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Restriction fragment length polymorphism (RFLP) of wild perennial relatives of soybean

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Summary. Total DNA from callus tissue of 28 accessions representing seven wild perennial *Glycine* species was compared using recombinant genomic probes derived from *G. max,* the soybean. Using two probes, we show that this molecular approach both confirms and extends the model for the taxonomic relationships between the species derived from morphological and cytogenetic data, and that it provides clear evidence that RFLP analysis of genomic sequences has the potential for revealing the derivation of the member species of the wild perennial *Glycine* taxon. Although, in this preliminary report, the sample size for each species is small, it is clear that the greatest between-accession variation occurs in *G. tabacina* (B_2B_2) and *G. clandestina* (A_1A_1), suggesting that these may be the taxa from which further speciation occurred in the subgenus.

Key words: *Glycine* spp. – RFLP – Taxonomy – Wild perennial species

Introduction

Restriction fragment analysis and restriction site mapping of nuclear DNA have been used to detect and assess phylogenetic relationships in several plant genera (Doyle 1987; Doyle and Beachy 1985; Helentjaris et al. 1985). These molecular methods of analysis employ restriction endonuclease digestion and hybridization with specific cloned probes to compare homologous DNA fragments within and between species. These DNA techniques give a direct analysis of the genome not provided by morphological and protein analytical methods, and are thus powerful tools for biosystematic studies.

The subgenus *Glycine,* as currently defined (Singh et al. 1988), comprises 15 wild perennial herbaceous species distributed mainly in Australia, north to South China, and in the southwest rim of the Pacific (Broué et al. 1977; Hymowitz and Singh 1987; Newell and Hymowitz 1978; Tindale and Craven 1988). The 15 species have no known commerical value, but their wide phenotypic variation and taxonomic proximity to the cultivated soybean, *Glycine max* (L.) Merr., make them a potential source of germ plasm for the improvement of the cultigen. Indeed, soybean has been successfully crossed with several of the wild perennial species (Singh et al. 1987). A wide variety of classical systematic approaches have been used to define relationships within the taxon (Constanza and Hymowitz 1987; Singh and Hymowitz 1985; Singh et al. 1988). Although these have refined the classification of the subgenus, they can only give a partial analysis of the limited set of characters available for scoring.

The only existing report of RFLP analyses showing species relationships in the genus *Glycine* subgenus *Glycine* used a cytoplasmic ribosomal gene as the probe (Doyle and Beachy 1985). These results showed considerable variation in repeat size and presence of EcoRI sites between accessions of five of the species examined (only single accessions of the other two species were analyzed). Since, however, few of the fragments could be matched between species, little could be concluded about the relationships between the species. This pilot study was, therefore, undertaken to determine whether single-copy genomic sequence RFLP analysis would prove to be a more flexible approach to use in biosystematic studies of the subgenus. We have used the existing classification based on morphological and karyotypic characters as a reference. The results, even with a very limited set of heterologous probes, isolated from the soybean, show that

this technique is capable of revealing considerable detail about the relationships between the species and also about the amount of variation within individual species. Of particular interest is the observation that *G. tabacina* and *G. clandestina* show more variation than any of the other species examined and may be the progenitor species from which many of the others evolved.

Materials and methods

Plant material and tissue culture

Twenty-eight accessions representing seven diploid species in the subgenus *Glycine* (Table 1) were cultured to produce callus for DNA isolation. Five seeds per accession were germinated in MS (Murashige and Skoog 1962) medium without hormones, in the dark for 10–14 days. Hypocotyls were cut into 1-cm sections and placed on either MS basal medium containing 2.0 mg/l 2,4-D and 1.0 mg/l benzoyl amino purine (BAP) or the basal medium of Phillips and Collins (1979) containing 0.6 mg/l picloram and 0.1 mg/l BAP. Subcultures were made every 2 weeks until at least 10 g of callus were obtained per accession.

Table 1. Accessions of the wild *Glycine* species used in RFLP analysis and their location and origin

Species	IL No. [a]	Origin
G. canescens F. J. Herm.	379 ¥	Alice Springs, NT
	438	Mt. Hopeless, SA
	461	Condobolin, NSW
	670	Stanley Chasm, NT
G. clandesina Wendl.	306 *f*	Purgatory Creek, NSW
	471	Ingebyra, NSW
	473 ¥	Clyde Mountain, NSW
	490 *f*	Grenfell, NSW
	507	Fineflower, NSW
	578	Tasmania
	631 ¥	Jerangle, ACT
G. cyrtolona Tind.	480	Brampton Is., Qd
	482 ¥	Brampton Is., Qd
G. latifolia (Benth.) Newell and Hymowitz	373	'Inverelle', NSW
	375	Capella, Qd
	491 ¥	Rockdale, NSW
G. microphylla (Benth.) Tind.	449	Cook Dist., Qd
	502 *f*	Grafton, NSW
	503	Wahroonga, NSW
	541	Kangaroo Valley, NSW
	542	Clyde Mountain, NSW
G. tabacina (Labill.) Benth.	336 ¥	'Cangai' 'No. 1', NSW
	342/342-1	Purgatory Creek, NSW
	343	Grafton, NSW
	347	Chinchilla, Qd
	370	Toorooka, NSW
G. tomentella Hayata	367 [b]	Condobolin, Qd
	398 [b]	Condamine, Qd
	601 *f*	Port Moresby, PNG

[a] IL numbers are temporary University of Illinois accession numbers

[b] All accessions have *2n* chromosome numbers of 40 except *G. tomentella* accession nos. 367 and 398, which have a *2n* number of 38

¥ – Only assayed with the pE15/EcoRI probe-enzyme combination

f – Only assayed with the NN-8/DraI probe-enzyme combination

DNA isolation

The total callus tissue derived from all five seeds of each accession was harvested and excess water was removed by suction drying. Total DNA was extracted using a rapid protocol developed by Dr. R. Hauptman (personal communication). Ten to twenty grams of tissue was frozen in liquid nitrogen and ground into a fine powder in a mortar and pestle. The tissue was allowed to thaw on ice and 1 ml/g tissue weight of TESE extraction buffer was added (50 m*M* *Tris*-HCl, pH 8.0; 50 m*M* EDTA, pH 8.0; 50 m*M* NaCl; 400 µg/ml ethidium bromide; 2% sarkosyl). The slurry was shaken vigorously and centrifuged at 10,000 rpm in a Sorvall SS34 rotor for 30 min. The supernatant was collected and the DNA banded in cesium chloride (0.975 g/ml initial concentration) in the presence of ethidium bromide (400 µg/ml) at 55,000 rpm in a Sorvall T1270 angle rotor for 20 h at 15 °C. The DNA band was removed from the tube and extracted at least five times with 20 × SSC (SSC is 0.15 *M* NaCl; 0.015 *M* sodium citrate) saturated isopropanol to remove the ethidium bromide. The DNA was recovered by ethanol precipitation (2 vol) after dilution of the solution three times with distilled water. The resulting pellet after centrifugation was dried under vacuum and redissolved in TE (10 m*M* *Tris*-HCl, pH 8.0; 1 m*M* EDTA). Yields were of the order of 5 µg/g fresh wt callus.

Genomic blots

Ten micrograms of total DNA were digested at 37 °C with the restriction endonucleases EcoRI or DraI (Anglian Biotech. Ltd.) in the buffers recommended by the manufacturer. When trace agar contamination of the DNA preparations inhibited restriction endonuclease digestion, spermidine was added to a final concentration of 1 m*M* (Bouché 1981). After digestion, the DNA was precipitated, dissolved and fractionated in TPE (80 m*M* *Tris*-phosphate; 8 m*M* EDTA) on 1.0% agarose gels (Seakem, LE). After electrophoresis, the fractionated DNA was transferred to nylon membrane filters (Gelman Biotrace RP) in 0.4 *M* NaOH (Reed and Mann 1985).

Source of probes

Two probes from different sources were used in this pilot study. pE15 is a plasmid-vector genomic clone of the soybean urease gene from Dr. J. Polacco (Krueger et al. 1987). NN-8 is a bacteriophage M13 clone of soybean genomic DNA from Dr. K. G. Lark.

Preparation of radioactive probes

The 11 kb EcoRI insert fragment from the plasmid pE15 was gel purified following digestion with EcoRI and fractionation on 1.0% low-melting point agarose gels (BRL) in glycine buffer (0.2 *M* glycine, 15 m*M* NaOH, 2 m*M* EDTA; final pH 9.0). Gel slices containing the insert fragment were cut from the gel, boiled for 5 min and the DNA was radiolabeled according to the procedure of Feinberg and Vogelstein (1983, 1984). The labeled probe was separated from unincorporated nucleotides by column chromatography on Sephadex G100 in TE.

The M13 probe (NN-8) was labeled by primed synthesis using the single-stranded phage DNA as template. The primer

annealing reaction was done using 1 µg of phage DNA in 50 m*M* NaCl, 10 m*M* *Tris*-HCl (pH 7.5), 10 m*M* $MgCl_2$ and 5 ng universal sequencing primer. Primer annealing was done for 10 min at 70 °C followed by cooling to room temperature for 15 min. Primed synthesis was done in annealing buffer containing 10 m*M* dithiothreitol, 3 m*M* each of dATP, dGTP, and dTTP, 50 µCi ^{32}P-dCTP (3,000 Ci/mmol; NEN or ICN), and 2.5 units of Sequenzyme (Anglian Biotech. Ltd.). The mixture was incubated for 1.5 h at room temperature. Unincorporated nucleotides were removed by column chromatography on Sephadex G100 in TE.

Hybridization and autoradiography

Blots were pre-washed for 1–3 h at 42 °C in 10 ml/100 cm^2 of filter of 1 *M* NaCl, 0.5% sodium dodecyl sulphate (SDS), 50 m*M* *Tris*-HCl (pH 8.0) and 1 m*M* EDTA. Prewashed filters were prehybridized overnight at 42 °C in a mixture of 50% formamide (Aldrich), 5 × SSC, 20 m*M* phosphate buffer (pH 6.5), 5 × Denhardt's (1966) reagent, 1% SDS, 2.5% Dextran sulfate (Sigma) and 100 µg/ml sheared, heat-denatured salmon sperm DNA (Sigman Type III). The prehybridization mixture was heated to 90 °C for 15 min and cooled to 42 °C prior to use. After prehybridization, the solution was removed and replaced with 10 ml hybridization solution per 100 cm^2 of filter. Hybridization solution differed from prehybridization solution in that the phosphate buffer concentration was reduced to 5 m*M* and the dextran sulfate concentration was increased to 5%. Heat-denatured radioactive probe (at least 1×10^7 cpm/ml) was added and hybridization was carried out overnight at 42 °C. The blots were then washed twice in 2 × SSPE (SSPE is 0.18 *M* NaCl, 10 m*M* phosphate buffer (pH 7.4), 1 m*M* EDTA) containing 0.5% SDS for 2 h at 42 °C and 1 h at 65 °C. They were then washed in 0.2 × SSPE containing 2% SDS for 1 h at 65 °C, blotted dry, wrapped in plastic wrap, and autoradiographed overnight at −70 °C with intensifying screens (Dupont).

After autoradiography, hybridized probes were stripped from the membranes by washing with 0.4 *M* NaOH at 42 °C for 1 h and rinsed in 0.2 *M* *Tris*-HCl (pH 8.0), 0.5% SDS, 0.1 × SSC. Blots were probed up to four times without noticeable loss of resolution or signal intensity.

Results

It is not practical to use leaf material for the isolation of genomic DNA from many of the wild perennial *Glycine* species because of their small leaf size and slow growth rate. For this reason, fresh callus was initiated from hypocotyls of the accessions to be examined. This approach generated 10 g of tissue for DNA isolation faster than did cultivating the plants themselves. A problem inherent in the use of cultured tissue is that alterations in the genome can occur during culture, which can manifest themselves in regenerated plants as somaclonal variation (Larkin and Scowcroft 1983; Lee and Phillips 1987; Singh 1986). The observation that, for several species, there is no observable variation in fragment pattern between accessions suggests that such variation is either not a problem over the time of culture used here (up to 10 weeks) or that the particular sequences that we have analyzed are not prone to variation of this type. We are currently assaying replicate DNA samples from separate callus initiations of the same accession for several of the species with probes isolated from *Glycine clandestina* Wendl., to confirm this observation. For this pilot study, only single culture initiations were used for DNA isolation and only two probe/enzyme combinations are presented. Genomic DNA samples digested with EcoRI were probed with the 11-kb insert fragment of pE15 and those digested with DraI were probed with NN-8.

B-genome species group analysis

The B-genome species group comprises *Glycine latifolia* (Benth.) Newell and Hymowitz (B_1B_1), *Glycine microphylla* (Benth.) Tind. (BB), and *Glycine tabacina* (Labill.) Benth. (B_2B_2). *Glycine cyrtoloba* Tind. (CC), although not a member of the B-genome group per se, is believed to be more closely related to B-group than to A-group species (Singh et al. 1988). Analysis of the EcoRI-fragment patterns with the probe pE15 and the DraI-fragment patterns with probe NN-8 confirms these conclusions (Fig. 1). *Glycine microphylla* shows a highly conserved pattern with both probe/enzyme combinations over the 1,130 km range within which the accessions were collected (New South Wales to the southern part of Queensland; Table 1). Only accession 502 shows a pattern variant in that it appears to lack the major 2.0-kb NN-8/DraI fragment (Fig. 2). The pE15/EcoRI pattern seen in three *G. latifolia* accessions is identical to that seen in *G. microphylla*. In contrast, the two *G. latifolia* accessions that were assayed with the NN-8/DraI combination show a pattern identical to that shown by two of the *G. tabacina* accessions. Both *G. cyrtoloba* accessions show identical pE15/EcoRI patterns, which share three out of five fragments with the *G. microphylla* pattern. The two extra bands are unique to this species. The fragment pattern seen in *G. cyrtoloba* with the NN-8/DraI probe/enzyme combination is, however, identical to that seen in the majority of the *G. microphylla* accessions. This similarity in fragment pattern clearly confirms *G. cyrtoloba's* close relationship to the B-genome group despite its lack of adventitious roots, the character currently taken to be diagnostic of B-genome group members (Constanza and Hymowitz 1987). This supports earlier conclusions on the relationship between *G. cyrtoloba* and B-genome group species based on leaf venation and pod length (Newell and Hymowitz 1983; Tindale 1984).

Glycine tabacina, in contrast to the other B-genome species, shows a high level of within-species polymorphism when assayed with either probe/enzyme combination. Fragment pattern identities vary with the probe/enzyme combination used. pE15/EcoRI analysis shows two accessions (370 and 336) with identical fragment patterns to that of *G. microphylla* and *G. latifolia,* while NN-8/DraI analysis shows identity of accessions 343 and 370 only with the *G. latifolia* pattern. Since accession 336 was

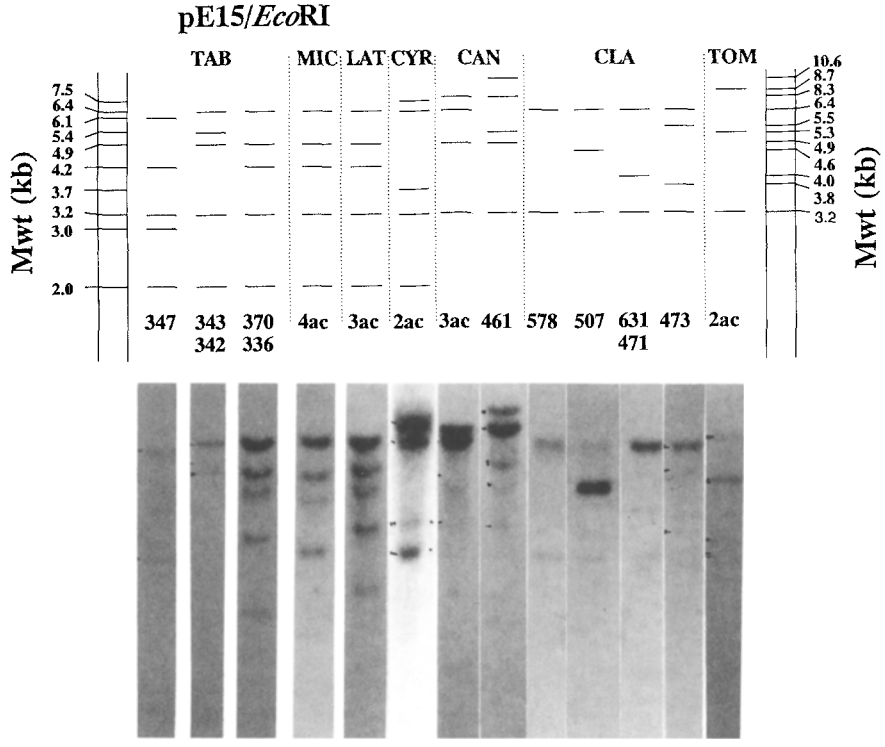

Fig. 1. Analysis of wild perennial *Glycine* species using the pE15/EcoRI probe/enzyme combination. Autoradiographs show the fragment patterns seen when EcoRI digests of genomic DNA from accessions of the wild perennial *Glycine* species were probed with an 11-kb fragment from the soybean urease gene. Since the samples shown were analyzed on more than one gel, the diagram shows the fragments aligned on the basis of their mobilities relative to those of the HindIII fragments of bacteriophage lambda. CAN = *G. canescens*; CLA = *G. clandestina*; CYR = *C. cyrtoloba*; LAT = *G. latifolia*; MIC = *G. microphylla*; TOM = *G. tomentella*. The codings *2ac*, *3ac* and *4ac* indicate the number of accessions which showed the pattern depicted. The catalog numbers of the accessions involved in each case can be derived from the listings in Table 1

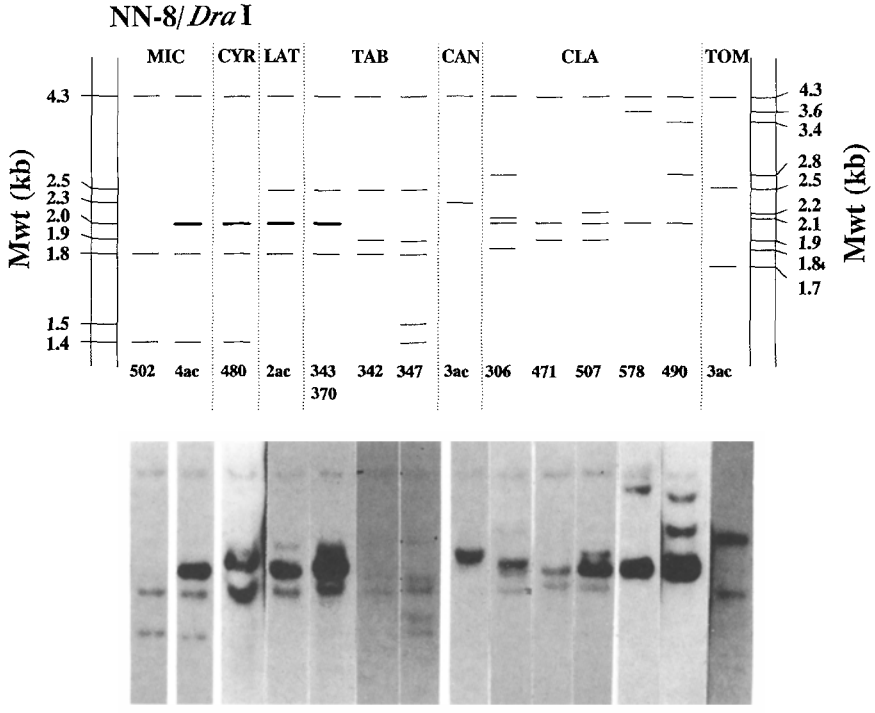

Fig. 2. Analysis of wild perennial *Glycine* species using the NN-8/DraI probe/enzyme combination. Autoradiographs show the fragment patterns seen when DraI digests of genomic DNA from accessions of the wild perennial *Glycine* species were probed with a random genomic clone from soybean. Since the samples were analyzed on more than one gel, the diagram shows the fragments aligned on the basis of their mobilities relative to those of the HindIII fragments of bacteriophage lambda. CAN = *G. canescens*; CLA = *G. clandestina*; CYR = *G. cyrtoloba*; LAT = *G. latifolia*; MIC = *G. microphylla*; TOM = *G. tomentella*. The codings *2ac*, *3ac*, and *4ac* indicate the number of accessions which showed the pattern depicted. The catalog numbers of the accessions involved in each case can be determined from the listings in Table 1

not tested with the NN-8/DraI combination, it is not known whether it shows the same relationship. Accession 343 is different from both *G. microphylla* and *G. latifolia* when assayed with the pE15/EcoRI combination.

From these data, it is clear that *G. tabacina* contains significantly more variation than the other members of the B-genome group. In early classification schemes, *G. microphylla* and *G. latifolia* were considered pod-shape and size variants of *G. tabacina*, and only more recently have they been defined as separate species on the basis of more rigorous morphological and cytogenetic analyses. This suggests that these species (and indeed the

less closely related *G. cyrtoloba*) may have recently evolved from the *G. tabacina* gene pool.

A-genome species group analysis

Two of the three principal species in the A-genome group (*Glycine canescens* F. J. Herm. and *Glycine clandestina* Wendl.) were assayed with the two probe/enzyme combinations. The three *G. canescens* accessions show no variation in fragment pattern when assayed with the NN-8/DraI probe/enzyme combination. Two of these accessions show identical pattern when assayed with the pE15/EcoRI probe/enzyme combination, as does a fourth assayed only with this combination. One of the accessions (461), although showing the typical NN8/DraI pattern, shows an atypical pattern using the pE15/EcoRI combination.

Glycine clandestina shows considerable variation in fragment pattern when assayed with either probe/enzyme combination. With each combination, the one (NN-8/DraI: 4.3 kb) or two (pE15/EcoRI: 6.4 and 3.2 kb) fragments that are common to most of the species can be seen. The patterns of additional fragments are, in general, unique to individual accessions and there are few similarities between these accession-specific fragments and fragments appearing in analyses of any of the other A- or B-genome group species.

These data show that there appears to be more diversity in the A- than in the B-genome group. *Glycine canescens* has been classified cytogenetically as AA (Singh et al. 1988). In the analyses presented here, however, the fragment pattern observed in the *G. canescens* accessions show as much similarity to those of the B-genome species as they do to the other A-genome species. As with the B-genome species analyses, one species in the A-genome group (*G. clandestina*; A_1A_1) appears to contain significantly higher levels of variation than does the other. The third confirmed member of the A-genome group, *Glycine argyrea* (A_2A_2), of which only three accessions are known, was not tested in this pilot program.

The final species tested in this limited survey, *Glycine tomentella* Hayata, shows complex cytological and molecular variation (Doyle and Brown 1989). The accessions assayed here are of the $2n=2x=40$ (DD genome) or $2n=2x=38$ (EE genome) classes, yet they show simple fragment patterns with little or no variation and little or no similarity to any of the other patterns seen in the A- and B-genome species. None of the other diploid or tetraploid accessions of this species were studied in this limited survey.

Discussion

The data we have presented here show clearly that the use of single-copy genomic probes has the potential for providing considerable information on the evolution and species relationships between the wild *Glycine* species. Even with the small sample size that we have analyzed using heterologous probes, the relationships deduced from analysis of cytogenetic (Singh and Hymowitz 1985; Singh et al. 1988), isozyme (Menancio 1988), and morphological (Constanza and Hymowitz 1987) characters have been both confirmed and expanded. It is clear that within the B-genome species group, *G. tabacina* contains more variation than do any of the other species. In the A-genome species group, it is *G. clandestina* which shows the highest level of variation. It appears that the A-genome group, in general, shows less similarity between species than does the B-genome group. The D- and E-genome groups (represented here by *G. tomentella*) cannot, with the limited analyses presented here, be shown to be clearly related either to the B- or A-genome groups.

In order to confirm and expand these preliminary conclusions, we have isolated a library of PstI clones from *G. clandestina* using the approach of Burr et al. (1988), and are using these in a systematic study of the well-characterized accessions of the wild species that are available in the Illinois germ plasm collection.

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