Identification of Plastome Variants useful for Cytoplasmic Selection and Cultivar Identification in Onion

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ADDITIONAL INDEX WORDS. *Allium cepa*, cytoplasmic male sterility, intraspecific variation, noncoding chloroplast region, sublimons

ABSTRACT. The relatively low evolution rate of the chloroplast DNA has made it an ideal tool to study evolutionary processes in plants above the species levels. However, recent studies have demonstrated that intraspecific variation in the chloroplast DNA is also common. This variation has provided useful insights into population level evolutionary processes. The polymerase chain reaction and sequencing of a noncoding chloroplast region used to classify onion lines for cytoplasmic type facilitated the identification of one sterile and two normal plastome variants in onion (*Allium cepa* L.). Sequence comparison revealed that differences between plastome variants included the presence of single-nucleotide polymorphisms associated with cytoplasmic type and variable numbers of tandem repeats, possibly resulting from slipped-strand mispairing. Our observations support the use of chloroplast-specific markers to assist in the selection of specific cytoplasmic types, suggest the potential to facilitate genotype determination, and demonstrate the presence of additional variation within cytoplasm type which gives insight into plastome evolution and may facilitate more accurate genotyping and selection.

The chloroplast genome (plastome) of plants exists as a single circular chromosome ranging from 120 to 217 kb in length (Palmer, 1987). The plastome is organized into two single-copy regions (large and small) separated by an inverted repeat. Much of the size variation between species is accounted for by variation in the size of the inverted repeat. The structure and gene content of the chloroplast genome is well conserved among plants, with the exception of one group of legumes, who are missing the inverted repeat (Palmer, 1987). The chloroplast genome also exhibits a conservative rate of nucleotide substitution relative to plant nuclear genes (Clegg and Zurawski, 1992). This relatively low evolutionary rate of the cpDNA has made it an ideal tool to study plant systematics and evolution above the species level (Palmer, 1987; Palmer et al., 1988; Soltis et al., 1992).

Despite the low evolutionary rate of the chloroplast genome, intraspecific chloroplast DNA (cpDNA) variation has been documented in a wide array of plant families (Soltis et al., 1992). The genetic nature of intraspecific cpDNA variation is typically limited to base substitution changes and insertion–deletion mutations (Soltis et al., 1992). It has been observed that there is a high incidence of sequence length mutation within single species. However, species differ considerably in the occurrence of length mutations versus base substitutions. The prevalence of length variation may provide insights into evolutionary processes within and among populations (Jordan et al., 1996; Soltis et al., 1992).

The plant mitochondrial genome differs from that of other higher eukaryotes by its large genome size, variability, and pres-

ence of large direct repeats and consequent multipartite structure (Mackenzie et al., 1994). Numerous abnormalities, including duplication of partial sequences of known coding regions, presence of chloroplast sequences, and evidence of unusual recombination events are also present (Lonsdale, 1989). Mitochondrial DNA (mtDNA) recombination events that occur among homologous repeated sequences as well as among apparently nonhomologous sequences result in a highly variable genome configuration in higher plants relative to other eukaryotic systems (Mackenzie et al., 1994). Some of these rearrangements are associated with cytoplasmic male sterility (Lonsdale, 1989).

It has been demonstrated that substoichiometric DNA fragments (sublimons) are present in low abundance relative to the main mtDNA. Sublimons have been detected in wheat (*Triticum* sp.) (Kück et al., 1993), maize (*Zea mays* L.) (Small et al., 1987, 1989), and *Brassica* mtDNA (Shirdazegan et al., 1989). Recent evidence suggests that these substoichiometric molecules may be the substrates for which new mitochondrial genomic configurations are generated (Hanson and Folkerts, 1992). Similar observations of substoichiometric organelle DNA organizations have been observed in chloroplasts of higher plants (Moon et al., 1987) and in animal mitochondria (Hauswirth and Laipis, 1986).

Sublimons may be maintained and passed from generation to generation through maternal inheritance and may differentially accumulate in specific progeny and populations (Small et al., 1989). According to Leaver et al. (1988) persistent substoichiometric fragments could provide a means for rapid cryptic genome evolution, only revealed in subsequent generations by transmission, amplification, and resulting fixation. Nuclear genes can also have an influence on the amplification or loss of subgenomic circular mtDNAs as demonstrated previously in *Zea* and *Phaseolus* (Covello and Gray, 1991; Escote-Carlson et al., 1990).

Cytoplasmic male sterility (CMS) is a common trait in many plant species, controlled at least in part by cytoplasmically inherited genes, and results in the inability of a plant to shed or produce viable pollen (Hanson and Conde, 1985; Laughnan and Gabay-Laughnan, 1983). CMS is agriculturally valuable for the production of hybrid seed by allowing for controlled pollination and

Received for publication 24 June 1998. Accepted for publication 3 Dec. 1998. We gratefully acknowledge the technical assistance of Roger Horn and Avutu S. Reddy. Richard W. Jones (Asgrow Vegetable Seeds, Saticoy, Calif.) is acknowledged for providing genotypes 1630 A and TEG 951 C. J. Alcalá was funded through an assistantship from the Vegetable Improvement Center. Use of trade names does not imply endorsement of the products named nor criticism of similar ones not named. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked advertisement solely to indicate this fact.

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eliminating the need for hand emasculatation.

In onion (*Allium cepa*), two types of cytoplasmic male sterility have been described. Cytoplasmic male sterility type T was discovered in the onion cultivar Jaune Paille des Vertus (Berninger, 1965) and is controlled by a cytoplasmic determinant [T] and three independently segregating restorer loci (Schweisguth, 1973). Cytoplasmic male-sterility type S was discovered in the onion cultivar Italian Red and is conditioned by the interaction of the cytoplasm (sterile, [S]) with a single recessive male-fertility nonrestoring allele (*ms*). (Jones and Emsweller, 1936). Hybrid onion production primarily relies on CMS type S because of the simpler inheritance of fertility restoration (Jones and Clarke, 1943).

Although the biochemical and genetic bases underlying CMS are not well understood (Hanson and Conde, 1985; Levings, 1983, 1993), this trait has been associated with changes in the genomes of the mitochondria and chloroplast (Boeshore et al., 1983; Conde et al., 1982; Levings and Pring, 1976, 1977; Pring et al., 1982). In onion, restriction patterns of chloroplast and mitochondrial DNA have permitted distinction between [S], [N], and [T] cytoplasms (Courcel et al., 1989; Havey, 1993; Holford et al., 1991; Satoh et al., 1993). Since the onion plasmon shows maternal inheritance, polymorphisms observed in the chloroplast genome can be used to identify [S] and [N] cytoplasms (Courcel et al., 1989; Havey, 1993; Holford et al., 1991; Satoh et al., 1993). Havey (1995) proposed the use of plastome-specific DNA amplification to distinguish between [S] and [N] cytoplasms and to aid in the identification of maintainer lines from open-pollinated populations.

The amplification of a noncoding chloroplast sequence (intergenic spacer) between *trnT* (UGU) and *trnL* (UAA) in onion yields either a 1.0 kb or a 1.1 kb amplicon associated with [S] or [N] cytoplasm, respectively (Havey, 1995). Havey (1995) demonstrated that plants with [N] cytoplasm show a 100-bp insertion in the cpDNA. The use of the polymerase chain reaction (PCR) and chloroplast-specific primers have allowed rapid and accurate cytoplasm distinction. The utility of cytoplasm determination in onion breeding has been demonstrated previously by Havey (1995) and Alcala et al. (1997). Here we report DNA sequence variation within the intergenic spacer of different [N] cytoplasm breeding lines and as compared to [S] cytoplasm lines. DNA sequence variation was observed both as single base substitutions and at the level of numbers of entire repeat sequences suggesting possible slip-strand mispairing during replication as one origin of variability. The observed sequence variation may be useful in the selection of specific cytoplasmic types and in the identification of genotypes.

Materials and Methods

PLANT MATERIAL. Onion seedlings were grown at 22 °C for 2 weeks under fluorescent lights in MetroMix 700 (Kinney Bonded, Donna, Tex.). Two-week-old leaves were harvested and frozen immediately in liquid nitrogen and stored at -80 °C until needed for DNA isolation.

DNA ISOLATION AND ANALYSIS. Two-week-old leaf samples of 15 onion breeding lines (24 samples/line) were used to extract total DNA by the method of Fulton et al. (1995). Concentration was determined by agarose gel electrophoresis using known amounts of lambda DNA as standards and by UV spectrophotometry.

PCR AND GEL ELECTROPHORESIS. A ± 1000 -bp cpDNA region (intergenic spacer between *trnT* (UGU) and *trnL* (UAA) 5') was amplified by PCR using the oligonucleotides SM1 (5'-CATTACAAATGCGATGCTCT-3') and SM2 (5'-

TCTACCGATTTCGCCATATC-3') as primers (Havey, 1995) and amplification conditions as described previously (Alcala et al., 1997). Amplified DNAs were separated by agarose gel electrophoresis using neutral gel buffer and 1.5% agarose gels as in Fulton et al. (1995). The presence of a 1.0 kb or a 1.1 kb amplicon was used as an indicator of [S] or [N] cytoplasms, respectively. The appearance of an amplicon of different mobility compared to the controls prompted the sequencing and molecular analysis of additional breeding lines. Amplifications which when repeated twice and resulted in identical results were considered accurate.

DNA SEQUENCE ANALYSIS. Control clones, 1630 A ([S] cytoplasm, 1.0 kb amplicon) and TEG 951 C ([N] cytoplasm, 1.1 kb amplicon) were cloned previously (Alcala et al., 1997). One clone per control was sequenced. Sequencing of the amplicon was performed using primers SM1, SM2, and SM3 (5'-CTGGAAGGGTTCGATATTCTTC-3') to encompass the entire region. To verify that the noncoding chloroplast region and single-nucleotide polymorphisms (SNPs) were conserved among several genetic backgrounds, and that the sequences accurately reflect the majority of sequences in the respective plastomes, seven [N] cytoplasmic lines and six [S] cytoplasmic lines (1 plant/line) were amplified with primers SM1 and SM2 and the resulting amplified products were directly sequenced without cloning. Direct sequencing of the PCR products will not reflect rare sequence nor errors resulting from polymerase misincorporation as such variants will represent only a small fraction of the larger pool even if generated in early rounds of amplification. Sequencing was performed on an ABI 377 automated sequencer (Perkin Elmer, Norwalk, Conn.) with fluorescent labeling reagents and protocols provided by Invitrogen (Carlsbad, Calif.).

Results

The sequencing of amplicons of lines 1630 A and TEG 951 C revealed that these fragments were 1088 and 1184 bp long, (GenBank accession numbers AF007065 and AF007066) respectively (Fig. 1). The [S] cytoplasm control and all six [S] cytoplasm lines tested had identical sequence over the entire amplicon and all seven yielded agarose gel bands of identical size. Cytoplasmic classification of [N] cytoplasm onion lines via electrophoretic mobility in agarose gels also revealed a third uniquely sized amplicon of 1141 bp (Fig. 1) present in the fertile [N] cytoplasm

Fig. 1. Specific amplification of onion DNA using primers SM1 and SM2. Lane 1: 1-kb DNA ladder (Life Technologies). Lanes 2-3: [S] and [N] cytoplasm controls. Lanes 4-15: 12 onion plants from breeding line Dorada x Kadavan showing [S] and [N] cytoplasms. Note that plants 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, and 15 possess a different amplification product. Arrow denotes novel cytoplasm size fragment.

of two short-day onion cultivars, Dorada and Perla (GenBank accession numbers AF011392 and AF011393, respectively). It is important to note that sequence variation in the actual amplicon priming sites would not have been detected.

Several notable differences were observed between [S] and [N] plastome variants. Four single-nucleotide polymorphisms (SNPs 1, 3, 4, and 5) associated with cytoplasmic type were observed between controls 1630 A and TEG 951 C (Table 1, Fig. 2). The four SNPs observed in the control lines were also conserved as differences among all [S] versus [N] lines screened, thus confirming the association of the single-nucleotide differences with [S] and [N] cytoplasm in onion. Cultivars Dorada and Perla shared one nucleotide polymorphism, SNP2, which was exclusive to these cultivars (Table 1, Fig. 2). In addition, a single base-pair substitution (SNP 6) was found to be exclusive to 'Dorada'.

Sterile and normal plastome variants also differed in the number and position of tandem repeats. Tandem repeat 1 was present twice in all [S] cytoplasmic lines whereas both [N] cytoplasmic variants showed only one copy (Table 1, Fig. 2). However, sequence analysis of seven breeding lines classified with [N] cytoplasm through conventional cytoplasmic screening (Table 1) (Havey, 1995) showed that three unique tandem repeats (TR 2; 45, 45, and 42 bp long, respectively) were present in five of the lines whereas [S] cytoplasmic lines showed only one repeat 42 bp long (Fig. 2). Interestingly, [N] cytoplasm cultivars Dorada and Perla showed only two repeats (45 and 42 bp) instead of three repeats typical of the other five cultivars with [N] cytoplasm (Table 1, Fig. 2). The presence of additional tandem repeats in cultivars with [N] cytoplasm accounted for the majority of the difference in the fragment sizes observed between [N] cytoplasmic variants and among [S] and [N] cytoplasmic lines. A third tandem repeat (TR3) was observed in all cultivars. This repeat was 17 bp long and was present twice in all cultivars with [N] cytoplasm (Table 1, Fig. 2). Cultivars with [S] cytoplasm showed this repeat only once.

In summary, four single nucleotide polymorphisms distinguished all [N] from all [S] cytoplasm types analyzed. One

additional single nucleotide polymorphism distinguished the two [N] cytoplasmic types. In addition, tandem repeat sequences served as more robust indicators of plastome variation. Tandem repeat 1 occurred as two copies in all [S] cytoplasm lines tested and one copy in all [N] cytoplasm lines. Conversely, tandem repeat 3 occurred as two copies in all [N] cytoplasm lines and one copy in all [S] cytoplasm lines. The largest repeat, tandem repeat 2, also varied among all [S] cytoplasm (1 copy) versus [N] cytoplasm lines, however there was internal variation in [N] cytoplasm with two lines harboring two copies of this repeat while the remaining five lines each contained three copies (Fig. 2).

Discussion

PLASTOME VARIATION AS A BASIS FOR CYTOPLASM SELECTION. Chloroplast DNA is frequently used in systematic studies of species divergence (Palmer, 1987) and has been useful in elucidating evolutionary relationships within and among populations (Soltis et al., 1992; Wagner et al., 1987). The chloroplast genome has been reported to show a lower average mutation rate than specific regions of plant or animal nuclear DNA (Wolfe et al., 1987). Although it has been suggested that the chloroplast genome shows a low evolutionary rate, the noncoding regions of the plastome tend to evolve more rapidly than do coding regions (Clegg et al., 1991; Palmer et al., 1988; Wolfe et al., 1987).

In this study, sequence analysis of approximately 1 kb of a noncoding chloroplast genome region between *trnT* genes allowed for the distinction of one sterile and two normal plastome variants in onion. The sequence information of the different plastomes obtained in this study is similar to that obtained by Taberlet et al. (1991) who used the same universal primers (SM1 and SM2) to successfully amplify cpDNA noncoding regions over a wide taxonomic range.

Several single-nucleotide polymorphisms and variable numbers of tandem repeat sequences of 8 to 45 bp associated with cytoplasmic type were characterized in fifteen onion breeding

Table 1. Molecular differences of a noncoding chloroplast intergenic spacer region between *trnT* and *trnL* among short-day onion breeding lines.

Breeding line	Cytoplasm	Tandem repeat ^z (TR) (no. copies)			Single nucleotide polymorphism (SNP) ^y					
		TR 1	TR 2	TR 3	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6
TEG 951 C	[N]	1	3	2	C	G	T	G	A	T
Dorada ^x x Kadavan	[N]	1	2	2	C	A	T	G	A	C
Perla ^x x Kadavan	[N]	1	2	2	C	A	T	G	A	T
Chona x (βαYellow x Ringold)	[N]	1	3	2	C	G	T	G	A	T
Ben Shemen x TG 502	[N]	1	3	2	C	G	T	G	A	T
(βαWhite x NMYG) x TG 1015 Y ^x	[N]	1	3	2	C	G	T	G	A	T
Ringer x TG 1015 Y	[N]	1	3	2	C	G	T	G	A	T
TG 1015 W	[N]	1	3	2	C	G	T	G	A	T
1630 A	[S]	2	1	1	A	G	A	T	G	---
TG 1015 Y x Ringer	[S]	2	1	1	A	G	A	T	G	---
TG 502-2 x 4172 RB	[S]	2	1	1	A	G	A	T	G	---
Ringold Yellow Selection	[S]	2	1	1	A	G	A	T	G	---
Texas Early White ^x x TG 1015 Y	[S]	2	1	1	A	G	A	T	G	---
TG 1025-85 x TG 1025 ^x	[S]	2	1	1	A	G	A	T	G	---
Kadavan x Perla	[S]	2	1	1	A	G	A	T	G	---

^zTR 1 = tandem repeat of 8 bp (5'-CTTTTAGA-3') 31 bp from primer SM2; TR 2 = tandem repeat of 42 to 45 bp (5'-GGACTGATCCGCTATAATATGAATATAATTAATCTTATTGTTTA-3') 49 bp from primer SM2; TR 3 = tandem repeat of 17 bp (5'-TATATTATTAATATTA-3') 839 bp from primer SM2 in genotype 1630 A (Fig. 2).

^ySNP1 = 142 bp from primer SM2, SNP2 = 178 bp from primer SM2, SNP3 = 618 bp from primer SM2, SNP4 = 633 bp from primer SM2, SNP5 = 697 bp from primer SM2, SNP6 = 924 bp from primer SM2 (Fig. 2).

^xPlant variety protected cultivars developed by the Texas A&M Onion Breeding Program.

1184 bp fragment (N cytoplasm)

1141 bp fragment, corresponding to cultivars Dorada and Perla (N cytoplasm)

1088 bp fragment (S cytoplasm)

Fig. 2. Molecular differences between three plastome variants in onions. The sequenced region corresponds to a noncoding chloroplast region between *trnT* and *trnL* amplified by polymerase chain reaction using primers SM1 and SM2. Single nucleotide polymorphisms and tandem repeats are indicated.

lines. Until this decade reports of intraspecific variation among plastome sequences were infrequent. However, Soltis et al. (1992) summarized nearly 60 examples of intraspecific variation from >15 plant families. This report suggested that most examples of intraspecific cpDNA variation involve differences among, rather than within populations. Nevertheless, intrapopulational variation in cpDNA, has also been detected in populations in which only a few individuals are analyzed. For example, Banks and Birky (1985), reported intraspecific plastid DNA variation in two populations of *Lupinus texensis* even though only two and six individuals were sampled from each population. Intrapopulational variation has also been observed in one population of *Zea perennis* (Doebly, 1989), one population of *Pisum humile* (Palmer et al., 1985), and several populations of *Medicago sativa* (Johnson and Palmer, 1989). Intraplant cpDNA variation has also been observed in rice (*Oryza sativa* L.) (Moon et al., 1987), *Pinus banksiana* (Govindaraju et al., 1988), and *Medicago* (Johnson and Palmer, 1989).

We used one plant per breeding line (eight lines with [N]

-cytoplasm, seven lines with [S] cytoplasm) to identify differences among plastome variants. However, as all differences (except for one single base substitution between two [N] cytoplasm lines) were observed identically in individuals from two or more distinct lines it is highly probable that they are representative of intraspecific variation. Indeed the four single nucleotide polymorphisms and the variation in tandem repeat numbers for tandem repeats 1 and 3 were identical among the individuals examined from all [S] versus [N] cytoplasm lines and thus can be considered reliable indicators of intraspecific variation in the onion cpDNA. The identification of identical variation in copy number of tandem repeat 2 for two of the [N] cytoplasm lines (Dorada and Perla) relative to the remaining five [N] lines is also significant as 1) identical single plant genetic alteration in individuals from two distinct lines is highly improbable, 2) both lines are derived from a common parent, and 3) 24 individuals each of 'Dorada' and 'Perla' were amplified with primers SM1 and SM3 and yielded identically sized bands to those derived

from the sequenced 'Dorada' and 'Perla' individuals. Since the shift in gel mobility observed among [N] cytoplasm lines is due to the variation in tandem repeat 2 copy number, this assay is a highly reliable indicator of identical tandem repeat copy number in all 48 individuals. Thus, in total 25 of 25 individuals from 'Dorada' and 'Perla' were shown to have one less copy of tandem repeat 2 than 25 individuals each from the other 5 [N] cytoplasm lines tested based on PCR (data not shown).

POSSIBLE ORIGINS OF DNA VARIATION IN THE ONION *trnT*-*trnF* REGION. Addition-deletion mutations accumulate in noncoding regions at a rate that is at least equal to nucleotide substitution, accelerating the divergence of noncoding regions (Clegg and Zurawski, 1992). Many addition-deletion mutations are associated with short direct repeats, and probably arise from slipped-strand mispairing during replication (Takaiwa and Sugiura, 1982; Clegg and Zurawski, 1992). Due to the association of addition-deletion mutation with direct repeats, it has been suggested that particular noncoding regions may experience higher rates of these mutations because of local sequence features (Clegg and Zurawski, 1992). Therefore, the noncoding region between the *trnT* (UGU) and *trnF* (GAA) genes in the large single-copy region of onion may be prone to addition-deletion mutations as evidenced by the appearance of several tandem repeats (e.g., tandem repeat 2).

The short-day onion cultivar Dorada was developed from one self-pollinated bulb out of the cultivar Ben Shemen (R. Jones,

personal communication). This selected population showed many desirable characteristics when compared to the original 'Ben Shemen' and also showed segregation for color (white vs. yellow bulbs). This family was massed consecutively five times using selected yellow bulbs from each mass. The few white segregates present in the third mass of 'Dorada' were used to develop 'Perla'. The fact that these two lines share a common plastome variation in terms of tandem repeat 2 copy number in the noncoding intergenic spacer region is thus not surprising.

The plant mitochondrial genome is characterized by a multipartite structure (substoichiometric molecules known as sublimons) due to the active recombination between pairs of repeated DNA sequences (Leaver et al., 1988). Similar observations of substoichiometric organelle DNA organizations have been observed in chloroplasts of higher plants (Moon et al., 1987). The mitochondrial sublimons are generally stable and inherited from generation to generation (Lonsdale et al., 1984) and presumably the same would be true of plastome sublimons. A possible explanation for the appearance of TR2 in cultivars Dorada and Perla could be that the mutation giving rise to this repeat (probably by slipped-strand mispairing) is present on chloroplast sublimons. Since these substoichiometric DNA fragments are present at relatively low levels, mutational events in one of these molecules can eventually affect a relatively high proportion of the sublimon population (random genetic drift) (Leaver et al., 1988). Such sublimons could have become prevalent and eventually become fixed and amplified in the 'Dorada' and 'Perla' selections regardless of their genetic background (Leaver et al., 1988). Nevertheless, if the variant sequences observed among any of the lines examined were due to the presence of sequences known to exist on the plastome but duplicated on sublimons, the method of DNA sequencing used (direct sequencing of PCR products without cloning of individual amplicons) would have yielded overlapping (nonscoreable) sequences from the position of the missing repeat onward as both the plastome and hypothetical sublimon would have been simultaneously sequenced. Rather, the method of DNA sequence generation employed confirms that the variant sequences observed represent prevalent plastome DNA forms. In summary, the best explanation for the observed differences in intergenic spacer sequence among [N] cytoplasm lines is slipped-strand mispairing during replication of plastome DNA in an ancestor predating the split of the Dorada and Perla lines.

Depending on when this variation occurred one might predict that the noncoding chloroplast region of 'Ben Shemen' might contain an identical sequence variation. Ben Shemen was thus also analyzed for this variable region (Table 1, Fig. 2) and did not show the two exclusive tandem repeats of 'Dorada' and 'Perla' but showed instead three repeats commonly observed in other cultivars with [N] cytoplasm. The SNPs specific to 'Dorada' and 'Perla' were also not found in 'Ben Shemen'. One possible explanation for this observation could be that the 'Ben Shemen' seed stock used for this experiment was of a different source from the original stock from which 'Perla' and 'Dorada' were selected. Additionally, the original 'Ben Shemen' seed stock from which one self-pollinated bulb was selected to originate 'Dorada' and 'Perla' could have contained heterogeneous mixtures of plastomes. Therefore, with consecutive masses, plastomes with two copies of TR2 could have become fixed and amplified in the progeny populations. Finally, the sequence variation resulting in the Dorada-Perla variation may have arisen in or shortly after the original selection from Ben Shemen. The fact that as few as a single plastid can be transmitted through the ovule would allow for rapid fixation of a plastome variant (Mullet, 1988). Regardless of when the

variation arose it is a clear fingerprint of these two lines.

In summary, intraspecific cpDNA variation has been observed among populations, within populations, and within individual plants. It has been suggested that the prevalence of intraspecific variability in the chloroplast genome has been underestimated (Soltis et al., 1992). The results of this study suggest that intraspecific cpDNA variation in onion may be common and worth analyzing using noncoding chloroplast sequences. Unique sequences that flank the tandem repeats can potentially be used as targets or for making highly polymorphic PCR-based markers (Burr, 1994). Chloroplast intraspecific polymorphisms could be useful to classify onion cultivars by cytoplasmic type (Alcala et al., 1997) and to fingerprint or pedigree onion genotypes.

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