

Authors:

Dayou Cheng^{1,2}, Yu Yoshida¹, Kazuyoshi Kitazaki¹, Shinya Negoro¹, Hiroyuki Takahashi³, Dechang $Xu²$, Tetsuo Mikami¹, Tomohiko Kubo¹

Title:

Mitochondrial genome diversity in *Beta vulgaris* **L. ssp** *vulgaris* **(Leaf and Garden Beet Groups) and its implications concerning the dissemination of the crop**

Affiliations:

1: Laboratory of Genetic Engineering, Research Faculty of Agriculture, Hokkaido University, N-9, W-9,

Kita-ku, Sapporo, 060-8589, Japan

2: School of Food Science and Engineering, Harbin Institute of Technology, West Da-Zhi Street, Harbin, Heilongjiang, 150001, China

3: Memuro Upland Farming Station, National Agricultural Research Center for Hokkaido Region (NARCH), Shinsei, Memuro, 082-0081, Japan

Corresponding author:

Tomohiko Kubo

E-mail: tomohiko@abs.agr.hokudai.ac.jp

Tel/Fax: +81-11-706-2484

Abstract

Four mitochondrial minisatellites were used to study cytoplasmic diversity in leaf and garden beet germplasm resources. Eleven multi-locus haplotypes were identified, of which one (named mitochondrial minisatellite haplotype 4, hereafter min04) was associated with male-sterile Owen cytoplasm and two others (min09 and min18), with a normal fertile cytoplasm. European leaf beet germplasm exhibited the greatest haplotype diversity, with min09 and min18 predominating. In North African leaf beet accessions, only these two haplotypes were observed, making it likely that North African accessions were descended from European genotypes. The prevalence of min18 was also noted in leaf beet from the Middle East and western Asia. Such a pattern contrasts with that found in east Asian leaf beet where the two haplotypes were extremely rare. The geographical structure of the mitochondrial haplotypes allowed us to infer possible dissemination pathways of leaf beet. Additionally, we showed that mitochondrial genome diversity was low in garden beet germplasm, with min18 being highly predominant. An explanation of this limited diversity may lie in the geographically restricted origin of as well as relatively short cultivation histories of garden beet.

Key words

Plant mitochondria, variable number of tandem repeat, *Beta vulgaris*, leaf beet, garden beet, cytoplasmic male sterility

Introduction

Beets are believed to have been initially domesticated as a leafy vegetable in southern and eastern Europe and adjacent regions of Asia (Hancock 2004; Biancardi 2005). They probably resembled what we would describe today as leaf beet. Although various possible pathways for the domestication of cultivated beets (*Beta vulgaris* ssp. *vulgaris*) have been proposed (Bosemark 1979; de Bock 1986; Fischer 1989), it is now widely accepted that cultivated beets have direct relationships with the wild sea beet (*B. vulgaris* L. ssp. *maritima*) (Biancardi 2005). Through cultivation, variants with swollen parts, which consisted of the root and hypocotyl or of hypocotyl only, were selected. Such selection has resulted in four major groups of cultivars which differ from one another in their external features and common usage: the leaf beet group [*Beta vulgaris* L. ssp. *vulgaris* convar. *cicla* (L.) Alef.], garden beet group [*B. vulgaris* L. ssp. *vulgaris* var. *vulgaris*], fodder beet group [*B. vulgaris* L. ssp. *vulgaris* var. *rapacea* Koch], and sugar beet group [*B. vulgaris* L. ssp. *vulgaris* var. *altissima* Döll] (Lange et al. 1999; Hammer 2001).

Sugar beet is economically the most important cultivated form and its gene pool may be genetically narrow, mainly for two reasons. All current sugar beet cultivars are presumably descended from a single-source population, the White Silesian beet (Fischer 1989). In addition, the introduction of cytoplasmic male sterility (CMS) and the near ubiquitous use of three recessive nuclear genes, two involved in the restoration of male fertility (Owen 1942, 1945) and one for monogerm seeds (van Geyt et al. 1990), have likely further narrowed the genetic variation of the breeding stock. By contrast, leaf beet has never been bred intensively, thereby leading us to assume that a lot of variation is still present in leaf beet cultivars and landraces. Baranski et al. (2001) emphasized that leaf beet as well as garden beet can be considered as a potential, valuable source of desirable characters lacking in sugar beet breeding programs. It should also be noted that leaf and garden beets do not have the weedy characteristics of wild *Beta* species and hybridize readily with sugar beet (van Geyt et al. 1990; Lange et al. 1999).

Except for a few reports (Ecke and Michaelis 1990; Frese 1991; Shun et al. 2000; Jung et al. 1993; Senda et al. 1998), however, a systematic study with reference to genetic variation in leaf beet is missing. The same holds true for garden beet (Wang and Goldman 1999). With this in mind, we have decided to estimate the extent of genetic diversity within leaf and garden beet germplasm resources. As a first step, we examined here cytoplasmic diversity in leaf and garden beet accessions using polymorphisms in mitochondrial minisatellite loci. The results showed a considerable amount of diversity within the leaf beet germplasm, and gave insights into the dissemination route of leaf beet.

Materials and methods

Plant material

Accessions described as leaf beet or garden beet were obtained from three different *Beta* genetic resources holdings, three commercial companies and personal collections (Table S1). Sugar beet-lines TK81-MS (Owen cytoplasm)(Satoh et al. 2004), I-12CMS(2) (male-sterile cytoplasm derived from wild beets collected in Turkey), I-12CMS(3) (male-sterile cytoplasm derived from wild beets collected in Pakistan)(Mikami et al. 1985), TK81-O (normal fertile cytoplasm, a maintainer of Owen CMS)(Kubo et al. 2000) and NK-310mm-O (normal fertile cytoplasm, a maintainer of Owen CMS)(Taguchi et al. 2009) were used as references.

DNA analysis

Total genomic DNA was extracted from young fresh leaves of single plants using a CTAB protocol (Cheng et al. 2009). PCR amplifications, cloning, sequencing, and Southern blot hybridization were

performed as detailed elsewhere (Kubo et al. 2000). The sequences of four mitochondrial minisatellites (TR1, TR2, TR3, and TR4) were amplified using primers described in Nishizawa et al. (2000). Ambiguity of the number of repeats was solved by DNA sequencing. The primers used to amplify the *orf129* sequence were 5'-ATCCATGGTGATGAATCCTTATATTCTGC-3' and 5'-CTAGAGCTCTCACTGTGAGAGATAG-3'. A combination of 47 sets of primers was also synthesized (Table S2) and utilized to amplify contiguous fragments which were expected to cover the entire mitochondrial genomes from TK81-O and NK-310mm-O, using LA-Taq (Takara Bio, Ohtsu, Japan). The probe used in Southern hybridization was the DNA fragment containing TR1 of TK81-O (Nishizawa et al. 2000).

Protein analysis

Total proteins were extracted from young fresh leaves as described in Cheng et al. (2009). Proteins were fractioned by 15% SDS polyacrylamide gel electrophoresis, transferred to Hybond-P membranes (GE Healthcare UK, Amersham Place, England), and probed with antisera according to Yamamoto et al. (2005). Preparation of the antisera against preSATP6 and ORF129 followed the procedures of Yamamoto et al. (2005, 2008).

Results

Mitochondrial minisatellite loci

The analysis of four mitochondrial minisatellite loci (TR1 to TR4) is summarized in Tables S1 and S3 in the electronic supplementary material. Over the whole sample, TR1 showed the highest variability with a total of six alleles, TR4 also being polymorphic with three alleles, and both TR2 and TR3 with two alleles. By combining the data for these polymorphic loci, we were able to distinguish 11 mitochondrial haplotypes, of which eight (min04, min06, min07, min08, min09, min10, min11 and min18) were previously reported to be present in cultivated beets and relatives (Nishizawa et al. 2007), and three (min19, min20 and min21) were identified for the first time in this study. In leaf beet germplasm, all the mitochondrial haplotypes were widely distributed, except haplotypes min20 and min21, which were restricted to Greek and Portuguese accessions, respectively (Tables S1 and S3; Fig. 1).

It should be noted that haplotypes min04 and min06 are associated with male-sterility inducing Owen cytoplasm and I-12CMS(3) cytoplasm, respectively, and min18 with a normal fertile (cv. TK81-O-type) cytoplasm (Nishizawa et al. 2007; Cheng et al. 2009). We also found that the normal-cytoplasmic maintainer line of Owen CMS, NK-310mm-O, possessed haplotype min09 (see below). Despite the limited number of individual plants per accession (1-20 plants), 40 % of the accessions exhibited two or more haplotypes (Tables S1 and S3).

'NK-310mm-O'-type normal cytoplasm

We next wished to compare the physical organization of mtDNAs from NK-310mm-O and TK81-O. Total genomic DNA isolated from the two lines was digested with *Eco*RI and hybridized with four mitochondrial gene probes (*atp1, atp6, atp9,* and *cox2*) as well as with the TR1-specific probe. Only the TR1 probe showed a difference in the restriction fragments to which it hybridized, suggesting that the mitochondrial genomes of these lines were very similar (Fig. 2 and data not shown).

Forty-seven sets of primers were further designed based on the complete nucleotide sequence of TK81-O mtDNA (Kubo et al. 2000), and used to amplify via PCR, contiguous and overlapping fragments (2-11 kb in size) which were expected to cover the entire mitochondrial genomes from both TK81-O and NK-310mm-O. *Hap*II digestion of the fragments amplified from TK81-O and

NK-310mm-O revealed no differences in restriction patterns, except for three TR1-bearing fragments (122821-127820, 210011-220052, and 350041-361091) (Fig. 3). This indicates that the organizational differences between the two mitochondrial genomes are confined to the three regions (locations in TK81-O mtDNA: 125220-125637, 215568-215985, and 351541-351958, see DDBJ/EMBL/GenBank accession number BA000009) that encompass the TR1 locus.

Haplotypes min04, min06 and min21

A mitochondrial open reading frame, *orf129,* was found to be associated with male sterility caused by I-12CMS(3) cytoplasm originating from wild beet (Yamamoto et al. 2008). The protein product (12 kDa in size) of *orf129* was further implicated as the cause of pollen disruption of beets when tobacco plants expressing the gene in the nucleus with a mitochondrial transit sequence were observed to be male sterile (Yamamoto et al. 2008)

In this study, the *orf129* sequence was PCR-amplified from representatives of all the haplotypes detected in this study. As a result, we found that some of the plants with haplotype min06 and all the plants with min21 were *orf129* positive. From a total of 137 plants having the min06 haplotype, 87 plants yielded a 0.4kbp fragment as did the reference line I-12CMS(3), whereas no product was generated by the remaining 47 plants (Table S3, Fig. 4). An anti-ORF129 antiserum was utilized to identify the protein product of *orf129* on immunoblots. As far as we examined, the plants bearing *orf129* (i. e. min06/+*orf129* and min21) exhibited a single protein of 12 kDa, while no signal was detected in the plants lacking *orf129* (Fig. 5A and data not shown).

We also investigated whether the Owen CMS-associated mitochondrial protein (termed preSATP6, Yamamoto et al. 2005) was present in plants carrying the min04 haplotype. Two individual plants from a Chinese landrace 'Gongxianqingyebojincai' were tested by immunoblot analysis and both showed preSATP6 expression (Fig. 5B).

Distribution of the mitochondrial haplotypes in leaf beet

A first glance at the distribution pattern of the mitochondrial haplotypes makes it clear that the haplotypes are not geographically randomly distributed (Fig. 1; Table S1). For example, two haplotypes min09 and min18 (associated with 'NK-310mm-O'-type and 'TK81-O'-type normal cytoplasms, respectively) were highly predominant in Greek leaf beet accessions. In other European accessions (mainly from western Europe), min09 was the most-frequent haplotype, followed by min07. We also found that North African accessions examined possessed either the min09 or min18 haplotype. Furthermore, the prevalence of haplotype min18 was noted in the leaf beet from the Middle East and western Asia. Such a pattern contrasts with that found in Japanese and Chinese leaf beet accessions where the three haplotypes described were extremely rare; instead, haplotypes min06/+*orf129* (associated with I-12CMS(3)-type sterility cytoplasm) and min11 occurred abundantly.

Distribution of the mitochondrial haplotypes in garden beet

We detected six haplotypes in a sample of 17 garden beet accessions (Fig. 1, and details are shown in Tables S1 and S3). Haplotype min18 was the most-frequent one, being shared by 14 accessions. The other five haplotypes (min06/+*orf129,* min06/-*orf129,* min08, min09 and min19) were limited to a single accession. It is also worth mentioning that 16 accessions exhibited exclusively a single haplotype each while four haplotypes were apparent in an accession from Uzbekistan.

Discussion

Cytoplasmic diversity in leaf beet germplasm

We previously analyzed 50 accessions of cultivated and wild beets to assess their cytoplasmic diversity based upon polymorphisms in mitochondrial minisatellite loci (Nishizawa et al. 2007). The analysis revealed 18 mitochondrial haplotypes, of which eight were found among leaf beet accessions from geographically diverse origins in the present study. We also identified three additional haplotypes (min19, min20, and min21) within the leaf beet germplasm collection. This is not surprising, considering that wild beet samples in our previous study were limited in number (a total of 50 individuals). The results presented here indicate that the leaf beet germplasm still contains a relatively broad range of cytoplasmic genotypes.

By contrast, mitochondrial haplotype diversity was found to be low in the garden beet germplasm accessions examined. Historical evidence indicates a restricted origin of garden beet in parts of southern Germany and/or northern Italy in the 14th Century (Hammer et al. 1990). One can thus suppose that genetic bottlenecks have probably occurred during domestication, leading to the narrow cytoplasmic germplasm base of garden beet landraces and cultivars. However, we should also pay attention to the fact that an accession from Uzbekistan was a mixture of at least four distinct cytoplasms. It is thus of interest to investigate whether a relatively high diversity still remains in the Uzbekistan garden beet germplasm.

Probable dissemination pathways of leaf beet

Since mtDNA is strictly maternally inherited in beets (Mikami et al. 1985; Desplanque et al. 2000), mtDNA polymorphisms reflect maternal lineages, and the geographical structuring of mtDNA reflects seed-mediated gene dispersal and consequently allows the origin and dissemination routes of cultivated

9

beets to be inferred. In this study, the distribution of mitochondrial haplotypes in leaf beet germplasm accessions gave rise to several interesting observations.

First, the European germplasm was richest in haplotypes, an observation that agrees well with the postulation that leaf beet was primarily domesticated in the Mediterranean region where the wild ancestor is widely distributed (de Bock 1986). It is broadly accepted that a center of origin of a crop generally has the greatest genetic diversity (Harlan 1992; Vavilov 1997; Hancock 2004). Second, in the North African germplasm were observed only two haplotypes (min09 and min18) which predominated in the European germplasm. This suggests strongly that nearly all North African accessions may have been introduced from Europe and a marked reduction in mitochondrial genome diversity within the North African germplasm may have resulted from the founder effect associated with crop dissemination. A third notable observation is that the leaf beet accessions from the Middle East and western Asia represented a subset of the mitochondrial genome diversity present within the European germplasm. It remains uncertain whether this is attributed to a limited number of entries from the countries (e. g. Israel, Turkey) that are under-represented in this area, or whether several pre-existing mitochondrial haplotypes have been eliminated in this area due to genetic drift. Another explanation is that leaf beet has migrated from the place of origin to this area and crop dissemination has been associated with a reduction of mitochondrial genome diversity.

When the Japanese and Chinese accessions were examined, their mitochondrial haplotype diversity was found to be somewhat reduced relative to the diversity observed in the European germplasm. In particular, leaf beet accessions from southern China were characterized by much lower levels of diversity, suggesting crop dispersal from eastern China to southern China. Shun et al. (2000) described that leaf beet has been cultivated in China for about 2500 years. They also assumed that leaf beet was introduced by at least two routes into China: Arab traders may have brought the crop from Iran to China, and leaf beet may have been also transported along the Silk Road or by sea. A possible scenario comes to mind. After leaf beet made its first appearance in China (probably in areas with growing conditions similar to its original birth place), cultivation spread eastward. The crop subsequently reached Japan from eastern China. However, the present-day genetic diversity in Japanese germplasm resources might be the result of multiple transportations of different genotypes from Europe and China, as well as of selection by humans. Analysis of additional landraces using mitochondrial and nuclear DNA polymorphisms would assist in resolving the evolutionary history of leaf beet.

Male-sterile cytoplasm in beet germplasm resources

This paper presents evidence that at least two different sources of male-sterile cytoplasm (Owen and I-12CMS(3) cytoplasm) were distributed in leaf beet germplasm. Owen cytoplasm was rare, occurring in three of 77 leaf beet accessions: one French and two Chinese accessions. Our result is in line with reports that Owen cytoplasm was rarely found in wild sea beet populations (Laporte et al. 2001; Dufay et al. 2009). Meanwhile, I-12CMS(3) cytoplasm occurred frequently in Japanese and Chinese leaf beet accessions. Interestingly, 10 accessions were revealed to exclusively have I-12CMS(3) cytoplasm, though the number of individual plants per accession was limited. The seeds of these accessions are considered to have mostly been multiplied using a small number of seed parent plants by farmers (H. Takahashi, K. Tabata, and M. Tanaka, unpublished). The maintenance of I-12CMS(3) cytoplasm at high frequency raises the possibility that the nuclear restoring (*Rf*) alleles required for fertility restoration in I-12CMS(3) cytoplasm have been predominant in these accessions.

In conclusion, one observation merits comment. It is known that the *Rf* alleles involved in the complete restoration of fertility in Owen CMS are scarce in sugar beet breeding materials (Owen 1945; Theurer and Ryser 1969). On the other hand, several authors (Bliss and Gabelman 1965; Theurer 1971) reported that some garden beet cultivars carried strong *Rf* alleles for Owen CMS, leading us to surmise that Owen cytoplasm may be often detected in garden beet cultivars and landraces. Nevertheless, our survey failed to find Owen cytoplasm within the garden beet germplasm accessions examined; the accessions almost exclusively had a TK81-O-type normal cytoplasm. If *Rf* alleles were prevalent in

garden beet accessions with normal cytoplasm, one could speculate that these alleles have a function separate from the restoration of fertility. The distribution of *Rf* alleles in garden beet as well as leaf beet germplasm is worthy of further investigation.

Acknowledgements D. C. was a recipient of grants from a Chinese Government Supported Researchers Program and the Japan International Science and Technology Exchange Center (JISTEC). The authors wish to thank The Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), USDA Agriculture Research Service, Sakata Seed Co. Ltd, Takii & Co. Ltd, Kajita Seed Co. Ltd (Japan), NIAS (National Institute of Agrobiological Sciences) Genebank, and Dr. S. Kurihara-Yonemoto (National Agricultural Research Center for Hokkaido Region, Sapporo, Japan) for providing seeds of cultivated *Beta* germplasm. This work was supported in part by Grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, a Grant from the Program for Promotion of Basic Research Activities for Innovative Biosciences, Japan, and the Program for Promotion of Basic and Applied Researches for Innovation in Bio-oriented Industry (BRAIN).

References

- Baranski R, Grzebelus D, Frese L (2001) Estimation of genetic diversity in a collection of the Garden Beet Group. Euphytica 122**:** 19-29
- Biancardi E (2005) Brief history of sugar beet cultivation. In: Biancardi E, Campbell LG, Skaracis GN, de Biaggi M (eds) Genetics and Breeding of Sugar Beet, Science Publishers, Enfield, pp 3-9
- Bliss FA, Gabelman WH (1965) Inheritance of male sterility in beets, *Beta vulgaris* L. Crop Sci 5**:** 403-406
- Bosemark NO (1979) Genetic poverty of the sugar beet in Europe. In: Zeven AC, van Harten AM (eds) Proc Conf Broadening Genet Base Crops, Pudoc, Wageningen, pp 29-35.
- Cheng D, Kitazaki K, Xu D, Mikami T, Kubo T (2009) The distribution of normal and male-sterile cytoplasms in Chinese sugar-beet germplasm. Euphytica 165**:** 345-351
- de Bock TSM (1986) The genus *Beta:* domestication, taxonomy and interspecific hybridization for plant breeding. Acta Hort 182**:** 335-343
- Desplanque B, Viard F, Bernard J, Forcioli D, Saumitou-Laprade P, Cuguen J, van Dijik H (2000) The linkage disequilibilium between chloroplast DNA and mitochondrial DNA haplotypes in *Beta vulgaris* ssp *maritima* (L.): the usefulness of both genomes for population genetic studies. Mol Ecol 9**:** 141-154
- Dufay M, Cuguen J, Arnaud J-F, Touzet P (2009) Sex ratio variation among gynodioecious populations of sea beet: can it be explained by negative frequency-dependent selection? Evolution 63: 1483-1497
- Ecke W, Michaelis G (1990) Comparison of chloroplast and mitochondrial DNA from five morphologically distinct *Beta vulgaris* cultivars: sugar beet, fodder beet, beet root, foliage beet, and Swiss chard. Theor Appl Genet 79**:** 440-442
- Fischer H E (1989) Origin of the 'Weisse Schlesische Rube' (white Silesian beet) and resynthesis of sugar beet. Euphytica 41**:** 75-80
- Frese L (1991) Variation patterns in a leaf beet (*Beta vulgaris, Chenopodiaceae*) germplasm collection. Plant Syst Evol 176**:** 1-10
- Hammer K, Stanarius A, Kuhne T (1990) Differential occurrence of beet cryptic viruses A new tool for germplasm characterization and evolutionary studies in beets? Euphytica 45**:** 23-27
- Hammer K (2001) Chenopodiaceae. In: Hanelt P, Institute of Plant Genetics and Crop Plant Research (eds) Mansfield's Encyclopedia of Agricultural and Horticultural Crops, vol. 1, Springer, Berlin, pp 235-264
- Hancock JF (2004) Plant Evolution and the Origin of Crop Species. 2nd edn. CABI Publishing, Cambridge
- Harlan JR (1992) Crops and Man. American Society of Agronomy, Madison, Wisconsin
- Jung C, Pillen K, Frese L, Fahr S, Melchinger AE (1993) Phylogenetic relationships between cultivated and wild species of the genus *Beta* revealed by DNA "fingerprinting". Theor Appl Genet 86**:** 449-457
- Kubo T, Nishizawa S, Sugawara A, Itchoda N, Estiati A, Mikami T (2000) The complete nucleotide sequence of the mitochondrial genome of sugar beet (*Beta vulgaris* L.) reveals a novel gene for tRNACys(GCA). Nucl Acids Res 28**:** 2571-2576
- Lange W, Brandenburg W, de Bock TSM (1999) Taxonomy and cultonomy of beet (*Beta vulgaris* L.). Bot J Linn Soc 130**:** 81-96
- Laporte V, Viard F, Bena G, Valero M, Cuguen J (2001) The spatial structure of sexual and cytonuclear polymorphism in the gynodioecious *Beta vulgaris* ssp *maritima:* I- at a local scale. Genetics 157**:** 1699-1710
- Mikami T, Kishima Y, Sugiura M, Kinoshita T (1985) Organelle genome diversity in sugar-beet with normal and different sources of male sterile cytoplasms. Theor Appl Genet 71**:** 166-171
- Nishizawa S, Kubo T, Mikami T (2000) Variable number of tandem repeat loci in the mitochondrial genomes of beets. Curr Genet 37**:** 34-38

Nishizawa S, Mikami T, Kubo T (2007) Mitochondrial DNA phylogeny of cultivated and wild beets:

relationships among cytoplasmic male-sterility-inducing and nonsterilizing cytoplasms. Genetics 177**:** 1703-1712

Owen FV (1942) Male sterility in sugar beets produced by complementary effects of cytoplasmic and mendelian inheritance. Am J Bot 29**:** 692

Owen FV (1945) Cytoplasmically inherited male-sterility in sugar beets. J Agr Res 71**:** 423-440

- Satoh M, Kubo T, Nishizawa S, Estiati A, Itchoda N, Mikami T (2004) The cytoplasmic male-sterile type and normal type mitochondrial genomes of sugar beet share the same complement of genes of known function but differ in the content of expressed ORFs. Mol Genet Genom 272**:** 247-256
- Senda M, Onodera Y, Mikami T (1998) Cytoplasmic diversity in leaf beet cultivars as revealed by mitochondrial DNA analysis. Hereditas 128**:** 127-132
- Shun ZF, Chu SY, Frese L (2000) Study on the relationship between Chinese and East Mediterranean *Beta vulgaris* L. subsp. *vulgaris* (leaf beet group) accessions. In: Maggioni L, Frese L, Germeier C, Lipman E (ed,) Report of a Working Group on *Beta.*, International Plant Genetic Resources Institute, Rome, pp 65-69
- Taguchi K, Ogata N, Kubo T, Kawasaki S, Mikami T (2009) Quantitative trait locus responsible for resistance to Aphanomyces root rot (black root) caused by *Aphanomyces cochlioides* Drechs. in sugar beet. Theor Appl Genet 118**:** 227-234
- Theurer JC, Ryser GK (1969) Inheritance studies with a pollen fertility restorer sugarbeet inbred. J Am Soc Sugar Beet Tech 15**:** 538-545
- Theurer JC (1971) Inheritance studies of a pollen restorer from Ruby Queen table beet. J Am Soc Sugar Beet Tech 16**:** 354-358

van Geyt JPC, Lange W, Oleo M, de Bock TSM (1990) Natural variation within the genus *Beta* and its

possible use for breeding sugar beet: A review. Euphytica 49**:** 57-76

Vavilov NI (1997) Five Continents. International Plant Genetic Resources Institute, Rome

- Wang M, Goldman IL (1999) Genetic distance and diversity in table beet and sugar beet accessions measured by randomly amplified polymorphic DNA. J Am Soc Hort 124**:** 630-635
- Yamamoto MP, Kubo T, Mikami T (2005) The 5'-leader sequence of sugar beet mitochondrial *atp6* encodes a novel polypeptide that is characteristic of Owen cytoplasmic male sterility. Mol Genet Genom 273**:** 342-349
- Yamamoto MP, Shinada H, Onodera Y, Komaki C, Mikami T, Kubo T (2008) A male sterility-associated mitochondrial protein in wild beets causes pollen disruption in transgenic plants. Plant J 54**:** 1027-1036

Figure legends

Figure 1 Geographical distribution of mitochondrial minisatellite haplotypes of leaf beets. Each circle represents regional pool of leaf beets. Total number of plants is shown in the center of the circles. Ratio of haplotypes is color-coded. Number of haplotypes is in parenthesis. Data of garden beets are shown in the box. See Tables S1 and S3 for details.

Figure 2 DNA gel blot analysis of total cellular DNA from leaf tissue of TK81-O and NK-310mm-O. The blot was probed with a TR1-containing DNA fragment (see Materials and methods). Five micro-grams of total cellular DNA was electrophoresed in a 0.8% agarose gel. The size of the signal band is shown in kb. As TR1 is located on the repeated sequences of TK81-O-mitochondrial gnome, two 4.9 kb *Hin*dIII fragments and one 2.8 kb *Hin*dIII fragment can be expected from the master circle (see BA000009).

Figure 3 Results of a CAPS (cleaved amplified polymorphic sequence) analysis of TK81-O and NK-310mm-O. Regions of mitochondrial genome (nucleotide coordinate in BA000009 is shown at the top of each panel) were amplified by PCR using primer sets shown in Table S2. PCR amplicons were digested with the restriction endonuclease *Hap*II, then electrophoresed in a 1% agarose gel. Size markers are shown on the right (kb).

Figure 4 Detection of *orf129* sequences from *Beta* germplasm. The name of the strain/accession is shown at the top of the panel, as well as the plant ID (146-4 to 148-5, see Table S3). PCR amplicons were electrophoresed in a 2% agarose gel. Size markers are shown on the left (kb).

Figure 5 Western blots of total cellular protein of leaf from sugar- and leaf beets. The name of the strain/accession is shown at the top of the panel, as well as the plant ID (76-1, 76-2, 92-1 and 92-2, see Table S3). As a loading control, images of membrane stained with Ponceau S are shown below, **Panel a.** The blot was probed with anti-ORF129 antiserum (Yamamoto et al. 2008). It should be noted that I-12CMS(2) harbors a variant *orf129* whose translation products are exactly the same as I-12CMS(3) in amino acid sequence but accumulated five times more abundantly than I-12CMS(3) (Onodera et al*.* manuscript in preparation). **Panel b.** The blot was probed with anti-preSATP6 antiserum (Yamamoto et al. 2005).

TR1

Table S1 Leaf and garden beet germplasm accessions examined in this study and their mitochondrial minisatellite haplotypes

1, Each haplotype is based on the combination of alleles from TR1, TR2, TR3 and TR4 loci. Eight haplotypes were named min04, min06, min07, min08, min09, min10, min11, and min18, using the same nomenclature as described in TABLE 3 of Nishizawa et al. (2007). The remaining three haplotypes were determined according to the phenotypes (number of the repeat units) obtained with the four TR loci: 10 (TR1)/ 6 (TR2)/ 3 (TR3)/ 1 (TR4) for min19, 10/ 3/ 3/ 3 for min20, and 5/ 6/ 2/ 3 for min21 (see Table S3).

2, The symbols + and - refer to the presence or absence of orf129.

Table S2 Forty-seven primer sets which were expected to cover the entire mitochondrial genomes from both TK81-O and NK-310

Table S3 Number of repeat units in each of the four mitochondrial minisatellite loci and presence/absence of orf129 in leaf beet- and

garden beet germplasms used in this study

*, no data