Theor Appl Genet (2001) 102:78–82

J.W. Lilly and M.J. Havey

Sequence analysis of a chloroplast intergenic spacer for phylogenetic estimates in *Allium* section *Cepa* and a PCR-based polymorphism detecting mixtures of male-fertile and male-sterile cytoplasmic onion

Received: 29 November 1999 / Accepted: 28 April 2000

Abstract Restriction-enzyme analysis of the chloroplast (cp) DNA yielded maternal phylogenies supporting a close phylogenetic relationship among normal (N) male-fertile and male-sterile (S) cytoplasmic bulb onion (*Allium cepa*), *Allium altaicum*, *Allium fistulosum*, *Allium galanthum*, *Allium roylei*, and *Allium vavilovii*. The S cytoplasm of onion is most likely an alien cytoplasm introduced in antiquity into onion populations. We previously showed that size differences in an intergenic spacer in the cp DNA distinguish N and S cytoplasms of onion. We cloned and sequenced this intergenic spacer from the N and S cytoplasms of onion, *A. altaicum*, *A. fistulosum*, *A. galanthum*, *Allium pskemense*, *Allium oschaninii*, *A. roylei*, and *Allium ampeloprasm* (outgroup) to identify the nature of previously described RFLPs and to develop a PCR-based marker revealing N-cytoplasmic contamination of S-cytoplasmic hybrid seed lots. Phylogenies based on restriction-enzyme analysis of the entire cp DNA were similar, but not identical, to those based on sequence divergence in this intergenic region.

Keywords Cytoplasmic male sterility · Polymerase chain reaction · *Allium cepa*

Introduction

Restriction-enzyme analysis of the chloroplast (cp) genome is a useful tool to estimate maternal phylogenies below the family level (Palmer 1987; Clegg and Durbin 1991). Phylogenetic resolution among more-closely related species using the cp DNA may be low because of relatively few synaptomorphies (Sandbrick et al. 1990). Regions of the cp genome amass polymorphisms at different rates. For example, introns and intergenic spacer regions more quickly accumulate nucleotide differences or structural rearrangements, as compared to coding regions (Posno et al. 1986; Wolfe et al. 1987; Kelchner and Wendel 1996). Phylogenetic estimates based on nucleotide substitutions or indels (insertion or deletion events) in these rapidly evolving regions may (Mes et al. 1997; Sang et al. 1997) or may not (Goldenberg et al. 1993; Morton and Clegg 1993) yield homoplasious characters, affecting comparisons with the cp genome as a whole or other specific cp regions.

Phylogenetic estimates based on polymorphisms at or between restriction-enzyme sites in the cp DNA among species in *Allium* section *Cepa* revealed a well-supported maternal clade, comprised of normal (N) male-fertile and male-sterile (S) cytoplasms of onion, *Allium altaicum*, *Allium fistulosum*, *Allium galanthum*, *Allium rolyei*, and *Allium vavilovii* (Havey 1992). The close phylogenetic relationships among species within this clade were consistent with previously characterized karyotypes (Saini and Davis 1970) and crossabilities (van Raamsdonk et al. 1992). Interspecific hybrids can be generated among bulb onion and these closely related *Allium* species (Emsweller and Jones 1935a; Maeda 1937; Saini and Davis 1967; McCollum 1982; van der Meer and de Vries 1990; van Raamsdonk et al. 1992); however, these interspecific hybrids or their backcross progenies are often sterile (Emsweller and Jones 1935b; McCollum 1971; van der Valk et al. 1991; Ulloa et al. 1995; Havey 1999).

S cytoplasm is an alien cytoplasm introduced in antiquity into onion populations (Havey 1993). The N and S cytoplasms of onion can be distinguished by polymorphisms in or near the intergenic spacer between tRNAs T and L in the cp genome (Havey 1995). N cytoplasm and *A. vavilovii* possess approximately 100 base pairs (bp) more of DNA than the other studied species. A polymorphic *Bgl*II site distinguishing *A. fistulosum* and *A. altaicum* from the other related species is also located in this intergenic region. To further characterize these polymorphisms and to identify new ones, we cloned and se-

Communicated by H.C. Becker

J.W. Lilly · M.J. Havey (✉)
Vegetable Crops Unit, United States Department of Agriculture, Agricultural Research Service, Department of Horticulture, 1575 Linden Drive, Univ. of Wisconsin, Madison, WI 53706, USA
Fax: +1 608–262–4743
e-mail: mjhavey@facstaff.wisc.edu

quenced this intergenic region for seven *Allium* species. We estimated phylogenetic relationships and compared them with those based on the entire cp genome. We also developed a PCR-based marker revealing low levels of N cytoplasm in hybrid seed lots of onion.

Materials and methods

Total genomic DNAs from eight Alliums in the section *Cepa*, *Allium ampeloprasum* (section *Porrum*), and *A. roylei* (section *Rhiziridеum*) (Table 1) were purified over CsCl gradients as previously described (Havey 1991). *A. ampeloprasum* is an appropriate outgroup for phylogenetic estimates within the section *Cepa* (Havey 1991, 1992). *A. roylei* was included because previous studies placed it with species in the section *Cepa* (Havey 1992). The DNAs were the same as those used in previous phylogenetic studies based on restriction-enzyme analyses (Havey 1992, 1993). Oligonucleotide primers A and B were synthesized and used to amplify the intergenic region between the coding regions for transfer RNAs T and L in the cp genome (Taberlet et al. 1991). Reaction conditions were 10–50 ng of total genomic DNA, 1 µM of each primer, 2.5 mM of $MgCl_2$, 1 × reaction buffer as supplied by the manufacturer (Perkin-Elmer), 0.2 mM of each dNTPs, and a 0.5 unit of *Taq* polymerase. PCR reactions were performed with an initial heating at 94°C for 1 min followed by 30 cycles of 1 min at 94°C, 1 min at 42°C, and 2 min at 72°C. The last extension cycle was for 10 min to allow cloning of PCR products in the pCR-II vector (Invitrogen) by AT tailing. PCR products were diluted and ligated into the pCR-II vector as recommended by the manufacturer. Competent bacteria of strain DH5α (Gibco-BRL) were transformed with ligation products and plated on LB medium with ampillicin (Sambrook et al. 1989). Plasmids from two well-isolated colonies from each accession were individually purified using the QiaPrep Spin mini-prep kit (Qiagen) according to the manufacturer's directions. The inserts were released by digestion of plasmids with *Eco*RV and *Hin*dIII, run on 1.0% agarose gels, and determined to be of correct sizes. Gels were blotted to a Zetaprobe (Bio-Rad) membrane as previously described (Havey 1991) and hybridized with orchid clones 17 a&b (Chase and Palmer 1989) to establish homology with the correct cp region (Havey 1992). At least two independent clones from each cytoplasm were isolated.

Three to five randomly selected bacterial colonies for each clone were harvested from selective media (LB with 50 µg/ml of ampicillin) and grown overnight in the same liquid medium. Plasmid DNA was purified as described before. Sequencing reactions were with 1 µg of plasmid DNA, 10–15 pM of pUC forward or reverse sequencing primer, 1 × BIG-DYE (Perkin-Elmer) terminator PCR Buffer, 4 µl of BIG-DYE sequencing enzyme in a total volume of 20 µl. Cycle conditions were 95°C for 2.5 min; 50 cycles of 96°C for 20 s, 50°C for 25 s, 60°C for 5 min; and a 4°C hold. Unincorporated dye terminators were removed by passing the reaction through the DyeEX Spin kit (Qiagen). Reactions were then dried under vacuum and delivered to the University of Wisconsin (UW) Biotechnology Center for sequencing on an ABI377. At least two well-resolved runs in each direction were completed for each of three to five clones from two independent clones of each cytoplasm. Sequences were edited and aligned using Sequencer 3.0 (GeneCodes) software and deposited into NCBI genebank under the accession numbers listed in Table 1.

Polymorphic restriction enzyme sites within the intergenic region were identified using Sequencer 3.0. Unique oligonucleotide primers distinguishing N and S cytoplasmic onion were identified using Oligo 6.1 (Molecular Biology Insights) with high-stringency searches to eliminate oligonucleotides with common priming sites within different cytoplasms. Oligonucleotides were synthesized at the UW Biotechnology Center. Mixtures of N and S cytoplasmic DNAs were prepared at the concentrations listed in Fig. 3. PCR reactions revealing N-cytoplasmic contamination in S-cytoplasmic backgrounds had 10 ng of template DNA, 1 µM of each primer, 2.5 mM of each dNTP, 2.5 mM of $MgCl_2$, 1 × gold buffer (Perkin Elmer), and 1 unit of Ampli*Taq* Gold polymerase. Cycling conditions were 95°C for 10 min; 40 cycles of 94°C for 30 s, 55°C for 60 s, 60°C for 2 min; followed by a 4°C hold. Reactions were run on 1.0%-agarose gels at 10 mA for 14 h. Fragment sizes were estimated by comparison with a standard 100-bp ladder (Promega).

Sequence similarities were created with software from the Wisconsin Package (Genetics Computer Group) using PILEUP with a 30-bp comparison window and plotted using PLOTSIMILARITY. Phylogenetic analyses were completed using PAUPSEARCH / PAUPDISPLAY (Wisconsin Package, Genetics Computer Group) using branch and bound searches of Wagner parsimony with *A. ampeloprasum* as the outgroup. The most-parsimonious phylograms were generated and the bootstrap method used to estimate confidence intervals for the phylogenies. Phylogenies based on the intergenic region and restriction-enzyme analysis of the entire cp molecule (Havey 1992) were compared visually and by the number of additional steps required to generate user-defined trees.

Results

Cloning and sequence analysis

Two independent PCR products were cloned after amplification of genomic DNA from each cytoplasm (Table 1). Gel-blot analyses demonstrated that the cloned fragments hybridized with orchid clones 17 A&B and were of the correct size (data not shown). As expected, sequence analyses revealed that the intergenic region between tRNAs T and L in the cp DNA was larger for N-cytoplasmic onion (1201 bp) and *A. vavilovii* (1200 bp) than for the other cytoplasms (averaging 1089 ± 13 bp). This size difference was expected and the approximately 111 bp of extra sequence is the basis of a PCR-based marker distinguishing the N and S cytoplasms of onion (Havey 1995). The intergenic region is AT rich, possess-

Table 1 Origin of accessions and sequence assession numbers for *Allium* species

Allium species	Accession[a]	NCBI Accession Numbers[c]
A. altaicum	Tax 33	AF184335
A. cepa	B3350 B (N cytoplasm)	AF184337
A. cepa	Spartan Banner 80 (S cytoplasm)	AF184338
A. fistulosum	Ishikura	AF184339
A. galanthum	Tax 698	AF184340
A. oschaninii	Tax 978	AF184341
A. pskemense	Tax 514	AF184342
A. roylei	8127	AF184334
A. vavilovii	PI406677	AF184343
A. ampeloprasum[b]	PI280574	AF184336

[a] PI = USDA Plant Introduction Number. Origins and vouchers have been described by Havey (1992, 1993)
[b] Outgroup for phylogenetic analyses
[c] Sequences are available from NCBI website at http://www.ncbi.nlm.nih.gov/

ing an average of 75.2 ± 0.9% AT. Previously described polymorphic *Bgl*II sites (Havey 1991, 1992) distinguishing species in section *Cepa* from *A. fistulosum* and *A. altaicum* (cpDNA-8) and *A. ampeloprasum* (cpDNA-9) are located at positions 314 and 283, respectively.

The average similarity score among species (excluding the outgroup *A. ampeloprasum*) using a 30-bp window was 0.854 (Fig. 1), indicating that sequence homology was generally conserved across the intergenic region. When the average similarity score is above the dashed line in Fig. 1, the sequences among the analyzed species were more similar. Greater sequence divergence occurs at approximately position 261 with differently sized AT tracks flanked by direct repeats; loss of a 17-bp repeat by *A. altaicum* and *A. fistulosum* at position 375; loss of 15 bp beginning at position 541 by S-cytoplasmic onion, *A. altaicum*, *A. fistulosum*, *A. galanthum*, and *A. roylei*; and 34-bp direct repeats starting at positions 639, 684, and 729 in N-cytoplasmic onion and *A. vavilovii*.

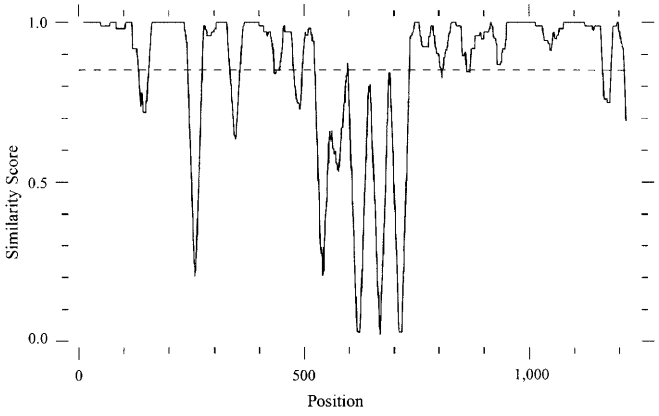

Fig. 1 Similarity plot among the sequences in the noncoding intergenic spacer between tRNAs T and L in the chloroplast genomes of *A. altaicum*, *A. cepa* (N and S cytoplasms), *A. fistulosum*, *A. galanthum*, *A. oschaninii*, *A. pskemense*, *A. roylei*, and *A. vavilovii*. The outgroup *A. ampeloprasum* was not included in this analysis. Comparisons were made using a 30-bp window. The *dashed line* (0.854) is the mean sequence similarity among the analyzed species

Phylogenetic analyses

Sequencing of the intergenic region revealed 872 informative characters for phylogenetic analyses. A single most-parsimonious Wagner tree of 3146 steps (Fig. 2 A) was generated with consistency and retention indices of 0.587 and 0.419, respectively. This tree confidently placed N-cytoplasmic onion with its likely progenitor *A. vavilovii* (Havey 1992, 1997) and *A. fistulosum* with its likely progenitor *A. altaicum* (Friesen et al. 1999). *A. galanthum*, *A. roylei*, and S-cytoplasmic onion were placed close to N-cytoplasmic onion and *A. vavilovii*. *Allium pskemense* was placed with *A. fistulosum* and *A. altaicum* (Fig. 2A). *Allium oschaninii* was placed outside of the clade containing these other species. The placement of species was similar, but not identical, to the single most-parsimonious maternal phylogeny based on the entire cp genome (Fig. 2B). In this analysis, *A. oschaninii* and *A. pskemense* were placed outside of a well-supported clade containing N- and S- cytoplasmic onion, *A. altaicum*, *A. fistulosum*, *A. galanthum*, *A. roylei*, and *A. vavilovii* (Havey 1992). Using only informative characters from the intergenic region, only three additional steps were required to generate a Wagner tree identical to the tree based on restriction-enzyme analysis of the entire cp genome (Fig. 2B).

PCR-based polymorphism revealing N-cytoplasmic contamination of S-cytoplasmic seed lots

We used the sequence of the tRNA T to L intergenic region in the cp DNA to design oligonucleotide primers re-

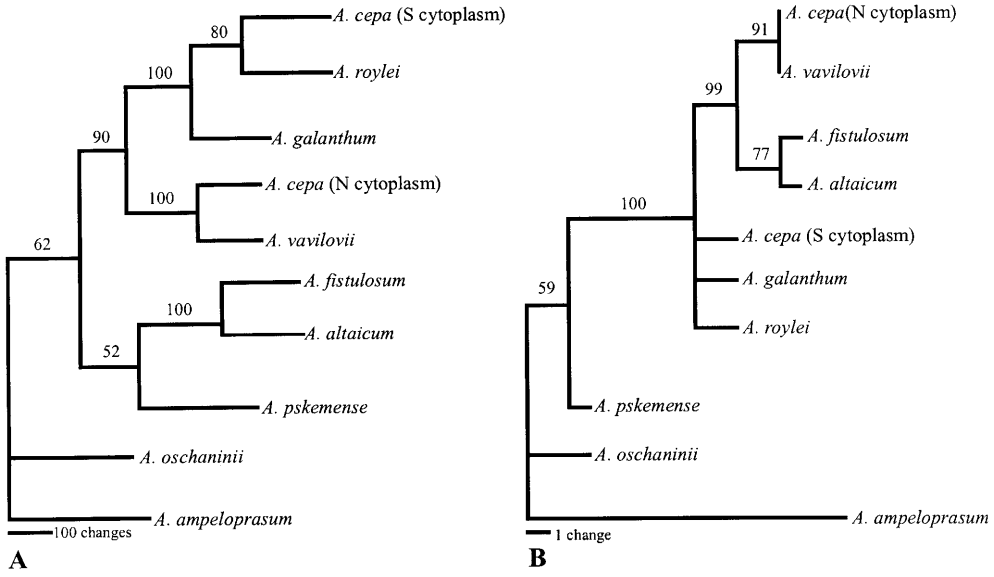

Fig. 2 Single most-parsimonious Wagner tree generated from sequence divergence in the intergenic spacer between tRNAs T and L in the chloroplast DNA (**A**) of species in *Allium* section *Cepa*. The single most-parsimonious Wagner tree based on restriction-enzyme analysis of the entire chloroplast genome (**B**) has been previously published (Havey 1992) and is included here for convenience only. *A. ampeloprasum* is the outgroup. *Numbers* above lines indicate percent of bootstrap replicates possessing the node

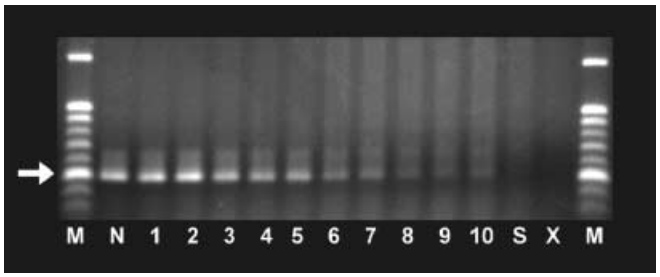

Fig. 3 Ethidium bromide-stained agarose gel of PCR products obtained using normal cytoplasm-specific PCR primers. *Lanes M* are the 100 base pair (bp) marker (Promega); *lanes N and S* are normal (*N*) male-fertile and male-sterile (*S*) cytoplasm DNAs (10 ng total), respectively; *lanes 1–10* are mixtures of N and S DNAs: *1* = 7.5 ng N/2.5 ng S, *2* = 5.0 ng N/ 5.0 ng S, *3* = 2.5 ng N/ 7.5 ng S, *4* = 1.25 ng N/ 8.75 ng S, *5* = 0.63 ng N/ 9.37 ng S, *6* = 0.32 ng N / 9.68 ng S, *7* = 0.16 ng N/ 9.84 S, *8* = 0.08 ng N/ 9.92 S, *9* = 0.04 ng N/9.96 ng S, *10* = 0.01 ng N / 9.99 ng S. *Lane X* is the control with no plant DNA. Arrow indicates the 500-bp marker fragment

vealing small amounts of N cytoplasm contaminating S-cytoplasmic inbreds or hybrids. The N cytoplasm-specific 5' primer (5'-ATA GCG GAT CCA GTC TTA AAC AAT-3') starts at position 662 and the 3' primer starts at position 1176 (5'-GCT TTC TAC CGA TTT CGC CAT ATC-3'). These primers amplify a 514-bp fragment from total genomic DNA isolated from N-cytoplasmic populations; no fragment is amplified from S-cytoplasmic DNA (Fig. 3). Using our reaction conditions, less than 1% N-cytoplasmic contamination was revealed (Fig. 3).

Discussion

Sequence and phylogenetic analyses

The sequence of the tRNA T to L intergenic cp region was previously studied for N and S cytoplasms of onion (Alcala et al. 1999). Their goal was to refine a previously identified size difference distinguishing these two cytoplasms (Havey 1995) for genetic-bit analyses in a selection program. Our results agree that this region is AT rich and size differences are most likely due to replication slippage (Alcala et al. 1999). Our goal was to sequence this region and develop PCR-based markers revealing low levels of N cytoplasm in hybrid or male-sterile inbred lines. We also used the sequence differences to estimate phylogenetic relationships among closely related *Allium* species of the section *Cepa*.

General agreement among phylogenies estimated using the cp DNA molecule as a whole and sequence analysis of specific regions of the cp DNA would be evidence of congruence among phylogenies based on different molecules or regions of molecules. Morton and Clegg (1993) reported that sequence analysis of localized hot spots in the cp genome may provide misleading phylogenies. Phylogenies based on restriction-enzyme analysis of the entire cp molecule and sequence analysis of an intergenic region were similar; only three additional steps separated the trees based on the intergenic sequence data. These analyses clearly support close relationships between N-cytoplasmic onion and *A. vavilovii*; between *A. fistulosum* and *A. altaicum*; and among *A. galanthum*, *A. roylei*, and S-cytoplasmic onion (Figure 2).

PCR-based polymorphism revealing cytoplasmic mixtures

The production of hybrid-onion seed requires at least two generations of seed increase. Production of the male-sterile female parent requires isolation with its N-cytoplasmic maintainer parent (Jones and Davis 1944). This male-sterile inbred parent may be used directly to produce a two-way hybrid or crossed with another maintainer inbred line to produce a male-sterile F_1 seed parent, which is further crossed with the male-fertile paternal parent to produce a three-way hybrid. In each of these seed production cycles, contamination of the male-sterile parent can occur by physical seed mixtures with maintainer lines or the pollen parent of the hybrid. Physical mixture could occur by mistakes during the harvest, cleaning, or packaging of the seed. In the case of the male-sterile inbred line or F_1 seed parent, one would observe male-fertile plants in the normally male-sterile inbred line. Grow-outs of the male-sterile line to check male sterility take 2 years. If male-fertile plants were observed, PCR determinations of the cytoplasm (Havey 1995) can be used to distinguish between outcrossing with a restorer line or physical mixture with the maintainer line.

Producers of hybrid-onion seed would benefit from a technique that reveals low levels of N-cytoplasmic contamination of male-sterile inbred or F_1 seed parents avoiding the 2 years required for grow outs. Assuming that the male parent of a commercial hybrid is N-cytoplasmic, this technique would also reveal mixtures of hybrid seed with its male parent. We identified oligonucleotide primers in the tRNA T to L intergenic region in the cp DNA revealing small amounts of N cytoplasm contaminating S-cytoplasmic inbreds or hybrids. Although the specific lesion conditioning CMS in onion most likely resides in the mitochondrial DNA, we chose to use the cp DNA because there is, on average, more cp than mitochondrial DNA extracted from leaf samples (Havey unpublished) and both the mitochondrial and cp genomes show maternal transmission in onion (Havey 1995). The presence of approximately 1% N cytoplasm can be revealed in a mixture of N and S cytoplasmic DNAs (Fig. 3). These oligonucleotide primers may be useful for quality control during the production of male-sterile inbred lines and hybrid-onion seed using S cytoplasm as the source of CMS.

Acknowledgements The generous financial support of Gilroy Foods, Novartis, Rio Colorado, Sakata, and Seminis is gratefully acknowledged. Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable. These experiments were carried out in accordance with US laws

References

Alcala J, Pike L, Giovannoni J (1999) Identification of plastome variants useful for cytoplasmic selection and cultivar identification in onion. J Am Soc Hort Sci 124: 122–127

Chase M, Palmer J (1989) Chloroplast DNA systematics of lilioid monocots, feasibility, resources and an example from the Orchidaceae. Am J Bot 76: 1720–1730

Clegg M, Durbin M (1991) Molecular approaches to the study of plant biosystematics. Aust Syst Bot 3: 1–8

Emsweller S, Jones H (1935a) An interspecific hybrid in *Allium*. Hilgardia 9: 265–273

Emsweller S, Jones H (1935b) Meiosis in *Allium fistulosum*, *Allium cepa*, and their hybrid. Hilgardia 9: 277–294

Friesen N, Pollner S, Blattner F, Bachmann K (1999) RAPDs and non-coding chloroplast DNA reveal a single origin of the cultivated *Allium fistulosum* from *A. altaicum*. Am J Bot 86: 554–562

Goldenberg E, Clegg M, Durbin M, Doebley J, Ma D (1993) Evolution of a non-coding region of the chloroplast genome. Mol Phylog Evol 2: 52–64

Havey M (1991) Phylogenetic relationships between cultivated *Allium* species from restriction enzyme analysis of the chloroplast genome. Theor Appl Genet 81: 752–757

Havey MJ (1992) Restriction enzyme analysis of the chloroplast and nuclear 45S ribosomal DNA of *Allium* sections *Cepa* and *Phyllodolon*. Plant Syst Evol 183: 17–31

Havey M (1993) A putative donor of S-cytoplasm and its distribution among open-pollinated populations of onion. Theor Appl Genet 86: 128–134

Havey M (1995) Cytoplasmic determinations using the polymerase chain reaction to aid in the extraction of maintainer lines from open-pollinated populations of onion. Theor Appl Genet 90: 263–268

Havey M (1997) On the origin and distribution of normal cytoplasm of onion. Genet Res Crop Evol 44: 307–313

Havey M (1999) Seed yield, floral morphology, and lack of male-fertility restoration of male-sterile onion (*Allium cepa*) populations possessing the cytoplasm of *Allium galanthum*. J Am Soc Hort Sci 124: 626–629

Jones H, Davis G (1944) Inbreeding and heterosis and their relation to the development of new varieties of onions. USDA Tech Bull No 874

Kelchner S, Wendel J (1996) Hairpins create minor inversions in non-coding regions of chloroplast DNA. Curr Genet 30: 259–262

Maeda T (1937) Chiasma studies in *Allium fistulosum*, *Allium cepa*, and their F_1, F_2 and backcross hybrids. Jap J Genet 13:146–159

McCollum G (1971) Sterility of some interspecific *Allium* hybrids. J Am Soc Hort Sci 96: 359–362

McCollum G (1982) Experimental hybrids between *Allium fistulosum* and *A. roylei*. Bot Gaz 143:238–242

Meer Q van der, de Vries J (1990) An interspecific cross between *Allium roylei* Stearn and *Allium cepa* L. and its backcross to *A. cepa*. Euphytica 47: 29–31

Mes T, Friesen N, Fritsch R, Klaas M, Bachmann K (1997) Criteria for sampling in *Allium* based on chloroplast DNA PCR-RFLPs. Systematic Bot 22: 701–712

Morton B, Clegg M (1993) A chloroplast DNA mutational hotspot and gene conversion in a noncoding region near rbcL in the grass family (Poaceae). Curr Genet 24: 357–365

Palmer J (1987) Chloroplast DNA evolution and biosystematic uses of chloroplast DNA variation. Am Nat 130 (suppl): S6-S29

Posno M, van Vliet A, Groot G (1986) The gene for *Spirodela oligorhiza* chloroplast ribosomal protein homologous to *E. coli* ribosomal protein L16 is split by a large intron near its 5' end: structure and expression. Nucleic Acids Res 14: 3181–3195

Raamsdonk L van, Wietsma W, de Vries J (1992) Crossing experiments in *Allium* L. section *Cepa*. Bot J Linn Soc 109: 293–303

Saini S, Davis G (1967) Compatibility in some *Allium* species. Hort Sci 91: 401–409

Saini S, Davis G (1970) Karyotypic analysis of some *Allium* species. J Am Soc Hort Sci 95: 102–105

Sambrook J, Maniatis T, Fritsch E (1989) Molecular cloning (2nd edn). Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Sandbrink J, van Bruggen A, van Brederode J (1990) Patterns of infraspecific chloroplast DNA variation in species of Silene. Biochem Syst Ecol 18: 233–238

Sang T, Crawford D, Stuessy T (1997) Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Peonia* (Paeoniaceae). Am J Bot 84: 1220–1136

Taberlet P, Gielly L, Pautou G, Bouvet J (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. Plant Mol Biol 17: 1105–1109

Ulloa M, Corgan J, Dunford M (1995) Evidence for nuclear-cytoplasmic incompatibility between *Allium fistulosum* and *A. cepa*. Theor Appl Genet 90: 746–754

Valk P van der, de Vries S, Everink J, Verstappen F, de Vries J (1991) Pre- and post-fertilization barriers to backcrossing the interspecific hybrid between *Allium fistulosum* L. and *A. cepa* L. with *A. cepa*. Euphytica 53: 201–209

Wolfe K, Li W, Sharp P (1987) Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. Proc Natl Acad Sci USA 84: 9054–9058