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Genomic relationships among diploid wild perennial species of the genus *Glycine* Willd. subgenus *Glycine* revealed by crossability, meiotic chromosome pairing and seed protein electrophoresis *

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Summary. The nomenclature of species beased on classical taxonomy can be verified from cytogenetic, biochemical and molecular studies. The objective of the study presented here was to provide further information on genomic affinities among species of the genus *Glycine* Willd. based on crossability, meiotic chromosome pairing of F_1 hybrids and seed-protein profiles. Meiotic chromosome pairing data revealed no genomic similarity between *G. microphylla* (BB) and *G. falcata* (FF), nor between *G. tomentella* (2n = 38; EE) and *G. microphylla* (BB). Despite morphological similarity between *G. cyrtoloba* (CC) and *G. curvata* no F_1 hybrid was obtained, although 748 flowers were pollinated. The seed-protein banding patterns showed *G. latrobeana* to be closer to the A-genome species than to others. Based on these results we assign genome symbol A_3A_3 to *G. latrobeana*. Likewise, *G. curvata* was allotted the designation C_1C_1 because the seed-protein banding patterns of *G. curvata* and *G. cyrtoloba* are similar. The genome designations of *Glycine* species based on cytogenetic investigations may be further extended by results obtained from biochemical and molecular approaches.

Key words: *Glycine* spp. – Chromosome pairing – Cytogenetics – Seed protein electrophoresis

Introduction

Phylogenetic relationships among species of the genus *Glycine* Willd. are being established by means of cytogenetic (Grant et al. 1984a, b; Singh and Hymowitz 1985a, b, 1988; Singh et al. 1988), biochemical (Grant et al. 1984a; Brown 1990) and molecular (Doyle et al. 1990a, b) approaches. On the basis of cytogenetic analysis, genome symbols have been assigned to 9 of the 15 taxonomically identified diploid (2n = 40) wild perennial species of the subgenus *Glycine* and also to both annual species, *G. soja* Sieb. and Zucc. and *G. max* (L.) Merr., of the subgenus *Soja* (Moench) F. J. Herm. (Singh and Hymowitz 1985a; Singh et al. 1988; Hymowitz et al. 1990).

Cytogenetic investigations have demonstrated that genomically similar *Glycine* species hybridize readily and set mature pods. The F_1 hybrids germinate normally, producing viable, vigorous, fertile plants that occasionally differ by a paracentric inversion. By contrast, in genomically dissimilar species the crossability rate is low and pod abortion is common. Immature seeds must be germinated aseptically; hybrids are weak, slow growing and totally sterile. Sometimes hybrid seed inviability, seedling lethality and vegetative lethality are observed (Singh et al. 1988).

The number of studies on genomic relationships among wild diploid (2n = 40) perennial *Glycine* species have declined since the publication of Singh et al. (1988). A few genomically unlike species remain to be studied. Further progress has been impeded because we have only a few accessions of these species, in some only one, and these are extremely difficult to grow at Urbana, Illinois. Thus, the results of another approach, that of seed-protein profile (electrophoresis), was attempted. The seed-protein profile technique has proven to be an excellent tool for assessing species relationships and it has been utilized to resolve taxonomic and evolutionary problems in several other plant species (Ladizinsky and Hymowitz 1979; Crawford 1983). This method has not

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Table 1. Accessions used in interspecific hybridization and seed-protein profile analysis in the subgenus *Glycine*

Species	PI[a]	2n	Genome	Collection sites
G. argyrea	505151	40	A_2A_2	Cooloola National Park, Qld Australia
G. canescens	440932	40	AA	Birdville, Goyders Lagoon, SA, Australia
G. clandestina	440958	40	A_1A_1	Iandra Lane, NSW, Australia
G. curvata	505166	40	??	Red Bank Creek, Qld, Australia
G. cyrtoloba	440962	40	CC	Brampton Island, Qld, Australia
	440963	40	CC	Brampton Island, Qld, Australia
	505168	40	CC	Ten Mile Creek, Qld, Australia
G. falcata	505179	40	FF	Lanogan, Qld, Australia
G. latrobeana	483196	40	??	Inverleigh, V, Australia
G. microphylla	440956	40	BB	Cook District, Qld, Australia
G. tomentella	505222	40	DD	Lockhart River Airport, Qld Australia
	440998	38	EE	Condamine, Qld, Australia
G. max cv Williams 82		40	GG	Urbana, IL, USA

[a] PI, Plant introduction

been explored previously in establishing genomic relationships among species of the genus *Glycine*.

Materials and methods

Table 1 lists accessions of nine wild perennial species of the subgenus *Glycine* and of a soybean, *G. max* (L.) Merr. cv 'Williams 82'. All the accessions contained 2n = 40 chromosomes, except PI 440998 (*G. tomentella* Hayata), which had 2n = 38. The plant growing conditions, hybridization technique and cytological procedures described by Singh and Hymowitz (1985b) were used. Putative hybrid gynoecia were sprayed with a growth hormone solution containing 100 mg gibberellic acid (GA3), 25 mg naphthalene acetic acid (NAA), and 5 mg kinetin per liter distilled water 24 h post pollination (Jena and Khush 1989) and once a day for 19–21 days (Singh et al. 1990). Immature seeds from aborted pods, 19–21 days after pollination (DAP), were aseptically cultured in artificial media (Singh et al. 1987).

All the hybrids were identified morphologically. Voucher herbarium specimens of accessions and hybrids were deposited in the herbarium of the Crop Evolution Laboratory, University of Illinois, Urbana.

For seed-protein profiles a single accession each (standard determined from cytogenetic studies) was chosen to represent the A (*G. argyrea* Tind, *G. canescens* F. J. Herm., *G. clandestina* Wendl.), B [*G. microphylla* (Benth.) Tind.], C (*G. cyrtoloba* Tind.), D (*G. tomentella*), E (2n = 38) (*G. tomentella*) and F (*G. falcata* Benth.) genomes. Furthermore, *G. curvata* Tind. and *G. latrobeana* (Meissn.) Benth. were also included (Table 1) because previously we failed to establish their genomic relationships cytogenetically (Singh et al. 1988). A soybean cultivar, 'Williams 82', was added as a control.

The seeds were crushed finely after removal of the seed coats. The flour was mixed in a buffer (20 mg/ml) consisting of 62.76 m*M* TRIS-HCl (pH 6.8), 1% sodium dodecyl sulfate (SDS), 1% 2-mercaptoethanol, 10% glycerol and 0.001% bromophenol blue.

The samples were boiled for 5 min and centrifuged in a microcentrifuge at maxiumum speed for 2 min. A 10-µl aliquot of the supernatant was applied to each lane of the gel. Denaturing discontinous polyacrylamide slab gel electrophoresis in the presence of SDS (SDS-PAGE) was conducted according to Laemmli (1970) using Bio-Rad's mini-PROTEANII Dual slab cell and PROTEANII xi slab cell. The gels were stained with Coomassie brilliant blue R-250 and were scanned on a LKB UltroScan XL laser densitometer.

Results

Genomic relationships among Glycine species based on cytogenetic analysis

Since the publication of Singh et al. (1988), three new interspecific F_1 hybrids among genomically distinct diploid species have been obtained (Table 2). The origin, morphology, meiosis and seed fertility of these hybrids are described below.

Crossability. Pod set in crosses between *G. argyrea* (A_2A_2) and *G. tomentella* (2n = 40; DD) was 5.0%. A total of 14 mature pods and 2 aborted pods (24DAP) were harvested. In *G. microphylla* (BB) × *G. falcata* (FF), 7 pods of the 125 flowers pollinated developed, but all aborted 17–22 DAP. All of the immature seeds (26) were cultured and only two plants were recovered. Of the 118 flowers pollinated in the *G. tomentella* (2n = 38; EE) and *G. microphylla* (BB) cross, 1 pod containing four seeds was harvested that aborted 19 DAP (Table 2).

We expected to obtain normal pod set and fertile F_1 hybrids among morphologically and genomically similar species. However, this is not always true. For example, *G. cyrtoloba* and *G. curvata* carry curved pods and their morphological features are very much alike (Tindale 1984, 1986). In the *G. cyrtoloba* and *G. curvata* cross a

Table 2. Parental accessions, crossability rate, origin of F_1 plants, meiotic chromosome pairing at metaphase I, and seed set in interspecific hybrids in the subgenus *Glycine*

Hybrid	Crossability (%)	Origin of F_1	Number of F_1 plants	2n	Chromosome associations		Total PMCs	Seed set
					I	II		
G. argyrea × *G. tomentella* 505151 (40) × 505222 (40)	14+2[a]/322 (5.0)	SC[b]	3	40	18.6 (8–24)[c]	10.7 (8–16)	39	Sterile
G. microphylla × *G. falcata* 440956 (40) × 505179 (40)	7[b]/125 (5.6)	SC	2	40	38.6 (32–40)	0.7 (0–4)	25	Sterile
G. tomentella × *G. microphylla* 440998 (38) × 440956 (40)	1[b]/118 (0.9)	SC	1	39	38.2 (35–39)	0.4 (0–2)	45	Sterile

[a] Pods set (aborted)/total numbers of pollinated flowers
[b] SC, Seed culture
[c] Mean (range)

total of 748 flowers were pollinated. All of the gynoecia died 2–3 DAP, and pod set was not recorded.

Morphology and fertility of F_1 hybrids. Thirty seeds produced from mature pods in the *G. argyrea* and *G. tomentella* ($2n=40$) cross germinated normally, but all of the seedlings died. Likewise, of the three seedlings obtained from the seven immature cultured seeds 2 died; however, a third seedling produced a few flower buds before dying. The vegetative growth of all F_1 plants was extremely slow; the plants were stunted and did not set pods (Table 2).

Singh et al. (1988) recorded vegetative lethality in F_1 hybrids of *G. microphylla* and *G. falcata.* Later crosses, however, produced 1 aborted pod that contained four seeds. Of the four seeds cultured, two seeds germinated after 6 months. At the beginning, both of these F_1 plants were weak and slow in the vegetative growth, but once the roots were well established, growth was rapid and the plants exhibited hybrid vigor for leaf size and internode length and were totally sterile. The F_1 plants possessed a twining stem type similar to that of *G. microphylla* while the stem type in *G. falcata* is erect to scrambling.

Two seedlings germinated through in vitro culture in *G. tomentella* (2n=38) × *G. microphylla* were moved to the greenhouse after 1½ years in culture. One seedling survived, and it was very slow growing. At maturity, the hybrid plant was intermediate between both parents for several morphological traits, but did not inherit adventitious roots and was totally pollen and seed sterile. *Glycine microphylla* contains adventitious roots, a characteristic feature of the B-genome species (Costanza and Hymowitz 1987).

The degree of chromosome synapsis at metaphase I was higher in *G. argyrea* × *G. tomentella* than in *G. microphylla* × *G. falcata* and *G. tomentella* (2n=38) × *G. microphylla* (Table 2). In *G. microphylla* × *G. falcata*, at metaphase I the number of univalents ranged from 32 to 40 with a mean of 38.6 and the number of bivalents ranged from zero to four (Table 2). Figure 1a shows a metaphase-I sporocyte with one loosely associated rod bivalent and 38 univalents, and Fig. 1b contains 40 scattered univalents exhibiting no genomic similarity.

The only F_1 plant of *G. tomentella* (2n=38) and *G. microphylla* derived from the immature embryo culture technique contained 2n=39 chromosomes. At metaphase I, a majority of the sporocytes (32/45=71.1%) showed 39I (Fig. 1c) and the frequencies of one (8/45=17.7%) and two (5/45=11.1%) loosely associated rod bivalents (Fig. 1d) were low (Table 2).

Genomic relationships among Glycine species based on seed protein profiles

The seed protein profile data shown in Fig. 2 corroborate the cytogenetic results. A high similarity in both the number and the relative mobility of various major seed proteins (heavily stained bands) was observed among A-genome species (Fig. 2). It should also be noted that *G. latrobeana* is closer to the A-genome than the other species (Fig. 2; lane D). This was further demonstrated by comparing the densitometric scans of the protein profiles of *G. latrobeana* with that of one of the A-genome species, *G. cladestina.* Both species showed similar banding patterns except at the 40–50 kDa molecular weight range (Fig. 3). Furthermore, a relative similarity was clearly obvious between *G. cyrtoloba* and *G. curvata* as resolved by 10% SDS-PAGE for the high-molecular-weight seed proteins (95–20 kDa; Fig. 4) and 17% gel for the low-molecular-weight ones (20–5 kDa; Fig. 5).

The banding patterns of *G. falcata* (Fig. 2; lane J) and *G. max* (Fig. 2; lane K) were distinctly different than the other *Glycine* species, which again supports the cytogenetic results.

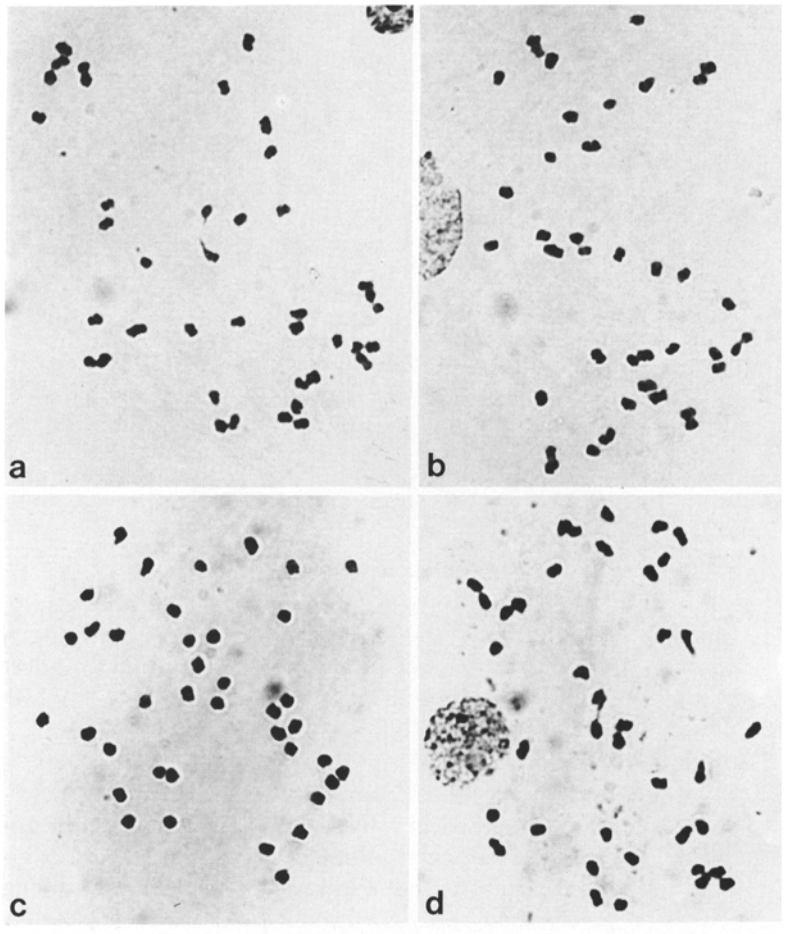

Fig. 1 a–d. Meiosis in interspecific *Glycine* hybrids. **a, b** *G. microphylla* (2n = 40) × *G. falcata* (2n = 40). **a** Metaphase I with 38I + 1II, **b** Metaphase I with 40I. **c, d** *G. tomentella* (2n = 38) × *G. microphylla* (2n = 40) **c** Metaphase I with 39I, **d** Metaphase I with 35I + 2 rod II. × 1,900

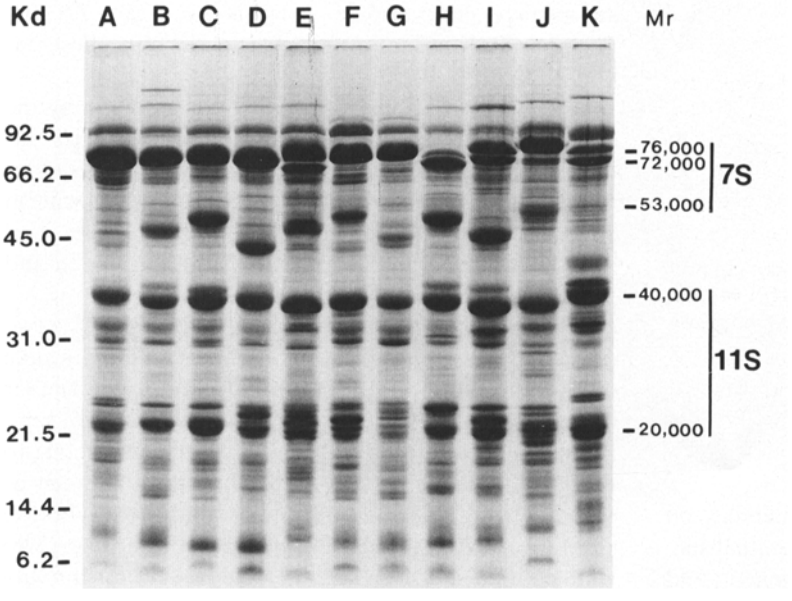

Fig. 2. Analysis of total seed proteins of various species of the genus *Glycine* by gradient (12–17%) SDS-PAGE under reducing conditions using 2-mercaptoethanol. *Lanes A G. argyrea* (PI 505151), *B G. canescens* (PI 440932), *C G. clandestina* (PI 440985), *D G. latrobeana* (PI 483196), *E G. microphylla* (PI 440956), *F G. cyrtoloba* (PI 440962), *G G. curvata* (PI 505166), *H G. tomentella* (PI 505222), *I G. tomentella* (PI 440998), *J G. falcata* (PI 505179), *K G. max* cv 'Williams 82'. Storage globulins, 11S (glycinins) and 7S (B-conglycinins), and the apparent Mr values of these subunits are indicated on the *right* (based on Shotwell and Larkins 1989)

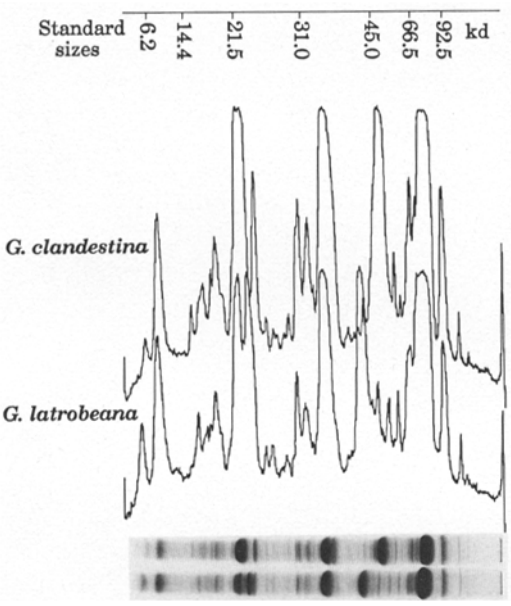

Fig. 3. Comparison of the seed-protein profiles resolved on a gradient (12–17%) SDS-PAGE of *G. clandestina* (PI 440958) and *G. latrobeana* (PI 483196) with their densitometric scans shown *above*

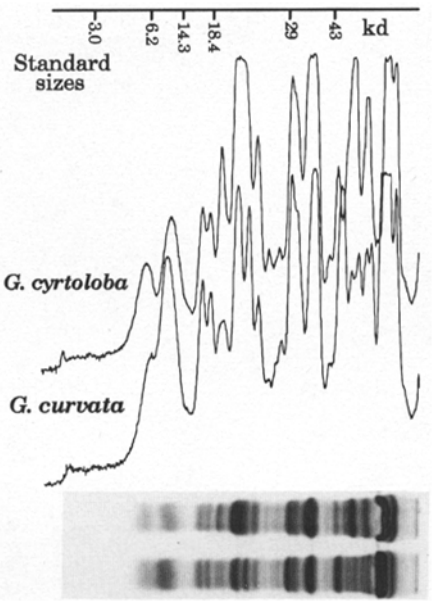

Fig. 5. Comparison of low-molecular-weight seed-protein profiles resolved on 17% SDS-PAGE of *G. cyrtoloba* (PI 440962) and *G. curvata* (PI 440962) and *G. curvata* (PI 505166) with their densitometric scans shown above

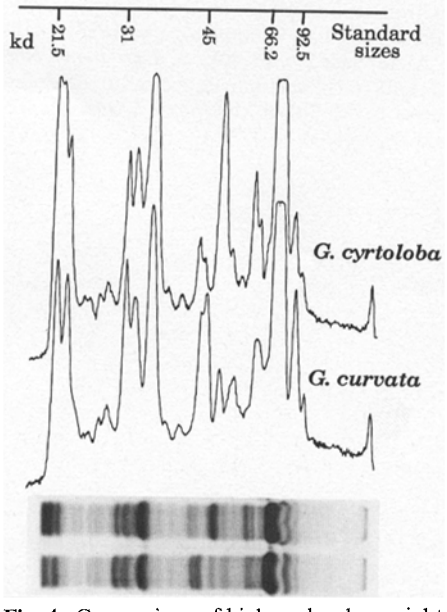

Fig. 4. Comparison of high-molecular-weight seed-protein profiles resolved on 10% SDS-PAGE of *G. cyrtoloba* (PI 440962) and *G. curvata* (PI 505166) with their densitometric scans shown *above*

Discussion

The objective of this study was to provide further data on genomic relationships among diploid wild perennial species of the subgenus *Glycine* based on cytogenetic and seed-protein electrophoresis. We were unable to determine cytogenetically the genome of *G. latrobeana* because this particular species would not grow satisfactorily at Urbana, Illinois. However, the seed-protein banding patterns shown in Figs. 2 and 3 and the chloroplast DNA data of Doyle et al. (1990a, 1990b) reveal clearly that *G. latrobeana* belongs to the A-genome species (*G. argyrea, G. canescens, G. clandestina*). These findings support the observations of Grant et al. (1984b) who reported an F_1 plant, derived from embryo culture, between *G. latrobeana* (G1252) and *G. canescens* (G1115) that showed an average chromosome synapsis of 5.25I + 17.38II. These results led us to assign the genome symbol designation A_3A_3 to *G. latrobeana*.

Crossability is an excellent measure for assessing the degree of genomic affinity between parental species. Interspecific crosses involving *Glycine* species with similar genomes usually result in mature pods and seeds, while in crosses involving genomically dissimilar species pod abortion is common (Grant et al. 1984a, b; Singh and Hymowitz 1985a, b; Singh et al. 1988).

The only exception so far to this rule involves the two species *G. cyrtoloba* and *G. curvata*. Both species are alike morphologically (Tindale 1984, 1986), carry similar chloroplast DNA (Doyle et al. 1990a, b) and have similar seed-protein profiles (Figs. 2, 4, 5). It is interesting to note that in spite of such a close similarity between *G. cyrtoloba* and *G. curvata*, we were unable to hybridize these species even though a large number of flowers (748) were pollinated. This result suggests that the crossability

barrier between *G. cyrtoloba* and *G. curvata* is probably physiological or genic rather than chromosomal. Therefore, we assign the genome symbol designation C_1C_1 to *G. curvata*.

Seed-protein electrophoresis is a very reliable technique by which, to determine species affinities and has been utilized effectively with several plant species (Ladizinsky and Hymowitz 1979; Crawford 1983). In the genus *Glycine*, a wide variation in trypsin inhibitors was found in raw mature seed (Mies and Hymowitz 1973). Our results further substantiate the existence of considerable differences in the seed-protein profiles among the wild diploid perennial species of the subgenus *Glycine* (Fig. 2). The variation was highly visible in the high-molecular-weight proteins (>40 kDa), whose migration pattern falls within the range of the α, α_1 and β subunits (Mr 76,000, 72,000, and 53,000, respectively) of the 7S globulins (conglycinins) of the soybean (Fig. 2).

In the subgenus *Glycine*, *G. falcata* is a unique species because it differs from the other species with regards to several morphological traits (Hermann 1962, Newell and Hymowitz 1978), seed-protein composition (Mies and Hymowitz 1973; Fig. 2), oil and fatty acid content (Chavan et al. 1982), leaf flavonoids (Vaughan and Hymowitz 1984), ribosomal gene variation (Doyle and Beachy 1985) and phytoalexin production (Keen et al. 1986). Chloroplast DNA data suggest that *G. falcata* should be grouped with species carrying the A chloroplast (plastome) genome (Doyle et al. 1990a, b). However, cytogenetic results do not support this conclusion. *Glycine falcata* showed negligible chromosome homology with the A- and B-genome species, since a majority of the sporocytes showed 40 univalents (Putievsky and Broué 1979; Singh et al. 1988; Table 2).

Studies on genomic relationships among wild perennial species of the subgenus *Glycine* are far from complete. The extremely low crossability rate, a high rate of pod abortion, the very low recovery of hybrid plants through in vitro culture, hybrid seed inviability and hybrid seedling lethality have hampered progress. Sometimes the hidden genomic diversity within a diploid species, not revealed by classical taxonomy, will further slow determination of species relationships. For example, differences between 38- and 40-chromosome *G. tomentella* accessions are not morphologically conspicuous, but isozyme banding patterns have shown seven distinct groups (Doyle and Brown 1985; Doyle et al. 1986). Aneudiploid (2n=38) *G. tomentella* was assigned to D1 and D2 groups, whose genomes are probably similar (Singh, unpublished results). The 40-chromosome *G. tomentella* accessions have been separated into five groups [D3 (ABC), D4, D5, D6, D7]. Tindale (1986) removed D6 from these groups and assigned a new species, *G. arenaria* Tind. The D7 group accession (G1545) was misidentified because its plants develop adventitious roots – a characteristic feature of the B-genome species (AHD Brown personal communication). The D4 *G. tomentella* accessions are genomically closer to the A-genome species than to the D3B and D5 *G. tomentella* (Singh et al. 1988; Doyle et al. 1990c). The D5 *G. tomentella* contains an accession, PI 505203, that was collected from Stonewall Creek, Western Australia. This accession has produced aborted pods in crosses with D3 and D4 tomentellas, and so far hybrids have not been recovered, furthermore, it differs in seed-protein profiles (Kollipara, Singh and Hymowitz, unpublished results). These results indicate that the 40-chromosome tomentellas harbor a considerable amount of genetic diversity, although all are included in one species.

In conclusion, the results presented here for the *Glycine* species and also the one previously reported by Jauhar (1990) for the diploid species of the Triticeae suggest that the multidisciplinary approach to genome analysis will enhance our understanding of the genomic affinities among species better than a reliance on a single technique.

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