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Chloroplast DNA variation in the genus *Glycine* subgenus *Soja*

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ABSTRACT: Chloroplast DNAs from 26 soybean (*Glycine max*) cultivars or plant introductions (PIs) and from five PIs each of *G. soja* and *G. gracilis* have been compared by using the restriction endonucleases *Ava* I, *Bam*H I, *Cla* I, *Eco*R I, and *Xho* I. Only the enzymes AVA I, CLA I, and ECOR I distinguished restriction fragment-length polymorphisms. Five unique plastome types have been identified, including three types within *max* and three within *gracilis*. Despite the apparent diversity within the soybean germplasm collection, no variation was observed among the chloroplast DNAs of the predominant maternal ancestors of the currently grown soybean cultivars. Hybridization studies demonstrated that the restriction fragment-length polymorphisms were not the result of changes that occurred in the small single-copy region, the rRNA genes, or the *psb* A border region of the chloroplast genomes. By inference, we can postulate that the changes probably occurred in the large single-copy region. Considering the wide phenotypic overlap, the ease of interspecific crossing, and the existence of common plastome types, we believe that it might be appropriate to consider the subgenus *Soja* to be comprised of two highly variable species (*G. max* and *G. soja*) rather than to attempt to delimit a third intermediate species (*G. gracilis*).

SEQUENCE VARIATION among organelle genomes can be detected by type II restriction endonucleases. Restriction endonucleases recognize and cleave at a specific four to eight base-pair sequence[22]. Thus, restriction endonucleases can be used to detect: base changes within a recognition sequence, creation of new cleavage sites, and molecular rearrangements altering the distance between cleavage sites. The specificity of restriction endonucleases has provided a fundamental tool for the analysis of organelle DNA variation.

Restriction endonuclease fragment-pattern analyses have been used to detect sequence variation among chloroplast DNAs (cpDNAs) of many plant genera. Timothy et al.[26] detected sequence variation among the teosinte. Gordon et al.[7] found differences among five wild-type chloroplast genomes of *Oenothera*, and Lebacq and Vedel[13] were able to resolve three different types of cpDNAs among the *Brassica*. cpDNA variation also was observed among six isonuclear lines of male-sterile *Nicotiana*[6]. Using a slightly modified approach to restriction endonuclease fragment-polymorphism analysis, Clegg et al.[3] found cpDNA variation among pearl millet and related species. Other studies have uncovered intraspecific cpDNA variation[2,16,19].

Studies of the evolution of cpDNA consistently have shown that the inverted repeat region is highly conserved[10,19]. Much of the sequence conservation of the inverted repeat is accounted for by the slow rate of evolution of the rRNA sequences[17,18]. A correlation of restriction fragment-length polymorphisms to specific regions of the chloroplast genome of soybeans and related species could provide insight into the nature of cpDNA evolution at the intrageneric level.

Some overlap is found among the phenotypes of *Glycine max*, *G. soja*, and *G. gracilis*[1]. Much of the evidence concerning the taxonomic relationship of these species is circumstantial. They may represent a single, highly variable species, or *G. gracilis* may be an introgression product of crosses between *G. max* and *G. soja*, or *G. gracilis* may be an evolutionary intermediate between *G. max* and *G. soja*. The divergent taxonomic views of the relationship of these species have been discussed by Hadley and Hymowitz[8]. A molecular comparison of the chloroplast genomes of representatives of each of these species would yield valuable information about their relationships to each other.

The objectives of this research were to 1) identify potential sources of cytoplasmic diversity within the soybean germplasm collec-

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tion and to assess the degree of cytoplasmic uniformity among commonly grown soybean cultivars, 2) correlate restriction fragment-pattern polymorphisms to mutational events occurring in specific regions of the cpDNA molecule, and 3) gain insight into the evolutionary interrelatedness of *G. max*, *G. soja*, and *G. gracilis*.

Materials and Methods

The cultivars and plant introductions (PIs) used in this study represent the geographically diverse origins of most members of the soybean germplasm collection. Twenty-six cultivars or PIs of *Glycine max* (L.) Merr., including the maternal ancestors occurring most frequently in the parentage of commercially grown cultivars (Table I) were analyzed. We also analyzed the cpDNAs of five PIs of *G. gracilis* Skvortz. and five PIs of *G. soja* Sieb. & Zucc. (Table II).

Seed from northern cultivars and from *G. gracilis* were increased in field plots at Ames, Iowa, and Isabela, Puerto Rico. Seed from *G. soja* were increased in greenhouse plantings at Ames, Iowa. Seed from southern cultivars were provided by Dr. T. C. Kilen, Soybean Production Research, USDA-ARS, Stoneville, Mississippi.

Glycine max and *G. gracilis* seedlings were grown in a sandbench for 21 to 27 days. *Glycine soja* seedlings were grown for 27 to 35 days. Primary trifoliolates and unifoliates were harvested and prepared as reported elsewhere[20].

DNA was digested with the endonucleases *Ava* I, *Bam*H I, *Cla* I, *Eco*R I, and *Xho* I (New England Biolabs). Digestions were carried out in the reaction buffer recommended by the manufacturer. Electrophoresis was carried out in a 0.8 percent agarose gel made up in TAE running-buffer (0.4 M tris-acetate, 0.002 M Na_2EDTA). Electrophoresis was for 18 hours at 1.1 V/cm (25–35 mA). After electrophoresis, the gels were stained in distilled water containing 0.5 μg/ml ethidium bromide. Stained gels were photographed over short-wave UV light by using a Polaroid MP4 camera with UV filter and type 665 film.

DNA transfer to GeneScreen (New England Nuclear) was done according to the method of Southern[23]. The filters were hybridized to ^{32}P-labeled recombinant plasmids Ch9.M (cprDNA) containing a 12-kbp fragment carrying the 23S, 16S, and 5S rRNA genes of pearl millet cpDNA, pMCS 1, which contained a 21-kbp fragment covering the small single-copy region and portions of the inverted repeats of pearl millet cpDNA[3], and pZRB 427, which contained the *psb* A coding sequence of maize cpDNA (Dr. L. McIntosh, DOE/Michigan State University, pers. comm.). The *psb* A gene has been mapped to the large single-copy region adjacent to the inverted repeat region of soybean cpDNA[25]. Hybridization reactions were carried out according to the protocol of Maniatis et al.[15].

Autoradiograms were produced by exposing the hybridized filters to Kodak X-Omat R film with Dupont Cronex Lightning-Plus intensifying screens for 16-72 hours at -70°. After exposure of the autoradiograms, the hybridized probes were stripped from the GeneScreen by washing in 60 percent formamide in 2 × SSC at room temperature for one hour with two changes of SSC. After this treatment of the filter, rehybridization was carried out.

Table I. Maternal origin of *Glycine max* cultivars and plant introductions assessed for chloroplast DNA restriction fragment-length polymorphism

Cultivar or accession	Maturity group	Maternal origin	Cultivar or accession	Maturity group	Maternal origin
Clark	IV	China	Kingston	IV	Japan
CNS	VII	China	Medium Green	I	Japan
Dorman	V	China	Tokyo*	VII	Japan
Dunfield*	III	China	Minsoy	0	France
Hark	I	China	Arksoy	VI	Korea
Harosoy	II	China	PI 424078	III	Korea
Manchu	III	China	PI 290114	0	Hungary
Mandarin*	I	China	PI 290136	0	Hungary
Mukden*	II	China	PI 323551	VIII	India
Peking	IV	China	A.K.	III	(unknown)
Richland	II	China	Illini*	III	(unknown)
Sooty	IV	China	Polysoy	IV	(unknown)
Virginia	IV	China	Roanoke*	VII	(unknown)

*Cultivars identified as maternal ancestors of commonly grown soybean cultivars

Table II. Maternal origin of Plant Introductions of *Glycine soja* and *Glycine gracilis* assessed for chloroplast DNA restriction fragment-length polymorphism

Glycine soja			*Glycine gracilis*		
PI*	MG†	MO‡	PI	MG	MO
PI 65549	II	China	PI 65388	II	Northeast China
PI 81762	II	USSR	PI 79593	II	Northeast China
PI 424004A	II	Korea	PI 153292	III	Belgium
PI 423997	00	USSR	PI 326580	I	India
PI 101404B	II	China	PI 82278	III	Korea

*PI = Plant Introduction; †MG = maturity group; and ‡MO = maternal origin

Results

Restriction fragment-pattern (RFP) analysis

Using the endonucleases *Ava* I, *Bam*H I, *Cla* I, *Eco*R I, and *Xho* I, we were able to routinely distinguish a minimum of 26, 29, 20, 16, and 14 bands, respectively. Although many low-molecular-weight fragments frequently were resolved, these were not used in determining cytoplasmic groupings. Each of the enzymes used recognized a six-base-pair sequence. Therefore, our cytoplasmic groupings are based on a sampling of less than 0.5 percent of the soybean cpDNA base sequence.

Limit digestion with *Bam*H I and *Xho* I failed to distinguish differences among any of the cpDNAs assayed. However, limit digestion with the endonucleases *Ava* I, *Cla* I, and *Eco*R I allowed us to distinguish variation at both the interspecific and the intraspecific level. *Ava* I resolved two RFPs (Figure 1). The pattern depicted in lane B is unique to PI 79593, a *G. gracilis* accession. The pattern in lane B is characterized by a slow-migrating fragment of about 5.0 kbp and is missing a fragment corresponding to about 1.7 kbp.

The restriction endonuclease *Cla* I resolved three restriction-fragment patterns (Figure 2). The patterns shown in lanes A and B were relatively common. The pattern shown in lane C was found only in entry PI 79593. With the pattern in lane A as the standard, the pattern in lane B is missing a band corresponding to about 3.45 kbp and has an extra band corresponding to about 2.3 kbp. The pattern in lane C is characterized by a slower-migrating fragment corresponding to about 3.55 kbp. The distribution of the cultivars and PIs among

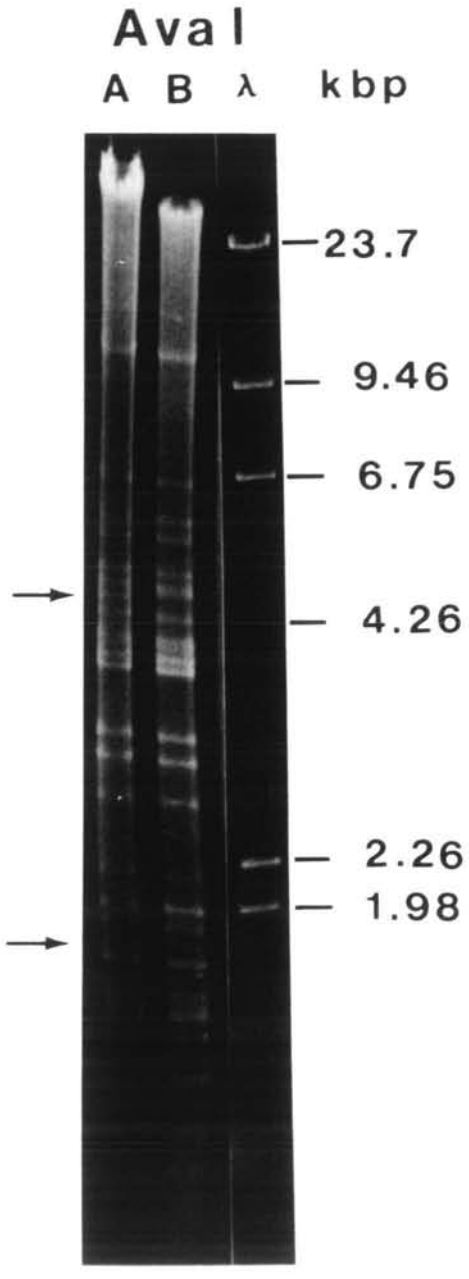

FIGURE 1 Restriction fragment-length polymorphism of soybean cpDNA digested with the restriction endonuclease *AVA* I. Lane A = 'Dunfield'; lane B = PI 79593; molecular weight marker is lambda DNA-*Hind* III digest.

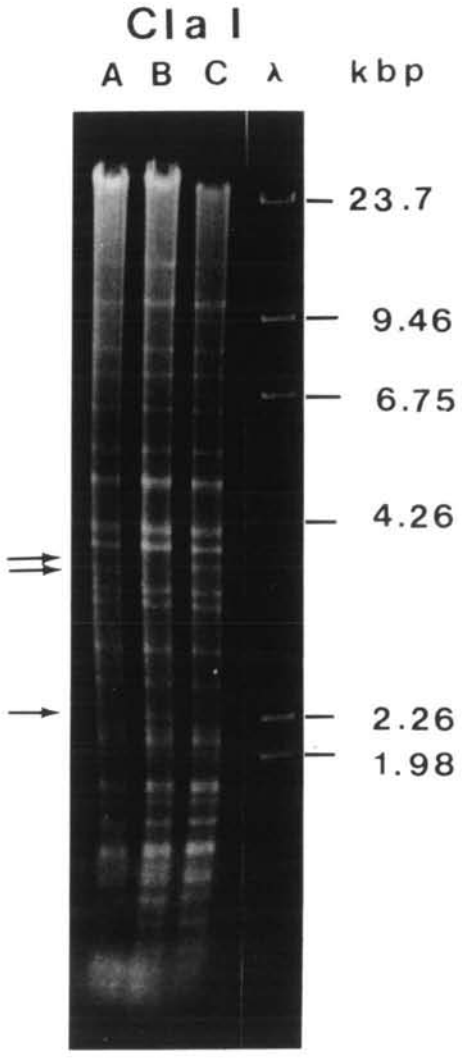

FIGURE 2 Restriction fragment-length polymorphism of soybean cpDNA digested with the restriction endonuclease *Cla* I. Lane A = 'Medium Green'; lane B = 'Dorman'; lane C = PI 79593; molecular weight marker is lambda DNA-*Hind* III digest.

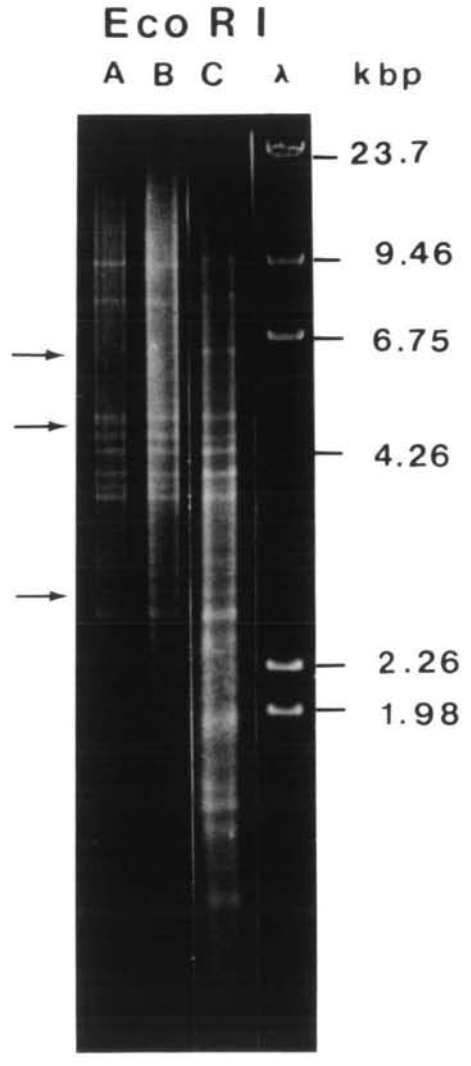

FIGURE 3 Restriction fragment-length polymorphism of soybean cpDNA digested with the restriction endonuclease *Eco*R I. Lane A = 'Clark'; lane B = PI 65549; lane C = PI 79593; molecular weight marker is lambda DNA-*Hind* III digest.

these cpDNA groupings is shown in Table III. All the *G. soja* accessions had the pattern shown in lane A, whereas *G. max* accessions and cultivars were present in both lanes A and B. The two accessions from Hungary, PIs 290136 and 290114, while very similar morphologically, were separated into patterns A and B by *Cla* I.

The endonuclease *Eco*R I also resolved three types of cpDNA (Figure 3). The patterns shown in lanes A and B are relatively common patterns. However, most *G. max* cultivars and accessions were in lane A, and all *G. soja* accessions were in lane B. The pattern in lane C is found only in two PIs, PI 79593 and PI 153292 (Table IV). Relative to pattern A, pattern B is missing a fragment of about 5.1 kbp and contains an extra band of about 2.9 kbp. Pattern C seems to be identical to pattern B with the exception of an extra 6.6-kbp fragment. The presence of the 6.6-kbp fragment in lane C is not readily accounted for. Extensive digestion with excess enzyme failed to lessen the intensity of this band. It is not immediately clear whether this fragment results from the creation of an *Eco*R I site in a larger fragment or the deletion of an *Eco*R I cleavage site between smaller fragments. Failure to detect concurrent changes in fragment migration rates is probably the result of their co-migration with other bands.

Filter hybridizations

Hybridizations with ^{32}P-labeled cloned segments of cpDNA to filters containing the bound restriction-fragment patterns of *Ava* I, *Cla* I, and *Eco*R I were carried out to determine if the restriction fragment-length polymorphism could be correlated with a specific region of the chloroplast genome. Hybridization patterns should indicate whether any of the fragments involved in pattern polymorphism encompassed regions covered by Ch9.M (cp-rDNA), pMCS 1, or pZRB 427. However, none of the unique soybean cpDNA bands hybridized to any of these probes (data not shown). Therefore, the mutational events

leading to the restriction fragment-pattern polymorphisms observed in this study probably did not occur in regions of the chloroplast genome corresponding to these cloned fragments.

Discussion

Glycine max accessions in the soybean germplasm collection number in the thousands. A definitive study involving each of these accessions is not feasible. Therefore, we sampled the soybean germplasm collection by selecting cultivars and PIs from geographically diverse origins. We analyzed the cpDNA from 26 cultivars and PIs of *G. max* and from five PIs each of *G. gracilis* and *G. soja*. Our analyses showed intraspecific cpDNA variation within *G. max* and *G. gracilis*. This suggests that the soybean germplasm collection may contain a broad range of cytoplasmic types.

This study identified five plastome groupings within the soybean germplasm collection (Table V). Members of each grouping appear to have similar cpDNAs, on the basis of having the same combination of restriction fragment patterns. Plastome grouping 1 includes mainly 'modern' soybean cultivars. Plastome grouping 2 includes several older cultivars and PIs of *G. max*. One type of cpDNA (group 3) appears to be typical of the subgenus *Soja*. This grouping includes all of the *G. soja* PIs analyzed, as well as primitive representatives of *G. max* and *G. gracilis*. Plastome groupings 4 and 5 contain only one PI each, both *G. gracilis*. Plastome groupings 1, 2, and 3 are each represented by four countries, with China the only country in common.

A limited number of soybean lines have been used to develop the soybean cultivars commonly grown today[5,9,21,24]. A Special Committee on Genetic Vulnerability[4] has calculated that the maternal ancestors of northern and southern cultivars are Mandarin, Illini, Tokyo, Dunfield, Mukden, and Roanoke, and that their combined frequencies of occurrence in the parentage of northern and southern cultivars are 51 percent, 23 percent, 11 percent, 8 percent, 4 percent, and 4 percent, respectively. Because of this, the cytoplasmic germplasm base for commercially grown soybean cultivars may be dangerously narrow. Our results clearly support this. Even though five unique combinations of restriction-fragment patterns were identified, the modern soybean cultivars, including those cultivars identified as maternal ancestors of the cultivated soybean, all yielded the same combination of patterns. Therefore, we found no variation among the cpDNAs of the maternal ancestors of currently grown soybean cultivars and conclude that cytoplasmic uniformity exists within the collection of commercially grown cultivars.

Hybridization data suggest that the genomic modifications resulting in the restriction fragment-length polymorphisms did not occur in the cpDNA small single-copy region, in the rRNA genes, or in the border region containing the *psb* A coding sequence. Although the restriction fragment-length polymorphism observed in this study may be due to changes that occurred in the portions of the cpDNA inverted repeat region not covered by the Ch9.M (cp-rDNA) probe, it is more probable that they occurred in the large single-copy region. No attempt has been made in this study to characterize the specific mutational basis

Table III. Restriction fragment-length-pattern groupings of *Glycine* spp. cpDNA based on *Cla* I digestion

Restriction patterns		
A	B	C
Arksoy	A.K.	PI 79593 (g)
CNS	Clark	
Kingston	Dorman	
Medium Green	Dunfield	
Peking	Hark	
Sooty	Harosoy	
Virginia	Illini	
PI 290136 (m)	Manchu	
PI 424078 (m)	Mandarin	
PI 323551 (m)	Minsoy	
PI 65549 (s)	Mukden	
PI 81762 (s)	Polysoy	
PI 424004A (s)	Richland	
PI 101404B (s)	Roanoke	
PI 423997 (s)	Tokyo	
PI 65388 (g)	PI 290114 (m)	
PI 153292 (g)		
PI 326580 (g)		
PI 82278 (g)		

Plant Introductions of *G. max*, *G. soja*, and *G. gracilis* are denoted by (m), (s), and (g), respectively; restriction fragment-length-pattern groupings are as shown in lanes A, B, and C of Figure 2

Table IV. Restriction fragment-length-pattern groupings of *Glycine* based on *Eco*R I digestion

Restriction patterns*		
A	B	C
Most *G. max* except Peking, Virginia, and PI 323551 (m)	All *G. soja* PIs PI 65388 (g) PI 326580 (g) PI 82278 (g) Peking Virginia PI 323551 (m)	PI 79593 (g) PI 153292 (g)

*Plant Introductions of *G. max* and *G. gracilis* are denoted by (m) or (g), respectively; restriction fragment-length-pattern groupings are as shown in lanes A, B, and C of Figure 3

Table V. Plastome groupings within the genus *Glycine* based upon *Ava* I, *Bam*H I, *Cla* I, *Eco*R I, and *Xho* I restriction fragment-length polymorphism

Plastome groupings				
1	2	3	4	5
A.K.	Arksoy	Peking	PI 153292 (g)	PI 79593 (g)
Clark	CNS	Virginia		
Dorman	Kingston	PI 323551 (m)		
Dunfield*	Medium Green	PI 65549 (s)		
Hark	Sooty	PI 81762(s)		
Harosoy	PI 290136 (m)	PI 424004A (s)		
Illini	PI 424078 (m)	PI 101404B (s)		
Manchu		PI 423997 (s)		
Mandarin*		PI 65388 (g)		
Minsoy		PI 326580 (g)		
Mukden*		PI 82278 (g)		
Polysoy				
Richland				
Roanoke*				
Tokyo*				
PI 290114 (m)				

*Cultivars identified as a maternal ancestor of commonly grown soybean cultivars. *G. gracilis*, *G. soja*, and *G. max* Plant Introductions are denoted by (g), (s), and (m), respectively

for the observed changes in restriction fragment patterns. Note, however, that the magnitude of the increase in size of the 5.0 kbp *Ava* I band (Figure 1) appears equal to that of the 3.55 kbp *Cla* I band (Figure 2), suggesting that a single deletion/addition mutation is responsible for the observed fragment alterations for both enzymes. In no case does a unique fragment from any one pattern give rise to two visible fragments from another pattern, whose sizes sum to the size of the 'original' fragment. This also suggests that addition/deletion mutations may be involved in creating the observed fragment-pattern polymorphisms. Thus, the creation or loss of a restriction site due to a simple point mutation can be ruled out.

A comparison of chloroplast DNA provides some insight into the relationships between *G. max*, *G. gracilis*, and *G. soja*. Phenotypically, *G. gracilis* plants are intermediate between *G. soja* and *G. max*, although some overlap does occur[1]. Some question has arisen as to whether *G. gracilis* is an evolutionary intermediate between *G. soja* and *G. max* or whether it is an introgression product between the two[8]. Upon the basis of restriction fragment patterns of mtDNA with *Bam*H I and *Sal* I, Sisson et al.[21] favored the 'introgression' hypothesis. They found that the mtDNA from a single PI of *G. gracilis* was indistinguishable from the mtDNA of the maternal ancestors of the commonly grown soybean cultivars. However, the complexity of the mitochondrial genome[14,28] and its rapid rate of evolution[27] make analysis of restriction fragment-pattern polymorphism among all but very closely related species difficult. The low level of base-sequence diversity among cpDNAs[27] and the rarity of major rearrangements during cpDNA evolution[12] might make the chloroplast genome a more suitable measure of cytoplasmic diversity and taxonomic relatedness.

It is well established that interspecific crosses do occur between the annual *Glycine* species[11]. Thus, the *G. gracilis* type could result from introgression of *G. max* and *G. soja*. However, if introgression accounts for the existence of *G. gracilis*, then it also might account for the existence of the primitive *G. max* representatives 'Virginia', 'Peking', and PI 323551 because these *G. max* representatives seem to contain cytoplasm common to all three species (Table V). The classification scheme shown in Table V indicates that a single cpDNA type is central to the subgenus *Soja*. The presence of all five PIs of *G. soja* analyzed in this study in plastome group 3 (Table V) lends support to the hypothesis that *G. soja* is the putative wild progenitor of *G. max* and *G. gracilis*.

It might be correct to consider *G. max* and *G. soja* as two highly variable species, rather than to consider *G. gracilis* as an intermediate species. If we consider the complications arising from introgression among the species, it also might be practical to consider the entire subgenus as a single highly variable species.

In summary, this research has demonstrated that the commonly grown soybean cultivars represent a restricted cytoplasmic pool relative to the cytoplasmic diversity present among related lines within the subgenus *Soja*. Although hybridization data suggest that restriction site changes have occurred in the large single-copy region of the soybean-chloroplast genome, the specific mutational basis for the observed changes has not been investigated. On the basis of phenotypic overlap, ease of interspecific crossing, and plastome groupings, we feel that it might be practical to consider *G. max* and *G. soja* as two variable species within the subgenus, or even to consider the entire subgenus *Soja* as one highly variable species.

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