY541616
<i>B. montana</i>	Spain	AY541617
<i>B. oleracea</i>	Spain	AY541618
<i>B. rapa</i> (syn. <i>campestris</i>)	Italy	AY541619
<i>B. rupestris</i>	Italy	AY541620
<i>B. villosa</i> subsp. <i>bivoniana</i>	Italy	AY541621
<i>B. villosa</i> subsp. <i>drepanensis</i>	Italy	AY541622

Data analyses

Nucleotide sequences were aligned manually using DNASIS version 3.0 (Hitachi Software Engineering Co., Ltd., Tokyo, Japan), with manual modification to minimize the number of gaps. The number of polymorphic sites (P) and the nucleotide diversity value (π) (Nei and Li 1979) in *Raphanus* were calculated using DnaSP version 4.00 (Rozas and Rozas 1997).

The *trnK/matK* sequence data were analyzed for phylogenetic relationships by the neighbor-joining (NJ) method (Saitou and Nei 1987) and maximum parsimony (MP) method using PAUP* version 4.0 (Swofford 1999, 2002). The insertions/deletions were treated as missing data in both methods. The number of nucleotide substitutions per site was estimated based on Kimura's two-parameter method (1980) and used to determine the genetic distance to construct the NJ tree. All the informative substitutions were used to construct the MP trees. For the MP trees, heuristic search was carried out 100 times with random-addition-sequence (RANDOM) and tree-bisection-reconnection (TBR) branch swapping. The reliability was evaluated using bootstrapping with PAUP (Felsenstein 1985) by performing 1000 replications.

Results

Variation of *trnK/matK* sequences in *Raphanus*

The 2198–2211 bp long *trnK/matK* sequences were obtained from 27 accessions, including 18 *Raphanus*, 7 *Brassica*, 1 *Sinapis* and 1 *Isatis* accessions (GenBank No. AB354252–AB354278) (Table 1). The results of the alignment revealed two insertions/deletions and 9 substitutions in *Raphanus*. Two insertions/deletions were found at the *trnK* 3' and 5' introns. At the 3' intron, one 7 bp insertion/deletion was detected only in *R. raphanistrum* (Accession number I8). Four substitutions were found in the *trnK* intron regions and all the 5 substitutions in the coding region were nonsynonymous (Table 3).

Nucleotide diversity was calculated for all the *trnK/matK* sequences, and for the 5' *trnK* intron, the *matK* coding region and the 3' *trnK* intron separately, for the cultivated accessions, wild accessions and all the accessions separately (Table 3). The values of nucleotide diversity were 0.00184 and 0.00134 for the cultivated and wild accessions, respectively, and 0.00154 for all the accessions. The highest value

was detected in the 3' *trnK* region in the cultivated accessions (0.00455), and the lowest value (0.00081) in the coding region of the wild accessions. In the *trnK/matK* sequence, the diversity value in the *trnK* 3' region was always higher than those in the *matK* coding region and the *trnK* 5' intron region.

Phylogenetic analyses of the genus *Raphanus* and its related genera

The maximum parsimony (MP) and neighbor-joining (NJ) methods enabled to reconstruct almost the same phylogenetic trees, and only the NJ tree is shown in Fig. 1. With the MP method, only one shortest tree was detected by the heuristic search based on site-change data for the *trnK/matK* sequence. The tree length was 227, and the consistency index and homoplasy index were 0.9515 and 0.0475, respectively. For both MP and NJ trees, high bootstrap values were assigned to the main branches (Fig. 1)

The phylogenetic tree showed that all the accessions of *Raphanus* formed one cluster and were located in the Rapa/Oleracea lineage of *Brassica*, which included *B. rapa*, *B. juncea* and *B. oleracea*. This lineage had apparently diverged from the Nigra lineage that contained *B. carinata*, *B. nigra* and *S. alba*. *B. barrellieri* was the phylogenetically closest *Brassica* species to *Raphanus*. This species formed a sister group with *Raphanus*. This topology of the phylogenetic tree revealed the paraphyletic relations of *Brassica* species.

In the *Raphanus* cluster, two groups with high bootstrap values, 86 and 95% were detected. One group (group C in Fig. 1) included the East Asian cultivated radish *R. sativus* var. *hortensis* and the wild accessions, *R. landra* and *R. sativus* var. *raphanistroides*. The other group consisted of two subgroups (A and B). The subgroup A contained *R. sativus* var. *sativus*, *R. sativus* var. *caudatus* and the wild accessions *R. raphanistrum*, *R. landra* and *R. sativus* var. *raphanistroides*. An intermediate group (B) between groups A and C contained only one cultivated variety, the black Spanish radish *R. sativus* var. *niger* and the wild accessions *R. raphanistrum* and *R. landra*. The phylogenetic tree indicated that all the wild radish species were at polyphyletic positions, e.g. the accessions of *R. raphanistrum* and *R. sativus* var. *raphanistroides* belonged to two groups and the accessions of *R. landra* belonged to all the three groups. As for the *Brassica* species, the Nigra lineage species, *B. nigra*

Table 3. Number of polymorphic sites (P) and nucleotide diversity value (π) of *Raphanus*

	Total	5' intron	<i>matK</i>			3' intron
			Total	Synonymous	Nonsynonymous	
Insertions/deletions	2	1	0	–	–	1
Number of polymorphic sites (P)	9	2	5	0	5	2
Nucleotide diversity (π) ($\times 10^{-3}$)						
all accessions	1.54	2.37	1.01	0	1.31	4.08
wild accessions	1.34	2.17	0.81	0	1.05	3.83
cultivated accessions	1.84	2.53	1.27	0	1.64	4.55

Fig. 1. Neighbor-joining tree based on the *trnK/matK* sequences. Numbers above the branches denote the bootstrap value (above 50%). *Isatis tinctoria* was used as an outgroup. The accession number, country and whether the accession was a wild (W) or a cultivated species (C) are indicated in parentheses for *Raphanus*. The GenBank number is indicated in parentheses for the accessions quoted from EMBL/GenBank/DDBJ.

and *B. carinata* formed a cluster with *S. alba*, whereas in the Oleracea/Rapa lineage, *B. oleracea*, *B. rapa* and their related species, *B. juncea*, *B. napus* formed a subcluster, *B. rupestris* and *B. villosa* formed another subcluster and *B. macrocarpa* formed a third subcluster by itself.

Discussion

Phylogenetic position of *Raphanus* species

Warwick and Black (1991, 1997) studied cpDNA variations in *Brassica* and its related species, including two *Raphanus* species, *R. sativus* and *R. raphanistrum* and concluded that the two *Raphanus* species formed a cluster and belonged to the Rapa/Oleracea group in the two major groups of *Brassica* (Rapa/Oleracea group and Nigra group).

Raphanus displays large variations in morphology, as cultivated species are classified into many varieties. It also shows large molecular variations in cultivated and wild species, in particular between the European and East Asian species, which were detected in mtDNA by Yamagishi and Terachi (2003) and Yamagishi (2004) and in cpDNA by Yamane *et al.* (2005). Therefore, we were originally suspicious of Warwick and Black (1991, 1997)'s conclusion, because only two European samples of *Raphanus* species were included in their study. However, the present phylogenetic tree (Fig. 1) was in complete agreement with the results obtained by Warwick and Black (1991, 1997). Furthermore, *B. barrelieri* was most closely related to the *Raphanus* species and formed a sister clade with the *Raphanus* accessions, indicating that *B. barrelieri* was different from other species of *Brassica*. The position of the *Raphanus* species was included in the paraphyletic relationships among the *Brassica* species. The results of the present study did not contradict the existence of phylogenetic relationships among the species of *Brassica* and were also in complete agreement with the results obtained by Warwick and Black (1991, 1997). The phylogenetic relationships among *B. oleracea* and its related wild species such as *B. cretica* and *B. incana* (these species are frequently treated as subspecies of *B. oleracea*) were described in detail by Gómez-Campo (1999).

Cultivated radish displays larger variations than wild species

In the process of domestication, selection and bottleneck effects reduce the genetic variations in cultivated species. Hence, the genetic variations of cultivated species are usually less pronounced than those of the wild ancestral species, as observed in barley (Neale *et al.* 1988), soybean (Xu *et al.* 2002) and water melon (Dane *et al.* 2004). In radish, cultivated radish showed a higher value of nucleotide diversity (0.00184) than the wild species (0.00134) (Table 3), presumably due to the insufficient collection of wild samples. However, based on our chloroplast SSR data (Lü *et al.* 2005) the same number of haplotypes was detected in cultivated and wild radish, and all the mitochondrial DNA types in wild radish were detected in cultivated radish (Yamagishi and Terachi 2003). Hence, the high diversity in cultivated radish could be ascribed to the frequent hybridization between cultivated radish and wild species and reticulate evolution of the varieties of cultivated radish.

Song *et al.* (1990) suggested that *R. sativus* was derived from a bridge species, resulting from the hybridization of a species of *B. oleracea* lineage and a species of *B. nigra* lineage. Since only one sample of *Raphanus* species, *R. sativus* var. *hortensis* (Chinese radish) was used in their study, we were suspicious of their conclusion, which, however, does not contradict the present results on variability at the molecular level in cultivated and wild radish species. Further studies should be carried out on the role of the *Brassica* species in the evolution of cultivated radish. Actually, many *Brassica* species can be crossed with cultivated and wild radish

(Harberd 1972). Fig. 1 shows that *B. barrelieri* is a sister of *Raphanus*. At present, no one suggested that *B. barrelieri* had been involved in the domestication of radish. However, since any hypothesis on the development of cultivated radish has to explain why cultivated radish displays as much variation as all of the wild species, this possibility should be seriously considered.

Phylogenetic relationships among Raphanus accessions

The origin of cultivated radish has been investigated by many authors. In early morphological studies, it was postulated that cultivated radish originated from a single species (*R. raphanistrum*, de Candolle 1883, Kobabe *et al.* 1959, *R. martimus*, Henslow 1898) or through hybridization (*R. landra* × *R. martimus*, Schulz 1919, Clapham *et al.* 1962). Recently, Yamagishi and Terachi (2003), Yamagishi (2004) and Yamane *et al.* (2005) have suggested that cultivated radish in Europe and in East Asia was derived from different ancestors, assigning a multiple origin to cultivated radish. The present phylogenetic analysis shown in Fig. 1 suggests that *R. sativus* var. *sativus* (European small radish), *R. sativus* var. *niger* (Spanish black radish) and *R. sativus* var. *hortensis* (East Asian big long radish) belonged to different clusters, indicating that it is highly probable that they originated independently, presumably from different wild species. A similar result was also obtained, based on the analysis of chloroplast SSR variations in *Raphanus* (Lü *et al.* 2005). As for the candidate for the wild ancestor, *R. sativus* var. *raphanistroides* (East Asian wild radish) was closely related to East Asian cultivated radish, based on numerous experimental results (Yamagishi *et al.* 1998, Yamane *et al.* 2005). If this East Asian wild radish was an endemic true wild species in Asia, it may be reasonable to consider that it is the wild ancestor of East Asian cultivated radish, as suggested by Shinskaya (1931). However, East Asian wild radish had not been considered to be an endemic true wild species, but an escaped form of East Asian cultivated radish (Makino 1961). Recently, this hypothesis on escaped form has not been confirmed by studies on mtDNA variation (Yamagishi and Terachi 2001, Yamagishi 2004). Presently, the origin of East Asian wild radish remains to be determined. The phylogenetic tree in Fig. 1 suggests that one accession of East Asian wild radish belonged to group A, together with other wild species from Europe, that is, East Asian wild radish showed a genetic relationship with the wild radish from Europe. Since the European wild species are distributed in all the three groups (A, B, C) it is reasonable to consider that the East Asian wild radish originated in Europe as a weed of radish or a wild species, as proposed by many authors (Aoba 1989, Kitamura 1958). As for the phylogenetic position of *R. sativus* var. *niger*, both sequence analysis and SSR studies (Lü *et al.* 2005) on cpDNA suggested that the var. *niger* shows a unique position in the phylogenetic tree, which is quite different from that of other cultivated radish varieties, pointing to an independent origin of this variety. Since the accessions of European cultivated

radish, *R. sativus* var. *sativus*, exclusively belonged to group A, along with three wild species, *R. raphanistrum*, *R. landra* and *R. sativus* var. *raphanistroides*, the present results failed to identify the wild ancestral species of European cultivated radish. However, the hypothesis that *R. raphanistrum* is the parental species of European cultivated radish is valid, if the origin of the morphological characteristics of cultivated radish which are critically different from those of the wild species, i.e., flower color and silique morphology, could be reasonably explained (Yamane *et al.* 2005).

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