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Intra- and inter-specific variations in the mitochondrial gene *orf138* of Ogura-type male-sterile cytoplasm from *Raphanus sativus* and *Raphanus raphanistrum*

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Abstract In order to gain a better understanding of the evolution of Ogura male-sterile cytoplasm in radish, a large-scale sequence analysis of mitochondrial *orf138* was conducted using 107 Japanese wild radishes, 29 cultivated radishes and seven *Raphanus raphanisturum*. A single approximately 0.8-kb fragment containing the *orf138* locus was amplified from each plant by PCR, and the nucleotide sequence of an entire coding region of *orf138* was determined by direct-sequencing procedures. An identical sequence to the published *orf138* (Type A) was identified in Japanese wild radish, including a single plant in a population near Kagoshima prefecture where Ogura (1968) first found 'Ogura male-sterile radish'. Thus, it was confirmed that the 'Ogura male-sterile cytoplasm' was derived from Japanese wild radish, with a Type A *orf138* sequence, growing in this area. A total of six nucleotide changes and a single insertion/deletion (indel) were found in *orf138* from both wild and cultivated radishes. By a combination of mutations, the *orf138* sequences of the 143 radish plants were classified into nine types. Based on the pattern of mutations and the distribution of *orf138* variants, it was concluded that the *orf138* variants are derived from Type B or C, after Ogura-type cytoplasm was introduced from *R. raphanistrum* into Japanese wild radish.

Keywords Radish · Male sterility · Ogura cytoplasm · Mitochondrial gene · *orf138*

Introduction

Cytoplasmic male sterility is a maternally inherited trait that is characterized by the inability of a plant to produce functional pollen. Since the application of this trait to breeding programs eliminates labor-intensive hand emasculation, it has been used for F_1 seed production in various crops such as maize, sunflower, sorghum, onion and sugar beet and among others (Hanson 1991).

Apart from the practical interest, cytoplasmic male sterility provides us with an invaluable opportunity to study the interaction between mitochondrial and nuclear genes. Although little is known about the nuclear factor(s) which restore the male-fertile phenotype, molecular analyses have often identified unusual mitochondrial chimeric genes which are causally related to the male-sterile phenotype. *T-urf13* in Texas male-sterile cytoplasm of maize (Dewey and Levings 1986), *orfH522* (*orfc*) in PET1 male-sterile cytoplasm of sunflower (Kohler et al. 1991; Laver et al. 1991), and *orf107* in A3 male-sterile cytoplasm of sorghum (Tang et al. 1996) are some of the many examples. In cruciferous plants, four open reading frames (orfs), i.e. *orf224* (Singh and Brown 1991) in 'Polima' and *orf222* (L'Homme et al. 1994) in 'nap' cytoplasms of *Brassica napus*, *orf263* (Landgren et al. 1996) in *Brassica tournefortii* cytoplasm and *orf138* (Bonhomme et al. 1992) in 'Ogura' cytoplasm of *Raphanus sativus*, have been identified as 'male-sterile genes'. Interestingly, except for *orf224* and *orf222* which exhibit some sequence similarity (79% at the amino-acid level), these male-sterile genes are completely different from each other. How these mitochondrial genes were created in the cruciferous lineage is not known.

To our knowledge, no systematic studies on intraspecific variation using plant mitochondrial genes have been presented. In *orf138*, however, fragmental evidence showing sequence polymorphisms has been reported. A deletion of 39 nucleotides which consist of one of three repeats in the 3′ part of the coding region was demonstrated in a Japanese cultivar 'Kozena' (Iwabuchi et al. 1993) and Japanese wild radish (Yamagishi and Terachi 1996), whereas a deletion of 78 nucleotides including two of the three repeats was recently found in some rapeseed cybrids (Bellaoui et al. 1998). Also, two

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orf138 variants containing single or double nucleotide substitutions from the published Ogura *orf138* sequence were revealed in wild radish, *Raphanus raphanistrum* (Yamagishi and Terachi 1997).

Our previous studies showed that many populations of Japanese wild radish and some strains of *R. raphanistrum* contained plants having *orf138*, whereas few cultivated radishes possessed it (Yamagishi and Terachi 1996, 1997). From these results, we concluded that a direct ancestor of 'Ogura male-sterile radish' is Japanese wild radish, and inferred a close relationship between Japanese wild radish and *R. raphanistrum*. We also found that all of *orf138* from *R. raphanistrum* (Yamagishi and Terachi 1997) and Japanese wild radish (our unpublished data) so far sequenced had a few base substitutions from the published Ogura *orf138* sequence. This fact made it impossible for us to pinpoint the origin of this 'Ogura' cytoplasm, that was spread worldwide as a breeding material. However, the wide distribution of *orf138* in the wild radishes across the different species suggests the existence of further variants, and systematic data on intra- and inter-specific variations in *orf138* will provide useful information on the origin and differentiation of Ogura-type cytoplasm. In the present report we thus conducted a large-scale sequence analysis of *orf138* using 114 wild and twenty nine cultivated radishes, in order to gain a better understanding of the evolution of Ogura-type cytoplasm in radishes.

In the present analysis, a total of six nucleotide changes and a single insertion/deletion (indel) were found in the coding region of *orf138* from wild and cultivated radishes. By a combination of these mutations, *orf138* sequences were classified into nine types. Among them, an identical *orf138* sequence to the published one was first identified in Japanese wild radish. Based on the pattern of mutations and the distribution of *orf138* variants, the ancestral types of *orf138* were inferred in *R. raphanistrum* and the differentiation of Ogura-type cytoplasm among wild and cultivated radishes was discussed.

Material and methods

Plant material

Table 1 lists the Japanese wild radish studied. Traditionally, Japanese wild radish has been classified as *R. sativus* forma *raphanistroides*, and our previous PCR-aided survey showed that 19 out of 26 populations of Japanese wild radish contained plants possessing *orf138* (Yamagishi and Terachi 1996). Two to eleven plants collected from each of the 19 populations (a total of 107 plants) were subjected to sequence analysis.

Table 2 shows the radish cultivars analyzed here. Except for the Japanese cultivar 'Kozena', the distribution of Ogura-type cytoplasm in cultivated radishes had been earlier restricted to the three Chinese cultivars, 'Chibetto Kei Daikon', 'Daibaika' and 'Soubaika Kansaitou' (Yamagishi and Terachi 1996). Further extensive surveys, however, identified plants with Ogura-type cytoplasm in two Japanese cultivars, 'Noushi-No.1' and 'Sabaka', and in three Chinese cultivars, 'Koui', 'Meiri' and 'Taihei'. Two to four plants (a total of 28 plants) in each of the nine cultivars were used in the present study. A single plant of 'MS-Gensuke', which is a male-sterile variety having 'Ogura cytoplasm', was used as a control.

Table 3 indicates the strains of *R. raphanistrum* investigated. Our previous study showed that three out of six strains of *R. raphanistrum* collected in USA and Turkey contained *orf138* (Yamagishi and Terachi 1997). One to four plants (a total of seven plants) in three strains, RS-12, RS-15 and RS-17, were analyzed.

PCR amplification and nucleotide sequencing of *orf138*

The *orf138* sequence is specific to the mitochondrial DNA of Ogura-type cytoplasm in radishes. As a major configuration of the Ogura mitochondrial genome, *orf138* is located between the *trfM* and *orfB* genes (Fig. 1, Bellaoui et al. 1998). Primers A and F which are homologous to *trfM* and the 3′-flanking region of *orf138*, respectively, were synthesized. PCR using primers A and F amplified a 789-bp DNA fragment that contained an entire coding and the 5′/3′-flanking regions of *orf138*. The detailed PCR

Table 1 Japanese wild radish used for DNA sequencing analysis of *orf138*

Abbrev.	Population	Prefecture	Island	Number of plants[a]
Irm	Iriomote	Okinawa	Okinawa	2 (12)
Znp	Zanpazaki	Okinawa	Okinawa	6 (10)
Tng	Tanegashima	Kagoshima	Kyushu	7 (7)
Ksk	Kushikino	Kagoshima	Kyushu	2 (2)
Tmk	Tomioka	Kumamoto	Kyushu	9 (15)
Fke	Fukue	Nagasaki	Kyushu	2 (4)
Imj	Imajuku	Fukuoka	Kyushu	9 (16)
Srh	Shirahama	Miyazaki	Kyushu	11 (15)
Dai	Daiei	Tottori	Honshu	7 (16)
Hms	Hamasaka	Hyogo	Honshu	2 (3)
Mhm	Mihama	Wakayama	Honshu	3 (15)
Int	Inatori	Shizuoka	Honshu	3 (3)
Cts	Chitose	Chiba	Honshu	11 (16)
Hkt	Hikatae	Toyama	Honshu	2 (15)
Kkz	Kakizaki	Niigata	Honshu	8 (16)
Atm	Atsumionsen	Yamagata	Honshu	11 (15)
Iws	Iwasaki	Aomori	Honshu	5 (12)
Tns	Tanesashi	Aomori	Honshu	4 (4)
Erm	Erimomisaki	Hokkaido	Hokkaido	3 (9)
			Total	107 (205)

[a] The number of plants possessing *orf138*. The DNA sequence was determined for each of these plants. The numbers in parentheses indicate the total number of plants for which the presence of *orf138* was surveyed by a PCR-aided assay [Yamagishi and Terachi (1996), and unpublished results]

conditions employed were described by Yamagishi and Terachi (1997).

A PCR product was purified using a SUPRECO2 filter (Takara Shuzo), according to the manufacturer's instruction. Direct-sequencing was performed using a Thermo Sequenase fluorescent-labelled primer cycle sequencing kit (Pharmacia) with 7-deaza-dGTP and Cy5-labelled primers G and H (Fig. 1). The nucleotide sequence was determined using the ALF express DNA sequencer (Pharmacia) as described in the specification supplied. By using Cy5-labelled primers G and H, both strands covering an entire coding region of *orf138* (at least 450 bp) were sequenced. The sequence data were deposited in the DDBJ/GENEBANK/EMBL databases under nos. AB055435–AB055443. The sequence data were analyzed with GeneWorks software (Oxford Molecular Group), and the DnaSP software package (Rozas and Rozas 1999).

Table 2 The radish cultivars used for DNA sequencing analysis of *orf138*

Cultivar	Origin	Number of plants[a]
Kozena	Japan	2 (4)
Noushi-No.1	Japan	3 (3)
Sabaka	Japan	2 (2)
Chibetto Kei Daikon	China	2 (4)
Daibaika	China	4 (4)
Soubaika Kansaitou	China	4 (4)
Koui	China	3 (3)
Meiri	China	4 (4)
Taihei	China	4 (4)
MS-Gensuke	Japan	1 (1)
	Total	29 (34)

[a] The number of plants possessing *orf138*. The DNA sequence was determined for each of these plants. The numbers in parentheses indicate the total number of plants for which the presence of *orf138* was surveyed by a PCR-aided assay [Yamagishi and Terachi (1996, 1997), and unpublished results]

Table 3 *R. raphanistrum* used for DNA sequencing analysis of *orf138*

Strain	Collection site	Number of plants[a]
RS-12	California, USA	2 (10)
RS-15	Turkey	4 (4)
RS-17	Turkey	1 (3)
	Total	7 (17)

[a] The number of plants possessing *orf138*. The DNA sequence was determined for each of these plants. The numbers in parentheses indicate the total number of plants for which the presence of *orf138* was surveyed by a PCR-aided assay (Yamagishi and Terachi 1997)

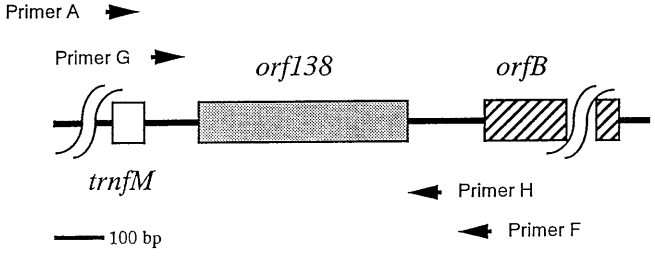

Fig. 1 Organization of the *orf138* locus from Ogura cytoplasm of radish. *Horizontal arrowheads* indicate positions of the primers used for PCR amplification (primers A and F) and DNA sequencing (primers G and H)

Results

Intra- and inter-specific variations in the *orf138* from wild and cultivated radishes

The nucleotide sequence of an entire coding region of *orf138* was examined in 143 different plants. PCR amplification using the primers A and F was made in 107 Japanese wild radishes, 29 cultivated radishes (including a plant of 'MS-Gensuke' used as a control for the Ogura male-sterile cytoplasm), and seven plants of *R. raphanistrum*. By direct-sequencing we identified a 417-bp open reading frame (i.e. *orf138*) encoding a 138 amino acid-long polypeptide in 126 plants. A 378-bp open reading frame (*orf125*) encoding a 125 amino acid-long polypeptide was found in 'Kozena' and the 15 plants of Japanese wild radish, Iws, Atm and Tmk. The nucleotide sequence of the *orf138* from 'MS-Gensuke', hereafter designated as Type A, was completely identical to the *orf138* deposited in the public databases under accession no. Z12626 (Bonhomme et al. 1992) and no. Z18896 (Krishnasamy and Makaroff 1993).

Compared with the Type A sequence, six nucleotide changes and one deletion were found (Fig. 2, Table 4). Five out of the six changes were clustered in the first 100-bp region of *orf138*, whereas a deletion was located in the 3′ half of the coding region of *orf138*. Among the six nucleotide changes, five (A to C at the 7, 61 and 90 sites, and T to A at the 95 and 329 sites) are non-synonymous and the remaining one (A to G at site 99) is synonymous. With the non-synonymous (replacement) changes, Thr, Lys, Leu, Phe and Ile residues at amino-acid positions 3, 21, 30, 32 and 110 in Type A, respectively, are replaced by Pro, Gln, Phe, Tyr and Lys residues, respectively, at the corresponding positions in the *orf138* variants. If RNA editing (C to U conversion) is considered, a nucleotide change at site 7 in the variants may create Ser, not Pro, at amino-acid position 3. It is also possible that RNA editing at site 61 creates a new stop codon in a variant instead of the 21st Gln.

The variant found in 'Kozena' and some Japanese wild radishes was explained by a deletion (39 bp) of the middle or the last repeat of the three tandemly repeated sequences, corresponding to a Lys- and Glu-rich motif in *orf138*. The deleted or smaller version of the *orf138* sequence had been reported in 'Kozena' as *orf125* (Iwabuchi et al. 1993) or *orf138-S* (Yamagishi and Terachi 1996). The sequence of two 'Kozena' and 15 Japanese wild radish plants determined by us was completely identical to that deposited in the public databases (accession no. AB015327).

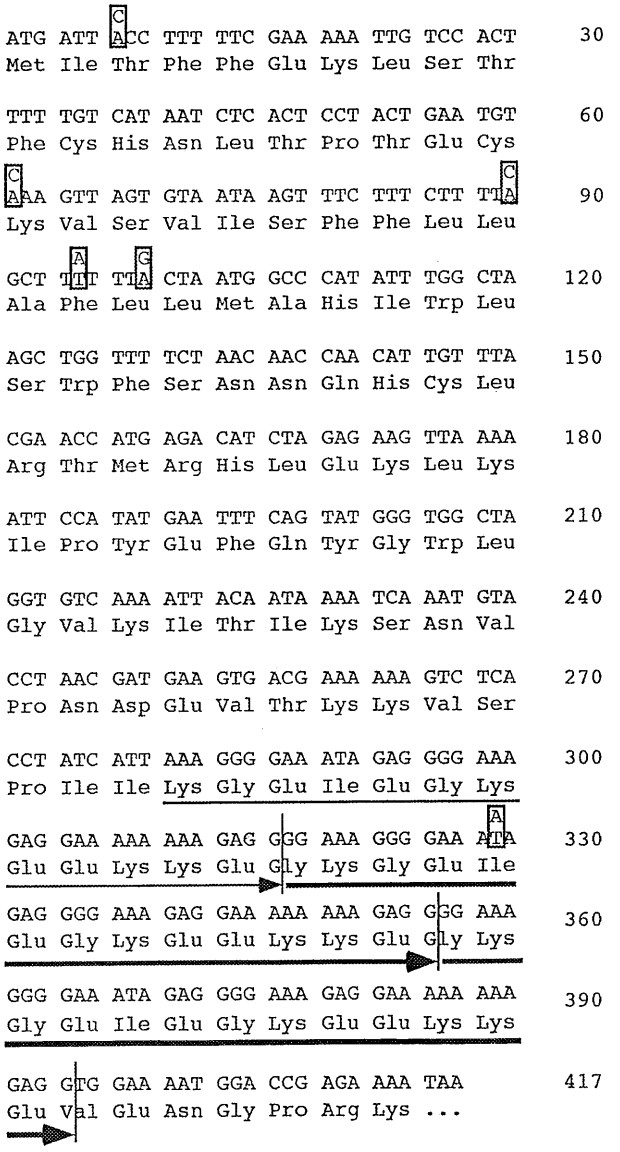

Fig. 2 Nucleotide and derived amino-acid sequence of the coding region of the Type A *orf138* sequence. Nucleotides which are substituted in the variants are *boxed.* The one *thin* and two *thick horizontal arrows* indicate three imperfect 39-bp repeats present in the C-terminus of *orf138*, whereas the *vertical lines* show the border between repeats. One of the two repeats represented by *thick arrows* is deleted in the Type F sequence

By the combination of the seven mutations mentioned above, the *orf138* sequences from 143 plants were classified into nine types (Type A to Type I, Table 4). Except for a 39-bp deletion, the sequence of Type F, represented by 'Kozena', was completely identical to that of Type C. The number of base changes between the different types was very small; at most four base changes were enough to explain their differences (e.g. between Type D and Type I).

The estimates of nucleotide diversity, π (Nei 1987), among a total of 107 Japanese wild radishes, was 0.0029 for the entire coding region of *orf138*, whereas that among 29 cultivated radishes was 0.0026. The estimate among six *R. raphanistrum* was 0.0007 for the entire coding region of *orf138.*

The distribution of orf138 variants in wild and cultivated radishes

Table 5 summarizes the distribution of *orf138* variants in wild and cultivated radishes. All but Type H were found in 107 Japanese wild radishes. In eight populations ('Irm', 'Znp', 'Mhm', 'Hkt', 'Cts', 'Ksk', 'Iws' and 'Int'), only one type of *orf138* sequence was identified. In the remaining 11 populations two to four types of *orf138* were found. In particular, four types of *orf138* (sequence Types B, C, D and F) were identified in the nine plants from the Tomioka population ('Tmk').

In contrast with Japanese wild radishes, only one type of *orf138* sequence was identified in all cultivated radishes except for 'Chibetto Kei Daikon'. The three, two and two plants in the Japanese cultivars 'Noushi-No.1', 'Sabaka' and 'Kozena', respectively, possessed only the Type B, E and F sequence. respectively. Five cultivars introduced from South China ('Daibaika', 'Soubaika Kansaitou', 'Koui', 'Meiri' and 'Taihei') contained only the Type H sequence. In 'Chibetto Kei Daikon', one plant contained the Type A sequence, whereas the other had Type B.

As for *R. raphanistrum*, all four plants in the strain RS-15 contained the Type B sequence. A single plant in the strain RS-17 also contained Type B, whereas two plants in the strain RS-12 had Types B and C (Table 5).

Table 4 Summary of DNA variation identified in the coding region of *orf138* from wild and cultivated radishes

Type of *orf138*	Nucleotide site in the coding region of orf138						
	7	61	90	95	99	329	Δ[a]
A	A(Thr)	A(Lys)	A(Leu)	T(Phe)	A(Leu)	T(Ile)	–
B	"[b]	"	"	"	G	"	–
C	"	"	"	A(Tyr)	G	"	–
D	C(Pro)	"	"	"	"	"	–
E	C(Pro)	"	"	A(Tyr)	G	"	–
F	"	"	"	A(Tyr)	G	"	+
G	"	"	C(Phe)	"	G	"	–
H	"	C(Gln)	"	"	G	"	–
I	"	"	"	A(Tyr)	G	A(Lys)	–

[a] + and – indicate the presence and absence of a 39-bp deletion, respectively

[b] The symbol, ", indicates an identical nucleotide sequence to the Type A sequence. Amino-acid residues which are replaced by DNA replacement changes are shown in parentheses

Table 5 Distribution of *orf138* variants among wild and cultivated radishes

	Type of orf138									
	A	B	C	D	E	F	G	H	I	Total
Japanese wild radish										
Irm	0	2	0	0	0	0	0	0	0	2
Znp	0	6	0	0	0	0	0	0	0	6
Mhm	0	3	0	0	0	0	0	0	0	3
Hkt	0	0	2	0	0	0	0	0	0	2
Cts	0	0	11	0	0	0	0	0	0	11
Ksk	0	0	0	2	0	0	0	0	0	2
Iws	0	0	0	0	0	5	0	0	0	5
Int	0	0	0	0	0	0	3	0	0	3
Imj	3	6	0	0	0	0	0	0	0	9
Dai	1	6	0	0	0	0	0	0	0	7
Fke	1	0	0	1	0	0	0	0	0	2
Kkz	0	2	6	0	0	0	0	0	0	8
Tns	0	1	3	0	0	0	0	0	0	4
Hms	0	1	0	0	1	0	0	0	0	2
Srh	0	10	0	0	0	0	1	0	0	11
Erm	0	2	0	0	0	0	0	0	1	3
Tng	1	3	0	3	0	0	0	0	0	7
Atm	0	1	0	2	0	8	0	0	0	11
Tmk	0	1	1	5	0	2	0	0	0	9
Subtotal	6	44	23	13	1	15	4	0	1	107
%	5.6	41.1	21.5	12.1	0.9	14.0	3.7	0.0	0.9	100
Cultivated radish										
MS-Gensuke	1	0	0	0	0	0	0	0	0	1
Noushi-No.1	0	3	0	0	0	0	0	0	0	3
Sabaka	0	0	0	0	2	0	0	0	0	2
Kozena	0	0	0	0	0	2	0	0	0	2
Daibaika	0	0	0	0	0	0	0	4	0	4
Soubaika Kansaitou	0	0	0	0	0	0	0	4	0	4
Koui	0	0	0	0	0	0	0	3	0	3
Meiri	0	0	0	0	0	0	0	4	0	4
Taihei	0	0	0	0	0	0	0	4	0	4
Chibetto Kei Daikon	1	1	0	0	0	0	0	0	0	2
Subtotal	2	4	0	0	2	2	0	19	0	29
%	6.9	13.8	0.0	0.0	6.9	6.9	0.0	65.5	0.0	100
R. raphanistrum										
RS-12	0	1	1	0	0	0	0	0	0	2
RS-15	0	4	0	0	0	0	0	0	0	4
RS-17	0	1	0	0	0	0	0	0	0	1
Subtotal	0	6	1	0	0	0	0	0	0	7
%	0.0	85.7	14.3	0.0	0.0	0.0	0.0	0.0	0.0	100
Total	8	54	24	13	3	17	4	19	1	143
%	5.6	37.8	16.8	9.1	2.1	11.9	2.8	13.3	0.7	100

Discussion

Polymorphism in the *orf138* of Ogura-type cytoplasm

This is the first report exploring intraspecific variations in a plant mitochondrial gene at the nucleotide-sequence level. Since the evolutionary rate of plant mitochondrial genes is much slower than that of nuclear genes (Wolfe et al. 1987; Laroche et al. 1997), low levels of polymorphism were expected in the coding region of *orf138*. However, a total of seven mutations (six nucleotide changes and one insertion/deletion) were identified in *orf138* of the Ogura-type cytoplasm. Excluding the deletion, the nucleotide diversity (π: Nei 1987) for the entire coding region of *orf138* from Japanese wild radish was 0.0029. Although no comparable data are available for any other plant mitochondrial genes, this value is within the range of nucleotide variation reported for nuclear genes of other wild plant species, e.g. 0.0056 for the *Adh* coding region of *Arabidopsis thaliana* (Innnan et al. 1996), and 0.0011 for the *Pgi* coding region of *Dioscorea tokoro* (Terauchi et al. 1998). Because of the low variability of the mitochondrial genes, they have been thought unsuitable for plant phylogenetic studies below family level (Hiesel et al. 1994). However, the aforementioned fact indicates that some mitochondrial genes are as divergent as the nuclear ones coding allozymes. This suggests, further, that some of them can be used to study

population and evolutionary genetics at inter- or intraspecific level in plants.

Maintenance of female (or male-sterile) plants in populations of plant species has been examined by Lewis (1941) and Ross and Shaw (1971). Charlesworth (1981) studied mathematically the cytoplasmic genic system of male sterility, and found that polymorphism is not maintained except in the very specific case where a nuclear restorer gene (Rf) is associated with reduced fitness. Indeed, most of wild radish populations are fixed with the Rf gene, whereas the cytoplasm shows a differentiation into normal and Ogura types (Yamagishi 1998). In such populations, mutations in *orf138* have no effect on seed performance. This implies that *orf138* is selectively neutral in wild radishes. In harmony with this idea, the tests of neutrality (Tajima 1989; Fu and Li 1993) using a set of 107 *orf138* sequences from Japanese wild radish did not reject the neutral mutation hypothesis (Fu and Li's test: D*=–0.0405, *P*>0.10; Tajima's test: D=0.2989, *P*>0.10). Of interest to studies in the future is how polymorphism, not only in *orf138* but also in the type of cytoplasm (i.e. Ogura vs normal), is maintained in wild radishes.

Differentiations and evolution of *orf138* in wild and cultivated radishes

The published *orf138* sequence of Ogura-type cytoplasm (Bonhomme et al. 1992; Krishnasamy and Makaroff 1993), designated as Type A in this report, was found in six plants of Japanese wild radish and in a single plant of the Chinese cultivar 'Chibetto Kei Daikon'. As expected, our control variety 'MS-Gensuke' possessed the Type A sequence (Table 5). It should be noted that plants with the Type A sequence were found on Tanegashima island ('Tng') near Kagoshima prefecture where Ogura (1968) originally found the male-sterile cytoplasm (Table 5). Thus, it is probable that the male-sterile radishes that Ogura (1968) found in escaped conditions were those with a Type A *orf138* sequence and, consequently, that the cytoplasm with the Type A *orf138* has been spread worldwide.

The frequency of Type A, however, was low; only 5.6% of Japanese wild radishes examined here possessed this type. Instead, Type B was the major one in Japanese wild radishes. The sequence of Type B is different from Type A by a single synonymous change at site 99 (Fig. 2, Table 4), and 41% of the Japanese wild radishes examined here possessed this sequence. That Type B was the major one was also demonstrated by the fact that it was found in 13 out of the 19 populations mapped in diverse areas of Japan. In addition, Type B was also the major one in *R. raphanistrum*; here all four, a single, and one of two plants in the strains RS-15, RS-17 and RS-12, respectively, had the Type B sequence (Table 5). These results clearly indicate that Type B is one of the ancestral types of *orf138*, and that Type A was derived from Type B in Japanese wild radish.

The Type C sequence is distinguished from Type B by a single replacement change, the site being at 95.

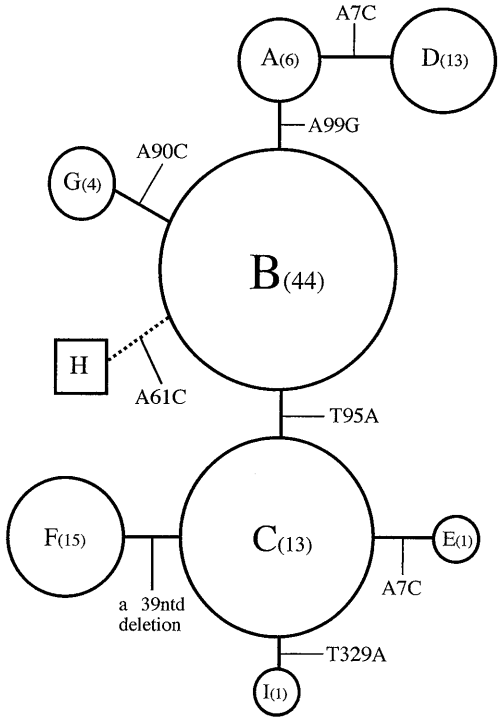

Fig. 3 Phylogenetic relationships among Ogura-type cytoplasms of radishes. An *alphabetical letter* in the circles (or a square) indicates the type of *orf138*, whereas the *number in parenthesis* shows the number of Japanese wild radishes containing the corresponding *orf138*. The size of circles is drawn to represent the relative frequency of each *orf138* variant, and a mutation accounting for the difference between the two variants is shown. Since Type H is identified only in the Chinese cultivars, the connection between Type B and Type H is only tentative (represented by a *dotted line*)

The frequency of Type C is relatively high (21.5%) in the present material. About 50% of the plants with this sequence were taken from a single population, Chitose ('Cts'). In Japanese wild radishes, the distribution of the Type C sequence is more limited than that of Type B. It is of interest, however, that a single plant in strain RS-12 of *R. raphanistrum* also contained the Type C sequence. This demonstrates that the polymorphism present in the *orf138* of *R. raphanistrum* is shared with Japanese wild radish, suggesting a close relationship of the cytoplasm between *R. raphanistrum* and Japanese wild radish. Type C is considered to be another ancestral type of *orf138*.

Type E and Type I were identified in a single plant in a restricted population (Type E in 'Hms', Type I in 'Erm'). These two types are distinguished from Type C by a single replacement change, and it is suggested that both of them were derived from Type C in Japanese wild radish. Similarly, the Type G and Type H variants can be explained by a single nucleotide change from Type B, whereas Type D evolved from Type A. As previously mentioned, Type F is considered to be derived from Type C by a 39-nucleotide deletion. Taken together, a plausible scenario for the differentiation of *orf138* in wild and cultivated radishes is depicted in Fig. 3.

Overall, the evolution of Ogura-type cytoplasm is as follows. While it is still unclear how *orf138* was created in wild radishes, the Ogura-type cytoplasm possessing Type B and Type C *orf138* sequences came into existence in *R. raphanistrum*. These cytoplasms were introduced, along with the nuclear Rf gene(s), into Japanese wild radish by an unknown process. Because of the presence of the Rf gene, Ogura-type cytoplasm was distributed to, and has been maintained in, various areas of Japan. Several independent mutations occurred in *orf138* of Japanese wild radish, and the variants including Type A have been maintained in populations. Ogura-type cytoplasms with particular types of *orf138* were picked up in cultivation as described below, and have been retained in some native varieties.

Ogura-type cytoplasm found in cultivated radishes

Although the number is restricted, a few Japanese cultivars have *orf138* and its variant, *orf125*. For each of them, the same *orf138* variants were found in Japanese wild radishes which were collected in sites near to the native locations of these cultivars; 'Iws' in the Aomori prefecture to 'Kozena' (Type F), 'Znp' in Okinawa island to 'Noushi-No.1' (Type B) and 'Hms' in the Hyogo prefecture to 'Sabaka' (Type E). These results suggest that the Ogura-type cytoplasm found in each of these cultivars was derived from Japanese wild radish growing in the corresponding area. It should be pointed out that the variation reported in this article is maternally inherited, and cannot be introduced into the cultivated radish by an accidental contamination of pollen from the wild-type. Hence, the cultivars with Ogura-type cytoplasm are the results of the intentional introduction of wild radish plants, or their progenies, into cultivation by farmers. The presence of the same *orf138* variants between the cultivars and the wild radishes is evidence of direct or indirect use of the latter for radish cultivation in Japan. Further studies on the nuclear genes will clarify their relationships in more detail.

Except for 'Chibetto Kei Daikon', all the Chinese cultivars studied here contained the Type H sequence which was not found in the Japanese wild radishes. It would be necessary to check the mitochondrial DNA of the Chinese wild radish in order to elucidate the origin of Type H.

The effect of mutations in the ORF138 protein on the male-sterile phenotype of the Ogura-type cytoplasm

Although the cDNA sequences were not examined in this study for each of the *orf138* variants, the absence of editing in *orf138* transcripts was shown by Krishnasamy and Makaroff (1994) using 'Ogura male-sterile radish'. All the replacement changes found here, therefore, should lead to normal amino acid changes at the corresponding positions. Interestingly, most of the replacement changes found in this study were clustered in the amino-terminus of the ORF138 protein. The exceptions were the difference between Ile in Type A and Lys in Type I at the 110th amino-acid position, and the absence of one of three 13 amino-acid repeats in the carboxyl-terminus of Type F (Table 4). Especially, three replacement changes occurred in the region between sites 61 and 95 (corresponding to the amino-acid positions between 21 and 33). The reason why replacement changes are clustered in the amino-terminus is unknown, but it should be noted that the amino-terminus of the ORF138 protein is hydrophobic and considered to be associated with the mitochondrial membrane (Grelon et al. 1994; Krishmasamy and Makaroff 1994). It would of interest to determine the effects of each amino-acid change on the structure and the function of the ORF138 protein.

Our previous crossing experiments (Yamagishi and Terachi 1994, 1997) showed that cytoplasm with the Type B and Type D *orf138* sequence, like Type A, induces male sterility. Further observations on the fertility of cultivated radishes (Yamagishi and Terachi 1996) indicated that Ogura-type cytoplasm with the Type H sequence also induces male sterility. The cytoplasm containing the Type F sequence (i.e. *orf125*) is known as 'Kozena male-sterile cytoplasm', and can induce male sterility not only in radish but also in *B. napus*. These facts indicate that the third and the 21st amino acid changes identified here, and the 13 amino-acid deletion, do not affect the male-sterile phenotype of Ogura-type cytoplasm. Further crossing experiments using *orf138* variants should reveal the effect of the other three replacements in *orf138* on the phenotype of Ogura-type cytoplasm.

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