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Identification of cytoplasms using the polymerase chain reaction to aid in the extraction of maintainer lines from open-pollinated populations of onion

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Abstract S-cytoplasm is the most common source of cytoplasmic-genic male sterility (CMS) used to produce hybrid-onion seed. Identification of the cytoplasm of a single plant takes from 4 to 8 years and is complicated by the segregation of a nuclear gene that restores fertility. Although CMS in onion may be due to an incompatibility between the mitochondrial and nuclear genomes, Southern analyses of DNA from individual plants from crosses of S- and N-cytoplasmic plants supported maternal inheritance of the chloroplast and mitochondrial DNA and, therefore, polymorphisms in the chloroplast DNA may be used to classify cytoplasms. Amplification by the polymerase chain reaction of a fragment that carries an autapomorphic 100-bp insertion in the chloroplast DNA of N-cytoplasm offers a significantly quicker and cheaper alternative to crossing or Southern analysis. Molecular characterization of N- and S-cytoplasms and frequencies of the nuclear non-restoring allele allow onion breeders to determine the proportion of plants in open-pollinated populations that maintain CMS and can significantly reduce the investment required to identify individual maintainer plants.

Key words *Allium cepa* · *Allium* × *proliferum*
Chloroplast DNA · Cytoplasmic male sterility
Mitochondrial DNA · Organellar transmission

Introduction

Cytoplasmic-genic male sterility (CMS) systems are used to produce hybrid-onion seed. Two different CMS systems have been described in onion, termed S- and T-cytoplasms (Jones and Emsweller 1936; Berninger 1965). S-cytoplasm was discovered in the cultivar 'Italian Red' and is conditioned by the interaction of the cytoplasm with a single nuclear gene; fertility is restored by a dominant (*Ms*) allele (Jones and Clarke 1943). Male-sterile plants possess S-cytoplasm and are homozygous recessive at the restorer locus (S *msms*). T-cytoplasm was discovered in the French cultivar 'Jaune paille des Vertus', and restoration of fertility is conditioned by three independently segregating loci (Schweisguth 1973).

Restriction-enzyme analyses of the chloroplast (cpDNA) and mitochondrial (mtDNA) DNAs of normal (N) fertile, S-, and T-cytoplasms have demonstrated fragment size differences (de Courcel et al. 1989; Holford et al. 1991; Havey 1993; Satoh et al. 1993). De Courcel et al. (1989) delineated four cytoplasmic groups (M_1 through M_4) based on *Bam*HI digests of the mtDNA. Restriction-enzyme digests of both the cpDNA and mtDNA distinguished S-cytoplasm from the M-cytoplasmic groups (de Courcel et al. 1989). Holford et al. (1991) were able to distinguish among N-, S-, and T-cytoplasms with *Bam*HI and *Hin*dIII digests of mtDNA and among N- and S-cytoplasms with *Eco*RI, *Hin*dIII, and *Xba*I digests of cpDNA. Havey (1993) digested genomic DNA with 15 restriction enzymes, probed with a complete set of chloroplast clones, and identified five polymorphisms between N- and S-cytoplasms. The cultivar 'Pran', a triploid viviparous onion (*Allium* × *proliferum* [Moench] Schrad.), was proposed as the putative donor of S-cytoplasm (Havey 1993).

Onion is a biennial and 4 to 8 years are required to establish cytoplasms by crossing. One can verify that a fertile plant is N *msms*, N *Msms*, or S *Msms* by testcrossing to a sterile plant (S *msms*) and separately scoring the fertility of progeny from the fertile and sterile parents. The same procedure can be used to determine if a fertile plant is N *MsMs* or S *MsMs* except that reciprocal crosses with a maintainer (N *msms*) are required. Characterization of polymorphisms in the cpDNA and mtDNA that distinguish N- and S-cytoplasms (de Courcel et al. 1989; Holford et al. 1991; Havey 1993; Satoh et al. 1993) are significantly faster than crossing, but it is still time-consuming to complete DNA isolations from single plants, restriction-enzyme digestions, blotting, and hybridizations. The poly-

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merase chain reaction (PCR) would allow a quick and confident identification of the cytoplasm of individual plants. In this report, I present molecular evidence for maternal inheritance of the chloroplast and mitochondrial genomes in onion, describe a PCR-detectable polymorphism in the cpDNA that distinguishes N- and S-cytoplasms, and summarize how molecular identification of the cytoplasm can aid in the extraction of maintainer lines from open-pollinated populations of onion.

Materials and methods

De Courcel et al. (1989), Holford et al. (1991), and Satoh et al. (1993) reported polymorphisms in the mtDNA that distinguish N- and S-cytoplasms. I evaluated for these and new polymorphisms with *Bam*HI, *Bgl*II, *Eco*RI, *Eco*RV, *Hin*dIII, *Sma*I, *Xba*I, and *Xho*I and with mitochondrial clones of apocytochrome b [*cob* (Dawson et al. 1984)], cytochrome c oxidase subunits I and II [*cox1* and *2* (Isaac et al. 1985; Fox and Leaver 1981)], *atp6* and *atp9* (Dewey et al. 1985) of maize, and cytochrome c oxidase subunit III (*cox3*) of *Oenothera* (Hiesel et al. 1987). Clones of *cob*, *cox1*, and *cox2* were the gift of C. Leaver, Oxford University, UK, and *cox3* was the gift of A. Brennicke, Institut für Genbiologische, Berlin, Germany; the clones were kindly provided by C. Newton, University of Missouri - Columbia. Clones of *atp6* and *atp9* were the gift of D. Pring, USDA/ARS, University of Florida. Plasmids were purified through CsCl gradients, and the inserts were removed by the appropriate restriction enzyme(s), electrophoresed through 0.7% low-melting-point agarose gels in 1 × TAE (Sambrook et al. 1989), cleanly cut out of the gel, and labeled by random-hexamer priming (Feinberg and Vogelstein 1983).

Inbreds YB986A and B3350A were used as sources of S-cytoplasm, B3350B as a source of N-cytoplasm, and RJ70A and RJ70B as a source of T-cytoplasm and its maintainer, respectively. Open-pollinated (OP) populations 'Downing Yellow Globe' (USA), 'Early Yellow Globe' (USA), 'Rijnsburger' (Holland), 'Strigonowskij' (Russia), 'Stuttgarter Reisen' (Germany), 'Senshu-ki' (Japan), 'Sweet Spanish Colorado #6' (USA), 'Italian Red' (USA), and 'Texas Early Grano 502' (USA), were previously shown to be in N-cytoplasm based on the cpDNA (Havey 1993), were assumed to represent the diversity present among onions in N-cytoplasm, and were included to assess the variability for the above mitochondrial-clone-enzyme combinations. *Allium* × *proliferum* cultivar 'Pran' was included to compare mitochondrial polymorphisms with S-cytoplasm. The origins of these accessions and procedures for isolation of DNA, restriction-enzyme digestions, blotting, hybridizations, and autoradiography have been described (Havey 1991, 1993).

The sterile F_1 line B3350A × B2352B was caged with the inbred B2215C known to carry the dominant fertility-restoring (*Ms*) allele (Jones 1953), pollinated by bees, and seed was harvested separately from the sterile and fertile lines. Plants were grown, vernalized, flowered, and scored for male fertility. From seed harvested off of the sterile line, fertile plants must be hybrids [(B3350A × B2352B) × B2215C = S *Msms*]. To establish the transmission of the cpDNA and mtDNA in onion, CsCl-banded DNAs were isolated from the scapes of 11 individual fertile hybrid plants and seedlings of B3350A × B2352B and B2215C and evaluated for polymorphisms in the cpDNA [characters 1 and 4 (Havey 1993)] and mtDNA (*cox2*/*Bgl*II) that distinguish N- and S-cytoplasms.

I previously reported that N-cytoplasm possesses an autapomorphic 100-bp insertion in the cpDNA [character 42 (Havey 1993)]. Oligonucleotides A (5′-CATTACAAATGCGATGCTCT-3′) and B (5′-TCTACCGATTTCGCCATATC-3′) which flank non-coding regions between tRNAs in the cpDNA (Taberlet et al. 1991), were commercially synthesized and used to amplify cpDNA fragments. Ten ng of genomic DNA of B3350A or B3350B were mixed with 50 m*M* KCl, 10 mM TRIS(HCl) pH 8.3, 2.5 m*M* $MgCl_2$, 0.2 m*M* each of dATP, dCTP, dGTP, and dTTP, 1.0 unit of *Taq* polymerase (Perkin-Elmer, Norwalk, Conn. USA), and 1 μ*M* of each primer in a 25 μl volume. After an initial period at 94°C for 2 min, 30 cycles were completed at 94°C for 1 min, 42°C for 1 min, and 72°C for 2 min. The entire sample was electrophoretically separated through a 1.5% agarose gel in 1×TBE (Sambrook et al. 1989). Amplified fragments were digested with each of 15 restriction enzymes (*Bam*HI, *Bgl*II, *Bst*EII, *Cla*I, *Cfo*I, *Dra*I, *Eco*RI, *Eco*RV, *Hae*III, *Hin*dIII, *Kpn*I, *Mbo*I, *Sst*I, *Xba*I, and *Xho*I) to survey for polymorphic sites. To confirm that amplified fragments showing a 100-bp difference were the same as character 42 (Havey 1993), Southern hybridizations with orchid clone 17 (Chase and Palmer 1989) were completed. To determine the concentrations at which PCR detected the presence of both cytoplasms in a DNA mixture, CsCl-purified DNAs from B3350A and B3350B were separately prepared (10 ng of either DNA), mixed in equal amounts (5 ng of each DNA), blended as ten-fold dilutions ($1:10^{-1}$, $1:10^{-2}$, $1:10^{-3}$, $1:10^{-4}$, $1:10^{-5}$, $1:10^{-6}$, respectively), and subjected to PCR as described above.

The frequencies of N-cytoplasm in the OP populations 'Brigham Yellow Globe' (BYG), 'Mountain Danvers' (MD), 'Sapporo-Ki' (SK), and 'Texas Grano 1015Y' (TG1015Y) have been previously reported (Havey 1993; Havey and Bark 1994). For the inbred B2215C, the frequencies of N- and S-cytoplasms were estimated using two replicates of 50 plants. Seedlings were grown in the greenhouse and DNA extracted from leaf tissue of single plants using a commercial minipreparation (Gentra, Minneapolis, Minn., USA). Ten g of DNA was subjected to PCR and electrophoresis as described above.

To estimate the frequency of the nuclear nonrestoring (*ms*) allele, OP populations BYG, MD, and SK were caged with the sterile F_1 lines B3350A × B2352B, MSU611-1A × MSU611B, and B1731A × B5785B and pollinated by bees. Seed was harvested from the sterile lines. Samples of 86 (BYG) and 69 (MD and SK) bulbs were flowered in 1993 and the frequency of male-sterile plants estimated the frequency of the *ms* allele in the OP population.

Results

Molecular characterization of organellar inheritance

The six mitochondrial clones detected RFLPs between N- and S-cytoplasms for 29 of the 42 probe-enzyme combinations (6 gave poor signal-to-noise ratios); those reported neither by Holford et al. (1991) nor Satoh et al. (1993) were *cox1* with *Eco*RV, *Xho*I, and *Xba*I; *cox2* with *Bgl*II, *Eco*RV, *Sma*I, and *Xba*I; *cox3* with *Bam*HI, *Eco*RV, *Sma*I, and *Xba*I, and *Xho*I; *cob* with *Eco*RI and *Xba*I; *atp6* with *Bgl*II and *Eco*RV; and *atp9* with *Eco*RI, *Eco*RV, and *Xba*I. Holford et al. (1991) reported that *cob*-*Hin*dIII distinguished N-, S-, and T-cytoplasms; however, Satoh et al. (1993) and I observed no RFLPs among these cytoplasms. *Cob*-*Xba*I and *atp9*-*Bam*HI detected polymorphisms between T-cytoplasm (RJ70A) and its maintainer (RJ70B) (autoradiograms not presented). For all other probe-enzyme combinations, fragment sizes were the same for B3350B, RJ70A, RJ70B, 'Downing Yellow Globe', 'Early Yellow Globe', 'Rijnsburger', 'Strigonowskij', 'Stuttgarter Reisen', 'Senshu-ki', 'Sweet Spanish Colorado #6', 'Italian Red', and ''Texas Early Grano 502 (Fig. 1). These OP populations were classified as N-cytoplasmic by polymorphisms in the cpDNA and only possessed mitochondrial fragments of N-cytoplasm. 'Pran' always possessed identically sized fragments as the S-cytoplasm, further supporting my proposal

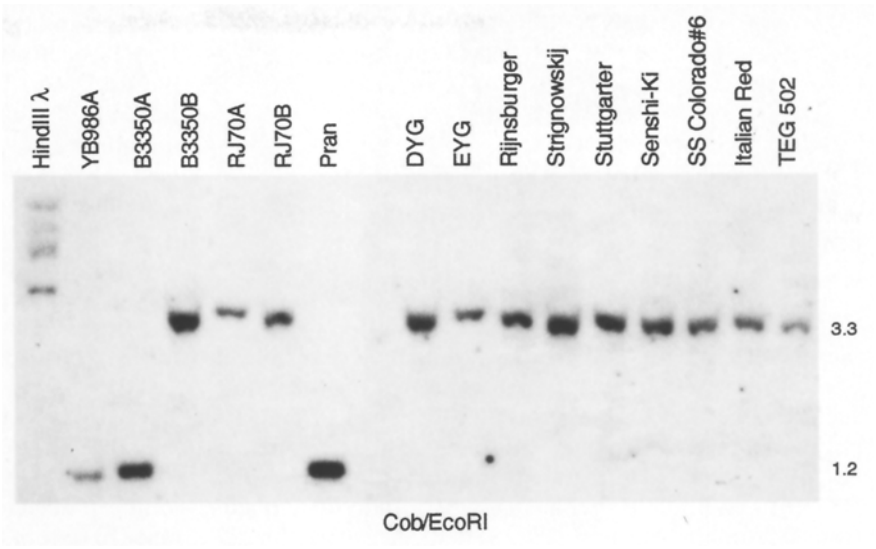

Fig. 1 Hybridization of apocytochrome b clone of maize to *Eco*RI digests of genomic DNA of onion detects a polymorphism between normal fertile (B3350B) and sterile (YB986A and B3350A) cytoplasms. The 1.2-kb S-cytoplasmic band was also observed in 'Pran', the putative donor of S-cytoplasm. T-cytoplasm (RJ70A) and its maintainer (RJ70B) possessed the same band as N-cytoplasm. The 9 other DNAs are from open-pollinated populations of onion previously shown to be N-cytoplasm from restriction-enzyme analysis of the chloroplast genome (Havey 1993)

that this viviparous onion is the donor of S-cytoplasm (Havey 1993).

Restriction fragment length polymorphisms (RFLPs) in the mtDNA and cpDNA established that B3350A×B2352B and B2215C were in S-cytoplasm and a mixture of N- and S-cytoplasms, respectively. The proportion of N-cytoplasm in B2215C was estimated by PCR evaluations of single plants to be 0.79±0.05 (see below). In addition, 35% of the (B3350A × B2352B) × B2215C progenies were sterile, which estimates the frequency of the *ms* allele in B2215C. Jones (1953) reported that B2215C was derived from the self pollination of a single plant and uniformly restored fertility in sterile lines (either N *MsMs* or S *MsMs*). A bulb or seed mixture must have occurred during maintenance of B2215C. We have previously documented the problematic introduction of S-cytoplasm into lines with the *Ms* allele at high frequency (Havey and Bark 1994). Because B2215C has been maintained by random pollination among caged bulbs for at least one generation, I assumed that N- and S-cytoplasmic plants possess the nuclear fertility-restoring allele at equal frequencies.

To date, most descriptions of polymorphisms at or between restriction-enzyme sites in the organellar genomes have used DNA pooled from numerous plants (de Courcel et al. 1989; Holford et al. 1991; Havey 1993). The possibility exists that populations in a mixture of N- and S-cytoplasms possess individual plants with organelles from both cytoplasms. Satoh et al. (1993) isolated DNA from individual plants of 'Sapporo-ki', an OP population possessing both N- and S-cytoplasms (Havey 1993), and presented autoradiograms showing no evidence of mtDNA mixtures. I evaluated for the transfer of cpDNA and mtDNA from the pollen parent to individual fertility-restored S-cytoplasmic plants to verify maternal inheritance. Because B2215C possessed both N- and S-cytoplasm, I had to evaluate at least 3 fertile hybrid plants ([B3350A × B2352B] × B2215C) to be 99% confident that at least 1 had an N-cytoplasmic pollinator (estimated using the binomial distribution as described by Mansur et al. 1990). For all of 11 plants, polymorphisms in the cpDNA and mtDNA were unique to the S-cytoplasm. The autoradiograms were allowed to overexpose and no evidence of occasional paternal transmission of the cpDNA [patterns analogous to autoradiograms presented by Havey (1993) and Havey and Bark (1994)] or mtDNA (Fig. 2) was observed.

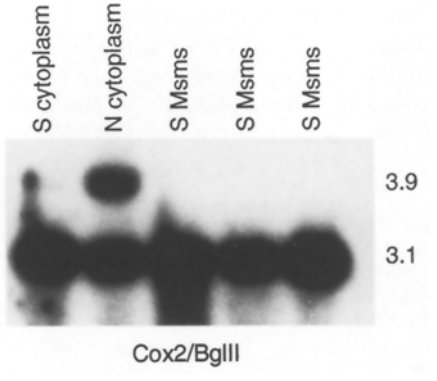

Fig. 2 Hybridization of the *cox2* clone of maize to *Bgl*II digests of genomic DNA of onion detects a polymorphism between normal (N) and sterile (S) cytoplasms. The 3.9-kb band found in N-cytoplasm was not observed in 11 (3 shown here) S-cytoplasmic plants restored to fertility by the nuclear *Ms* allele (S *Msms*), supporting maternal inheritance of the mitochondrial genome

PCR distinction of N- and S-cytoplasms

Because the cpDNA and mtDNA show maternal inheritance, oligonucleotide primers that preferentially amplify the autapomorphic 100-bp insertion in the cpDNA of N-cytoplasm (Havey 1993) can be used to distinguish N- and S-cytoplasms. This region was chosen because the poly-

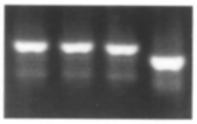

Fig. 3 Differentiation by PCR of normal (1.1 kb) and sterile (1.0 kb) cytoplasms from 4 plants of the onion open-pollinated population 'Mountain Danvers' using minipreparations of DNA and primers A and B as described by Taberlet et al. (1991)

morphism can be scored directly after gel electrophoresis of the PCR reaction and requires no further manipulation, such as digestion with a restriction enzyme, to detect a polymorphic site. Oligonucleotides A and B (Taberlet et al. 1991) amplified fragments from genomic DNA of B3350A and B3350B (gel not shown) or individual plants from 'Mountain Danvers' (Fig. 3) that carry a 100-bp difference (1.0 versus 1.1 kb for S- and N-cytoplasms, respectively). Digestion of the amplified fragments with 15 restriction enzymes revealed the presence of *Bgl*II, *Eco*RI, and *Mbo*I sites; however, none of the sites were polymorphic, as was expected from previous studies (Havey 1993). The amplified fragments hybridized with orchid clone 17, confirming that they were the same as character 42 (autoradiogram not shown). Importantly, PCR analysis of pooled DNA samples did not detect the presence of a cytoplasm if it represented less than 10% of the DNA mixture (gel not shown).

Discussion

Maternal inheritance of cpDNA in onion has been demonstrated by Tatabe (1968) studying transmission of leaf variegation and by Corriveau and Coleman (1988) using epifluorescence microscopy to document the exclusion of plastid DNA from the male gametophyte. Maternal inheritance of the mtDNA must predominate because CMS is maintained across many genetic backgrounds. Jones and Clarke (1943) self-pollinated individual fertile plants in S-cytoplasm (S *Msms*) and observed segregation for sterility, indicating that nuclear restoration of fertility does not result from a mixture of organelles. Molecular evaluations supported maternal inheritance of both organellar genomes in onion; RFLPs unique to the N-cytoplasm were absent in fertility-restored plants from controlled crosses (Fig. 2), and Satoh et al. (1993) observed mtDNA markers exclusively from N- or S-cytoplasm in DNA isolated from individual plants of 'Sapporo-ki'. In no case has any evidence of paternal transmission of the cpDNA or mtDNA of onion been detected. These results support maternal inheritance of the organellar genomes and, therefore, we can molecularly identify the cytoplasm of individual plants.

For populations possessing both N- and S-cytoplasms, random pollination would insure that the frequency of maintaining genotypes among plants in N-cytoplasm equals the frequency of sterile plants among those in S-cytoplasm. The frequency of a maintaining genotype is the product of the frequency of the *msms* genotype by the frequency of N-cytoplasm; e.g. in BYG, the frequency of maintainers equals (proportion in N-cytoplasm)(proportion *msms*) = $(0.63)(0.59)^2 = 0.219$ (Table 1). For OP populations in a mixture of N- and S-cytoplasms, molecular identification of cytoplasms can significantly reduce the number of individual pairings with a sterile tester required to identify a maintaining genotype (Table 1). In the case of TG1015Y, a breeder could expect that 85% fewer pairwise crosses would identify a maintaining genotype (N *msms*) among N-cytoplasmic plants as compared to plants from the OP population.

Table 1 Onion populations possessing both N- and S-cytoplasms and the nuclear fertility-nonrestoring (*ms*) allele and the numbers of plants that must be evaluated to be 95% confident of identifying one maintainer using classical and molecular approaches

Population[a]	Frequency of N-cytoplasm[a]	Frequency of *ms* allele[b]	Frequency of maintainers[c]	Number of OP plants to identify one maintainer[d]	Number of N-cytoplasmic plants to identify one maintainer[e]	Reduction[f]
Brigham Yellow Globe	0.63 ± 0.06	0.59	0.219	13	8	0.38
B2215C	0.79 ± 0.05	0.35	0.097	30	23	0.23
Mountain Danvers	0.80 ± 0.03	0.35	0.098	30	23	0.23
Sapporo-Ki	0.60 ± 0.08	0.41	0.101	29	17	0.41
Texas Grano 1015Y	0.15 ± 0.05	0.11	0.002	1663	247	0.85

[a] Origin of populations other than B2215C and proportion of normal fertile cytoplasm previously reported by Havey (1993) and Havey and Bark (1994) and are included for convenience only

[b] Frequency of nuclear fertility-nonrestoring allele was estimated by scoring the fertility of plants from testcrosses to sterile lines (see Materials and methods). For 1015Y, sterile plants occur at 1% (L. Pike, personal communication). The frequency of *ms* allele was estimated at (frequency of sterile plants) = (frequency of *ms* allele)^2(frequency of S-cytoplasm); $0.01 = (x)^2(0.85)$; $x = 0.11$

[c] Frequency of maintainer (N *msms*) plants estimated by the product of proportion in N-cytoplasm by the frequency of *ms* allele squared

[d] Number of plants of the open-pollinated population that must be evaluated to be 95% confident of identifying one maintainer (N *msms*) plant [estimated using binomial distribution (Mansur et al. 1990)]

[e] Number of N-cytoplasmic plants that must be evaluated to be 95% confident of identifying one maintainer (N msms) plant

[f] Reduction in the number of testcrosses to sterile plants required to identify one maintaining genotype from the open-pollinated populations versus a subset classified as N-cytoplasmic

Cytoplasmic identifications are especially important given that OP populations may be exclusively in S-cytoplasm (Havey 1993, Havey and Bark 1994). Such populations may be more commonly encountered in countries where hybrid seed has been retained and used to develop OP populations; e.g. in India, the OP population 'Pusa Red' may have been selected from hybrid 'Red Granex' (Currah 1992). After receiving seed of an uncharacterized OP population, the classical approach would be to pair individuals with sterile plants and evaluate for a maintaining genotype by scoring the fertility of progeny from the sterile parent, this requires at least 4 years and is complicated by segregation of the nuclear fertility-restoration gene. The oligonucleotide primers that preferentially amplify a cpDNA fragment carrying an autapomorphic 100-bp insertion in N-cytoplasm provide onion breeders with an alternative method to determine if maintainers can be extracted and at what cost. An uncharacterized population should be testcrossed to a sterile line. The frequency of sterile progeny from the sterile-tester line estimates the frequency of the *ms* allele. The cytoplasm(s) present can be identified by isolating pooled DNA from seedlings and completing Southern analyses for the gain of restriction-enzyme sites in the cpDNA (Havey 1993) and/or mtDNA (Holford et al. 1991, Satoh et al. 1993, Fig. 2). An alternative approach would be to use PCR to identify the cytoplasm of individual plants from the OP population. If the population possessed both N- and S-cytoplasms, the breeder could flower those plants in S-cytoplasm and score the frequency of sterile plants, equaling the square of the frequency of the *ms* allele. This latter approach avoids the 2 years required to score the fertility of testcrosses to a sterile line. Given the presence of the *ms* allele and N-cytoplasm, the frequency of maintaining genotypes can be calculated and the decision made whether or not to attempt extraction of maintainers. Although molecular identification of the cytoplasm does not shorten the onion-breeding cycle (2 years per generation are still required), the technique represents a more judicious use of resources.

Under the conditions employed, I do not recommend PCR analyses of pooled DNA samples because a cytoplasm was not detected if it represented less than 10% of the DNA mixture. A similar result was reported by Michelmore et al. (1991) for DNA bulks subjected to PCR using random decimer primers and Ulrich et al. (1993) for internal controls used in PCR-based diagnostics; competitive PCR may not confidently amplify fragments when the concentrations of competing sequences differ by an order of magnitude.

Although CMS is attributed to an incompatibility between the mitochondrial and nuclear genomes (Hanson 1991), the PCR-detectable polymorphism is in the cpDNA. Previous work (Jones and Clarke 1943; Tatabe 1968; Corriveau and Coleman 1988; Satoh et al. 1993) and that which I present here support maternal inheritance of both the cpDNA and mtDNA in onion; therefore, polymorphisms in either organelle should be useful for distinguishing N- and S-cytoplasms. However, infrequent biparental or paternal transmission of the organellar DNA would, over time, generate individual plants possessing a mosaic of organelles and complicate the molecular-aided extraction of maintainer lines from OP populations.

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