# MOLECULAR TAGGING OF MALE-FERTILITY RESTORATION LOCUS AND ITS SELECTION IN ONION (*Allium cepa* L.)

by

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# ABSTRACT

## MOLECULAR TAGGING OF MALE-FERTILITY RESTORATION LOCUS AND ITS SELECTION IN ONION (*Allium cepa* L.)

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Cytoplasmic-genic male sterility (CMS) is used to produce hybrid-onion seed. For the most widely used source of onion CMS, male sterility is conditioned by the interaction of the male-sterile (S) cytoplasm and the homozygous recessive genotype at a single nuclear male-fertility restoration locus (*Ms*). Maintainer lines possess normal (N) male-fertile cytoplasm and are homozyous recessive at the *Ms* locus. It takes four to eight years to establish if maintainer lines can be extracted from an uncharacterized onion population. Polymorphisms distinguishing N and S cytoplasms of onion have been identified and significantly reduce the time required to establish the cytoplasms of single plants. Segregations of restriction fragment length polymorphisms (RFLPs) revealed RFLPs flanking the *Ms* locus at 0.9 and 8.6 cM. The RFLPs and *Ms* were at or near linkage equilibrium in three open-pollinated onion populations.

Using the cDNA sequence (AOB272) revealing the most closely linked RFLP, we designed oligonucleotides and used the polymerase chain reaction to

amplify genomic DNAs from seven commercial onion cultivars and the parents (Ailsa Craig 43 and Brigham Yellow Globe 15-23) of our mapping family. We cloned and sequenced these amplified genomic DNAs to identify single nucleotide polymorphisms (SNP). Oligonucleotides were designed flanking the SNPs and were used as allele-specific primers to distinguish genotypes in the AOB272 genomic region. These organellar and nuclear markers can be used to select maintainer genotypes from onion populations or segregating families and should reduce the number of testcrosses required to identify maintaining genotypes.

The bulb onion is a diploid species with natural outcrossing rates ranging between 70% to 100% in the field. Male sterile plants must receive the male gametes from male-fertile plants in order to set seed. Therefore, the frequency of the *ms* allele will change over generatons due to natural selection of male gametes on male-sterile (S-*msms*) plants in onion populations possessing S cytoplasm or mixtures of N and S cytoplasms. I developed a model to predict changes in allelic frequencies due to failure of male gamete production from S-*msms* genotypic plants in onion.

# DEDICATION

I would like to dedicate my dissertation:

To my parents, Mavis and Ibrahim Gokce, for their endless love and encouragement, and to my brothers and sisters for their support.

To my lovely wife, Gulay Gokce, for her help, patience, and encouragement.

To my children, Cemre Mavis, and Emre Faik Gokce, for the love they give me and for helping me to relax by being good playmates.

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# Chapter One

## Origin and taxonomy of *Allium*

The bulb onion (*Allium cepa* L.) has been cultivated for thousands of years and is thought to have been first domesticated in the mountainous areas of Afghanistan, Pakistan, Tajikistan, or northern Iran (Brewster 1994). Vavilov (1951) indicated that onion originated in his Central Asiatic Center, including northwest India, all of Afghanistan, the former Soviet republics of Tajikistan and Uzbekistan, and western Tian-Shan. Secondary centers for onion are the Near-Eastern Center, the interior of Asia Minor, Trans-Caucasus, Iran, and the highlands of Turkmenistan. The Mediterranean center may be the origin of large-sized onions (Vavilov 1951).

The onion is a member of the genus *Allium*, which contains more than 500 species (Jones and Mann 1963, Traub 1968a, Stearn 1944) and includes important vegetable and ornamental types (Brewster 1994). The genus *Allium* is widely distributed over the warm-temperate and temperate zones of the northern hemisphere where its greatest diversity exists in Turkey, Iran, Iraq, Afghanistan, Kazakhstan, and western Pakistan (Hanelt 1990). The Alliums typically grow in generally open, sunny, dry sites in fairly arid climates, and many species are found in steppes, dry mountain slopes, rocky or stony open sites, or summer-dry vegetation (Hanelt 1990).

Being one of the earliest cultivated crops, the Alliums are also one of the earliest classified plants. The first known classification schemes are those based on morphological characters such as bulb size, shape, color, and pungency. The first named onion cultivars and descriptions of many diverse types go back to Hypocrites' time of 430 B. C. (McCollum 1976). The first extensive listing of *Allium* types dates back to Carolus Clusius' "Rariorum Plantarum Historia" (1601). Clusius' illustrations include several *Allium* species organized under the taxonomic headings *Victoralis, Scorodoprasum, Moly, Allium sive Moly montanum,* and *Moly Narcissi foliis.* The Swiss botanist Albrecht von Haller was the first one to use the name *Allium* to refer to all onions and their relatives in his publication "De allii Genere Naturali Libellus" (1745), in which he described in detail the nomenclature and morphological characteristics of 24 *Allium* species (Stearn 1944). Another classification was put forward by Philip Barker Webb in his "Phytographia Canariensis" in 1848. He separated species into two groups, the first *Melanocrommyum* for those species with more than two ovules in each chamber of the ovary, and the second *Crommyum* for the species with only two ovules (Stearn 1944). In the former Soviet Union, Vvedensky (1944) published a detailed description of *Allium*. He classified the cultivated Alliums into four sections, Cepa (bulb onion), Porrum (garlic and leek), Phyllodolon (Japanese bunching onion), and Rhiziridium (chive and Chinese chive). Havey (1991) supported the division of cultivated species into different sections and estimated phylogenetic relationships consistent with Vvedensky's taxonomic classification. Furthermore, using morphological criteria, crossability, and karyotypes, Traub (1968b) also divided *Allium* into four sections, Allium, Cepa, Fistulosa, and Rhiziridium with further division into subsections.

The botanical classification of the genus *Allium* has been reviewed and summarized by several authors (e.g. Bentham and Hooker 1883, Hutchinson 1934, 1973). The genus *Allium* is presently classified as: Class Monocotyledones, Superorder Liliiflorae, Order Asparagales, Family Alliaceae, Tribe Alliae, Genus *Allium* (Brewster 1994, Hanelt 1990). Formerly *Allium* was included in both the Liliaceae (Cronquist 1968, Cronquist et al. 1977, Takhtajan 1973) because of its ovary free and above the attachment of the other flower parts (Jones and Mann 1963), and the Amaryllidaceae (Hutchinson 1934 and 1973) due to an umbellate inflorescence surrounded by a spathe (Jones and Mann 1963).

The most recent taxonomic classification was proposed by Hanelt (1990), who divided the genus into five subgenera: *Rhizirideum* (G. Don ex Koch) Wendelbo, *Allium* L., *Bromatorrhiza* Ekberg, *Melanocrommyum* (Webb et Berth.) Rouy, and *Amerallium* Traub. Within each subgenus, he distinguished a number of sections containing closely related species. His five subgenera were based on groups differing in phylogenetic history, geographic affinity, evolutionary state, and morphological differences.

Subgenus *Rhizirideum* (G. Don ex Koch) Wendelbo includes *A. tuberosum* Rottl. Ex. Spr (Chinese chive or Nira), cultivated for edible, young inflorescence and garlic-flavored leaves. *Allium schoenoprasum* L. (chive) is grown for green, onion-flavored leaves in Europe. *Allium cepa* L. (onion and shallot) is of two types, the Common Onion group forms large sized bulbs and the Aggregatum group which forms smaller bulbs with laterals, thus producing cluster of bulbs. *Allium fistulosum* L. (Welsh onion, Japanese bunching onion, and Negi) is very similar in appearance to the common onion but does not form dormant bulbs. *Allium chinense* G. Don (rakkyo) is mainly grown in China and Japan for small bulbs mostly used in pickles (Brewster 1994). Subgenus *Rhizirideum* (G. Don ex Koch) is a heterogeneous group of Eurasian distribution. All taxa form rhizomes, although sometimes these are reduced to disc-like corms, similar to that in the common bulb onion.

Subgenus *Allium* includes *A. sativum* L. (garlic) is usually considered as a sterile species propagated vegetatively; however, recently some fertile, seed producing plants have been reported (Etoh 1986, Pooler and Simon 1994). *Allium ampeloprasum* L. (leek, kurrat, and great-headed garlic) originates from the Old World. Leeks, especially Turkish and Bulgarian, form long, edible thin pseudostems at harvest, while those from western Europe form shorter and thicker pseudostems and are autotetraploids (Meer and Hanelt 1990). Kurrat (autotetraploid) is common in Egypt and is grown for its leaves. Great-headed garlic is like a very robust garlic plant and is an asexually propogated hexaploid (Brewster 1994). The section *Allium* shows the greatest diversity and mainly ranges from the Mediterranean to the Caucasus.

Subgenus *Bromatorrhiza* Ekberg originates from southwest China and the Himalaya district and is not very common.  Cultivated species of this group are used as decorative plants (e.g., *A. cyathophorum* Bur. et Fr.).

Subgenus *Melanocrommyum* (Webb et Berth.) Rouy is from the Irano-Turanian floristic region from the Turan in Middle Asia to Afghanistan, Iran, and the Near East countries, and is not common and as well known as the other subgenera.  Like the subgenus *Bromatorrhiza* Ekberg, species of *A. cristophii* Trautv, *A. stipitatum* Rgl, and *A. giganteum* Rgl. have ornamental values.

The fifth subgenus is *Amerallium* Traub and, as its name indicates, contains mainly New World species.  The sections *Amerallium* Traub, *Lophioprason* Traub, *Caulorhizideum* Traub, and *Rophetoprason* Traub are indigenous to North America, while the section *Molium* G. Don Koch is Mediterranean.  Species of the section *Molium* G. Don Koch are known as decorative plants like *A. neapolitanum* Cyr.  American species of the subgenus *Amerallium* generally produce bulbs on rhizomes and these rather primitive characters resemble the subgenus *Rhizirideum*.  Although all the other subgenera have basic chromosome numbers of eight (2n=2x=16) or rarely nine (2n=2x=18), the subgenus *Amerallium* has a basic chromosome number of seven (2n=2x=14) (Traub 1968a and 1968b, Ved Brat 1965).  The subgenus *Amerallium* may contain different phylogenetic branches, since the basic chromosome numbers, leaf anatomy, and other characters indicate a separate status for this group (Hanelt 1990).

## Cultivation and economic significance of onion

The edible Alliums are grown worldwide and have been historically maintained as open pollinated populations. The genus *Allium* includes several cultivated economically and dietarily important species, the bulb onion (*A. cepa*), the closely related shallot (*A. cepa* var. *ascalonicum*) and potato onion (*A. cepa* var*. aggregatum*), chive (*A. schoenoprasum*), Chinese chive (*A. tuberosum*), Japanese bunching onion (*A. fistulosum*), garlic (*A. sativum*), leek (*A. ampeloprasum* var*. porrum* syn. *A. porrum*), and rakkyo (*A. chinense*) (Havey 1995a). The common onion (*A. cepa*) and garlic (*A. sativum*) are the most important and are grown, traded, and consumed in most countries. The bulb onion, Chinese chive, chive, Japanese bunching onion, and leek are mainly seed propagated; whereas garlic, great headed garlic, and cultivated forms of rakkyo are propagated vegetatively by cloves and bulb multiplication (Havey 1995a).

The bulb onion is grown as fresh shoots for green salad onions and as bulbs for consuming uncooked, cooked, and pickled or production of seed and sets. The average world production of the bulb onion in the last three years was approximately $45x10^6$ metric tons (MT) (FAO 1998, 1999, and 2000). Leading five countries in 1998, 1999, and 2000 average production were China $11.4x10^6$ MT, India $4.7x10^6$ MT, USA $3.2x10^6$ MT, Turkey $2.3x10^6$ MT, and Iran $1.6x10^6$ MT. Russian Federation, USA, Germany, and Malaysia were the leading bulb onion importer countries; the leading bulb onion exporter countries were

Netherlands, Argentina, USA, and Spain (FAO 1998 and 1999). In 2000, worldwide production of garlic was about one-fourth of the bulb onion ($10x10^6$ MT). The next most valuable Alliums are leek, concentrated in Europe, and Japanese bunching onion, mostly in Asia.

Turkey is one of the countries where onion has been cultivated for thousands of years. Anatolia (Asia Minor) is the origin of many bulbous plants. The Hittites lived there as early as 2000 BC and grew bulbous plants like onion, garlic, and leek, giving the names Sum-sar and Sum-sikil-sar to garlic and onion, respectively (Baytop and Mathew 1984). They celebrated the arrival of spring, as the flowers began to appear and snow melted away, by holding spring festivals (Ar 1943). This spring festival is still celebrated in Turkey on May 6 under the name “Hidirellez” (Spring-feast) and people go for picnics. A special pilav (Cigdem pilavi: Crocus pilav) is prepared with bulgur (cracked wheat) and Cigdem (Crocus) bulbs, and is eaten at Hidirellez in some areas such as Adana, Diyarbakir, and Gaziantep in Turkey. Furthermore, some local places are named after bulbous plants, such as Soganli Dag (Mountain of the bulbs) near Kayseri and Soganli Daglari (Mountains of the bulbs) between Rize and Erzurum (Baytop and Mathew 1984).

There are about 150 *Allium* species that grow in Turkey (Kolmann et al. 1983), some of which are commonly cultivated as vegetables although not native to Turkey and are found in forsaken areas of cultivation (Baytop and Mathew 1984). Some wild species, *A. ampeloprasum, A. macrochaetum, A. rotundum,* *A. schoenoprasum, A. zebdanense,* and *A. vineale*, are used under the name of wild onion or wild garlic. The scape and leaves of *A. vineale* (Sirmo) are used with some other herbs to prepare a kind of cheese known as "otlu peynir" (cheese with herbs) in the city of Van. In the region of Tunceli and Adana, the bulbs of *A. ampeloprasum* and *A. macrochaetum* subsp. *tuncelianum* are consumed under the name "yabani sarmisak" (wild garlic). *Allium rotundum* is known as "kormen" and its leaves are consumed in Mersin and Adana regions. The leaves of *A. zebdanense*, known as "geyik kormeni", are used as food in the Taurus mountains. Kaya sarmisagi (*A. macrochaetum*) is consumed instead of garlic in the Kahramanmaras and Adana regions. Instead of onion for flavoring, the fresh leaves of *A. schoenoprasum* are consumed in Hakkari and Van (Baytop and Mathew 1984).

Onion is a very old crop in Turkey and is used daily in cooking by all Turkish families year-round. It is one of the most important vegetable crops in Turkey, and is grown for consumption in the green stage and as mature bulb used as a main vegetable dish or as a flavorful ingredient of the main meat dishes. The onion production methods used in Turkey are direct seeding or sets. Currently, use of direct seed sowing continues to increase since the labor is getting more expensive. Most of the onion seed used in Turkey are hybrid cultivars imported either from Israel or the Netherlands. The hybrid cultivars are more uniform for maturity time, bulb size, and bulb shape. On the other hand, the use of sets still occupies about fifty percent of onion production area of

Turkey (Gunay 1992), although the yield from sets is less than that from hybrid seed in Turkey. As a result, the dry bulb onion production of Turkey ($2.3x10^6$ MT) is slightly lower than onion production of USA ($3.2x10^6$ MT) even though the planted area (105,000 ha) of Turkey is about two times greater than the area (67,250 ha) used for onion production in USA (FAO 2000).

**Cytoplasmic male sterility (CMS)**

The discovery of the cytoplasmic male sterility (CMS) and its first use in production of hybrid onion had a great effect on breeding of onion (Jones and Clarke 1943) and on many other crops. The bulb onion is an outcrossing species; natural outcrossing rates in onion range between 70% to 100% in the field (Meer and Bennekom 1968, 1972). Due to protandry, selfing is not possible for individual perfect flowers but possible between the flowers on the same umbel, causing lower selfing rates. The inflorescence may consist of a few to hundreds of perfect flowers per umbel (Havey 1993, Pike 1986). Individual flowers have six stamens, in two whorls of three. Inbreeding depression occurs in bulb onion (Jones and Davis 1944, Kotowski 1926). Jones and Emsweller (1936) reported that the bulb yields of hybrids, generated by crossing Italian Red 13-53 with various male-fertile cultivars, were greater than that of either parent.

Sterile cytoplasm used to produce hybrid onion in the USA traces back to single plant discovered in the cultivar Italian Red at the University of California Davis in 1925. Bulbs from Italian Red were selected from a commercial lot, planted, and self pollinated. One of those bulbs, Italian Red 13-53, flowered normally, but failed to produce seed and was propagated by small bulbs in the inflorescence. In 1943, Jones and Clarke showed that the sterility of the plant was conditioned by the interaction of the cytoplasm, normal (N) male-fertile versus male-sterile (S) cytoplasm, and a single nuclear gene (*Ms*). The recessive allele (*ms*) conditions male sterility in plants possessing S cytoplasm, yet has no effect on plants possessing N cytoplasm (Jones and Clarke 1943). Thus, male sterile plants are S *msms*. All plants with N cytoplasm are male-fertile regardless of the genotype at the *Ms* locus. In addition, plants of genotypes S *MsMs* and S *Msms* are male-fertile (Jones and Clarke 1943) since they possess the dominant allele. The cytoplasm of onion shows maternal inheritance (Jones and Davis 1944, Havey 1995b). CMS is maintained by crossing S *msms* plants with a maintainer line (N *msms*) genetically similar but differing in cytoplasm. The plants of genotype S *msms* are used as the female parent in hybrid production. The male-sterile character of Italian Red 13-53 has been incorporated into important commercial onion inbreds by backcrossing.

Another form of onion CMS is T cytoplasm discovered in the French cultivar Jaune Paille des Vertus (Berninger 1965). Unlike S cytoplasm, male sterility is controlled by the interaction between the T cytoplasm and three independently inherited recessive nuclear genes *a*, *b*, and *c*. The *a* gene operates independently of *b* and *c*. All *aa* plants with the T cytoplasm are sterile. Loci *b* and *c* act complementarily; all *bbcc* plants with the T cytoplasm are sterile

(Schweisguth 1973). Some European and Japanese hybrids are produced on T or a similar cytoplasm (Havey 1999).

Recently, Havey (1999) developed a source of CMS by backcrossing the cytoplasm of *A. galantum* to bulb onion. Male sterility is associated with a complete lack of anthers. No nuclear restorarion loci have been identified for this new CMS source.

## Onion breeding objectives

Bulbs can differ in size, shape, color, pungency, single center, firmness, and tightness of outer dry skins and neck. Plants can vary significantly in height, leaves, scape, morphology of scapes and inflorescence. Onion is biennial with alternative seed-to-bulb and bulb-to-seed generations. Consequently the genetic progression of the bulb onion is slow compared to annual crops. Cultivated onions are of two major types, known as short-day and long-day onions. However, a third group can be expressed as an intermediate day length type that forms bulbs between the two major groups. Onions that require 14 hours (hr) or more to bulb are classified as long-day, whereas those that bulb as day length exceeds 11-12 hr are considered short-day onions (Pike 1986). Onion bulbing is also affected by temperature.

Better quality, high yield, uniformity, and resistance to diseases are major breeding achievements in onion. Important bulb quality traits are bulb size, shape, color, pungency, firmness, dormancy, and amount of soluble solids. Bulb shape, size, and soluble solids show continuous phenotypic variation that suggest quantitative inheritance (McCollum 1971). In addition, McCollum (1966, 1968, 1971) reported large environmental effects and low heritability of bulb diameter and weight, high heritability of soluble solids (62-82%), and a negative genetic correlation between bulb size and soluble solids. Galmarini et al. (2001) identified a chromosome region on linkage group E controlling correlated traits of pungency, solids and antiplatelet activity.

Bulbs of various colors are preferred by consumers in different parts of the world. Rieman (1931) did the first extensive study of bulb color inheritance and described an incompletely dominant factor (*I* over *i*) as the inhibiting factor for the expression of color. When *I* homozygous dominant (*II*), bulbs are white regardless of the other genes. Three independent factors for the development of pigment in the onion bulb were proposed by Clarke et al. (1944); the *I* gene that was Rieman's inhibiting factor, the *C* gene completely dominant over *c* and needed for the production of any pigment, and the *R* gene for red color. When homozygous recessive at *I* and a dominant allele at *C*, bulbs with dominant allele at *R* are red (*iiC_R_*) and with recessive r alleles are yellow (*iiC_rr*). The genotype of *IiCCrr* is buff. There are two genotypes for white bulbs, recessive (*iiccR_*) and dominant (*II__*) whites. A golden color without pink is conditioned by a dominant allele at the *G* locus, and red pigment is conditioned by dominant *L* once the plant has the genotype *iiC-rrG-L-* (El-Shafie and Davis 1967).

Breeding disease resistant onion cultivars is becoming more important than ever before, primarily since there is growing consumer awareness and concern about the possibly adverse effects of pesticide residues on human health and the environment. Furthermore, some pesticides that were formerly used to control diseases are no longer registered and relatively few chemicals are becoming available for use. Pesticide sprays are also an added cost to growers that is ultimately passed on to the consumers. Onion diseases causing significant losses include *Fusarium* basal rot (*Fusarium oxysporum* f. sp. *cepae*), neck rot (*Botrytis allii*), white rot (*Sclerotium cepivorum*), pink root (*Pyrenochaeta terrestris*), downy mildew (*Peronospora destructor*), purple blotch (*Alternaria porri*), and leaf blight (*Botrytis squamosa*) (Havey 1993). Resistances to some of these diseases have been reported, such as purple blotch (Riollano 1943), downy mildew (Warid and Tims 1952), *Botrytis* neck rot (Meer et al. 1970, Miyaura et al. 1985), and white rot (Utkhede and Rahe 1978). Jones and Perry (1956) and Nichols et al. (1965) reported that resistance to pink root is inherited as a recessive allele at a single locus. Resistance to *Fusarium* basal rot has been reported (Gabelman 1988). Resistance to downy mildew is conditioned by dominant alleles at two genes (Warid and Tims 1952).

## Genetic Markers and Maps

Genetic maps and markers may speed up the process of plant breeding. Traits that are difficult to measure can be selected by using closely linked markers (Paterson and Wing 1993) since alleles tend to be inherited together. Detailed genetic maps have been developed for many crop plants, such as tomato, *Arabidopsis,* barley, peanut, and *Brassica rapa* (Paterson and Wing 1993). Tanksley et al. (1989) classified genetic markers as morphological, isozyme, or DNA markers. Morphological markers often have deleterious pleiotropic effects that are undesirable in breeding programs. Although isozymes have been used successfully in plant breeding and genetics (Tanksley et al. 1989), the number of isozymes is limited. On the other hand, molecular markers, such as restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), microsatellites, amplified fragment length polymorphism (AFLP), and single nucleotide polymorphism (SNP) occur more commonly and often in sufficient numbers to generate detailed genetic maps within single species (Botstein et al. 1980, Morgante and Olivieri 1993, Vos et al. 1995, Williams et al. 1990).

RFLPs are differences in the length of DNA fragments after digestion by restriction enzymes (Botstein et al. 1980). The restriction fragments are separated according to their molecular size by electrophoresis, denatured, transferred to solid supports such as nitrocellulose or nylon-based filters (Southern 1975), exposed to the radioactively labeled probes under conditions that promote DNA-DNA hybridization, and autoradiographed. RFLPs are generally codominant markers, allowing to distinguish all possible genotypes in any segregation generation (Beckmann and Soller 1983). Thus, it is possible to determine if a linked trait is present in a heterozygous or homozygous condition in an individual. Disadvantages are that RFLPs are time-consuming, expensive, use relatively large amounts of DNA, and require radioactivity.

RAPDs are based on the amplification of random DNA segments using single primers of arbitrary nucleotide sequence (Williams et al. 1990). The polymerase chain reaction (PCR) is used for the amplification reaction and fragments are analyzed by electrophoresis and detected by staining with ethidium bromide. Polymorphisms mainly result from the lack of priming at one of the two primer binding sites. This method has advantages of being technically simple, quick to perform, requiring small amounts DNA, and no radioactivity (Waugh and Powell 1992). However, RAPDs are dominant markers that cannot distinguish heterozygous loci from the homozygous dominant state (Williams et al. 1990). Winter and Kahl (1995) reported difficulties in reproducing RAPD patterns in different laboratories and in different thermocyclers.

Microsatellites are tandemly repeated simple short nucleotide repeats that are abundant, highly polymorphic, and inherited as a co-dominant marker (Morgante and Olivieri 1993). Repeated sequences are revealed by PCR amplification of short genomic regions and are generally visualized on acrylamide gels. The sequences flanking microsatellites are determined in order to produce unique PCR primers. Rafalski and Tingey (1993) reported that a considerable amount of effort and high start-up costs are required to identify enough microsatellite markers to create a reasonably complete genetic map.

Fischer and Bachmann (2000) developed 30 microsatellites in *Allium*, of which about 21 were polymorphic in onion populations.

AFLPs are based on a combination of restriction enzyme digestions and selective PCR amplification (Vos et al. 1995). The technique involves restriction of the DNA and ligation of oligonucleotide adapters, selective amplification of fragments, gel analysis on denaturing polyacrylamide gels, and scoring as the presence versus absence of bands on autoradiograms. AFLPs are the result of polymorphisms at or structural rearrangements between the restriction enzyme sites or differences at the additional nucleotide used to prime the DNA for amplification. Advantages of AFLPs are robustness and reliability, greater numbers of fragments per reaction than RFLPs or RAPDs, and many polymorphisms can be identified on a single autoradiogram. Disadvantages include dominance and the use of radioactivity (Vos et al. 1995). Heusden et al. (2000) developed an interspecific AFLP map between onion and *A. roylei*.

The research reported in this thesis was targeted toward the identification of molecular markers tightly linked to the *Ms* locus of onion and the conversion of one RFLP marker to PCR based system. I tested for linkage equilibrium between the *Ms* locus and linked markers using bulbs randomly selected from three open-pollinated (OP) onion populations. Finally, I developed a model to explain changes in allelic frequencies at the *Ms* locus for OP onion populations possessing both N and S cytoplasms.

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# Chapter Two

## MARKER-FACILITATED SELECTION OF MAINTAINER LINES IN ONION (*Allium cepa* L.)

**Abstract:**

Cytoplasmic-genic male sterility (CMS) is used to produce hybrid-onion seed. For the most widely used source of onion CMS, male sterility is conditioned by the interaction of the male-sterile (S) cytoplasm and the homozygous recessive genotype at a single nuclear male-fertility restoration locus (*Ms*). Maintainer lines are used to seed propagate male-sterile lines, possess normal (N) male-fertile cytoplasm, and are homozyous recessive at the *Ms* locus. Due to the biennial nature of onion, it takes four to eight years of crossing and scoring of progeny phenotypes to establish if maintainer lines can be extracted from an uncharacterized population or family. Polymorphisms distinguishing N and S cytoplasms of onion have been identified and significantly reduce the time required to establish the cytoplasms of single plants. Identification of nuclear markers tightly linked to the *Ms* locus would allow for molecular-facilitated selection of maintainer lines. We evaluated testcross progenies from a segregating family and randomly selected plants from three open-pollinated populations for nuclear restoration of male fertility over at least three environments. There were consistently too many male-sterile testcross progenies, indicating that the dominant allele conditioning fertility restoration for S cytoplasm may not show complete penetrance. Segregations of restriction fragment length polymorphisms (RFLPs) revealed RFLPs flanking the *Ms* locus

at 0.9 and 8.6 cM.  A sample of commercial onion germplasm was evaluated for putative allelic diversity at the RFLP loci linked to *Ms*.  The RFLPs and *Ms* were at or near linkage equilibrium in three open-pollinated onion populations.

## Introduction

The production of hybrid-onion seed is economically feasible using systems of cytoplasmic-genic male sterility (CMS). The male-sterile (S) cytoplasm, first described by Jones and Emsweller (1936), is the primary source of CMS used worldwide. Male fertility in plants with S cytoplasm is restored by a dominant allele at the nuclear male-fertility restoration locus *Ms* (Jones and Clarke 1943). Male-sterile plants possess S cytoplasm and are homozygous recessive at the nuclear locus (*S msms*). Male-sterile inbred lines are seed propagated by crossing with a maintainer line possessing normal (N) male-fertile cytoplasm and the homozygous recessive genotype at the restorer locus (*N msms*). Although examples of asexual propagation of individual male-sterile plants exist (Jones et al. 1949, Pike and Yoo 1990), the large-scale production of hybrid-onion (Jones and Davis 1944) seed is dependent on the availability of maintainer lines (*N msms*) to seed-propagate the male-sterile (*S msms*) lines. Extraction of maintainer lines from some onion populations, e.g. the cultivars Texas 1015Y (USA), Sapporo-Ki (Japan), or Pukekohe Longkeeper (New Zealand), has not been successful because of a high frequency of the dominant allele at *Ms* (Davis 1957, Havey and Randle 1996, Little et al. 1944) or the prevalence of S cytoplasm (Havey 1993, Havey and Bark 1994, Satoh et al.

1993). As a result, few hybrid cultivars have been developed from these economically important onion populations.

Due to the biennial nature of onion, four to eight years are required to determine if maintainer lines (*N msms*) can be extracted from an uncharacterized population or segregating family (Havey 1995). The nuclear and cytoplasmic genotypes can be classified as *N msms*, *N Msms*, or *S Msms* by testcrossing to a male-sterile plant (*S msms*), self pollinating the male-fertile plant, and separately scoring the male fertility of $S_1$ and testcross progenies. The same procedure will produce all male-fertile $S_1$ and testcross phenotypes for *N MsMs* and *S MsMs*. For these genotypes, a maintainer plant (*N msms*) must be crossed with *N MsMs* and *S MsMs* and progenies from these crosses self-pollinated and scored for male-fertility to establish their cytoplasms.

Polymorphisms in the chloroplast and mitochondrial DNAs have been identified that distinguish N and S cytoplasms of onion (Courcel et al. 1989, Holford et al. 1991, Havey 1993, Satoh et al. 1993) and allow breeders to establish the cytoplasm without crosses. Havey (1995) and Sato (1998) developed molecular markers revealed by the polymerase chain reaction (PCR) and it now takes only hours, as opposed to years, to establish the cytoplasm of a single onion plant. Cytoplasmic determinations are especially important given that open-pollinated populations may exclusively possess S cytoplasm (Havey 1993, Havey and Bark 1994). This scenario will be commonly encountered in countries where hybrid-onion seed has been retained and used to develop open-pollinated populations; for example in India the population Pusa Red was likely developed from the hybrid Red Granex (Currah 1992).

The development of maintainer lines would be greatly facilitated by identifying molecular markers flanking the nuclear *Ms* locus. Such markers would allow breeders to retain only those plants that are maintainers (*N msms*) or can be used to develop maintainer lines (*N Msms*), reducing the number of plants to be testcrossed to a male-sterile line. In this study, we evaluated restriction fragment length polymorphisms (RFLPs) for linkage to the *Ms* locus and assessed linkage equilibrium among linked molecular markers and *Ms* using plants randomly selected from three open-pollinated populations.

## Materials and Methods

A segregating population of 58 $F_3$ onion families from BYG15-23 x AC43 has been described (King et al. 1998a). Individual $F_2$ plants or $F_3$ families were testcrossed to one of five S-cytoplasmic lines (MSU611-1AxMSU611B, MSU5718AxMSU8155B, B1731AxMSU5785B, B3350AxB2352B, or B1828A) using standard techniques (Pike 1986). At least 20 testcross bulbs were grown, vernalized, randomly selected for flowering, and scored for male-fertility restoration by visual inspection of dehiscence over at least three years in Madison, WI (1994 to 2000). Genotypes of $F_2$ plants at the *Ms* locus were assigned based on segregations among testcross progenies (Appendix 1). A second segregating family derived from short-day onions (1064Bx1616C) was the gift of Dr. Richard Jones, Seminis Seed Company, and was used to establish segregation of additional polymorphic fragments revealed by AOB272/*Eco*RI. Putative allelic variation at RFLPs linked to *Ms* was identified using commercial inbreds or open-pollinated cultivars (Table 1, Appendices 2, 3, and 4).

To determine if linkage disequilibruim existed among RFLPs linked to the *Ms* locus, we randomly selected bulbs from the open-pollinated (OP) populations Brigham Yellow Globe (BYG), Mountain Danvers (MD), and Sapporo-Ki (SKI). Random male-fertile bulbs were self-pollinated and testcrossed to male-sterile (S *msms*) plants. Testcross seed from N-cytoplasmic parental bulbs was planted; bulbs harvested, vernalized, and flowered; and the frequency of male-fertile and male-sterile testcross progenies determined by visual inspection. The genotypes at the *Ms* locus for $S_0$ plants were determined from segregations of male fertility in the testcross families. Chi-square analyses were used to test goodness-of-fit between observed frequencies of genotypes at *Ms* and RFLPs (see below) and those expected at linkage equilibrium.

Genomic DNAs of parental inbreds (BYG15-23 and AC43), the $F_3$ families, and $S_1$ families from BYG, MD, and SKI were extracted from leaf tissue bulked from at least 50 seedlings and purified through CsCl gradients (Havey 1991, Sambrook et al. 1989). N and S cytoplasms were identified using chloroplast characters 2, 4, and 41 (Havey 1993); B3350B (N) and Spartan Banner 80 (SB80, S) were used as cytoplasmic controls. Genotypes at nuclear RFLPs were established for each $S_1$ family as described below. Segregations of RFLPs in the BYG15-23 x AC43 family were previously described (King et al. 1998a). We used a previous onion RFLP map developed by the former Native Plants, Inc. (Salt Lake City, UT) and made available by Gilroy Foods, Inc., to identify onion cDNAs [*Allium* Gilroy Food (AGF) 021 (API21), AGF027 (API27), AGF040 (API40), AGF 043 (API43) AGF045 (API45), AGF096 (API96), AGF109, AGF120, AGF122, AGF125, AGF136, AGF147, AGF148, AGF152, AGF158, AGF161, AGF166, AGF167, AGF173, AGF176, *Allium* Gilroy (AGI) 063, AGI101, AGI106, AGI128, AGI131, AGI151, AGI156, and AGI178 (Table 1)] revealing RFLPs putatively linked to the *Ms* locus. These cDNAs were hybridized firstly to *Eco*RI, *Eco*RV, and *Hin*dIII digests of BYG15-23 and AC43 DNAs; secondly to *Bam*HI, *Bgl*II, *Dra*I, *Kpn*I, *Pst*I, *Pvu*II, *Sac*I, *Xba*I, and *Xho*I digests of BYG15-23 and AC43 DNAs; and thirdly to *Alu*I, *Apa*I, *Asc*I, *Ava*I, *Ava*II, *Ban*I, *Bgl*I, *Bst*EII, *Dde*I, *Fsp*I, *Hae*III, *Hinc*II, *Mbo*I, *Msp*I, *Nae*I, *Nco*I, *Nde*I, *Not*I, *Sal*I, *Sca*I, *Spe*I, *Swa*I, and *Xmn*I digests to reveal RFLPs segregating in the mapping family and evaluated for linkage to *Ms* (Appendix 5). All cDNAs were sequenced as previously described (McCallum et al. 2001) and hybridized to DNAs of elite commercial inbreds or populations to determine the numbers of polymorphic fragments revealed by each probe-enzyme combination. Commercial populations were described by King et al. (1998b) or are listed in Table 1. RFLP loci were named using the probe (e.g. AOB272), restriction enzyme used to reveal the polymorphism (E1=*Eco*RI, E5= *Eco*RV, D1= *Dra*I, or H3=*Hin*dIII), and the sizes in kilobases of the segregating DNA fragments. Segregations of RFLPs and male-fertility restoration were tested for goodness-of-fit ratios by chi-square analyses. Linkage arrangements were estimated using Map Manager Xp version 0.10 (Manly 1993, Manly and Cudmore 1998).

## Results and Discussion

### *Segregation at the Ms locus*

King et al. (1998a) previously reported segregation at the *Ms* locus in the cross BYG15-23×AC43 scored for male-fertility restoration from at least 20 test cross bulbs (Mansur et al. 1990) at one location (Madison, WI) in one of three years (1994 to 1996). No RFLPs were linked closer than 13.8 cM to *Ms*. Because male-fertility restoration may be affected by the environment (Meer and Bennekom 1969), we evaluated male-fertility restoration using these same testcross families over at least three environments (1994-2000 at Madison, WI). The putative scores of 12 $F_2$ plants were changed, four from homozygous recessive to heterozygous, six from heterozygous to homozygous dominant, one from homozygous dominant to heterozygous and one from unclassified to homozygous dominant (Appendix 1). Segregation (14:28:13) at the *Ms* locus fit the expected 1:2:1 ratio ($p=0.973$).

Some families appeared male-sterile early, but shed pollen after over half the flowers in the umbel had dehisced. We highly recommend scoring male-fertility restoration in S cytoplasm over environments and over the entire dehiscence period of each umbel. Across environments, testcross families generated using paternal plants heterozygous at *Ms* from BYG15-23 x AC43 and from randomly selected plants from the three open-pollinated populations often segregated for more male-sterile than male-fertile progenies (Figure 1). We observed significant ($p < 0.05$) year effects, meaning that male-fertility restoration across populations (BYG15-23 x AC43, BYG, MD, and SKI) was affected by the environment. Jones and Clarke (1943) also reported the tendency to observe too many male-sterile segregants. Our experiences indicate that the consistent appearance of too few male-fertile plants may be due to scoring the restoration of male fertility too early, scoring within only one environment, incomplete dominance, and/or reduced penetrance of the dominant allele at *Ms*.

### ***Segregations and sequence analyses of RFLPs flanking the Ms locus***

We evaluated for linkage to *Ms* using the segregation of previously described (King et al. 1998a) and newly identified RFLPs (Figure 2). The *Ms* locus was placed at the highest confidence (LOD=3.0) between RFLPs

AOB272-E1-10.0/12.0 (0.9 cM) and two cosegregating RFLPs AOB186-E5-6.7/2.5 and AOB232-E5-24.0/30.0 (8.6 cM). Other closely linked RFLPs were revealed by cDNA clones AOB210L, API63, and API65L (Figure 2). Accession numbers and sequence homologies of cDNA clones revealing linkage to *Ms* are listed in Table 1. Hybridizations of AOB272, API63, API65L, and AOB210L to DNA-gel blots from a collection of elite onion populations revealed numerous polymorphic fragments (Table 1, Appendices 2, 3 and 4). For AOB272, fragments of 6.7, 10.0 and 12.0 kb are genetically characterized alleles; fragments of 10.0 and 12.0 kb segregated in BYG15-23 x AC43 (King et al. 1998a) and fragments of 6.7 and 12.0 kb segregated in the Seminis mapping family (not shown), among inbreds and/or open-pollinated onion populations (Figure 3). An additional fragment of 14.0 kb was observed among inbred onion populations and may be one more allele at this locus (Table 1 and Appendix 3). API63-E5-5.0/7.5 was linked at 1.7 cM from AOB272/*Eco*RI (Figure 2); putative alleles of 14 and 6.7 kb were identified among the elite onion germplasms (Table 1, Appendices 2, 3, and 4). RFLPs revealed by AOB210L-H3-8.0/9.0 and API65L-D1-3.0/3.5 were mapped 2.7 and 3.6 cM, respectively, from API63-E5-5.0/7.5 (Figure 2). However one must be careful when scoring polymorphisms revealed by AOB210 and API65; these clones showed significant homology to rubisco and revealed numerous fragments segregating independently of *Ms* (King et al. 1998a, Table 1).

### ***Linkage equilibrium among RFLPs and Ms locus***

Havey (1995) used densiometric scans of autoradiograms to estimate the frequencies of N and S cytoplasms and testcrosses to estimate allelic frequencies at the *Ms* locus in the open-pollinated populations BYG, MD, and SKI. We randomly selected 36, 69, and 91 bulbs from BYG, MD, and SKI populations, respectively, and established cytoplasms using polymorphisms in the chloroplast genome (Figure 4 and Table 2). We measured the frequency of genotypes at AOB272/*Eco*RI and *Ms* among plants from BYG, MD, and SKI and chi-square analyses demonstrated that these two loci are at linkage equilibrium (Table 2). Other RFLP loci linked to *Ms* (King et al. 1998a) were also found at linkage equilibrium with *Ms* locus (Table 3), indicating that adequate generations of random mating had occurred to reach linkage equilibrium assuming single *Ms* locus. Although the nuclear markers could not be used to predict genotypes at *Ms* in these open-pollinated populations, the number of testcrosses required to identify maintainer lines can be reduced by 53, 39, and 43%, respectively, by selecting only those plants possessing N cytoplasm (Havey 1995). These results conclusively demonstrate that molecular characterization of the cytoplasm is an effective technology to identify and isolate CMS-maintaining genotypes from OP onion populations possessing both N and S cytoplasms as suggested by Havey previously.

### *Use of cytoplasmic markers and RFLPs linked to the Ms locus*

Although the flanking RFLPs cannot be used to identify maintaining genotypes among plants from open-pollinated populations, these nuclear markers are useful when the breeder generates linkage disequilibrium by crossing among restoring and maintaining genotypes. To use the RFLP markers flanking the *Ms* locus, one should determine the fragments revealed by the flanking RFLPs for the parents used in the fertile-by-fertile cross. Fragments in coupling-phase linkage with the recessive *ms* allele can be identified. The segregation of the dominant and recessive alleles at *Ms* can then be predicted using these linked, phase-known markers. Although molecular identification of nuclear genotypes and the cytoplasm does not shorten the onion-breeding cycle (two years per generation are still required), the technique represents a more judicious use of resources by reducing the number of testcrosses required to identify maintainer plants.

## Future work

It is desirable to find markers more closely linked to *Ms* and, eventually, clone this locus. There are many classes of molecular markers that could be used, including RAPDs, AFLPs, microsatellites. We have not found RAPDs and AFLPs to work well in onion (Bradeen and Havey 1995, Sato and Havey unpublished). Fischer and Bachmann (2000) developed 30 onion microsatellites, too few to have a reasonable chance of tight linkage to *Ms*. High throughput identification and mapping of molecular markers remains difficult in onion due to its enormous nuclear genome.  Potentially, synteny (McCouch 2001) between onion and other monocots, such as rice, would allow for the identification of closely linked molecular markers in difficult species, such as onion.

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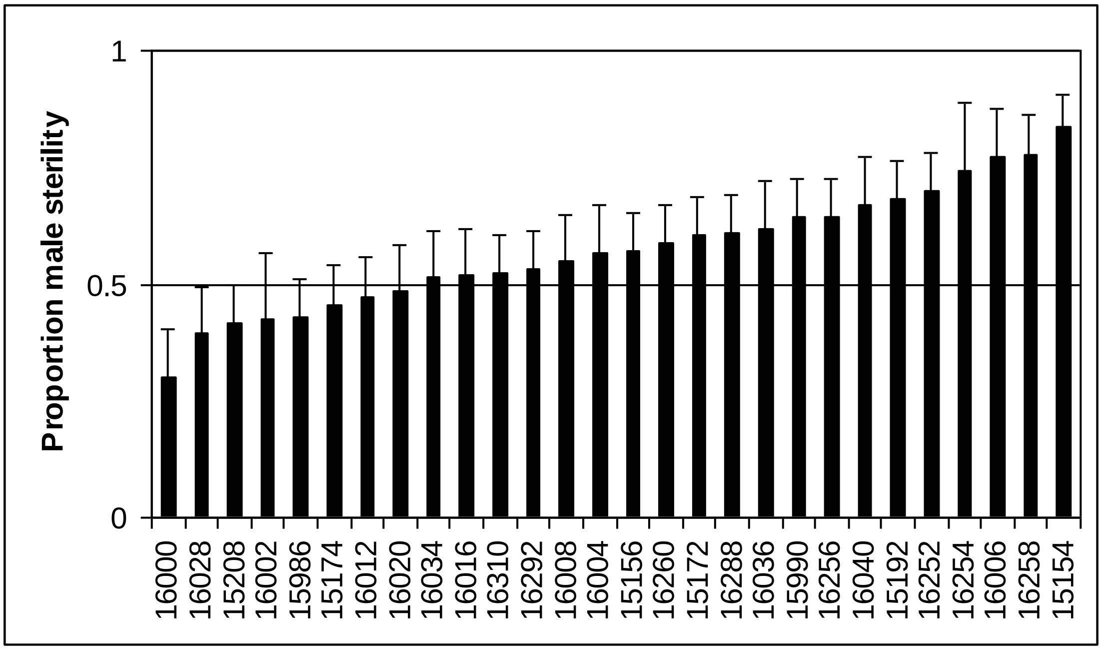

Figure 1. Mean proportion of male-sterile testcross progenies adjusted for years for testcross families generated from parental plants heterozygous at the *Ms* locus from the mapping family BYG15-23xAC43 (King et al. 1998a). Segregations of 1:1 were expected and too many male-sterile testcross progenies were observed for means from 20 out of 28 families. Familiy numbers are shown on the horizontal axis. Bars show standard errors.

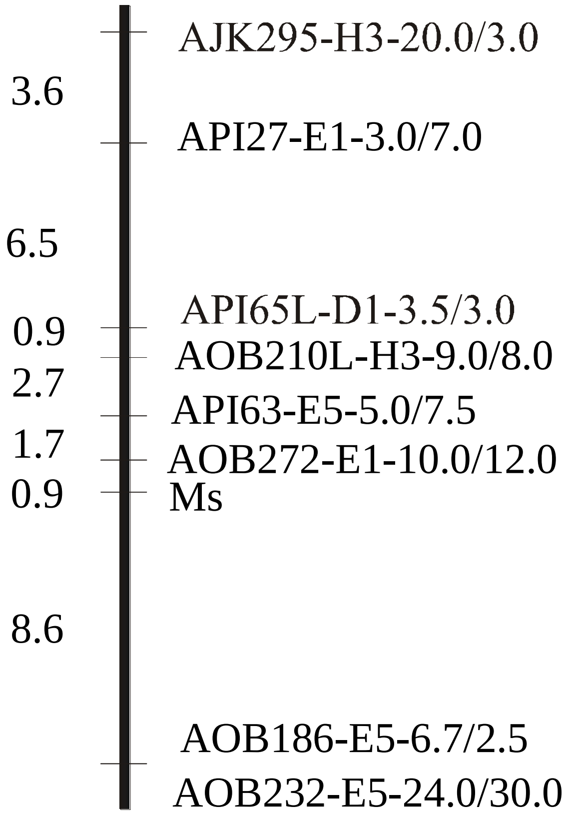

Figure 2. Linkages among restriction fragment length polymorphisms (RFLPs) and *Ms* locus of onion. Genetic distances in centi Morgans shown on left. Nomenclature for RFLPs is described in Materials and Methods. Only the center portion of linkage group B (King et al. 1998a) is shown.

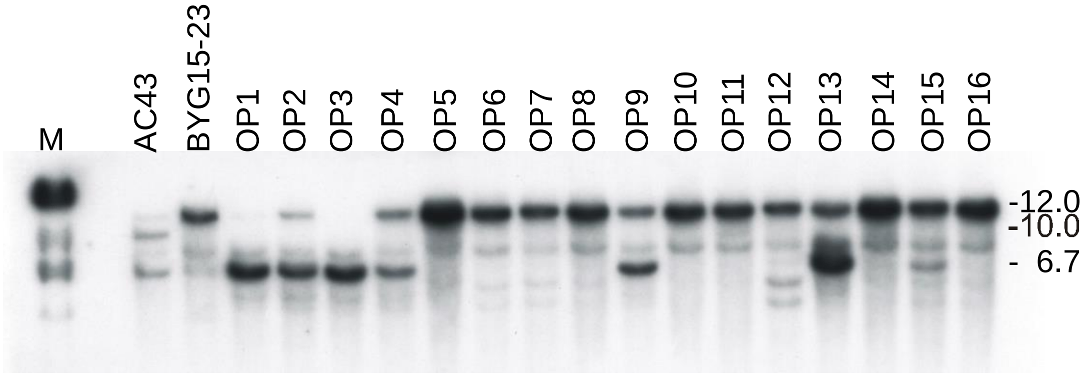

Figure 3. Allelic restriction fragment length polymorphisms revealed by AOB272-*Eco*RI for plants from the onion populations (origins are listed in Appendix 3). Fragment sizes in kilobases are shown on right. Size markers (M) and parents of mapping families [Alisa Craig (AC43) and Brigham Yellow Globe (BYG15-23)] were used as controls.

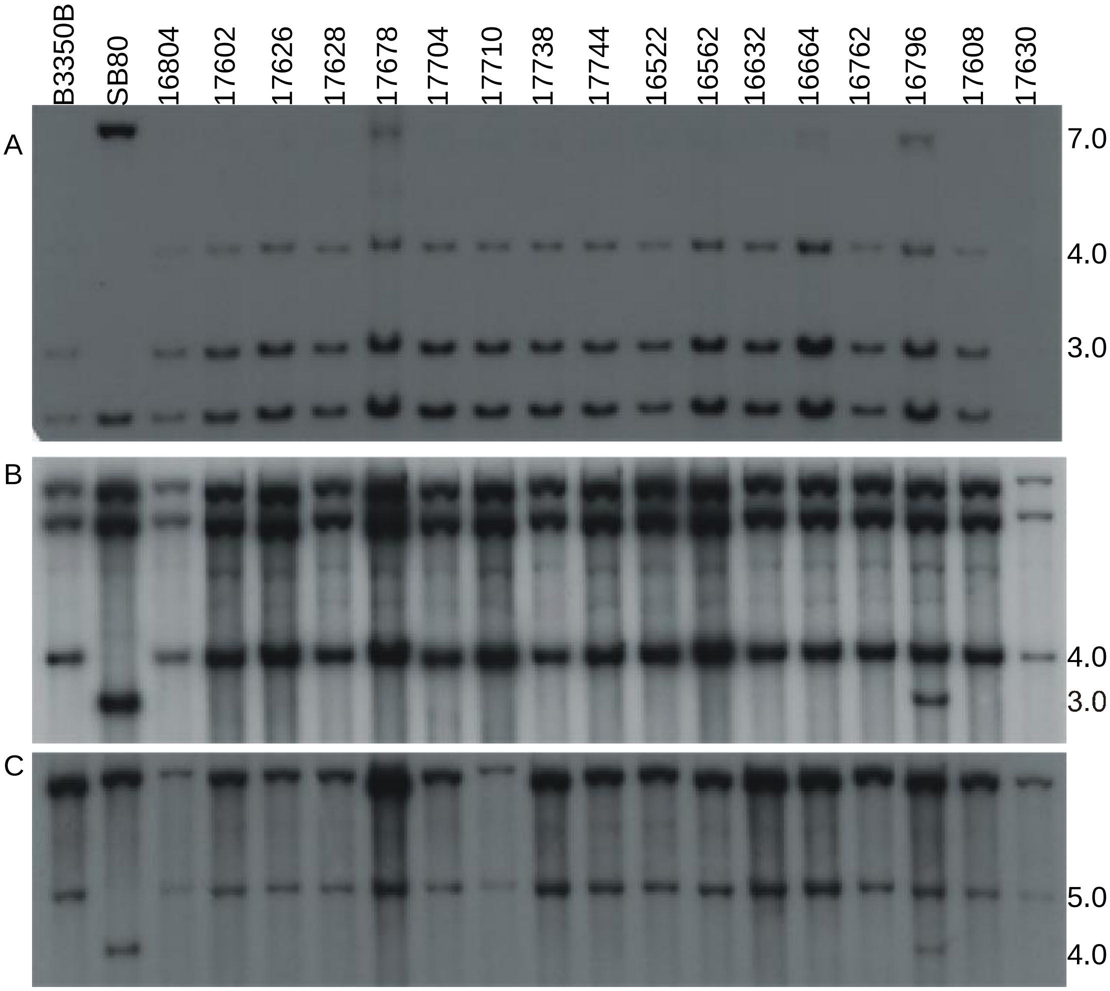

Figure 4. Autoradiograms establishing the cytoplasms of control [B3350B (N cytoplasm) and SB80 (S cytoplasm)] and $S_1$ families (origins listed in Appendix 1). A= Autoradiogram of *Eco*RV digested DNAs hybridized with orchid chloroplast clones 12 b&c (Havey 1993). Fragments of 3.0 and 4.0 kb indicate the presence of N cytoplasm. B= Autoradiogram of *Bgl*II digested DNAs hybridized with orchid chloroplast clones 3, 4 and 6a. Fragment of 3.0 kb indicates the presence of S cytoplasm. C= Autoradiogram of *Bam*HI digested

DNAs hybridized with orchid chloroplast clones 19 a&b. Fragment of 4.0 kb indicates the presence of S cytoplasm. The presence of both N and S cytoplasms in $S_1$ family 16796 was likely due to a seed mixture.

Table 1. Sequence homologies, Genbank accession numbers, and sizes of polymorphic DNA fragments revealed by cDNA clones revealing with restriction fragment length polymorphisms evaluated for linkage to the *Ms* locus in onion.

| cDNA Clone[a] | Restriction Enzyme[b] | Genbank Access. No. | Putative Homology | Sizes of Allelic Fragments[c] | Anonymous Fragments[c] |
|---|---|---|---|---|---|
| AGF045 (API45) | ND | AF366442 | Beta-tubilin | NA | NA |
| AGF096 (API96) | ND | AF366443 | No hits | NA | NA |
| AGF109 | NP | AF366444 | Spinacia oleracea (PsbY) | NA | NA |
| AGF120 | NP | AF366445 | No hits | NA | NA |
| AGF122 | NP | NA | Spinacia oleracea (PsbY) | NA | NA |
| AGF125 | EcoRI | AF366446 | No hits | 7.0, 20.0 | 5.0 |
| AGF136B (API27 | EcoRI | AF366447 | Arabidopsis | 3.0, 7.0 | 5.0 |
| AGF147 | NP | AF366448 | No hits | NA | NA |
| AGF148 | XbaI | NA | No hits | 4.0, 6.0 | 20.0, 7.0, 2.5, 2.0 |

Table 1. Continued.

| cDNA Clone[a] | RestrictionEnzyme[b] | Genbank Access. No. | Putative Homology | Sizes of Allelic Fragments[c] | Anonymous Fragments[c] |
|---|---|---|---|---|---|
| AGF152 | EcoRI | AF366449 | No hits | 10.0, 12.0 | |
| AGF158 | HindIII | AF366450 | No hits | 2.3, 7.0 | |
| AGF161 | HindIII | AF366457 | No hits | 2.8 | 3.0, 2.0 |
| AGF166&7 | EcoRV | AF366451 | Glutathione peroxidase | 2.0, 3.0 | 20.0, 5.0 4.0 |
| AGF173 | HindIII | AF366452 | Arabidopsis | 4.0, 3.0 | 10.0, 7.0, 6.0, 2.5 |
| AGF173 | HindIII | AF366452 | Arabidopsis | 8.0 | 10.0, 7.0, 6.0, 2.5 |
| AGF176 | NP | AF366453 | R. protein L30 (rpl30) | NA | NA |
| AGI063 | NP | NA | Phosphoglucomutase | NA | NA |
| AGI101 | EcoRI | NA | No hits | 7.0, 10.0 | 4.0 |
| AGI101 | EcoRI | NA | No hits | 5.0, 6.0 | 4.0 |
| AGI106 | NP | NA | No hits | NA | NA |

Table 1. Continued.

| cDNA Clone[a] | Restriction Enzyme[b] | Genbank Access. No. | Putative Homology | Sizes of Allelic Fragments[c] | Anonymous Fragments[c] |
|---|---|---|---|---|---|
| AGI131 | NP | NA | Arabidobsis | NA | NA |
| AGI151 | NP | NA | No hits | NA | NA |
| AGI156 | NP | NA | No hits | NA | NA |
| AGI178 | EcoRV | NA | No hits | 25.0, 24.0 | |
| AJK295 | HindIII | AA451599 | ACC oxidase | 3.0, 20.0 | 4.3, 5.0, 18.0 |
| AOB186 | EcoRV | AA451597 | Ubiquitin | 2.5, 6.7 | 6.0 |
| AOB210L | HindIII | AA451567 | Rubisco | 8.0, 9.0 | Numerous |
| AOB232 | EcoRV | AA451596 | Sterol methyl-transferase | 24.0, 30.0 | 4.0, 35.0 |
| AOB272 | EcoRI | AA451592 | No hits | 6.7, 10.0, 12.0 | 14.0, 5.0 |
| API27 | EcoRI | AA451547 | No hits | 3.0, 7.0 | 5.0 |

Table 1.  Continued.

| cDNA Clone[a] | Restriction Enzyme[b] | Genbank Access. No. | Putative Homology | Sizes of Allelic Fragments[c] | Anonymous Fragments[c] |
|---|---|---|---|---|---|
| API63 | EcoRV | AF366456 | Phospho-glucomutase 2 | 5.0, 7.5 | Numerous |
| API65L | DraI | AA451551 | Rubisco | 3.0, 3.5 | Numerous |

[a] B= fragment from putatively chimeric cDNA clone digested with *Nco* I.  AGF136B corresponds to fragment from 764 to 1266 bp of AGF136 clone.  L= Long fragment from putative chimeric cDNA clone digested with *Mbo*I restriction enzyme.  AOB210L corresponds to fragment from 178 to 407 bp; API65L corresponds to fragment from 427 to 746 bp.

[b] ND= Not determined, NP= not polymorphic with evaluated restriction enzymes.

[c] Known alleles at RFLP locus were established by King et al. (1998a) or this research.  Anonymous fragments were polymorphic among a sample of elite commercial inbred lines and may be alleles at these loci or at other loci.  Fragment sizes are in kilobases.  NA= not applicable.

Table 2. Frequencies of normal (N) male-fertile cytoplasm in the open-pollinated populations Brigham Yellow Globe (BYG), Mountain Danvers (MD), and Sapporo-Ki (SKI) and genotypes at the *Ms* locus and the restriction fragment length polymorphism AOB272/*Eco*RI for randomly selected N-cytoplasmic onion plants from these three populations. Linkage equilibrium between *Ms* and AOB272 was tested by goodness of fit of observed to expected genotypic frequencies based on allelic frequencies.

| | | | | Numbers of genotypes at AOB272/*Eco*RI[c] | | | |
|---|---|---|---|---|---|---|---|
| Pop. | Frequency of N cytoplasm[a] | Genotypes at *Ms* locus | Proportion[b] of genotypes at *Ms* | Homozygous for 12.0 kb | Heterozygous | Homozygous for 6.7 kb | Prob. |
| BYG | 0.78 | *MsMs* | 0.08 | 2 | 0 | 0 | 0.373 |
| | | *Msms* | 0.59 | 7 | 7 | 0 | |
| | | *msms* | 0.33 | 3 | 4 | 1 | |
| MD | 0.78 | *MsMs* | 0.37 | 16 | 1 | 0 | 0.217 |
| | | *Msms* | 0.39 | 13 | 4 | 1 | |
| | | *msms* | 0.24 | 7 | 4 | 0 | |
| SKI | 0.78 | *MsMs* | 0.42 | 17 | 5 | 0 | 0.209[d] |
| | | *Msms* | 0.42 | 1 | 21 | 0 | |
| | | *msms* | 0.16 | 1 | 7 | 0 | |

[a] Frequencies of N cytoplasm among male-fertile plants. Actual N cytoplasmic frequencies in the population are lower because male-sterile (*S*-*msms*) plants failed to set seed after self pollinations.

[b] Genotypic frequencies are shown only for plants possessing N cytoplasm.

[c] Alleles at AOB272/*Eco*RI were established by King et al. (1998a) or this research. Only the 12.0 and 6.7 kb fragments were observed among plants from these three open-pollinated populations.

[d] No homozygotes for the 6.7 kb fragment were observed and were excluded from the test.

Table 3. Numbers of plants scored for genotypes at *Ms* and fragment sizes in kilobases for nuclear restriction fragment length polymorphisms (RFLPs) and chi-square tests to estimate linkage equilibrium for random N-cytoplasmic $S_1$ families from the open-pollinated onion populations Brigham Yellow Globe, Mountain Danvers, and Sapporo-Ki.

| | | | | Brigham Yellow Globe | | | | Mountain Danvers | | | | Sapporo-Ki | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Probe[a] | Linked[b] | Frag[c] | Occur[d] | MSMS[e] | MSms | msms | Prob.[f] | MSMS | MSms | msms | Prob.[f] | MSMS | MSms | msms | Prob.[f] |
| AJB37 | 16.7 | 10 | 1 | 1 | 13 | 6 | 0.350 | 12 | 10 | 8 | 0.937 | 13 | 12 | 6 | 0.747 |
| | | | 0 | 0 | 0 | 1 | | 2 | 2 | 1 | | 2 | 2 | 2 | |
| | | 9 | 1 | 1 | 10 | 6 | 0.789 | 7 | 6 | 8 | 0.122 | 15 | 12 | 6 | 0.160 |
| | | | 0 | 0 | 3 | 1 | | 7 | 6 | 1 | | 0 | 2 | 2 | |
| | | 8 | 1 | 1 | 13 | 7 | - | 14 | 12 | 9 | - | 15 | 14 | 8 | - |
| | | | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | | 0 | 0 | 0 | |
| | | 6 | 1 | 1 | 12 | 4 | 0.143 | 13 | 10 | 8 | 0.748 | 12 | 7 | 5 | 0.236 |
| | | | 0 | 0 | 1 | 3 | | 1 | 2 | 1 | | 3 | 7 | 3 | |

Table 3. Continued.

| | | | | Brigham Yellow Globe | | | | Mountain Danvers | | | | Sapporo-Ki | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Probe[a] | Linked[b] | Frag[c] | Occur[d] | MSMS[e] | MSms | msms | Prob.[f] | MSMS | MSms | msms | Prob.[f] | MSMS | MSms | msms | Prob.[f] |
| | | 5 | 1 | 1 | 10 | 6 | 0.789 | 10 | 7 | 7 | 0.609 | 14 | 12 | 7 | 0.792 |
| | | | 0 | 0 | 3 | 1 | | 4 | 5 | 2 | | 1 | 2 | 1 | |
| | | 4 | 1 | 1 | 4 | 2 | 0.348 | 4 | 3 | 3 | 0.916 | 11 | 10 | 5 | 0.857 |
| | | | 0 | 0 | 9 | 5 | | 10 | 9 | 6 | | 4 | 4 | 3 | |
| AJK295 | 16.5 | 20 | 1 | 1 | 9 | 5 | 0.806 | 4 | 6 | 4 | 0.513 | 10 | 13 | 5 | 0.264 |
| | | | 0 | 0 | 4 | 2 | | 10 | 6 | 5 | | 6 | 2 | 3 | |
| | | 18 | 1 | 0 | 2 | 0 | 0.507 | 10 | 5 | 2 | 0.059 | 3 | 2 | 0 | 0.431 |
| | | | 0 | 1 | 11 | 7 | | 4 | 7 | 7 | | 13 | 13 | 8 | |
| | | 5 | 1 | 1 | 9 | 5 | 0.806 | 12 | 10 | 8 | 0.937 | 12 | 14 | 7 | 0.356 |
| | | | 0 | 0 | 4 | 2 | | 2 | 2 | 1 | | 4 | 1 | 1 | |

Table 3. Continued.

| | | | | Brigham Yellow Globe | | | | Mountain Danvers | | | | Sapporo-Ki | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Probe[a] | Linked[b] | Frag[c] | Occur[d] | MSMS[e] | MSms | msms | Prob.[f] | MSMS | MSms | msms | Prob.[f] | MSMS | MSms | msms | Prob.[f] |
| | | 3 | 1 | 1 | 10 | 7 | 0.341 | 5 | 9 | 4 | 0.121 | 7 | 7 | 4 | 0.958 |
| | | | 0 | 0 | 3 | 0 | | 9 | 3 | 5 | | 9 | 8 | 4 | |
| AOB41 | 72.3 | 10 | 1 | 2 | 8 | 4 | 0.621 | 2 | 11 | 3 | 0.085 | 7 | 8 | 2 | 0.701 |
| | | | 0 | 0 | 4 | 2 | | 5 | 4 | 5 | | 9 | 9 | 5 | |
| | | 8 | 1 | 1 | 4 | 6 | 0.027 | 5 | 8 | 7 | 0.242 | 9 | 13 | 6 | 0.272 |
| | | | 0 | 1 | 8 | 0 | | 2 | 7 | 1 | | 7 | 4 | 1 | |
| | | 4 | 1 | 0 | 4 | 2 | 0.784 | 1 | 4 | 3 | 0.598 | 3 | 2 | 3 | 0.221 |
| | | | 0 | 1 | 8 | 4 | | 6 | 11 | 5 | | 13 | 15 | 4 | |
| AOB116 | 25.9 | 5 | 1 | 1 | 10 | 7 | 0.439 | 6 | 12 | 6 | 0.175 | 18 | 15 | 3 | 0.155 |
| | | | 0 | 1 | 5 | 1 | 11.000 | 6 | 5 | | 6.000 | 8 | 5 | | |

Table 3. Continued.

| | | | | Brigham Yellow Globe | | | | Mountain Danvers | | | | Sapporo-Ki | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Probe[a] | Linked[b] | Frag[c] | Occur[d] | MSMS[e] | MSms | msms | Prob.[f] | MSMS | MSms | msms | Prob.[f] | MSMS | MSms | msms | Prob.[f] |
| | | 3.5 | 1 | 2 | 13 | 4 | 0.104 | 17 | 17 | 11 | 0.452 | 19 | 21 | 7 | 0.491 |
| | | | 0 | 0 | 2 | 4 | | 0 | 1 | 0 | | 5 | 2 | 1 | |
| | | 3 | 1 | 1 | 6 | 5 | 0.588 | 12 | 14 | 9 | 0.775 | 20 | 19 | 7 | 0.948 |
| | | | 0 | 1 | 9 | 3 | | 5 | 4 | 2 | | 4 | 4 | 1 | |
| | | 2.5 | 1 | 0 | 5 | 3 | 0.587 | 10 | 10 | 6 | 0.970 | 12 | 15 | 2 | 0.137 |
| | | | 0 | 2 | 10 | 5 | | 7 | 8 | 5 | | 12 | 8 | 6 | |
| AOB186 | 8.6 | 6.7 | 1 | 0 | 8 | 2 | 0.198 | 16 | 17 | 9 | 0.828 | 15 | 16 | 4 | 0.464 |
| | | | 0 | 2 | 6 | 5 | | 2 | 1 | 1 | | 6 | 6 | 4 | |
| | | 6 | 1 | 2 | 12 | 5 | 0.570 | 11 | 10 | 6 | 0.822 | 13 | 15 | 6 | 0.877 |
| | | | 0 | 0 | 2 | 2 | | 5 | 7 | 4 | | 7 | 7 | 2 | |

Table 3. Continued.

| | | | | Brigham Yellow Globe | | | | Mountain Danvers | | | | Sapporo-Ki | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Probe[a] | Linked[b] | Frag[c] | Occur[d] | MSMS[e] | MSms | msms | Prob.[f] | MSMS | MSms | msms | Prob.[f] | MSMS | MSms | msms | Prob.[f] |
| | | 2.5 | 1 | 2 | 10 | 7 | 0.211 | 2 | 0 | 0 | 0.170 | 5 | 4 | 3 | 0.544 |
| | | | 0 | 0 | 4 | 0 | | 14 | 17 | 10 | | 16 | 18 | 5 | |
| AOB232 | 8.6 | 30 | 1 | 2 | 13 | 5 | 0.330 | 15 | 16 | 8 | 0.780 | 15 | 18 | 7 | 0.643 |
| | | | 0 | 0 | 1 | 2 | | 2 | 2 | 2 | | 6 | 5 | 1 | |
| | | 24 | 1 | 1 | 2 | 2 | 0.452 | 5 | 7 | 5 | 0.562 | 11 | 10 | 3 | 0.013 |
| | | | 0 | 1 | 12 | 5 | | 12 | 11 | 5 | | 1 | 13 | 5 | |
| | | 4 | 1 | 1 | 12 | 7 | 0.176 | 17 | 18 | 10 | - | 18 | 21 | 8 | 0.496 |
| | | | 0 | 1 | 2 | 0 | | 0 | 0 | 0 | | 3 | 2 | 0 | |
| AOB262 | 32.4 | 9.5 | 1 | 0 | 1 | 2 | 0.376 | 11 | 14 | 7 | 0.896 | 13 | 15 | 5 | 0.902 |
| | | | 0 | 2 | 14 | 6 | | 4 | 4 | 3 | | 8 | 7 | 3 | |

Table 3. Continued.

| | | | | Brigham Yellow Globe | | | | Mountain Danvers | | | | Sapporo-Ki | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Probe[a] | Linked[b] | Frag[c] | Occur[d] | MSMS[e] | MSms | msms | Prob.[f] | MSMS | MSms | msms | Prob.[f] | MSMS | MSms | msms | Prob.[f] |
| | | 8 | 1 | 2 | 14 | 8 | 0.707 | 14 | 15 | 11 | 0.107 | 17 | 19 | 8 | 0.412 |
| | | | 0 | 0 | 1 | 0 | | 0 | 3 | 0 | | 4 | 3 | 0 | |
| AOB272 | 0.9 | 12 | 1 | 2 | 15 | 7 | 0.331 | 17 | 17 | 11 | 0.452 | 22 | 21 | 8 | 0.499 |
| | | | 0 | 0 | 0 | 1 | | 0 | 1 | 0 | | 0 | 1 | 0 | |
| | | 6.7 | 1 | 0 | 7 | 5 | 0.282 | 1 | 5 | 4 | 0.118 | 5 | 1 | 1 | 0.209 |
| | | | 0 | 2 | 8 | 3 | | 16 | 13 | 7 | | 17 | 21 | 7 | |
| API21 | 29.5 | 4.5 | 1 | 1 | 11 | 4 | 0.316 | 13 | 15 | 9 | 0.833 | 16 | 22 | 4 | 0.041 |
| | | | 0 | 1 | 2 | 3 | | 3 | 3 | 1 | | 5 | 1 | 3 | |
| | | 4.1 | 1 | 2 | 8 | 7 | 0.106 | 12 | 9 | 4 | 0.161 | 12 | 12 | 3 | 0.803 |
| | | | 0 | 0 | 5 | 0 | | 4 | 9 | 6 | | 9 | 11 | 4 | |

Table 3. Continued.

| | | | | Brigham Yellow Globe | | | | Mountain Danvers | | | | Sapporo-Ki | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Probe[a] | Linked[b] | Frag[c] | Occur[d] | MSMS[e] | MSms | msms | Prob.[f] | MSMS | MSms | msms | Prob.[f] | MSMS | MSms | msms | Prob.[f] |
| API27 | 12.7 | 7 | 1 | 1 | 6 | 3 | 0.549 | 2 | 3 | 4 | 0.108 | 4 | 3 | 1 | 0.717 |
| | | | 0 | 0 | 2 | 0 | | 4 | 2 | 0 | | 1 | 1 | 1 | |
| | | 6.5 | 1 | 0 | 3 | 0 | 0.368 | 4 | 3 | 0 | 0.090 | 1 | 3 | 1 | 0.255 |
| | | | 0 | 1 | 5 | 3 | | 2 | 2 | 4 | | 4 | 1 | 1 | |
| API51 | 87.8 | 6.7 | 1 | 1 | 11 | 7 | 0.704 | 11 | 5 | 5 | 0.619 | 6 | 5 | 6 | 0.591 |
| | | | 0 | 0 | 1 | 0 | | 1 | 1 | 0 | | 1 | 1 | 0 | |
| | | 5 | 1 | 0 | 5 | 5 | 0.270 | 11 | 1 | 5 | 0.000 | 3 | 4 | 3 | 0.684 |
| | | | 0 | 1 | 7 | 2 | | 1 | 6 | 0 | | 4 | 2 | 3 | |
| | | 3.5 | 1 | 1 | 11 | 7 | 0.704 | 10 | 6 | 4 | 0.966 | 6 | 5 | 5 | 0.991 |
| | | | 0 | 0 | 1 | 0 | | 2 | 1 | 1 | | 1 | 1 | 1 | |

Table 3. Continued.

| | | | | Brigham Yellow Globe | | | | Mountain Danvers | | | | Sapporo-Ki | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Probe[a] | Linked[b] | Frag[c] | Occur[d] | MSMS[e] | MSms | msms | Prob.[f] | MSMS | MSms | msms | Prob.[f] | MSMS | MSms | msms | Prob.[f] |
| API 63 | 2.6 | 9.5 | 1 | 1 | 9 | 5 | 0.806 | 11 | 11 | 5 | 0.147 | 13 | 12 | 7 | 0.907 |
| | | | 0 | 0 | 4 | 2 | | 3 | 1 | 4 | | 3 | 2 | 1 | |
| | | 7 | 1 | 1 | 13 | 7 | - | 14 | 12 | 9 | - | 14 | 12 | 7 | 0.988 |
| | | | 0 | 0 | 0 | 0 | | 0 | 0 | 0 | | 2 | 2 | 1 | |
| | | 6 | 1 | 0 | 4 | 1 | 0.604 | 7 | 5 | 6 | 0.521 | 7 | 7 | 3 | 0.847 |
| | | | 0 | 1 | 9 | 6 | | 7 | 7 | 3 | | 9 | 7 | 5 | |
| | | 4.3 | 1 | 0 | 8 | 5 | 0.388 | 3 | 3 | 7 | 0.014 | 0 | 3 | 1 | 0.159 |
| | | | 0 | 1 | 5 | 2 | | 11 | 9 | 2 | | 16 | 11 | 7 | |
| API 65 | 6.2 | 23 | 1 | 1 | 6 | 2 | 0.532 | 5 | 4 | 4 | 0.649 | 4 | 2 | 2 | 0.557 |
| | | | 0 | 0 | 2 | 2 | | 1 | 1 | 0 | | 1 | 2 | 2 | |

Table 3. Continued.

| | | | | Brigham Yellow Globe | | | | Mountain Danvers | | | | Sapporo-Ki | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Probe[a] | Linked[b] | Frag[c] | Occur[d] | MSMS[e] | MSms | msms | Prob.[f] | MSMS | MSms | msms | Prob.[f] | MSMS | MSms | msms | Prob.[f] |
| | | 10 | 1 | 1 | 7 | 4 | 0.713 | 6 | 5 | 4 | - | 5 | 4 | 2 | 0.070 |
| | | | 0 | 0 | 1 | 0 | | 0 | 0 | 0 | | 0 | 0 | 2 | |
| | | 9 | 1 | 0 | 2 | 2 | 0.532 | 2 | 2 | 2 | 0.870 | 3 | 1 | 2 | 0.568 |
| | | | 0 | 1 | 6 | 2 | | 4 | 3 | 2 | | 2 | 3 | 2 | |
| | | 7 | 1 | 1 | 6 | 4 | 0.478 | 2 | 0 | 0 | 0.177 | 0 | 1 | 2 | 0.208 |
| | | | 0 | 0 | 2 | 0 | | 4 | 5 | 4 | | 5 | 3 | 2 | |

[a] Onion cDNA clone.

[b] Linkage to *Ms* locus at distance in cM.

[C] Fragment sizes are in kilobases.

[d] Fragments on autoradiograms were scored as presence (1) versus absence (0).

[e] Genotypes at *Ms* locus were scored as homozygous dominant (*MSMS*), heterozygous (*MSms*), or homozygous recessive (*msms*) based on testcross segregations.

[f] Probabilities were calculated by Chi-square tests for expected ( at linkage equilibrium) versus observed values based on allelic frequencies. Probabilities could not be calculated when fragments were present or absent across all genotypes at *Ms*.

# Chapter Three

# CONVERSION OF A RESTRICTION FRAGMENT LENGTH POLYMORPHISM TIGHTLY LINKED WITH *MS* LOCUS IN ONION (*ALLIUM CEPA* L.) TO A MARKER REVEALED BY THE POLYMERASE CHAIN REACTION

## Abstract:

The most widely used source of cytoplasmic-genic male sterility (CMS) in onion is conditioned by the interaction of male-sterile (S) cytoplasm and the homozygous recessive (S-msms) genotype at a nuclear male-fertility restoration (*Ms*) locus. Maintainer lines are used to seed propagate male-sterile lines and possess normal (N) male-fertile cytoplasm and the homozygous recessive (N-msms) genotype at the *Ms* locus. Conventionally, determinations of the cytoplasm and the genotype at *Ms* for a single onion plant can take from four to eight years. Havey previously developed a PCR marker distinguishing N and S cytoplasms, and we identified restriction fragment length polymorphisms (RFLPs) flanking the *Ms* locus. Using the cDNA sequence (AOB272) revealing the most closely linked RFLP, we designed oligonucleotides and used the polymerase chain reaction to amplify genomic DNAs from seven commercial onion cultivars and the parents (Ailsa Craig 43 and Brigham Yellow Globe 15-23) of our mapping family. We cloned and sequenced these amplified genomic DNAs to identify single nucleotide polymorphisms (SNP). Oligonucleotides were designed flanking the SNPs and were used as allele-specific primers to distinguish genotypes in the AOB272 genomic region. These organellar and nuclear markers can be used to select maintainer genotypes from onion populations or segregating families and

should reduce the number of testcrosses required to identify maintaining genotypes.

## Introduction

The most widely used source of cytoplasmic-genic male-sterility (CMS) in onion is conditioned by the interaction of male-sterile (S) cytoplasm and the homozygous recessive (S-msms) genotype at a nuclear male-fertility restoration (*Ms*) locus (Jones and Clarke 1943, Jones and Emsweller 1936). Maintainer lines are used to seed propagate male-sterile lines and possess normal (N) male-fertile cytoplasm and the homozygous recessive (N-msms) genotype at *Ms*. Conventionally, cytoplasmic determinations of single onion plant can take from four to eight years and are complicated by segregation at the *Ms* locus. PCR markers have been developed distinguishing N and S cytoplasms (Havey 1995, Sato 1998). With the cytoplasmic PCR markers, one can determine the cytoplasm of a single onion plant in only a few hours, as opposed to years.

When RFLP markers flank a trait of interest, the coding sequence of RFLP probes can be used to reveal polymorphisms among individuals or populations using the polymerase chain reaction (PCR). After amplifying the genomic DNAs with specific oligonucleotide primers, PCR products can be digested by restriction enzymes (Graner et al. 2000, Hausner et al. 1999, Heusden et al. 2000) used to produce single-stranded conformational polymorphisms (Cespedes et al. 1999, Fischer and Lerman 1983, Hegedus and Khachatourians 1996, Orita et al. 1989a, 1989b, Sharpe et al. 1999), or analyzed for insertion-deletion (Indel) events (Lin and Davis 2000, Stappen et al.

1998, Xu et al 1995), microsatellites (Fischer and Bachmann 2000), or single nucleotide polymorphisms (Coryell et al. 1999, Gupta et al. 1999, Smith et al. 2000).

We identified nuclear RFLP markers flanking the *Ms* locus (Chapter 2) and determined the genotypes at these flanking molecular markers for commercial inbreds and the parents (Alisa Craig 43 and Brigham Yellow Globe 15-23) of our mapping family. These organellar and nuclear markers can be used to select maintainer genotypes from onion populations or segregating families and should reduce the number of testcrosses required to identify maintaining genotypes. In this study, we converted the closest linked RFLP (revealed by AOB272) to nonradioactive PCR-based markers.

## Material and Methods

AOB272-*Eco*RI is an RFLP tightly linked (0.9 cM) to *Ms* (Chapter 2). The cDNA (AOB272) was sequenced as described by McCallum et al. (2001). Specific oligonucleotide primers at the ends of the cDNA sequence were designed using Oligo 6.1 (Molecular Technology). CsCl purified genomic DNAs (gDNA) were isolated (Havey 1991) and primers were used to amplify genomic regions from AC43, BYG15-23, and seven onion populations (Table 4). PCR reaction conditions were 1.5 mM of $MgCl_2$, 0.2 mM each dNTPs, 1 $\mu$M of each primer, 1 unit Gold Taq DNA polymerase, and 100 ng of DNA in 50 $\mu$l final volume. PCR cycles were 10 minutes at 94 C, then 40 cycles at 30 seconds at 94 C, 45 seconds at 45 C, and 3 minutes at 68 C, and a final extension for 15 minutes at 72 C. PCR products were loaded on a 1% agarose gel and visualized by EtBr staining. Amplified gDNAs were cloned by TA tailing using the pCR 2.1 vector (Invitrogen), transformed into bacteria, and plated on ampicillin LB medium. Five transformed bacterial colonies from each of the nine onion populations were selected, plasmid DNA was isolated, and inserts were sequenced as previously described (Lilly and Havey 2001). Raw sequences in both directions were edited and contigs formed using Sequencer 3.1.2 (Genecodes).

Single nucleotide polymorphisms (SNP) among the nine onion populations were identified after sequence alignments. Oligonucleotide primers were designed to flank eight of these SNPs. Allele specific primer pairs were used to amplify specific alleles at AOB272 using PCR conditions of 2 mM of $MgCl_2$, 0.2 mM each dNTPs, 0.6 $\mu$M each primer, 0.6 unit Gold Taq DNA polymerase, 20 ng sample DNA in 30 $\mu$l final volume. Annealing temperatures were varied taking into consideration the melting temperatures of oligonucleotides at 1.2 mM $MgCl_2$ as calculated by the Oligo 6.1 program. Cycling conditions were 30 seconds at 94 C, 45 seconds at varied annealing temperatures (45 to 72 C), and 90 seconds at 72 C with cycles of 25, 30, 35, or 40 cycles.

Because primers with one mismatch at the 3’ end may still anneal to nontarget template DNA and amplify non specific alleles, we created modified single nucleotide polymorphic (MSNP) primers by substituting the second nucleotide from the 3’ prime ends with the same nucleotide on the template DNA, making primers with one mismatch at the 3’ minus one position for target allelic template and two polymorphic nucleotides for nontarget alleles. PCR reactions were described above with 40 cycles of 30 seconds at 94 C, 30 seconds at annealing temperatures 60 C varried over 10 C, and 60 seconds at 72 C.

We also attempted to identify alleles at AOB272 by single nucleotide extensions using the SNaPshot (ABI) system. Genomic DNAs from the nine onion cultivars (Table 4) were amplified using the AOB272 forward and reverse primers and purified by QIAquick PCR purification kits (QIAGEN). Seven primers flanking five SNPs were synthesized (Figure 5, Tables 5 and 6). Single nucleotide extensions used 1x SNaPshot Ready Reaction Premix, 4 μM of the primer, 20 ng preamplified template DNA in a 5 μl final volume. Cycling conditions were 96 C for 10 seconds, 50 C for 5 seconds, and 60 C for 30 seconds for a total of 25 cycles. After the single nucleotide extension, one unit of calf intestinal phosphatase was added to the PCR reaction mix, incubated for one hour at 37 C, and heat inactivated at 72 C for 15 minutes. At the UW Biotech Center, one μl of the final product was mixed with 1.2 μl of formamide buffer and 0.3 μl of size standard for analysis on the ABI377. Peaks representing single nucleotide extensions were identified using the GeneScan 3.1 software.

## Results and Discussion

We designed primers [5'-GCCAATTGCTAACTTACTCTT-3' (A272-2-Forward) and 5'-CATATTTTCTCTCATACCAA-3' (A272-5-Reverse)] to amplify the AOB272 genomic region. The forward and reverse primers produced a 1019 bp fragment as expected from 1375 bp from genomic DNA, and unexpected 388 or 376 bp fragments. These genomic fragments were cloned, sequenced, and used to identify polymorphisms among the nine onion populations. The 1019 bp sequence showed no homology to any sequences presently in the databases, indicating that AOB272 is a unique cDNA clone. The 388 bp and 376 bp fragments were identical except for a 12 bp deletion and showed no homology to the AOB272 region. We attempted to map the 388 and 376 bp fragments using RFLPs and PCR; however they were monomorphic in our mapping family. One can avoid amplifying this unrelated fragment by extending the forward and/or reverse primers to 25 bp since we often observed non-specific PCR products when we used primers less than 25 bp in length. We increased the A272-2F primer to 25 bp in length (5'-GCCAATTGCTAACTTACTCTTCAAG-3' =

AC-BYG-F) and used it with the A272-5R primer to specifically amplify AOB272 genomic region.

The genomic sequence amplified at AOB272 is 1375 bp in size and carries two introns (Figure 5). No INDELs, simple sequence repeats, or polymorphic restriction enzymes were present among the nine populations (Figure 5). However, the AOB272 genomic region possesses seven alleles based on 15 SNPs among the nine onion populations. Nine of these SNPs formed two alleles between the 324th and 1342nd nucleotides of the AOB272 genomic region (Figure 5 and Table 5). Because we used cDNAs as probes to develop our genetic map of onion, sequence conservation among elite onion populations would be expected.

### Allele specific primers

Primers were synthesized flanking specific SNPs (Figure 5). All primers amplified both the target and nontarget alleles using annealing temperatures of 45, 50, 55, and 60 C. We often observed weak signals corresponding to the nontarget allele at 60 C for primers pairing with the 02- and 03-reverse primers (Table 6). Only target alleles were amplified for primer pairs AC-09F & 05R, AC-10F & 03R, and AC-10F & 05R, BYG-09F & 05R, BYG-10F & 3R and 5R, BYG-11F & 4R and 5R at annealing temperature of 65 C, and primer pairs BYG-11F & 4R at 72 and 74 C. With 70 C annealing temperatures, AC-BYG-F

& 4R and 5R primers produced target fragments and weak nontarget products. The allele specific primers did not amplify any fragments at annealing temperatures at or above 65 C for primers pairing with either AC- or BYG-1R, 6R and 7R, and at 68 C for primers pairing with 8R.

We used two pairs of primers in one reaction tube to determine if codominant PCR products were amplified, but often one pair failed to produce any product or the other pair produced target and nontarget products (Figure 6). AC43 allele specific primers often produced weak products from BYG15-23 DNA samples, even though they did not produce products from other samples homozygous for the BYG15-23 allele. The most likely explanation is that BYG15-23 possesses the AC43 allele at low frequency. The AC43 and BYG15-23 allele specific primer pairs produced the target products at 74 to 70 C and 72 to 68 C with lower temperatures during earlier cycles (Figure 6). However, amplification was not consistent. We observed that individual allele specific primers consistently produced the target products, but that nontarget products were also produced occasionally at differing PCR cycling conditions. We concluded that SNP detection using flanking primers is not reliable. There will always be some nonselective amplifications and therefore errors when selecting specific genotypes. Flanking primers may successfully distinguish alleles at AOB272 if PCR cycling conditions are adjusted for each primer pair and reactions replicated to reduce the error rate. However, this may not be realistic because of variations in DNA template purity from minipreparations of large numbers of onion plants in a breeding program.

### Modified primers for single nucleotide polymorphisms (MSNPs)

Because we observed that primers possessing single mismatch at the 3' end often produced target and nontarget PCR products, we added one more polymorphic nucleotide to these SNP primers, thereby creating primers with two nucleotide mismatches for nontarget allele. For the AC43 allele specific modified (M) primers were AC-13M-F (5'-GTGAAGGGTTGCCCTGTTTTCAA-3') and AC-08M-R (5'-TCATACCAAACTTGATACTCAAAGATTTT-3'). BYG15-23 allele specific modified (M) primers were BYG-11M-F (5'-CCTGACAGTAGCCCATGGAGC-3') and BYG-04M-R (5'-ATCCAAAGCAGCAGACAGCAGA-3'). These primers possess the same nucleotide as the target genomic region at 3' minus one position. The MSNP primers amplified both target and nontarget alleles from AOB272 genomic region at 50 C (Figure 7). As annealing temperatures increased to 60 C nontarget products disappeared. AC43 allelic MSNP primer pairs produced weak products from BYG15-23 template DNA as observed previously (Figures 6 and 7), but never any products from plant homozygous for the BYG15-23 allele and recessive for *Ms* locus at annealing temperatures from 50 to 62 C. At annealing temperatures 64 C and above, AC43 specific modified primers did not produce any products. On the other hand, BYG15-23 specific MSNP primers amplified only from target alleles at 50, 52, 54 and 56 C. However, they produced weak AC43 products at 58, 60 and 62 C. Above 62 C, non specific amplifications were not observed and BYG MSNPs produced only target PCR products. Therefore, these MSNP primers are promising to amplify only target alleles at lower annealing temperatures. However at low annealing temperatures, MSNP primers occasionally produced products not homologous to AOB272. These can be eliminated by preamplifiying the AOB272 genomic region with cDNA specific primers, then these products can be used as template DNA for the MSNP primers.

### Single nucleotide extensions

Five SNPs were used to distinguish the AC43 and BYG15-23 alleles by single nucleotide extensions. We amplified the AOB272 genomic region and used these PCR products as templates for single nucleotide extensions. Oligonucleotide primers flanking SNPs were synthesized (Tables 5, 6 and 7). Dideoxynucleotides (ddNTP) carrying different flourescent tags (ABI SNaPshot kit) were mixed with primers flanking the SNPs and allowed to anneal to the amplified genomic fragments. A specific flourescent ddNTP was added to the primers, corresponding to the nucleotide at the specific SNP in the target DNA fragment. Specific extension products were well resolved on the ABI377;

Figure 8 shows the distinction of the SNP at the 1158th nucleotide position (Figure 5 and Tables 5 and 6) using primer AC-BYG-13s-F (5'-CAGTGAAGGGTTGCCCTGTTTTCT-3'). The single nucleotide extensions possess either A (green= ddATP) or G (blue= ddGTP) corresponding to the two alleles at position 1558 (Tables 5 and 6). This method is very sensitive to reveal specific alleles.

## Summary

Primers flanking individual SNPs in the AOB272 genomic region always amplified target fragments under optimal conditions. However, nontarget fragments were occasionally amplified causing incorrect classifications of genotypes (Figure 6). Modified primers flanking SNPs (i.e. two nucleotide different at 3' end for nontarget alleles) correctly amplified target DNA at lower annealing temperatures, but also occasionally amplified unrelated sequences (Figure 7). These unrelated amplification products can be eliminated by preamplification of the AOB272 genomic region and using this amplified DNA in the diagnostic PCR reaction. Although single nucleotide extensions worked the best to resolve alleles at AOB272 (Figure 8), sample runs on the ABI377 or ABI3700 quickly become cost prohibitive.

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Figure 5. Sequences for the onion cDNA AOB272 and genomic (g) DNA from nine commercial onion cultivars.

| Source[a] | 10 | 20 | 30 | 40 | 50 | 60 | 70 |
|---|---|---|---|---|---|---|---|
| cDNA | CAGTACCTCA | ATGGTGGTTC | CTAATTCTAC | TAGGTGGGAG | CGTTATTTTA | TCATTGATGC | TATCATTTGT |
| AC43 | | | | | | | |
| CN1 | | | | | | | |
| CN2a | | | | | | | |
| CN6 | | | | | | | |
| BYG | | | | | | | |
| CN2a | | | | | | | |
| CN8 | | | | | | | |
| PLK | | | | | | | |
| SKIa | | | | | | | |
| SKIb | | | | | | | |

Figure 5. Continued.

| Source[a] | 80 | 90 | 100 | 110 | 120 | 130 | 140 |
|---|---|---|---|---|---|---|---|
| cDNA | ATGGAAAGAG | CAAGTTCAAC | TTCCATGGTG | GGGTATGCTT | TTCGCATTTG | CATTGGCTTT | TGTTGTAACA |
| AC43 | | | | | TTCGCATTTG | CATTGGCTTT | TGTTGTAACA |
| CN1 | | | | | | | |
| CN2a | | | | | | | |
| CN6 | | | | | | | |
| BYG | | | | | ^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | | | | | | | |
| CN8 | | | | | | | |
| PLK | | | | | | | |
| SKIa | | | | | | | |
| SKIb | | | | | | | |

Figure 5. Continued.

| <u>Source</u>[a] | <u>150</u> | <u>160</u> | <u>170</u> | <u>180</u> | <u>190</u> | <u>200</u> | <u>210</u> |
|---|---|---|---|---|---|---|---|
| cDNA | CTCCCTATTG | GTGTTATTCA | AGCTACAACT | AATiiiiiii[b] | iiiiiiiiii | iiiiiiiiii | iiiiiiiiii |
|  |  |  |  |  | ATGTATACA |  |  |
| AC43 | CTCCCTATTG | GTGTTATTCA | AGCTACAACT | AATCAGGTAC | T |  TACCATATTG | CTATTTCAAA |
| CN1 |  |  |  |  |  |  |  |
| CN2a |  |  |  |  |  |  |  |
| CN6 |  |  |  |  |  |  |  |
| BYG | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a |  |  |  |  |  |  |  |
| CN8 |  |  |  |  |  |  |  |
| PLK |  |  |  |  |  |  |  |
| <u>SKIa</u> |  |  |  |  |  |  |  |
| SKIb |  |  |  |  |  |  |  |

Figure 5. Continued.

| Source[a] | 220 | 230 | 240 | 250 | 260 | 270 | 280 |
|---|---|---|---|---|---|---|---|
| cDNA | iiiiiiiiii | iiiiiiiiii | iiiiiiiiii | iiiiiiiiii | iiiiiiiiii | iiiiCAGCAA | CCTGGGTATG |
| AC43 | ATTTTTGTAA | TATCACTAGT | GCACTAAATC | TTTTATATTT | TGTTGATCAT | CTAACAGCAA | CCTGGGTATG |
| CN1 | | | | | | | |
| CN2a | | | | | | | |
| CN6 | | | | | | | |
| BYG | ^AA^^^^^^^ | ^^^^^^^^A^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | | | | | | | |
| CN8 | | | | | | | |
| PLK | | | | | | | |
| SKIa | | | | | | | |
| SKIb | | | | | | | |

Figure 5. Continued.

| Source[a] | 290 | 300 | 310 | 320 | 330 | 340 | 350 |
|---|---|---|---|---|---|---|---|
| cDNA | ATATCATTGC | TCAATTCATG | ATCGGTTATG | TACTTCCTGG | AAAGCCAATT | GCTAACTTAC | TCTTCAAGAT |
| AC43 | ATATCATTGC | TCAATTCATG | ATCGGTTATG | TACTTCCTGG | AAAGCCAATT | GCTAACTTAC | TCTTCAAGAT |
| CN1 | | | | | ^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | | | | | ^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN6 | | | | | ^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| BYG | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | | | | | ^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN8 | | | | | ^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| PLK | | | | | ^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIa | | | | | ^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIb | | | | | ^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |

Figure 5. Continued.

| Source[a] | 360 | 370 | 380 | 390 | 400 | 410 | 420 |
|---|---|---|---|---|---|---|---|
| cDNA | ATACGGCCGT | ATCAGCACTG | TGCATGCACT | GTCTTTCCTT | TCTGATCTTA | AATTAGGCCA | TTACATGAAG |
| AC43 | ATACGGCCGT | ATCAGCACTG | TGCATGCACT | GTCTTTCCTT | TCTGATCTTA | AATTAGGCCA | TTACATGAAG |
| CN1 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN6 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| BYG | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN8 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| PLK | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIa | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIb | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |

Figure 5. Continued.

| Source[a] | 430 | 440 | 450 | 460 | 470 | 480 | 490 |
|---|---|---|---|---|---|---|---|
| cDNA | ATTCCACCAA | GGGCCATGTA | CACTGCTCAG | iiiiiiiiii | iiiiiiiiii | iiiiiiiiii | iiiiiiiiii |
| AC43 | ATTCCACCAA | GGGCCATGTA | CACTGCTCAG | GTGACCACTT | CAGACTCTTT | TACTTTAGCA | GCAACTCCCA |
| CN1 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN6 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| BYG | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN8 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| PLK | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIa | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIb | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |

Figure 5. Continued.

| Source[a] | 500 | 510 | 520 | 530 | 540 | 550 | 560 |
|---|---|---|---|---|---|---|---|
| cDNA | iiiiiiiiii | iiiiiiiiii | iiiiiiiiii | iiiiiiiiii | iiiiiiiiii | iiiiiiiCTA | GTGGGAACTG |
| AC43 | TTTTACCTAT | AGAAAAAATT | GCTAACATTA | TCATTGTATG | GTATTTAACT | GTTGCAGCTT | GTGGGAACTG |
| CN1 | ^^^^^^^T^^ | ^^A^^^^^^^ | ^T^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^T | ^^^^^^^^^^ |
| CN2a | ^^^^^^^T^^ | ^^A^^^^^^^ | ^G^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^T | ^^^^^^^^^^ |
| CN6 | ^^^^^^^T^^ | ^^A^^^^^^^ | ^T^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^T | ^^^^^^^^^^ |
| BYG | ^^^^^^^A^^ | ^^G^^^^^^^ | ^T^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^A | ^^^^^^^^^^ |
| CN2a | ^^^^^^^A^^ | ^^G^^^^^^^ | ^G^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^A | ^^^^^^^^^^ |
| CN8 | ^^^^^^^A^^ | ^^G^^^^^^^ | ^C^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^A | ^^^^^^^^^^ |
| PLK | ^^^^^^^A^^ | ^^G^^^^^^^ | ^G^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^A | ^^^^^^^^^^ |
| SKIa | ^^^^^^^A^^ | ^^G^^^^^^^ | ^G^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^A | ^^^^^^^^^^ |
| SKIb | ^^^^^^^A^^ | ^^G^^^^^^^ | ^G^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^A | ^^^^^^^^^^ |

Figure 5. Continued.

| Source[a] | 570 | 580 | 590 | 600 | 610 | 620 | 630 |
|---|---|---|---|---|---|---|---|
| cDNA | TAGTAGCAGG | AATACTGAAC | CTTGGAGTAG | CATGGTGGAT | GTTGGAAAAC | ATAGAAAACA | TTTGTGACGA |
| AC43 | TAGTAGCAGG | AATACTGAAC | CTTGGAGTAG | CATGGTGGAT | GTTGGAAAAC | ATAGAAAACA | TTTGTGACGA |
| CN1 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN6 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| BYG | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN8 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| PLK | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIa | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIb | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |

Figure 5. Continued.

| Source[a] | 640 | 650 | 660 | 670 | 680 | 690 | 700 |
|---|---|---|---|---|---|---|---|
| cDNA | TTCTCTCCCT | GACAGTAGCC | CATGGACCTG | TCCAAAGTAC | AGGGTCACAT | TTGACGCATC | TGTAATCTGG |
| AC43 | TTCTCTCCCT | GACAGTAGCC | CATGGACATG | TCCAAAGTAC | AGGGTCACAT | TTGACGCATC | TGTAATCTGG |
| CN1 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^A^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^A^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN6 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^A^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| BYG | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^C^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^C^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN8 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^C^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| PLK | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^C^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIa | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^C^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIb | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^C^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |

Figure 5. Continued.

| Source[a] | 710 | 720 | 730 | 740 | 750 | 760 | 770 |
|---|---|---|---|---|---|---|---|
| cDNA | GGTCTGATTG | GGCCAAGTCG | GCTGTTCGGG | CATGGAGGGC | TGTACCGTAA | TCTCACCTGG | CTTTTCTTGG |
| AC43 | GGTCTGATTG | GGCCAAGTCG | GCTGTTCGGG | CATGGAGGGC | TGTACCGTAA | TCTCACCTGG | CTTTTCTTGG |
| CN1 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN6 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| BYG | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN8 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| PLK | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIa | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIb | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |

Figure 5. Continued.

| Source[a] | 780 | 790 | 800 | 810 | 820 | 830 | 840 |
|---|---|---|---|---|---|---|---|
| cDNA | TTGGTGCAGT | GCTGCCCGTA | CCCATTTGGG | TGCTGAGCAA | AGTATACCCG | GACAAGAAAT | GGATCCCACT |
| AC43 | TTGGTGCAGT | GCTGCCCGTA | CCCATTTGGG | TGCTGAGCAA | AGTATACCCG | GACAAGAAAT | GGATCCCACT |
| CN1 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN6 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| BYG | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN8 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| PLK | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIa | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIb | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |

Figure 5. Continued.

| Source[a] | 850 | 860 | 870 | 880 | 890 | 900 | 910 |
|---|---|---|---|---|---|---|---|
| cDNA | GATAAACATA | CCCGTTATCT | CTTATGGATT | TGCGGGCATG | CCACCCGCCA | CACCGACTAA | CATAGCGAGT |
| AC43 | GATAAACATA | CCCGTTATCT | CTTATGGATT | TGCGGGCATG | CCACCCGCCA | CACCGACTAA | CATAGCGAGT |
| CN1 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN6 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| BYG | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN8 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| PLK | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIa | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIb | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |

Figure 5. Continued.

| Source[a] | 920 | 930 | 940 | 950 | 960 | 970 | 980 |
|---|---|---|---|---|---|---|---|
| cDNA | TGGCTGGTGA | CTGGGACGAT | ATTCAACTAC | TTTGTCTTCA | GGTACAGGAA | GGATTGGTGG | AAGAAGTATA |
| AC43 | TGGCTGGTGA | CTGGGACGAT | ATTCAACTAC | TTTGTCTTCA | GGTACAGGAA | GGATTGGTGG | AAGAAGTATA |
| CN1 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^T^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^T^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN6 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^T^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| BYG | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^T^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^T^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN8 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^C^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| PLK | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^T^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIa | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^T^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIb | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^T^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |

Figure 5.  Continued.

| Source[a] | 990 | 1000 | 1010 | 1020 | 1030 | 1040 | 1050 |
|---|---|---|---|---|---|---|---|
| cDNA | ATTATGTGCT | GTCTGCTGCT | TTGGATGCGG | GTACGGCGTT | TATGGGAGTG | TTGCTGTTCC | TTGCTTTGCA |
| AC43 | ATTACGTGCT | GTCTGCTGCT | TTGGATGCGG | GTACGGCGTT | TATGGGAGTG | TTGCTGTTCC | TTGCTTTGCA |
| CN1 | ^^^^C^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^C^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN6 | ^^^^C^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| BYG | ^^^^T^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^T^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN8 | ^^^^T^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| PLK | ^^^^T^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIa | ^^^^T^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIb | ^^^^T^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |

Figure 5. Continued.

| Source[a] | 1060 | 1070 | 1080 | 1090 | 1100 | 1110 | 1120 |
|---|---|---|---|---|---|---|---|
| cDNA | GAATAATAAC | ATTAACCTGC | ATTGGTGGGG | CTCGGATGTG | GATCACTGCC | CGTTAGCCAA | ATGCCCGACT |
| AC43 | GAATAATAAC | ATTAACCTGC | ATTGGTGGGG | CTCGGATGTG | GATCACTGCC | CGTTAGCCAA | ATGCCCGACT |
| CN1 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN6 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| BYG | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN8 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| PLK | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIa | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIb | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |

Figure 5. Continued.

| Source[a] | 1130 | 1140 | 1150 | 1160 | 1170 | 1180 | 1190 |
|---|---|---|---|---|---|---|---|
| cDNA | TGGCCTGGAA | TTGCAGTGAA | GGGTTGCCCT | GTTTTCTGAA | TGCAGACTTT | AGTTGCAGGG | GCTCCCTTAA |
| AC43 | TGGCCTGGAA | TTGCAGTGAA | GGGTTGCCCT | GTTTTCTAAA | TGCAGACTTT | AGTTGCAGGG | GCTCCCTTAA |
| CN1 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^A^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^A^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN6 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^A^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| BYG | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^G^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^G^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN8 | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^G^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| PLK | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^G^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIa | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^G^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIb | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^G^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |

Figure 5.  Continued.

| Source[a] | 1200 | 1210 | 1220 | 1230 | 1240 | 1250 | 1260 |
|---|---|---|---|---|---|---|---|
| cDNA | GTGTTCAAGT | ATTTATATTG | ATGTTATTTC | TCGCTTTTTT | GAACAATTTA | GTAGAGAATG | GATTTAGATT |
| AC43 | ATGTTCAAGT | ATTTATATCG | ATGTTATTTC | TCGCTTTTTT | GAACAATTTA | GTAGAGAATG | GATTTCGATT |
| CN1 | A^^^^^^^^^ | ^^^^^^^^T^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^A^^^^ |
| CN2a | A^^^^^^^^^ | ^^^^^^^^T^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^A^^^^ |
| CN6 | A^^^^^^^^^ | ^^^^^^^^T^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^A^^^^ |
| BYG | G^^^^^^^^^ | ^^^^^^^^T^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^A^^^^ |
| CN2a | G^^^^^^^^^ | ^^^^^^^^T^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^A^^^^ |
| CN8 | G^^^^^^^^^ | ^^^^^^^^T^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^A^^^^ |
| PLK | G^^^^^^^^^ | ^^^^^^^^T^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^A^^^^ |
| SKIa | G^^^^^^^^^ | ^^^^^^^^T^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^A^^^^ |
| SKIb | G^^^^^^^^^ | ^^^^^^^^T^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ | ^^^^^A^^^^ |

Figure 5. Continued.

| Source[a] | 1270 | 1280 | 1290 | 1300 | 1310 | 1320 | 1330 |
|---|---|---|---|---|---|---|---|
| cDNA | AGGCTTTATG | AGAAACGAAT | ATATTACATG | ATGCGTTGTA | ATGTAATCTT | TGAGTATCAA | GTTTGGTATG |
| AC43 | AGGCTTTATG | AGAAACGAAT | ATATTACATG | ATGCGGTGTA | ATATAATCTT | TGAGTATCAA | GTTTGGTATG |
| CN1 | ^^^^^T^^^^ | ^^^^^^^^A^ | ^^^^^^^^^^ | ^^^^^G^^^^ | ^^A^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^^T^^^^ | ^^^^^^^^A^ | ^^^^^^^^^^ | ^^^^^G^^^^ | ^^A^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN6 | ^^^^^T^^^^ | ^^^^^^^^A^ | ^^^^^^^^^^ | ^^^^^G^^^^ | ^^A^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| BYG | ^^^^^C^^^^ | ^^^^^^^^A^ | ^^^^^^^^^^ | ^^^^^T^^^^ | ^^G^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN2a | ^^^^^T^^^^ | ^^^^^^^^A^ | ^^^^^^^^^^ | ^^^^^T^^^^ | ^^G^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| CN8 | ^^^^^T^^^^ | ^^^^^^^^A^ | ^^^^^^^^^^ | ^^^^^T^^^^ | ^^G^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| PLK | ^^^^^T^^^^ | ^^^^^^^^A^ | ^^^^^^^^^^ | ^^^^^T^^^^ | ^^G^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIa | ^^^^^T^^^^ | ^^^^^^^^A^ | ^^^^^^^^^^ | ^^^^^T^^^^ | ^^G^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |
| SKIb | ^^^^^T^^^^ | ^^^^^^^^C^ | ^^^^^^^^^^ | ^^^^^T^^^^ | ^^G^^^^^^^ | ^^^^^^^^^^ | ^^^^^^^^^^ |

Figure 5.  Continued.

| Source[a] | 1340 | 1350 | 1360 | 1370 | 1375 |
|---|---|---|---|---|---|
| cDNA | AGAGAAAATA | TGCTGAACAA | ATTACTTAAC | GGAATGTGTT | TTCGG |
| AC43 | AGAGAAAATA | TG | | | |
| CN1 | ^^^^^^^^^^ | ^^ | | | |
| CN2a | ^^^^^^^^^^ | ^^ | | | |
| CN6 | ^^^^^^^^^^ | ^^ | | | |
| BYG | ^^^^^^^^^^ | ^^ | | | |
| CN2a | ^^^^^^^^^^ | ^^ | | | |
| CN8 | ^^^^^^^^^^ | ^^ | | | |
| PLK | ^^^^^^^^^^ | ^^ | | | |
| SKIa | ^^^^^^^^^^ | ^^ | | | |
| SKIb | ^^^^^^^^^^ | ^^ | | | |

Figure 5. Continued.

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[a] AOB272 cDNA and corresponding genomic DNA sequences for populations listed in Table 4. CN2a and CN2b are two alleles in population of CN2 at AOB272 genomic region. Number of nucleotides from 1 to 1375 is for cDNA (including introns = i) and from 324 to 1342 bp is for nine onion populations (1019 bp).

[b] i= Sites of introns in the genomic DNA sequence.

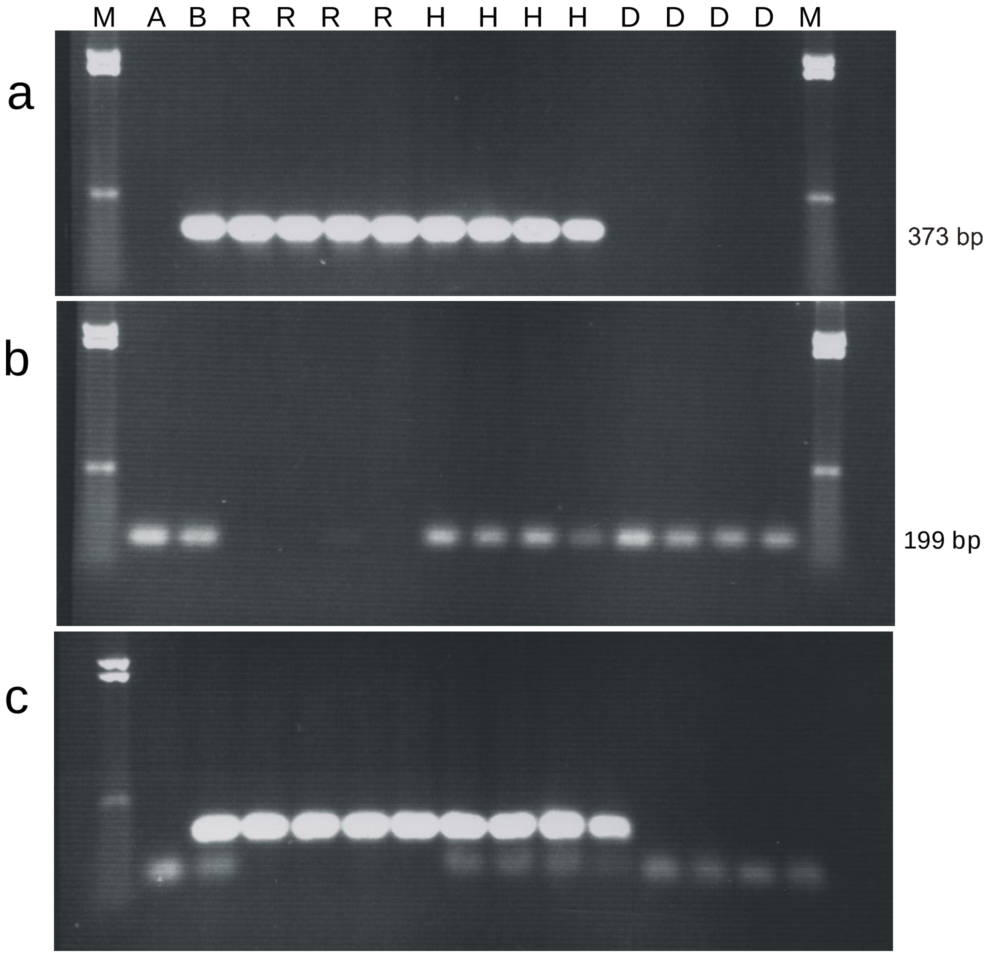

Figure 6. AC43 (A) and BYG15-23 (B) allele specific products amplified from four homozygous recessive (R), four heterozygous (H), and four homozygous dominant (D) genotypes at *Ms* locus from the onion mapping population. Fragment sizes in base pairs are shown on the right. a= BYG15-23 allele specific fragments amplified by primers BYG-11F versus BYG-04R at annealing 74 to 72 C during early cycles, then 72 C. b= AC43 allele specific primers AC-13-F vs AC-08ex-R with annealing temperatures from 72 to 68 during early

cycles, then at 68 C. c= Products from a and b ran on same gel after PCR amplification at differing annealing temperatures.

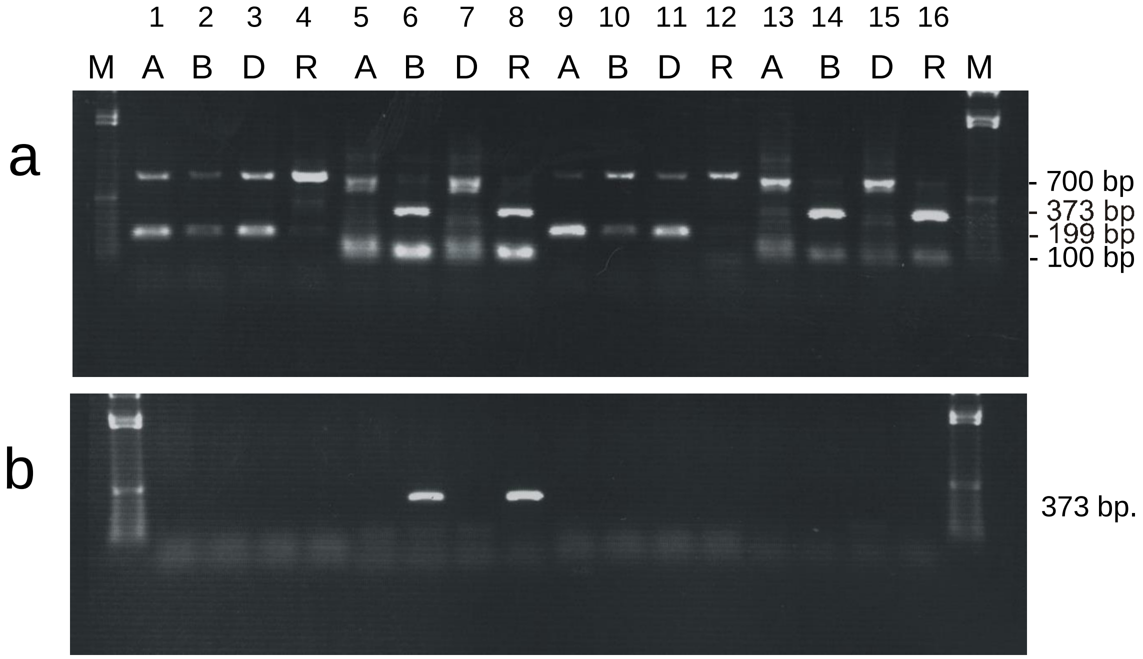

Figure 7. Allele specific fragments of AC43 (A), BYG15-23 (B), homozygous dominant (D) and recessive (R) genotypes at *Ms* from mapping population. The AC43 allele at AOB272 is in coupling phase with the dominant (*Ms*) allele; the BYG15-23 allele at AOB272 is in coupling phase with the recessive (*ms*) allele. Allele specific fragments were amplififed by modified single nucleotide polymorphic (MSNP) primers AC-13M-F & 08M-R and BYG-11M-F & 04M-R. Fragment sizes in basepairs are shown on right. a= 1-4 and 9-12 are AC43 specific primer product, 5-8 and 13-16 are BYG13-23 allele specific products. 1-8 are at annealing temperatures 54 C, 9-16 are at 56 C. b= Same order as in the a except that annealing temperatures were 64 C for samples 1-8 and 66 C for 9-16.

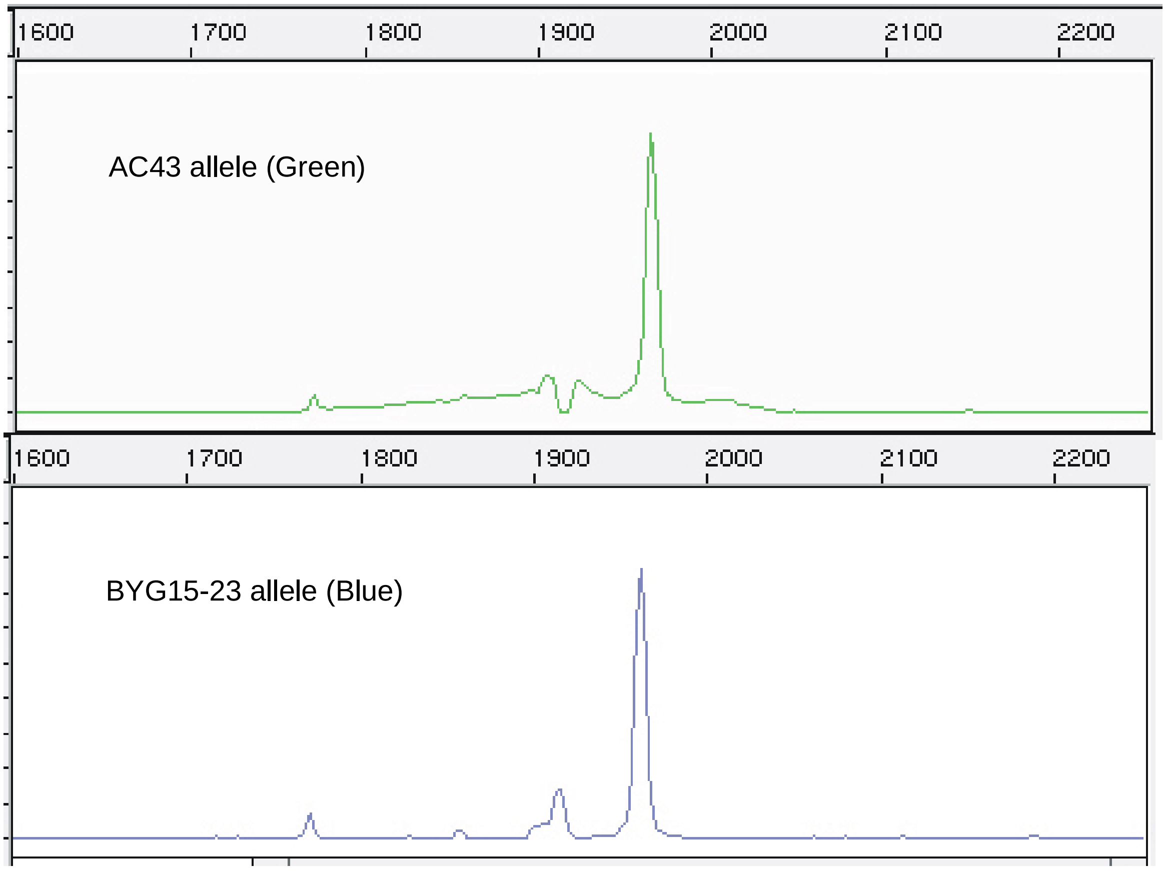

Figure 8. Chromagraphs of single nucleotide extensions for AC43 (Green) and BYG15-23 (Blue) alleles. The figure shows peaks for dideoxynucleotides carrying different flourescent tags. Peaks represent ddATP (upper green), and ddGTP (lower blue) with AC-BYG-13s-F primer flanking the SNP at the 1158th nucleotide (Table 5).

Table 4. Origins, cytoplasms, and genotypes at the *Ms* locus of onion populations used to develop allele specific markers at AOB272.

| No. | Population[a] | Origin | Source[b] | Cytoplasm and Genotype[c] |
|---|---|---|---|---|
| 1 | Ac43 | USDA | 417-2 | N-MSms |
| 2 | BYG15-23 | USDA | 125-1 | N-msms |
| 3 | SKIa | USDA | 16588 | N-MSMS |
| 4 | SKIb | USDA | 16664 | N-msms |
| 5 | CN1 | Asgrow Seed Corp. | 1604B | N-msms |
| 6 | CN2 | Asgrow Seed Corp. | 1607B | N-msms |
| 7 | CN6 | Asgrow Seed Corp. | 1673 | S-MSMS |
| 8 | CN8 | Asgrow Seed Corp. | 1616 | S-MSMS |
| 9 | PLK | May-Ryan Seed Comp. | NA | S-MSMS |

[a] AC43 and BYG15-23 are parents of mapping family (King et al. 1998), SKIa & b are $S_1$ families from Sapporo-ki, and CN1-8 are short-day onion inbreds from Asgrow.

[b] NA= not available.

[c] Cytoplasms are normal (N) male-fertile or male-sterile (S). Genotypes at *Ms* are homozygous dominant (*MSMS*), heterozygous (*MSms*), or homozygous recessive (*msms*).

Table 5. Summary of single nucleotide polymorphisms (SNPs) in the AOB272 genomic region for nine onion populations.

| | Location in AOB272 genomic sequence | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Population[a] | Ms[b] | 498 | 503 | 512 | 550 | 658 | 933 | 985 | 1158 | 1191 | 1209 | 1256 | 1266 | 1279 | 1296 | 303 |
| AC43 | H | T | A | C | T | A | T | C | A | A | C | C | T | A | G | A |
| CN1 | R | T | A | T | T | A | T | C | A | A | T | A | T | A | G | A |
| CN2a | R | T | A | G | T | A | T | C | A | A | T | A | T | A | G | A |
| CN6 | D | T | A | T | T | A | T | C | A | A | T | A | T | A | G | A |
| BYG15-23 | R | A | G | T | A | C | T | T | G | G | T | A | C | A | T | G |
| CN2b | R | A | G | G | A | C | T | T | G | G | T | A | T | A | T | G |
| CN8 | D | A | G | C | A | C | C | T | G | G | T | A | T | A | T | G |
| PLK | D | A | G | G | A | C | T | T | G | G | T | A | T | A | T | G |
| SKIa | D | A | G | G | A | C | T | T | G | G | T | A | T | A | T | G |
| SKIb | R | A | G | G | A | C | T | T | G | G | T | A | T | C | T | G |

[a] Populations are listed in Table 4.  CN2a and CN2b are two alleles in population CN2 at the AOB272 genomic region.

[b] Genotypes at *Ms* locus are homozygous dominant (D), heterozygous (H), or homozygous recessive (R).