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ORIGINAL PAPER

Identification of highly variable chloroplast sequences and development of cpDNA-based molecular markers that distinguish four cytoplasm types in radish (*Raphanus sativus* L.)

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Abstract Four types of cytoplasms (Ogura, DCGMS, DBRMF1, and DBRMF2) were identified in the previous studies using molecular markers based on mitochondrial genome variations in radish (*Raphanus sativus* L.). However, mtDNA markers have limitations in obtaining clear results due to complexity of radish mitochondrial genomes. To improve fidelity, molecular markers based on variation of chloroplast genome sequences were developed in this study. We searched for the sequence variations of chloroplast genome among the four cytoplasm types in 11 non-coding intergenic regions of ~8.7 kb. Highly variable intergenic regions between *trnK* and *rps16* were identified, and a couple of 4–34 bp indels were used to develop a simple PCR-based marker that distinguished the four cytoplasm types based on the PCR product length polymorphism. Two additional cpDNA markers were developed by using a single nucleotide polymorphism and 17-bp insertion. Analysis of 90 accessions using both mtDNA and cpDNA markers showed the perfect match of results of both the markers, suggesting strict co-transmission of mitochondria and chloroplast in radish. Phylogenetic trees showed that two male-sterility inducing cytoplasms, Ogura and DCGMS, were closely related to DBRMF1 and DBRMF2, respectively. Analysis of 120 radish germplasms introduced from diverse countries showed that the frequency of male-sterility inducing mitotypes of Ogura and DCGMS was very low, and DCGMS was predominately detected in eastern European countries. Majority of accessions from Europe and Asia were shown to contain DBRMF2 and DBRMF1 mitotypes, respectively.

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Introduction

Plant mitochondrial genomes have some peculiar structural features compared with small (15–18 kb) circular forms of animal mitochondrial genomes (Fauron et al. 1990; Budar et al. 2003; Hanson and Bentolila 2004; Knoop 2004). First, plant mitochondrial genomes are relatively large and variable, ranging from ~200 kb in *Brassica* species (Palmer and Herbon 1987) to ~2,400 kb in some Cucurbitaceae families (Ward et al. 1981). Second, the exact configuration of the plant mitochondrial genome remains unresolved (Oldenburg and Bendich 1998; Oldenburg and Bendich 2001), although circular sequences of entire mitochondrial genomes have been reported in *Arabidopsis* (Unseld et al. 1997), sugar beet (Kubo et al. 2000), rice (Notsu et al. 2002), rapeseed (Handa 2003), and tobacco (Sugiyama et al. 2005).

Unlike single circular animal mitochondrial genomes, plant mitochondrial genomes consist of multipartite forms, and even the stoichiometry of multiple forms is variable

within species (Sakai and Imamura 1993; Bellaoui et al. 1998; Arrieta-Montiel et al. 2001; Kim et al. 2007). Multipartite structures are caused by rearrangement of mtDNA; homologous recombination mediated by repeat sequences that are ubiquitously present in plant mitochondrial genomes is probably responsible for dynamic shuffling of plant mtDNAs (Palmer 1988; Albert et al. 1998; Notsu et al. 2002). Although variable stoichiometry of subgenomic mtDNA molecules exists in different cytoplasm types of a particular species, specific stoichiometry is constantly transmitted into the next generations over a short-term period (Janska et al. 1998; Kim et al. 2007). However, the stoichiometry changes over a long-term period by unresolved mechanisms of genomic shifting (Small et al. 1989; Arrieta-Montiel et al. 2001). Nuclear genes and stress from tissue culture are known to cause genomic shifting in some species (Fauron et al. 1990; Mackenzie and Chase 1990; Janska et al. 1998; Abdelnoor et al. 2003). Four mitochondrial genome variants (mitotypes Ogura, DCGMS, DBRMF1, and DBRMF2) containing unique stoichiometry of mtDNA substructures have been identified in radish (*Raphanus sativus* L.) (Kim et al. 2007; Lee et al. 2008). Among these, the Ogura and DCGMS mitotypes are involved in cytoplasmic male sterility (CMS) (Ogura 1968; Lee et al. 2008).

CMS is characterized by inability of pollen grain and is induced by chimeric mitochondrial genes (Hanson 1991; Hanson and Bentolila 2004). In addition to its usefulness in the study of interaction between nuclear and mitochondrial genomes, CMS has been used in F_1 hybrid seed production to prevent undesirable self-pollination, since Jones and Clarke (1943) first proposed the method of F_1 hybrid production using CMS in onions. To date, all known male-sterility inducing genes are found in mitochondrial genomes and are chimeric genes created by mtDNA rearrangement (Hanson 1991; Hanson and Bentolila 2004). A novel chimeric gene, *orf138*, is responsible for male-sterility in Ogura CMS of radish (Bonhomme et al. 1991; Grelon et al. 1994). Moreover, a nuclear gene involved in restoration of fertility of Ogura CMS was isolated (Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003). Like two other cloned restorer-of-fertility genes in petunia (Bentolila et al. 2002) and rice (Komori et al. 2004), its gene product encodes a pentatricopeptide repeat (PPR) protein. In contrast to the extensively studied Ogura CMS, the recently identified CMS conditioned by the DCGMS mitotype was shown to have a different male-sterility phenotype and its restorer-of-fertility loci do not appear to be related to that of Ogura CMS (Lee et al. 2008), indicating that maintainer and restorer lines for CMS of Ogura and DCGMS are different.

Therefore, when CMS induced by Ogura and DCGMS mitotypes is used in radish F_1 hybrid breeding, it is crucial to determine the correct mitotype to allow selection of appropriate maintainer lines and classification of the mitotypes of introduced germplasm. For determination of mitotypes, mtDNA-based markers were developed based on differential mtDNA rearrangement and stoichiometry between the four radish mitotypes (Kim et al. 2007; Lee et al. 2008). However, the PCR patterns of mtDNA-based markers were not clear enough for use in large-scale breeding materials due to ambiguity arising from the above-mentioned complexity of plant mitochondrial genomes. To overcome similar problems in onions, molecular markers based on variation of chloroplast genomes have been used (Havey 1995). In most angiosperms, mitochondria and chloroplasts are strictly co-transmitted to the next generations, thus variation of mtDNA is closely related to that of cpDNA (Reboud and Zeyl 1993; Olson and McCauley 2000).

In this study, we developed simple cpDNA markers based on variation of chloroplast genome sequences of four radish mitotypes to improve the fidelity of radish mitotype classification. Phylogenetic relationships and geographical distribution of four radish mitotypes are also discussed.

Materials and methods

Plant materials

Four representative accessions containing Ogura, DCGMS, DBRMF1, and DBRMF2 mitotypes used in the previous study (Lee et al. 2008) were used to sequence 11 chloroplast fragments (Fig. 1). Ninety breeding lines and cultivars whose mitotypes had been identified using mtDNA-based markers in a previous study (Kim et al. 2007) were used to test consistency of mtDNA and cpDNA-based markers. Geographic distribution of four radish mitotypes were surveyed using 120 radish germplasms requested from the National Plant Germplasm System, Agricultural Research Service, USA.

DNA extraction and PCR amplification

Total genomic DNA was extracted from the leaf tissue of three-leaf stage radishes using a commercial DNA extraction kit (DNeasy Plant Mini Kit, QIAGEN, Valencia, CA, USA) according to the manufacturer's manual. PCR was performed in a 10-μL reaction mixture containing 0.05 μg template, 1 μL 10× PCR buffer, 0.2 μL forward primer (10 μM), 0.2 μL reverse primer (10 μM), 0.2 μL dNTPs (10 mM each), and 0.1 μL polymerase mix (Advantage 2 Polymerase Mix, Clontech, Palo Alto, CA, USA). PCR amplification was carried out with an initial denaturation step at 94°C for 5 min followed by 40 cycles of 94°C for 30 s, 65°C for 30 s, and 72°C for 90 s, with a final 10-min extension at 72°C.

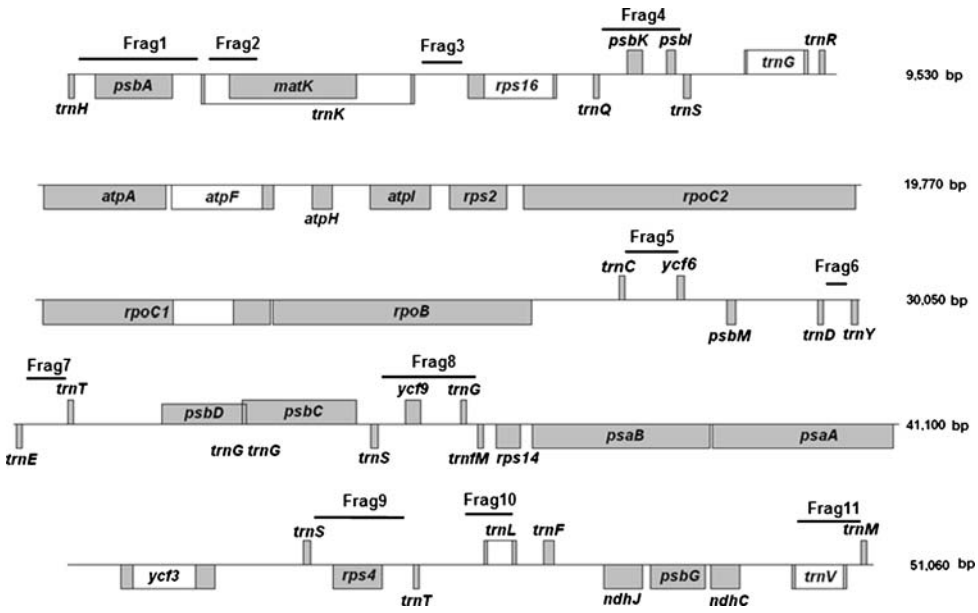

Fig. 1 Gene organization of *Brassica rapa* chloroplast genome. Partial regions of large single copy (LSC) are presented. The *filled rectangular boxes* indicate coding sequence and non-coding RNA genes. The *empty boxes* indicate introns. Genes *on the line* are transcribed from *left* to *right*, and genes *below the line* are transcribed from *right* to *left*. *Bars* on the map indicate the 11 fragments amplified from radish chloroplasts

Sequencing the chloroplast genomes of four radish mitotypes

Primers were mostly designed against tRNA genes of the *Brassica rapa* chloroplast genome to amplify 1–2 kb PCR products. Following successful PCR amplification from four radish mitotypes, the PCR products were purified using the QIAquick PCR Purification kit (QIAGEN) and directly sequenced. Sequencing reactions were carried out using Big Dye (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol, and sequences were obtained using an ABI 3700 Genetic Analyzer (Applied Biosystems).

Mitotype determination by mtDNA and cpDNA-based markers

Previously reported mtDNA-based markers (Kim et al. 2007) were used for PCR amplification, and the products were electrophoresed on a 1% w/v agarose gel to detect band patterns. PCR products amplified using cpDNA-based marker 1 were run on both 8% w/v acrylamide and 1.5% w/v agarose gels. For cpDNA marker 2, PCR products were digested with *Ava*II for 3 h at 37°C and the digested PCR products were run on a 1% w/v agarose gel. The PCR products of cpDNA marker 3 were run on a 2% w/v agarose gel for higher resolution. The primer sequences for the three cpDNA-based markers are presented in Table 1.

Construction of phylogenetic trees

Chloroplast sequences of other species whose entire chloroplast genome sequences were available were obtained from GenBank. The alignment of chloroplast sequence of radish and other species were performed by BioEdit (Hall 1999), and gaps were removed by Gblocks software (Castresana 2000). Phylogenetic trees were constructed using *MEGA* version 4 (Tamura et al. 2007).

Table 1 Sequences of primers used in this study

Primer name	Primer sequence (5′–3′)
M1-F	CTAAGCCGAATAGGATAATCTCAA
M1-R	CAAATATCCTGCTAAAAACTACCATG
M2-F	AGGGCGGTGCTCTGACCAATTGAACTA
M2-R	GAGCGGTTAATGGGGACGGACTGTAAA
M3-F	GCGGGTAGCTTACATATTCCTTCTTATG
M3-R	CGGCCTTGCTATCACTAAAGTGATATC

Results

Identification of highly variable intergenic regions on the chloroplast genomes of four radish mitotypes

In previous studies (Kim et al. 2007; Lee et al. 2008), we reported four radish mitotypes based on the variable gene organizations of mitochondrial genomes. For classification of mitotypes, we used a set of eight molecular markers; however, the banding patterns of PCR products from these eight molecular markers were not always clear due to the complexity and differential stoichiometry of mitochondrial genomes. In addition, many PCR reactions are required to classify a large number of samples. For these reasons, we developed chloroplast genome-based markers, based on the rationale that mitochondria and chloroplasts are transmitted together during reproduction.

Because few radish chloroplast sequences were available, we used sequence information from *B. rapa* (GenBank accession DQ231548), a close relative of radish. Although a complete sequence of the chloroplast genome of *B. rapa* was available at GenBank there was no annotation; therefore, we annotated the entire sequence using information from other species. To identify highly variable regions, we mainly selected intergenic sequences in the large single copy (LSC) region which are known to be more variable than inverted repeats (Maier et al. 1995; Asano et al. 2004) for sequencing (Fig. 1). Based on the *B. rapa* sequences, primers were designed against tRNA sequences since noncoding RNA genes in chloroplast genomes are known to be highly conserved among species (Daniell et al. 2006). Eleven fragments ranging from 490 to 1,465 bp were obtained from four radish mitotypes by direct sequencing of the PCR products (Fig. 1). A total of approximately 8.7 kb was analyzed in this study.

As expected, noncoding RNAs and coding sequences were highly conserved compared with intron and intergenic regions (Table 2). Furthermore, the level of polymorphism of intergenic regions varied depending on position. The majority of relatively large indels were observed in the intergenic regions between the *trnK* and *rps16* genes (Fig 2), suggesting that this intergenic region is a hotspot for insertion or deletion mutations in the radish chloroplast genome.

Development of molecular markers for distinguishing four radish mitotypes using polymorphic sequences of chloroplast genomes

To develop simple PCR-based markers, we first used relatively large indels in the *trnK-rps16* intergenic region. By designing primers to the flanking sequences of indels (Marker 1; Fig 2a), PCR products of different lengths were obtained from four radish mitotypes: DBRMF1 (331 bp), DCGMS (301 bp), DBRMF2 (294 bp), and Ogura (339 bp). When the PCR products were run on 8% w/v polyacrylamide gels, four mitotypes could be distinguished by length polymorphism (Fig. 3a); however, if the PCR products were run on a 1.5% w/v agarose gel, they could only be classified into two groups due to the lower resolution of agarose gels (Fig. 3b).

As a supplementary marker, a CAPS (cleaved amplified polymorphic sequence) marker was developed for detection of DBRMF1 based on a single nucleotide polymorphism in the *trnD-trnY* intergenic region unique to DBRMF1 (Fig. 4a). PCR products amplified using primers binding to the conserved *trnD* and *trnY* genes (Marker 2) were digested with *Ava*II. The DBRMF1 mitotype was not cut because it lacks the *Ava*II restriction site (Fig. 4b). A 17-bp insertion unique to the DCGMS mitotype was detected in the *trnD-trnY* intergenic region. This 17-bp insertion was repeated one more time in the DCGMS mitotype since an identical 17-bp sequence was tandem positioned immediately before this insertion. This repeat was not observed in eight other plant species belonging to the Brassicaceae family (data not shown), suggesting that the 17-bp repeat insertion occurred recently in the DCGMS mitotype. When PCR products amplified with primers against the flanking sequence of this 17-bp insertion (Marker 3) were run on a 2% w/v agarose gel, the DCGMS mitotype was clearly detected (Fig. 4b).

To test whether the results of mitotype classification performed by cpDNA-based markers matched those of mtDNA-based markers, 90 radish breeding lines and cultivars whose mitotypes had been previously identified using mitochondrial genome-based markers (Kim et al. 2007)

Table 2 Sequence fragments of radish chloroplast genomes analyzed in this study

Fragments	Length[a] (bp)	Positions	Polymorphic nucleotides between four radish mitotypes (%)		
			Coding and non-coding RNA genes	Introns	Intergenic regions
1	1,465	*trnH-psbA-trnK*	0.19	–[b]	1.93
2	539	*trnK-matK*	0.30	1.0	–
3	536	*trnK-rps16*	–	–	11.2
4	954	*trnQ-psbK-psbI-trnS*	0	–	0.61
5	644	*trnC-ycf6*	0	–	0.33
6	490	*trnD-trnY*	–	–	4.09
7	557	*trnE-trnT*	–	–	0.36
8	1,186	*trnS-ycf9-trnG-trnfM*	0	–	1.21
9	964	*trnS-rps4-trnT*	0	–	0.30
10	572	*trnT-trnL*	0	0	0.89
11	814	*trnV-trnM*	0	0.34	1.08
Total	8,721		0.11	0.36	2.27

[a] Fragment lengths of DBRMF1 mitotype are given. Fragment lengths of other mitotypes are slightly different due to indels

[b] No corresponding sequences are included in the fragment sequences

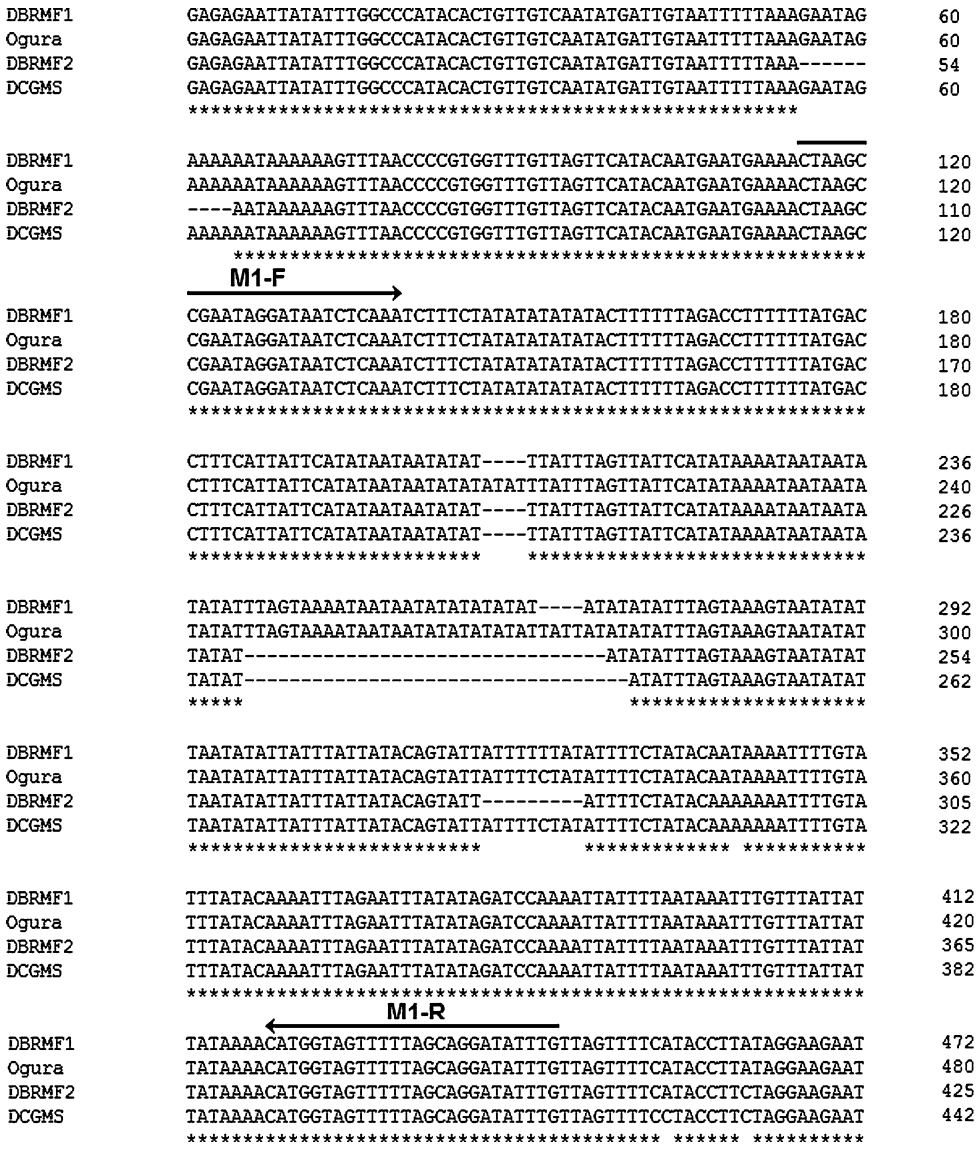

Fig. 2 Sequence alignment of chloroplast intergenic regions between *trnK* and *rps16* genes from four radish mitotypes: Ogura, DCGMS, DBRMF1, and DBRMF2. *Arrows* on the sequences indicate the position of primers for cpDNA-based marker 1

were analyzed using the three chloroplast genome-based molecular markers developed in this study. There was a perfect match between results obtained using mtDNA and cpDNA-based markers (Fig. 5).

Phylogenetic relationship and geographical distribution of four radish mitotypes

The phylogeny of radish was analyzed together with eight members of the Brassicaceae family whose entire chloroplast genome sequences are available. A phylogenetic tree was constructed using ~8.7 kb radish chloroplast sequences and homologous sequences of other species. This tree showed that radish is closely related to *B. rapa* (Fig. 6a). Furthermore, the phylogenic relationship between the four radish mitotypes was analyzed using *B. rapa* as an outgroup. The resulting phylogenic tree showed that the four mitotypes were divided into two subgroups (Fig. 6b), suggesting that the Ogura mitotype responsible for Ogura male-sterility (Ogura 1968) might be derived from DBRMF1, and the DCGMS mitotype causing another male-sterility phenotype (Lee et al. 2008) might originate from the DBRMF2 mitotype.

In addition, the geographic distribution of radish mitotypes was analyzed using 120 radish germplasms that were introduced from different countries. The mitotypes of each accession were identified using cpDNA-based markers (Supplementary Table 1). The majority of accessions belonged to DBRMF1 and DBRMF2 mitotypes, whereas the frequency of DCGMS and Ogura mitotypes was very low. In European counties, the DBRMF2 mitotype was twice as common as the DBRMF1 mitotype (Fig. 7). Conversely, DMRMF1 was twice as common as the DBRMF2 mitotype in Middle East and Eastern Asian countries. The

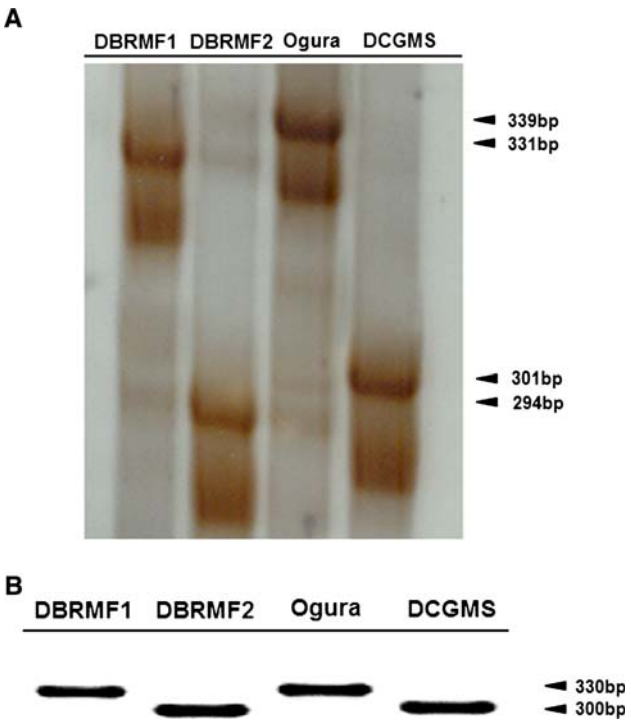

Fig. 3 PCR products of four radish mitotypes amplified using cpDNA-based marker 1. **a** Separation of PCR products on a 8% w/v acrylamide gel. **b** Separation of PCR products on a 1.5% w/v agarose gel

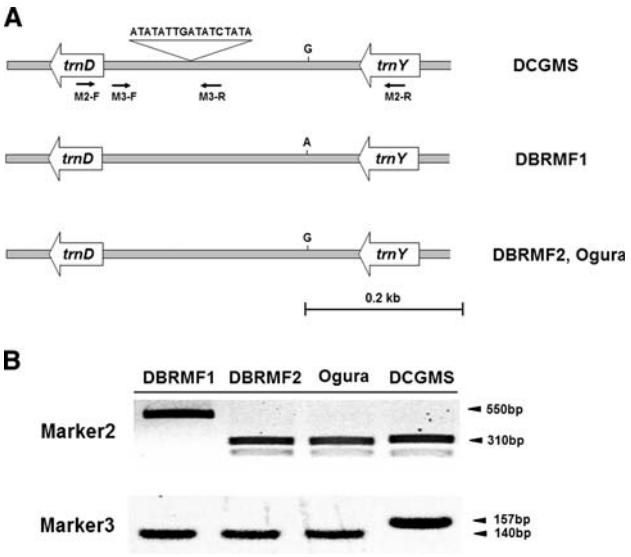

Fig. 4 Variation in the intergenic region between *trnD* and *trnY* and PCR patterns of cpDNA-based markers 2 and 3. **a** Sequence variation between four radish mitotypes. *Arrow-shaped boxes* indicate non-coding tRNA genes and the 5′–3′ direction. Nucleotide sequences on the triangles are inserted sequences. *Arrows below the sequence* indicate the primer positions of cpDNA markers 2 and 3. **b** Digested PCR products of cpDNA marker 2 and PCR products of cpDNA marker 3

DCGMS mitotypes were predominantly identified in accessions from eastern European countries such as Poland and Yugoslavia. Two previously reported DCGMS mitotypes

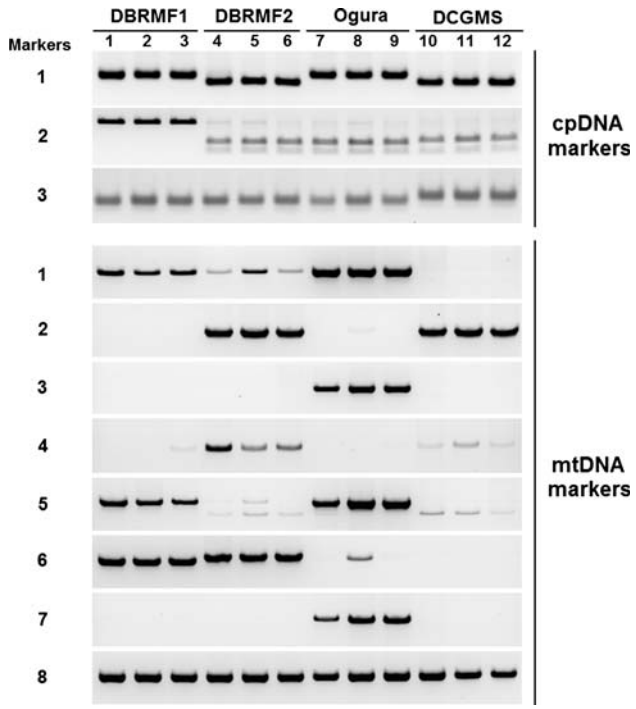

Fig. 5 Comparison of PCR patterns obtained using cpDNA and mtDNA markers. *Lanes 1–12* representative accessions of four radish mitotypes

(Lee et al. 2008) were also identified from accessions introduced from Uzbekistan and Russia. Only three accessions introduced from Kazakhstan contained Ogura mitotypes; however, these three accessions were heterogeneous because some individual plants in the three accessions were identified as DBRMF1 mitotypes.

Discussion

Variation of chloroplast genome sequence among four radish mitotypes

Many studies have been performed to reveal phylogeny of species, genera, and the higher level of taxonomy, but only a few studies on phylogeny based on cytoplasmic DNA variation at the intraspecific level in some forest species (Gao et al. 2007; Meng et al. 2007) and *Aegilops mutica* (Ohsako et al. 1996) have been reported. Here, we showed the phylogenetic relationship between four radish mitotypes based on variation in chloroplast sequences (Fig. 6b). Unexpectedly, sufficient variations existed in chloroplast DNA to resolve the phylogenetic relation of these four radish mitotypes, even though they are members of the same species.

The tree showed that two male-sterility inducing mitotypes, Ogura and DCGMS, were derived from DBRMF1 and DBRMF2 mitotypes, respectively (Fig. 6b). If we

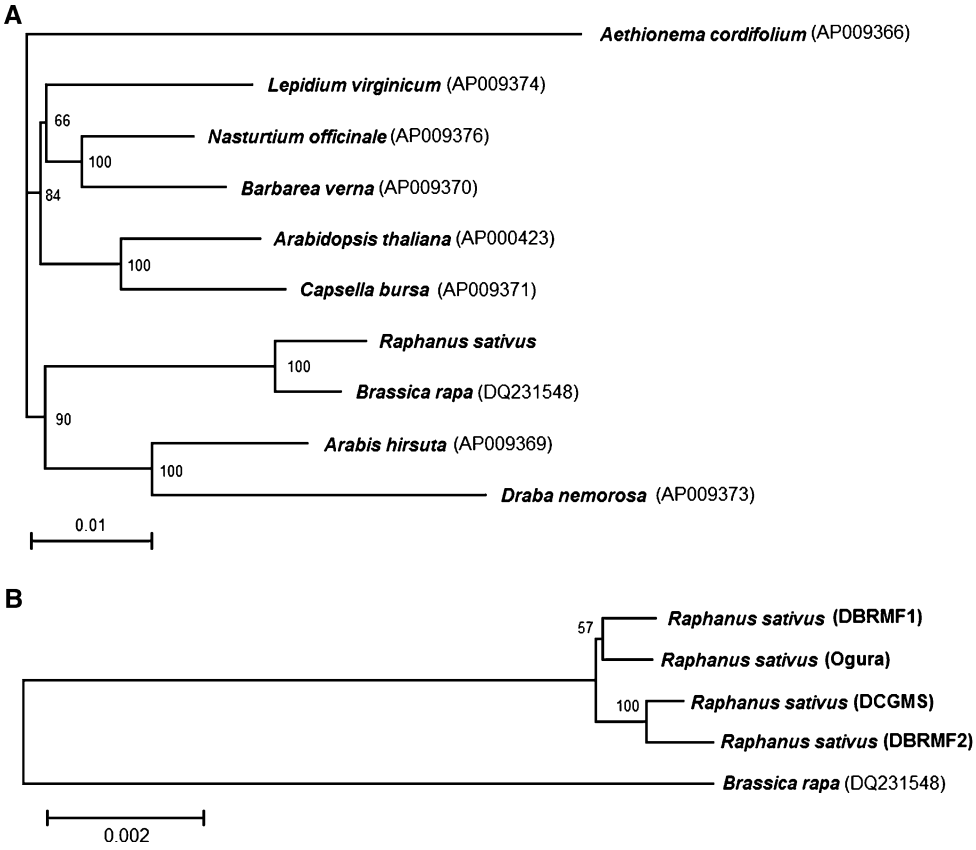

Fig. 6 Phylogenetic trees of chloroplast sequences of 11 selected regions shown in Fig. 1. The GenBank accession numbers are presented beside species names. The numbers at the *nodes* are the bootstrap probability (%) with 1,000 replicates. The *scale bars* indicate nucleotide substitutions per site. **a** A neighbor-joining tree showing the relationship between radish (DBRMF1) and other species belonging to the Brassicaceae family. *Aethionema cordifolium* was included as an outgroup. **b** A neighbor-joining tree showing the relationship between four radish mitotypes. *Brassica rapa* was included as an outgroup

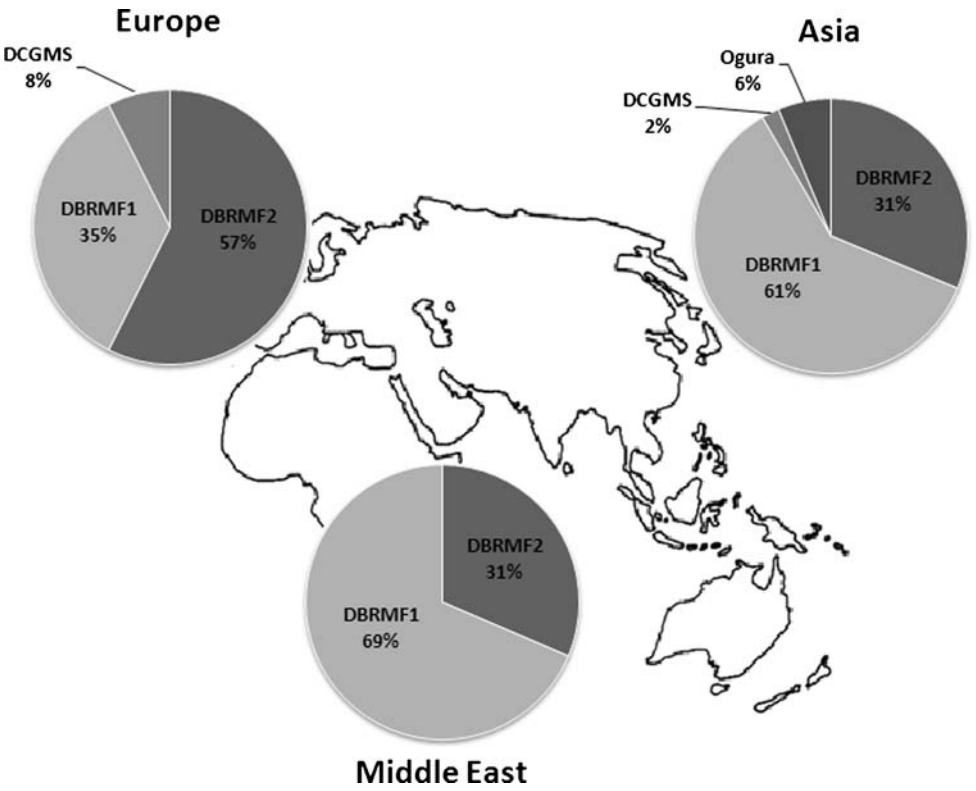

Fig. 7 Geographic distribution of four radish mitotypes analyzed using 137 radish germplasms introduced from diverse countries

assume that *Arabidopsis thaliana* and *B. rapa* diverged ~24 million years ago (Lysak et al. 2005), the Ogura and DCGMS mitotypes diverged from DBRMF1 and DBRMF2 mitotypes ~0.56 and ~0.51 million years ago based on the phylogenetic tree. Although this estimation of the divergence date is not sophisticated, we know that male-sterility phenotypes induced by Ogura and DCGMS mitotypes probably did not result from abrupt mutational events, but rather originated from male-fertile mitotypes hundreds and thousands of years ago. In addition, we speculate that the DCGMS mitotype was created from DBRMF2 in eastern Europe, since the frequency of DBRMF2 was high and DCGMS was predominantly found in Eastern Europe (Fig. 7). However, for a more accurate phylogeographic study, more samples of landraces collected from diverse regions must be analyzed.

Development of cpDNA-based molecular markers to distinguish radish mitotypes

Aside from a phylogenetic study on phylogeny of radishes, the practical goal of this study was to develop more reliable and economical molecular markers to distinguish cytoplasm types of radishes to assist the breeding process. Because nuclear genes involved in restoration of fertility in Ogura and DCGMS mitotypes are different (Lee et al. 2008), precise identification of mitotypes of radish germplasm or breeding lines is crucial for F_1 hybrid breeding in which male-sterility is used as a tool to prevent self-pollination. Without molecular markers, breeders have to resort to time-consuming and complex progeny tests to classify mitotypes; therefore, markers that distinguish mitotypes are invaluable tools in radish breeding. Such molecular markers can be optimized for accurate and high-throughput analysis of large-scale breeding samples.

The mtDNA-based markers developed in the previous study (Kim et al. 2007) were successfully used in classification of radish mitotypes, but analysis of PCR patterns of these markers was sometimes difficult due to the complexity of mitochondrial genomes (Palmer 1988; Albert et al. 1998; Kim et al. 2007). To overcome these problems, we developed simpler cpDNA-based markers based on variation of chloroplast genomes, since mitochondria and chloroplasts are strictly co-transmitted into next generation during reproduction in most angiosperms (Reboud and Zeyl 1993). Co-transmission of mitochondrial and chloroplast genomes in radish was supported by the perfect match of mitotype classification for 90 radish breeding lines and cultivars using cpDNA and mtDNA-based markers. In addition, PCR patterns of both cpDNA and mtDNA markers suggested that the DCGMS mitotype was more closely related to DBRMF2, whereas the Ogura pattern was similar to that of DBRMF1 (Fig. 4). This indicates that the degree of variation of the mitochondrial genome is correlated with that of the chloroplast genome, further indicating strict co-transmission of mitochondria and chloroplast in radish. Similarly, strict co-transmission over a long period was shown in *Silene vulgaris* by analysis of phylogenetic consistence between chloroplast and mitochondrial types (Olson and McCauley 2000).

Unlike complex radish mitochondrial genomes, the chloroplast genome is generally homogenous and circular. Therefore, the accuracy and expedition of radish mitotype determination are greatly enhanced by the development of cpDNA-based markers. Not only do these cpDNA-based molecular markers improve breeding methodology, but they will also be useful in future searches for new mitotypes in radish.

Phylogenetic relationships between *Raphanus sativus* and other species of Brassicaceae family

Polymorphisms in chloroplast genome sequences are a useful tool in phylogenetic studies. Although nuclear ribosomal ITS (internal transcribed spacer) sequences have been widely used (Yang et al. 1999), nuclear sequences are not applicable for phylogenetic study of radish mitotypes that belong to the same species. In early studies, a few coding genes in chloroplasts such as *rbcL* (Clegg 1993) and *ndhF* (Kim and Jansen 1995; Beilstein et al. 2006) were used in higher level taxonomic studies. However, these sequences are not suitable in lower levels of taxonomy, such as interspecific relationships, due to low resolution arising from conservation of coding genes. Therefore, to obtain sufficient variations for phylogenetic studies of lower level taxonomy, noncoding sequences of chloroplast sequences have been exploited (Gielly and Taberlet 1994; Shaw et al. 2007).

In this study, 11 chloroplast regions that were mostly noncoding sequences were used in a phylogenetic study of four radish mitotypes. Because the level under study is the intraspecific relationship, a relatively large number of noncoding sequences needed to be analyzed. Therefore, multiple regions with a total length of approximately 8.7 kb were analyzed to obtain sufficient variations between radish mitotypes. Although highly variable noncoding sequences were used, the tree showing the phylogenetic relationship between different genera of the Brassicaceae family (Fig. 6a) was in good agreement with previous phylogenetic studies of Brassicaceae family in which nuclear ITS sequences (Yang et al. 1999), chloroplast *matK* and nuclear *Chs* gene sequences (Koch et al. 2001), and chloroplast *ndhF* (Beilstein et al. 2006) were used to construct trees. As Scharanz et al. (2007) suggested, *Aethionema cordifolium* was successfully used as an outgroup to root the Brassicaceae family tree (Fig. 6a). *B. rapa* was closer to radish than

to any other genera. *B. rapa* and *Raphanus sativus* belong to the same tribe Brassiceae. Phylogenetic studies using the nuclear genome suggested that *R. sativus* was more closely related to *Brassica nigra* than to *B. rapa* (Yang et al. 1999), but studies using RFLP (restriction fragment length polymorphism) of chloroplast DNA showed that *R. sativus* was closer to *B. rapa* (Warwick and Black 1997). Indeed, we also found that *R. sativus* was more closely related to *B. rapa* when a phylogenetic tree was constructed using partial sequences of fragments 2, 6, 7, and 10 (data not shown). For these reasons, Song et al. (1990) assumed that *R. sativus* might be derived from interspecific hybridization between *B. rapa* and *B. nigra* in which *B. rapa* was the female parent.

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