The use of microsatellite markers has permitted the verification of the hypothesis that anther embryos are induced from different microspores or pollen grains, as it has been proved by the diverse genetic composition of embryos from the same anther. This confirms previous results obtained by isozymes and RAPDs (Bueno et al. 2000).

Parental Tree Analysis by Descendant Embryo Analysis

Allele segregation in the haploid descendants can be used for the heterozygosity analysis of an individual so that direct DNA analysis is not necessary for the parental tree. The high rate of polymorphism observed also permitted the identification of the parent tree by parental exclusion.

In this case locus ssQpZAG15 has two alleles, of 120 and 124 bp each, which are present in trees 1H, 3H, 4H, 7H, J, and 3M, excluding other trees as possible fathers. The locus ssQpZAG46 has two alleles present in the embryos, of 190 and 192 bp, which are present in trees 8H and 3M only. The combination of both exclusion criteria reveals 3M as the parental tree. The same result can be obtained with locus ssQpZAG110, with alleles of 222 and 238 bp, only present in trees 3H and 3M. The principle of parental exclusion could be applied in our embryo cultures and only two loci were sufficient for parental identification.

Conclusions

Nuclear DNA microsatellites are an adequate system for the tree identification in cork oak thanks to a high discrimination power among genotypes. Both alleles of each SSR locus were inherited by anther embryos, and Mendelian segregation (1:1) could be statistically proved by chi-squared test in one case. The homozygosity of both haploid and diploid anther embryos has been proved by microsatellite markers, revealing a certain rate of spontaneous DNA duplication. Embryo origin from multiple microspores or pollen grains inside a cork oak anther has been found by the different genetic composition of those embryos. The parent tree genome can be deduced from the haploid embryo progeny. SSR markers were used for the first time in cork oak, corroborating their applicability for many genetic analyses.

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FISH Mapping of the 5S and 18S-28S rDNA Loci in Different Species of *Glycine*

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Wild germplasms are often the only significant sources of useful traits for crops, such as soybean, that have limited genetic variability. Before these germplasms can be effectively manipulated they must be characterized at the cytological and molecular levels. Modern soybean probably arose through an ancient allotetraploid event and subsequent diploidization of the genome. However, wild *Glycine* species have not been intensively investigated for this ancient polyploidy. In this article we determined the number of both the 5S and 18S-28S rDNA sequences in various members of the genus *Glycine* using FISH. Our results distinctly establish the loss of a 5S rDNA locus from the "diploid" ($2n = 40$) species and the loss of two from the ($2n = 80$) polyploids of *Glycine.* A similar diploidization of the 18S-28S rDNA gene family has occurred in *G. canescens, G. clandestina, G. soja,* and *G. max* (L.) Merr. ($2n = 40$). Although of different genome types, *G. tabacina* and *G. tomentella* ($2n = 80$) both showed two major 18S-28S rDNA loci per haploid genome, in contrast to the four loci that would be expected in chromosomes that have undergone two doubling events in their evolutionary history. It is evident that the evolution of the subgenus *Glycine* is more complex than that represented in a simple diploid-doubled to tetraploid model.

Progress in soybean [*Glycine max* (L.) Merr.] improvement has been slow due to an overall lack of genetic variation in the germplasm, inherent difficulties in crossing, and a lack of cytogenetic and molecular markers (Keim et al. 1990). In a crop species with limited genetic variability, such as soybean (Delannay et al. 1983; Keim et al. 1989; Specht and Williams 1984), wild germplasms are often the predominant sources of genes for crop improvement.

The genus *Glycine,* contained within the tribe Phaseoleae, has been divided into two subgenera, *Glycine* and *Soja.* The subgenus *Glycine* consists of 15 wild perennial species, mostly diploid ($2n = 40$) and some allopolyploids ($2n = 80$) (Singh 1993). The subgenus *Soja* ($2n = 40$) contains the cultigen *Glycine max* (L.) Merr.

and its wild annual progenitor *G. soja* Sieb. and Zucc. (Singh 1993).

Nearly all the genera of tribe Phaseoleae have a chromosome number of $2n = 22$. As no members of the genus *Glycine* have a confirmed diploid chromosome number of 20 or 22, soybeans are thought to have arisen through an ancient allotetraploid event involving both chromosome doubling and chromosome loss, followed by the subsequent diploidization of the genome (Danna et al. 1996). However, the putative original progenitor species have not been identified (Hymowitz and Singh 1987; Kumar and Hymowitz 1989; Lackey 1980), nor have the wild species been closely investigated for evidence of this ancient polyploid event.

The next generation of evolutionary studies has moved beyond simple base addition/deletion frequency correlations and is focused on analysis of genome organization and synteny. However, despite the considerable attention and resources committed, the high-density, marker-saturated genetic maps and genomic DNA sequence data tell us relatively little about the large-scale physical organization of the chromosomes (Schmidt and Heslop-Harrison 1998). Probes for DNA repeats (e.g., ribosomal, microsatellite, telomeric, etc.) have become powerful tools for discerning chromosomal organization and have expanded our knowledge of evolutionary, genetic, and taxonomic relationships and have been used in practical applications such as agricultural forensics (individual identification) and cultivar tracking.

The nuclear genes encoding both 5S and 18S-28S ribosomal RNA (rRNA) consist of highly conserved repeat units arranged in one or more tandem arrays up to 10,000 bp long and variable nontranscribed spacer regions. In plants, the 5S rRNA genes are arrayed independently, while the 18S, 5.8S, and 26S rRNAs are produced together from a 45S rRNA precursor gene. In addition to multiple genes within an array, there may be multiple arrays (loci) on the same or different chromosomes.

Localization of multiple repetitive sequences by fluorescence in situ hybridization (FISH) provides a novel mechanism for viewing genomic organization and chromosome structure. These sequences can also act as landmarks for observing gene location, clustering, and orientation. Here we present results on the distribution, copy number, and location of both 18S-28S and 5S rDNA in species of wild perennial *Glycine*. The evolution of the agronomically important soybean, *Glycine max* (L.) Merr., turns out to be much more complex than a simple comparison of chromosome numbers would suggest.

Materials and Methods

Plant Material and Metaphase Preparation

Seeds of wild *Glycine* species—*G. canescens* (PI 440936 and 446937), *G. clandestina* (PI 339656 and 440958), *G. soja* (PI 81762), *G. tabacina* (PI 193232, 378704 and 440996), and *G. tomentella* (PI 441005), kindly provided by Dr. Theodore Hymowitz, Department of Agronomy, University of Illinois, Urbana, and of *G. max* (L.) Merr., cultivar Bedford (from the Alabama A&M Seed Laboratory)—were used as the sources for metaphase chromosome spreads. The terminal 1 cm of the roots was excised from individual seedlings, pretreated in 2.5 mM 8-hydroxyquinoline for 4 h at room temperature and fixed overnight in freshly prepared, room temperature, 3:1 (v/v) ethanol : glacial acetic acid. The root tips were treated with 0.1 N HCl for 5 min before incubation in a cell wall digestive enzyme cocktail of 5% R-10 cellulase and 1% pectolyase Y-23, for a duration of 5–20 min based on the length and thickness of the root tips, in a 37°C water bath. Metaphase spreads were prepared from the terminal 1 mm of the enzyme-treated root tips as described by Jewell and Islam-Faridi (1994).

Probe and Carrier DNA

Probes for 5S rRNA were generated from pAM033 which contained a 470 bp *Bam*HI-digested fragment of the 5S ribosomal RNA repeat of *Acacia melanoxylon* in pUC 118, provided by Dr. R. Appels, CSIRO, Australia. The plasmid pGMR3, containing a 4.5 kb *Eco*RI-digested fragment of the 18S-28S ribosomal RNA repeat of *G. max* in pBR325, provided by Dr. E. Zimmer, Smithsonian Institution, Washington, DC, was used for the 18S-28S rRNA site localization. Both the plasmids were isolated by the alkaline lysis plasmid maxiprep method as described by Silhavy et al. (1984). Whole plasmid DNA was labeled with biotin-14-dATP (BRL) using the Gibco BRL BioNick™ Labeling System or with digoxigenin-11-dUTP using the Boehringer Mannheim Nick Translation Kit. Commercially purchased *E. coli* DNA, sheared to an average fragment size of 200–500 bp, was used as the carrier DNA.

In situ Hybridization (Islam-Faridi and Mujeeb-Kazi 1995)

Slides were immersed in 30 μg/ml RNase/2× SSC for 45 min at 37°C, denatured in 70% formamide/2× SSC for 70 s at 70°C and then dehydrated in 70, 95, and 100% ethanol for 2 min each at −20°C. Probe mix (deionized formamide, 50% dextran sulfate, 15 μg/slide *E. coli* carrier DNA and 30 ng/slide labeled probe DNA in 2× SSC) was denatured at 80°C for 10 min, chilled on ice, applied to the slide, covered with a 20 mm × 40 mm coverslip, and sealed with rubber cement. Following overnight incubation at 37°C, the slides were rinsed at 40°C in 2× SSC twice for 5 min each, 2× SSC/50% formamide for 10 min, and 2×

Table 1. 18S-28S and 5S rDNA sites in species of genus *Glycine*

			Number of sites /metaphase spread			
			18S-28S rDNA		5S rDNA	
Species	2*n*	Genome symbol	Observed	Expected[a]	Observed	Expected[a]
G. canescens		AA				
PI 440936	40		2[b]	4	2	4
PI 446937	40		2	4	2	4
G. clandestina		A_1A_1				
PI 339656	40		2	4	2	4
PI 440958	40		2	4	2	4
G. soja		GG	2	4	2	4
PI 81762	40					
G. max		GG	2	4	2	4
Bedford	40					
G. tabacina		(complex)				
PI 440996	80		4 major 2 minor	8	4	8
PI 193232	80		N.D.	N.D.	4	8
PI 378704	80		N.D.	N.D.	4	8
G. tomentella		DD (complex)				
PI 441005	80		4 major 2 minor	8	4	8

[a] The number of sites expected in a tetraploid ($2n = 4x = 40$) and octaploid ($2n = 8x = 80$) based on an ancestral polyploid event in the evolution of *Glycine*.

[b] One pair of signals = two sites = one locus.

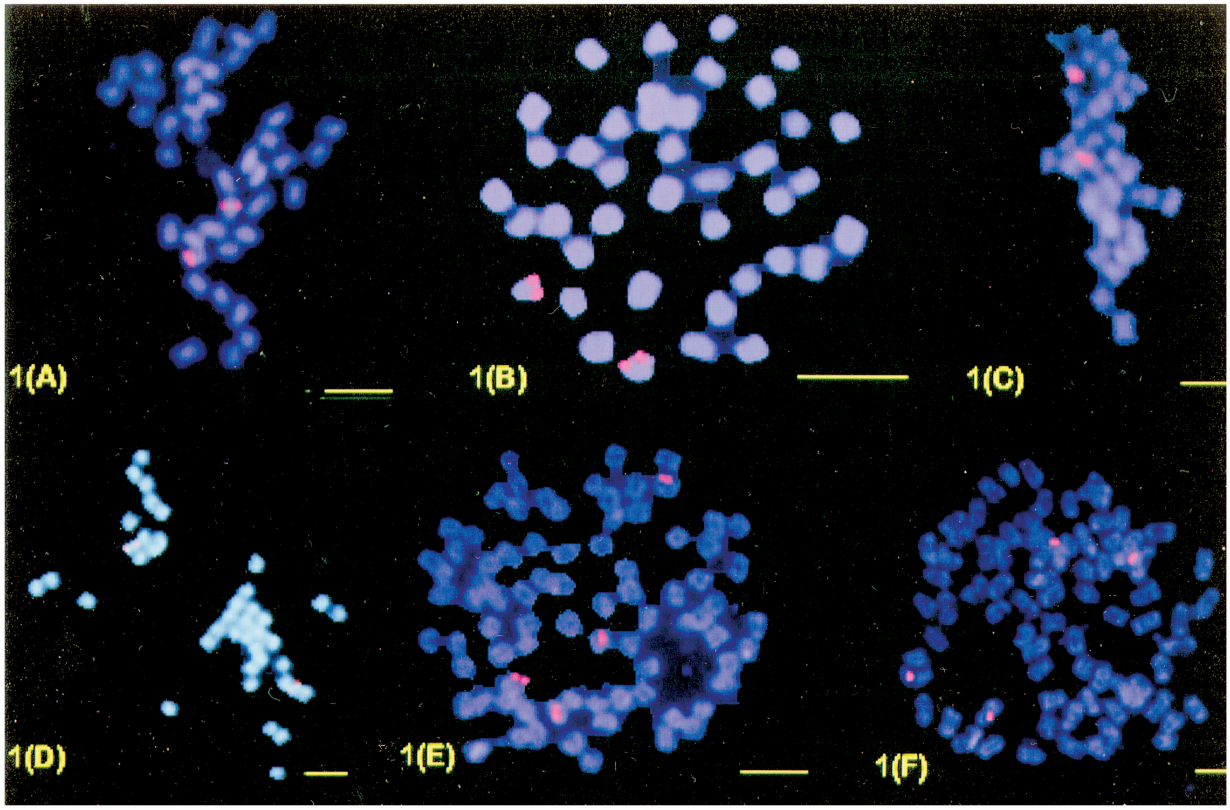

Figure 1. Fluorescence photomicrographs of metaphase chromosomes from various *Glycine* species hybridized with a digoxigenin-labeled 5S rDNA probe. Signals were detected using Cy3 and chromosomes were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Images were digitally captured in gray scale and the appropriate colors for the chromosomes and the signals were superimposed and contrast adjusted using the IPLab Spectrum P software. **(A)** *G. canescens* [(2*n* = 40), subgenus *Glycine*], showing one 5S rDNA locus. **(B)** *G. clandestina* [(2*n* = 40), subgenus *Glycine*], a single 5S rDNA locus. **(C)** *G. soja* [(2*n* = 40), subgenus *Soja*], one 5S rDNA locus. **(D)** *G. max* [(2*n* = 40), subgenus *Soja*], one 5S rDNA locus. **(E)** *G. tabacina* (2*n* = 80), two 5S rDNA loci. **(F)** *G. tomentella* (2*n* = 80), two 5S rDNA loci. Bar represents 5 μm.

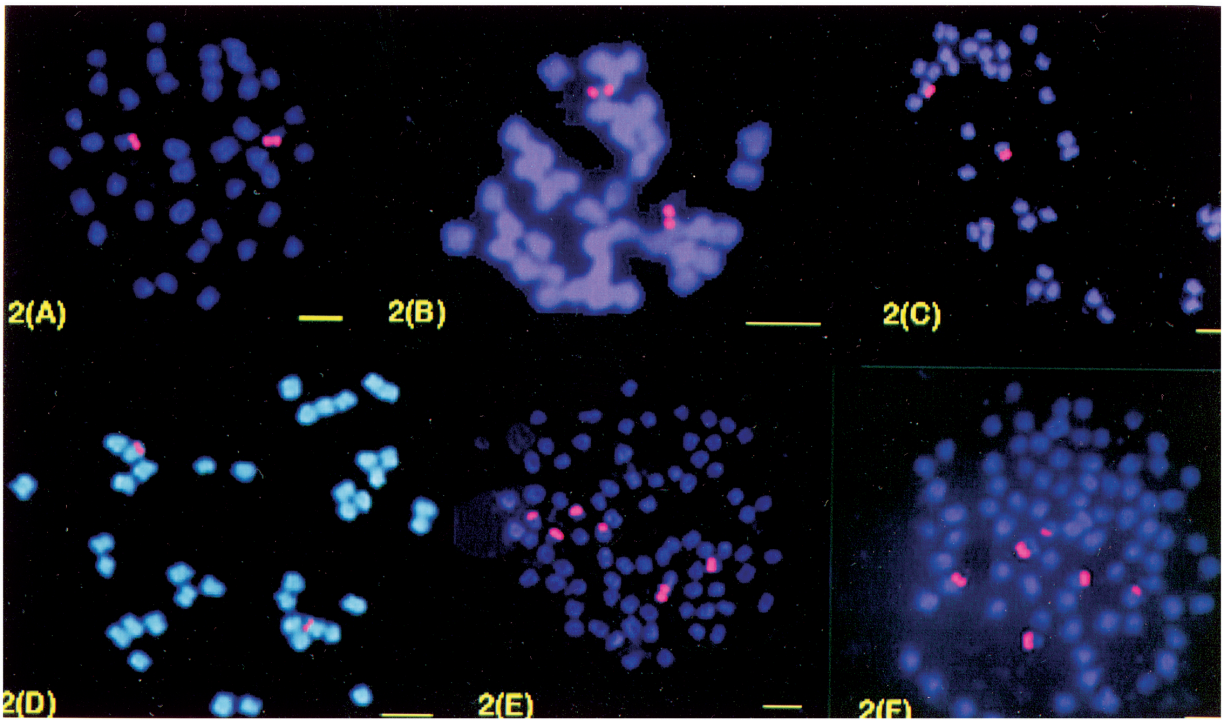

Figure 2. Fluorescence photomicrographs of metaphase chromosomes from various *Glycine* species hybridized with a digoxigenin-labeled 18S-28S rDNA probe. Signals were detected using Cy3 and chromosomes were counterstained with 4,6-diamidino-2-phenylindole (DAPI). **(A)** *G. canescens* [(2*n* = 40), subgenus *Glycine*], showing one 18S-28S rDNA locus. **(B)** *G. clandestina* [(2*n* = 40), subgenus *Glycine*], a single 18S-28S rDNA locus. **(C)** *G. soja* [(2*n* = 40), subgenus *Soja*], one 18S-28S rDNA locus. **(D)** *G. max* [(2*n* = 40), subgenus *Soja*], one 18S-28S rDNA locus. **(E)** *G. tabacina* (2*n* = 80), two major and one minor 18S-28S rDNA loci. **(F)** *G. tomentella* (2*n* = 80), two major and one minor 18S-28S rDNA loci. Both the major and minor loci in the polyploids are located on separate chromosomes. Bar represents 5 μm.

SSC for 5 min. The slides were incubated at room temperature in solutions of 2× SSC for 5 min, 1× SSC twice for 5 min each, and 4× SSC/0.2% Tween-20 for 5 min.

Signal from biotin-labeled probes was amplified and detected with sequential applications of 5 μg/ml FITC-avidin DCS in 5% BSA-4× SSC/0.2% Tween-20 for 30 min at 37°C, 5 μg/ml biotinylated-antiavidin D in 5% NGS-4× SSC/0.2% Tween-20 for 30 min at 37°C and 5 μg/ml FITC-avidin DCS in 5% BSA-4× SSC/0.2% Tween-20. Signal from digoxigenin-labeled probes was amplified with 2 μg/ml mouse antidigoxigenin (MAD) in 5% BSA-4× SSC/0.2% Tween-20 for 30 min at 37°C and detected with 5 μg/ml Cy3 anti-mouse in 5% NGS-4× SSC/0.2% Tween-20 for 30 min at 37°C. Between steps, slides were washed four times in 4× SSC/0.2% Tween-20 for 5 min each at 37°C. Chromosomes were stained with 3 μg/ml DAPI in McIlvaine's buffer (9 mM citric acid, 80 mM $Na_2HPO_4 \cdot H_2O$, 2.5 mM $MgCl_2$, pH 7.0) for 45 min at room temperature and destained in 4× SSC/0.2% Tween-20 for 20 s. Slides detected with FITC-avidin-DCS were further stained with 20 μg/ml of propidium iodide-2× SSC for 30 min at room temperature and destained for 20 s in 2× SSC. Vectashield™ antifade agent was applied to the slides before a 20 mm × 40 mm coverslip was placed over the slides.

Metaphase Observation and Photography

Images were digitally captured in gray scale using a Nikon cooled-CCD camera system and standard Olympus filter sets for ultraviolet (DAPI), triple band pass (DAPI/Cy3), and blue (PI/FITC) excitation. The appropriate colors for the chromosomes and the signals were superimposed and contrast adjusted using the IPLab Spectrum P software on an Apple Macintosh Power PC. The final images were printed using Adobe Photoshop, version 5.0.

Results

Single-label FISH, rather than dual-label FISH, was used to detect 5S rDNA and 18S-28S rDNA signals with the greatest possible sensitivity. Only sites at which two signals were visible, that is, one per chromatid, were scored. Major sites were defined as those giving very large pairs of signals observable in all interphase and metaphase cells. Smaller FISH signals, detectable in 30–40% of metaphase cells observed, were described as minor signals. The description and the number of sites for both 18S-28S and 5S rDNA signals are given in Table 1. Representative photomicrographs of the results are shown in Figures 1A–F and 2A–F.

Two high-stringency washes were effective in eliminating background hybridization and greatly enhanced the reliability of our results. The additional 1× SSC washes increased the signal : noise ratio by minimizing nonspecific hybridization. Signal intensity was not compromised by the higher stringency washes for both the 18S-28S and the 5S rDNA probes.

One major pair of 18S-28S rDNA FISH signals were observed in all of the interphase and metaphase spreads examined in the AA genome types of *G. canescens* and *G. clandestina* ($2n = 40$) (Figure 2A,B). *G. soja* and *G. max* ($2n = 40$), belonging to the GG genome type, also exhibited one major pair of 18S-28S rDNA FISH signals in all the spreads examined (Figure 2C,D). The polyploids *G. tabacina* and *G. tomentella* ($2n = 80$) (Figure 2E,F) exhibited a pattern of two major and one minor pair of FISH signals. All of the 18S-28S rDNA signals observed were telomerically or subtelomerically located.

A single major pair of 5S rDNA FISH signals was observed in all of the interphase and metaphase spreads in the ($2n = 40$) members (Figure 1A–D) of the genus *Glycine* that we examined. Only two major pairs of 5S rDNA FISH signals were seen in the polyploid *G. tomentella* and *G. tabacina* ($2n = 80$) (Figure 1E,F). The 5S rDNA loci were located distally on the chromosome pairs of all the species examined.

Discussion

The "diploid" ($2n = 40$) species examined in the subgenus *Glycine* and the subgenus *Soja,* irrespective of their genome type, have a single 5S rDNA locus per haploid genome. The polyploids ($2n = 80$) *G. tomentella* and *G. tabacina* clearly have two 5S rDNA loci per haploid genome. Previous FISH studies have shown *G. max* (L.) Merr as having a single 5S rDNA locus (Shi et al. 1996) and a single 18S-28S rDNA locus (Skorupska et al. 1989). CHEF gel electrophoresis detected a single 5S rDNA locus in *G. soja* (Danna et al. 1996) and Southern blot analysis showed two major 5S repeats in *G. tomentella* (Doyle and Brown 1989). However, this is the first confirmation of these results by FISH. If the genus *Glycine* has an allotetraploid origin (Hymowitz and Singh 1987; Kumar and Hymowitz 1989; Lackey 1980; Zhu et al. 1994) as has been postulated, then our results clearly demonstrate the loss of a 5S rDNA locus from the "diploid" species and loss of two from the polyploids of *Glycine.*

A similar diploidization of the 18S-28S rDNA array has apparently occurred in *G. canescens, G. clandestina, G. soja,* and *G. max* (L.) Merr. ($2n = 40$) since only half the expected number of loci per haploid genome were found. Both *G. tabacina* and *G. tomentella* ($2n = 80$), although of different genome types, showed two major loci per haploid genome in the metaphase spreads observed, as opposed to the four loci that would be expected in chromosomes that have undergone two doubling events in their evolutionary history. More than 50% of the spreads examined also detected an additional minor locus on a different chromosome from that of the major locus.

Evolutionary Implications

Both 5S and 18S-28S rDNA loci have experienced a diploidization event for members of both subgenera *Soja* and *Glycine* through physical loss of the sequences and not just loss of function. As these loci are on different chromosomes, at least in *G. max* (Shi et al. 1996), there must have been a separate deletion of each locus in the evolutionary past.

Loss or addition of rDNA loci during the evolution of a polyploid plant species has been documented in *Triticum* (Kim et al. 1993; Mukai et al. 1991), *Aegilops* (Badaeva et al. 1996), *Gossypium* (Crane et al. 1993; Hanson et al. 1996), and *Avena* (Jellen et al. 1988). Investigations of newly formed polyploids (Comai et al. 2000; Xu et al. 2000) show great genomic and phenotypic instability. Mechanisms responsible for the variation in the size and the number of the rDNA loci may include (1) translocation breakpoints near the locus that may have occurred and the sites may have been duplicated following polyploidization of the species (Hanson et al. 1996), (2) minor sites may have been added or deleted through nonhomologous unequal crossing over within the locus (Arnheim et al. 1980; Seperack et al. 1988), and (3) telomeric or subtelomeric positions of the rDNA loci would possibly allow significant rearrangements to occur without deleterious effects to the cells (Bennett 1982; Hanson et al. 1996). Additions, deletions, and rearrangements of genetic material, with concomitant phenotypic abnormalities, can occur but must eventually stabilize into genetically stable species (Grant

et al. 2000; Paterson et al. 2000; Wendel 2000). Such early instability could account for the presence of genome duplication (Shoemaker et al. 1996; Lee et al. 1999), satellite chromosomes (Huiyu and Ruiyang 1984) as well as diploidization (Grant et al. 2000; Hadley and Hymowitz 1973) within the genus.

Diploidization of both the rDNA loci could have occurred soon after the original polyploid event that resulted in the $2n = 40$ (4 FISH-detectable sites) *Glycine* ancestor, which was itself the result of a polyploidization of $2n = 20$ (2 FISH-detectable sites) species that then evolved into the present-day $2n = 40$ and $2n = 80$ *Glycine* spp. It can also be speculated, but less likely, that each *Glycine* member underwent deletion events independently after species radiation, indicating possible deletion hotspots in the chromosomes involved. The time frame of these diploidization events may be challenged if (1) more than one rDNA locus is found in the remaining ($2n = 40$) *Glycine* spp. or (2) if ($2n = 20$) members can be found (Kumar and Hymowitz 1989). If the report of a *G. max* ($2n = 20$) (Pillai 1976) can be confirmed, the number of 5S and 18S-28S rDNA loci must be investigated.

The number of 5S rDNA loci in the ($2n = 80$) *Glycine* members suggests an origin through polyploidy of two diploidized parents. However, the presence of major and minor 18S-28S rDNA loci complicate such a simple scenario. Confounding an explanation is the tremendous diversity found within both species, indicating possible multiple origins (*G. tabacina*) (Doyle et al. 1999) or active radiation/speciation (*G. tomentella*) (Singh et al. 1998). Multiple accessions within each species will have to be investigated to evaluate homologous relationships, if any, among their rDNA loci.

As FISH yields semiquantitative results, that a major and minor site are homologous or orthologous will depend on sequence information and the multiplicity of an array repeat unit within a locus. The 18S-28S rDNA minor sites that we have observed could either represent reduction of a major site through partial deletion, the addition of a smaller array from a larger rDNA array by unequal rearrangement, or partial array duplication.

Rapid evolution of multigene families is likely to produce readily detected polymorphisms between related species or among members of a species (Danna et al. 1996). Repeat units within a tandem array of a multigene family typically undergo concerted evolution (Dover 1986). Unequal crossing over can also change copy number of the array repeat unit as observed for the 5S rRNA genes in flax plants subjected to environmental stress (Schneeberger and Cullis 1991). Patterns of evolution in plants have been uncovered through analysis of such polymorphisms in the rDNA in species of the Triticeae (Kim et al. 1993; Mukai et al. 1991), *Arabidopsis* (Maluszynska and Heslop-Harrison 1993a), *Gossypium* (Crane et al. 1993), and *Brassica* (Delseny et al. 1990; Maluszynska and Heslop-Harrison 1993b).

It is evident that the evolution of the subgenus *Glycine* is much more complex than represented by a simple diploid → tetraploid (via chromosome doubling) model. Additional FISH studies on other species within the subgenus *Glycine* are necessary to completely decipher the evolutionary history of this important genus. And lastly, the restrained multiplicity of rDNA loci and their easy detection by FISH facilitates their use as a powerful tool for studies on the evolutionary behavior of repetitive gene families in soybean and other species.

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