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ASSESSING THE UTILITY OF ISOZYME NUMBER FOR
DETERMINING PLOIDAL LEVEL:
EVIDENCE FROM *HELIANTHUS* AND *HELIOMERIS* (ASTERACEAE)

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ABSTRACT

In order to evaluate the utility of isozyme number for estimating ploidal level in ancient polyploid (paleopolyploid) plant species, isozyme number was determined for species of the putatively paleopolyploid genus *Helianthus* with $n = 17$, and compared with those of a species of *Heliomeris* with $n = 8$. Electrophoretic examination of 13 enzymes revealed the presence of nine duplicated isozymes in *Helianthus annuus* and *Helianthus bolanderi* and six duplicated isozymes in *Heliomeris multiflora*. Thus, there is little difference in isozyme number between ploidal levels. It is suggested that the lack of strong concordance between isozyme number and ploidal level may be due to gene silencing in *Helianthus* and the presence of gene duplications not resulting from polyploidy in both taxa.

Key words: *Helianthus*, *Heliomeris*, polyploidy, isozyme number, chromosome number.

INTRODUCTION

Recent reviews and studies have discussed the potential value of isozyme number for estimating ploidal level in plants in cases where polyploidy is not directly inferable from chromosome number (Roose and Gottlieb 1976; Gottlieb 1981, 1982; Crawford 1983, 1985; Haufler and Soltis 1986; Soltis 1986). Isozyme number may be useful because the essence of polyploidy is not the number of chromosomes but the number of genomes (Gottlieb 1981). Thus, due to genome multiplicity, recently derived allopolyploid species generally display more isozymes per enzyme than do diploids (Roose and Gottlieb 1976; Gottlieb 1982; Hart and Langston 1977; Adams and Allard 1977).

It is not known, however, whether plant polyploids retain active function of duplicated genes over time. For example, putatively archaic woody angiosperms with high chromosome numbers are duplicated for a large number of enzymes (Soltis and Soltis unpublished) possibly suggesting that many of the loci duplicated through chromosomal doubling are not silenced. In contrast, presumably ancient polyploid (paleopolyploid) species of *Equisetum* (Soltis 1986), *Lycopodium* (Soltis and Soltis 1988), *Mabrya* (Elisens and Crawford 1988), *Galvezia* (Elisens and Crawford unpublished), and several homosporous fern genera (reviewed in Haufler and Soltis 1986) all are characterized by "diploid" isozyme patterns. To account

for the "diploid" isozyme patterns observed for many ferns and fern allies with high chromosome numbers, Soltis (1986), Soltis and Soltis (1988), and Haufler and Soltis (1988) question the hypothesized paleopolyploid origin of these taxa. Elisens and Crawford (1988), in contrast, suggest that silencing of loci duplicated through polyploidy is the most logical explanation for the "diploid" isozyme patterns in *Galvezia* and *Mabrya*, and that silencing of duplicated loci may not be an uncommon phenomenon in paleopolyploids. Their argument is strengthened by evidence of gene silencing in tetraploid *Chenopodium* (Wilson, Barber, and Walters 1983) and catostomid fish (Ferris and Whitt 1977).

Another factor complicating the use of isozyme number to estimate ploidal level in plants is the occurrence of gene duplications that do not result from polyploidy. Although these types of duplications appear to be infrequent in many angiosperm taxa (Gottlieb 1981, 1982), they are common in certain groups. For example, eight duplicated loci have been observed in diploid species of *Clarkia* (Gottlieb 1974, 1977; Gottlieb and Weeden 1979; Pichersky and Gottlieb 1983; Odrzykoski and Gottlieb 1984; Soltis, Soltis, and Gottlieb 1987), and at least three duplicated loci have been reported for diploid *Coreopsis* species (Crawford and Whitkus 1988).

In order to further evaluate the utility of isozyme number for estimating ploidal level in plant paleopolyploids, we compare isozyme numbers for species of *Helianthus* (Asteraceae) with $n = 17$, with those of a species of *Heliomeris* (Asteraceae) with $n = 8$. A description of the two genera and their cytogenetic relationship is given below.

The genus *Helianthus* is comprised of approximately 50 species of annual and perennial herbs (Schilling and Heiser 1981) native to North America. In the genus there are diploids, tetraploids, and hexaploids, all with the basic chromosome number of $\bar{x} = 17$. The basic chromosome number is considered to be of ancient allopolyploid origin for two reasons (Heiser and Smith 1955; Jackson and Murray 1983). First, lower chromosome numbers are found in three closely related genera of subtribe Helianthinae (Stuessy 1977; Robinson, Powell, King, and Weedin 1982): *Viguiera* ($\bar{x} = 12, 17, 18$), *Helianthopsis* ($\bar{x} = 9$), and *Heliomeris* ($\bar{x} = 8$). Second, Jackson and Murray (1983) induced quadrivalent formation in *Helianthus annuus* L. ($n = 17$) and *H. laciniatus* A. Gray ($n = 17$) by premeiotic colchicine treatment of microsporocytes—thus providing strong evidence for the hypothesized paleopolyploid origin of *Helianthus*.

The genus *Heliomeris*, recently re segregated from *Viguiera* by Yates and Heiser (1979), consists of six annual and perennial herbs native to North America. Chromosome counts have been reported for five species, and are nearly constant ($\bar{x} = 8$). One count of $n = 9$ is cited by Keil and Pinkava (1976). The most parsimonious explanation for the low base chromosome number of *Heliomeris* is that it has retained the ancestral chromosome number for subtribe Helianthinae (Heiser and Smith 1955; Yates and Heiser 1979). Robinson et al. (1982), however, suggest that $\bar{x} = 17$ may be the base chromosome number for this group, and that the $\bar{x} = 8$ and $\bar{x} = 9$ chromosome numbers observed in *Heliomeris* and *Helianthopsis* have been derived through polyhaploidy. Nevertheless, even if this were the case, a diploid isozyme condition would be anticipated in *Heliomeris* because the process of polyhaploidy is thought to involve the loss of an entire genome (DeWet 1971).

MATERIALS AND METHODS

Plants

Three species were examined electrophoretically: *Helianthus annuus*, $n = 17$ (Rieseberg 315, Columbia Co., Wash.); *Helianthus bolanderi* A. Gray, $n = 17$ (Rieseberg 99, Lake Co., Calif.); and *Helioomeris multiflora* (Nutt.) Blake, $n = 8$ (Rieseberg 384, Bannock Co., Idaho). For each population, 100 seedlings representing ten half-sib families were studied.

Electrophoresis

The following 13 enzymes were analyzed: acid phosphatase (ACPH), alcohol dehydrogenase (ADH), fructose-1,6-diphosphatase (F1,6DP), glutamate dehydrogenase (GDH), glyceraldehyde-3-phosphate dehydrogenase [NADP]G3PDH, isocitrate dehydrogenase [NADP](IDH), leucine aminopeptidase (LAP), menadione reductase (MNR), phosphoglucoisomerase (PGI), phosphoglucomutase (PGM), 6-phosphogluconate dehydrogenase (6-PGD), shikimate dehydrogenase (SKDH), and triosephosphate isomerase (TPI). These enzymes were selected for analysis because they were easily resolved, and the number of isozymes per enzyme for typical diploid seed plants is generally known (Gottlieb 1981, 1982).

Sample preparation and electrophoresis of enzymes followed the general methodology listed in Soltis et al. (1983). Enzyme extraction was accomplished by grinding whole seedlings in the tris-HCl grinding buffer of Soltis et al. (1983). Enzymes were best resolved using the following buffer systems: for ADH, GDH, and PGI, a modification of system 8 (Rieseberg and Soltis 1987); for LAP and TPI, system 6; for PGM, 6-PGD, MNR and SKDH, system 9; and for ACPH, [NADP]G3PDH, IDH, and F1,6DP, system 1. Staining methods followed Soltis et al. (1983) except for ADH, LAP, and MNR. ADH was stained following Gottlieb (1973), with MTT substituted for NBT. The enzyme assay utilized for LAP is given in Soltis and Rieseberg (1986). MNR was stained as follows: 75 ml 0.05 M tris-HCl pH 8.0, 20 mg menadione sodium bisulfite, 25 mg NADH, and 5 mg MTT.

Genetic Interpretation

Formal genetic analyses of many of the loci examined here have already been completed for *H. annuus* (Torres 1974, 1976; Kahler and Lay 1985), simplifying the process of inferring genotypes from electrophoretic phenotypes. However, the genetic basis of enzyme phenotypes not previously analyzed was determined by analysis of progeny arrays from half-sib families, and by general knowledge of enzyme substructure, number of loci encoding these enzymes in diploid plants, and isozyme compartmentalization (Gottlieb 1981, 1982).

To identify plastid isozymes, an enriched chloroplast fraction was prepared following Mills and Joy (1980), except that two additional washes were performed to remove all traces of cytosolic enzyme activity.

RESULTS

All plants examined had the same number of isozymes for ACPH, ADH, GDH, [NADP]G3PDH, F1,6DP, LAP, and PGM (Table 1). For ACPH, GDH,

Table 1. Enzymes examined and isozyme number for *Helianthus annuus*, *H. bolanderi*, and *Heliomeris multiflora*.

| Enzyme | Number of isozymes observed ¹ | | | Number of ² isozymes typical of diploid plants |
|-------------|--|---------------------|----------------------|---|
| | <i>H. annuus</i> | <i>H. bolanderi</i> | <i>H. multiflora</i> | |
| ACPH | 1 | 1 | 1 | 1-4 |
| ADH | 2* | 2* | 2* | 1-3 |
| F1,6DP | 3* | 3* | 3* | 2 |
| GDH | 1 | 1 | 1 | 1 |
| [NADP]G3PDH | 2 | 2 | 2 | 2 |
| [NADP]IDH | 2 | 2 | 3* | 1-2 |
| LAP | 2 | 2 | 2 | 1-2 |
| MNR | 4?* | 4?* | 2 | ? |
| PGI | 2 | 2 | 3* | 2 |
| PGM | 4* | 4* | 4* | 2 |
| 6-PGD | 3* | 3* | 2 | 2 |
| SKDH | 2* | 2* | 1 | 1-2 |
| TPI | 3* | 3* | 2 | 2 |
| | 31 | 31 | 28 | |

¹ When a single enzyme form was observed a "1" is given, although no isozymes are actually present (see Gottlieb 1982; Soltis 1986).

² (Gottlieb 1981, 1982; Haufler and Soltis 1986; Soltis 1986).

* Duplicated isozymes.

[NADP]G3PDH (Fig. 14), and LAP (Fig. 9-10), the number of isozymes observed was also the same as those typical of diploid seed plants (Table 1). Evidence for gene duplication, however, was observed for ADH (Fig. 16-17), F1,6DP, and PGM. Formal genetic analysis of the duplicated ADH isozymes has already been completed (Torres 1974, 1976).

F1,6DP is a tetrameric enzyme specified by two gene loci in diploid plants (reviewed in Gottlieb 1981, 1982). The minimum true-breeding pattern for three loci is six bands; five bands due to interaction between duplicated isozymes in the same subcellular compartment plus the product of a third locus which is localized in a different subcellular compartment. All plants exhibited six bands of activity for F1,6DP, suggesting that F1,6DP is specified by three loci in the species examined. Chloroplast isolations demonstrated that F1,6DP-1 is a plastid isozyme. Because F1,6DP is a glycolytic enzyme it can be inferred that the remaining two isozymes are cytosolic (Gottlieb 1981, 1982).

PGM, on the other hand, is a monomeric enzyme typically specified by two genes in diploid plants (reviewed in Gottlieb 1982, 1987 and Soltis et al. 1987). *Helianthus* and *Heliomeris* progeny exhibited a minimum true-banding pattern of four enzyme bands (Fig. 4) suggesting that PGM in these species is specified by four loci. Chloroplast preparations of this glycolytic enzyme revealed the presence of two plastid (PGM-1, PGM-2) and, by inference, two cytosolic (PGM-3, PGM-4) isozymes.

Helianthus annuus and *H. bolanderi* exhibited an increase in isozyme number relative to *Heliomeris multiflora* (Table 1) for MNR (Fig. 15), 6-PGD (Fig. 5-6), SKDH (Fig. 7-8) and TPI (Fig. 1-3). 6-PGD and TPI are glycolytic enzymes typically specified by two dimeric isozymes, one found in the plastids and the

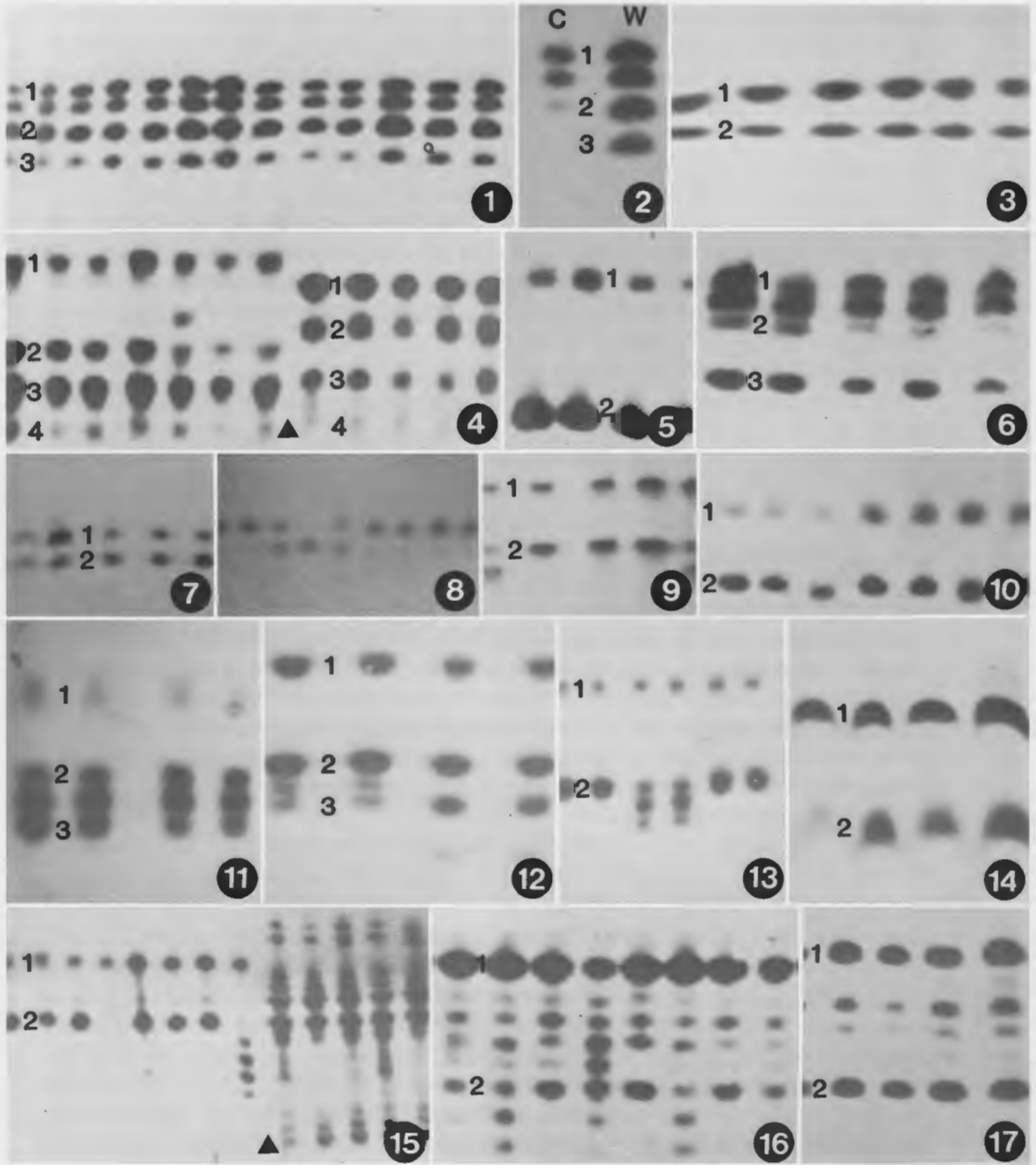


Fig. 1-17. Photographs of starch gels showing representative banding patterns for ten of the enzymes studied. Loci designations follow those used in text.—1. TPI: *H. annuus*.—2. TPI: *H. annuus*; C = chloroplast preparation; W = whole plant preparation.—3. TPI: *H. multiflora*.—4. PGM: samples left of triangle are *H. multiflora*; samples right of triangle are *H. annuus*.—5. 6-PGD: *H. multiflora*.—6. 6-PGD: *H. annuus*.—7. SKDH: *H. annuus*.—8. SKDH: *H. multiflora*.—9. LAP: *H. multiflora*.—10. LAP: *H. annuus*.—11. [NADP]IDH: *H. multiflora*.—12. PGI: *H. multiflora*.—13. PGI: *H. annuus*.—14. [NADP]G3PDH: *H. annuus*.—15. MNR: individuals left of triangle are *H. multiflora*; individuals right of triangle are *H. annuus*.—16. ADH: *H. multiflora*.—17. ADH: *H. annuus*.

other in the cytosol (reviewed in Gottlieb 1981, 1982). The minimum true-breeding pattern for two loci is two bands of activity, whereas the minimum true-breeding pattern for three loci is four bands because of interaction between duplicated isozymes in the same subcellular compartment to form an interlocus heterodimer. All *H. annuus* and *H. bolanderi* progeny displayed at least four bands of activity for 6-PGD, and TPI, indicating that these enzymes are specified by at least three loci in *Helianthus*. In contrast, a minimum true-breeding pattern of two enzyme bands was observed for these enzymes in *Heliomeris multiflora* (Fig. 3, 5), suggesting that 6-PGD and TPI are each encoded by two loci in this species (Table 1). Chloroplast preparations in *H. annuus* indicated that TPI-1, TPI-2 (Fig. 2), and 6-PGD-3 are plastid isozymes. Given the known subcellular localization of 6-PGD and TPI (Gottlieb 1982), it can be inferred that the remaining isozymes are cytosolic.

An increase in isozyme number was also observed in *Helianthus* for MNR and SKDH. MNR is a tetrameric enzyme in *Helianthus* and *Heliomeris* with heterozygous individuals displaying five bands of activity at *Mnr-2* (Fig. 15). In *Heliomeris multiflora* as a minimum true-breeding pattern of two enzyme bands was observed (Fig. 15), suggesting that MNR is specified by two genes in this species (Fig. 15). In contrast, the two *Helianthus* species exhibited complex banding patterns for MNR, with a single plant exhibiting a minimum of 10 enzyme bands (Fig. 15). These patterns suggest the presence of up to four MNR isozymes. Four MNR isozymes have also been reported in conifers (Ledig and Conkle 1983).

SKDH is a monomeric enzyme typically specified by one or two genes in diploid plants (Gottlieb 1982; Soltis 1986). *Helianthus annuus* and *H. bolanderi* segregated individuals with two to four enzyme bands for SKDH, suggesting that the SKDH was specified by two genes (Fig. 7). One- or two-banded enzyme phenotypes were observed for *Heliomeris multiflora* progeny, suggesting that a single gene encodes SKDH in this taxon (Fig. 8).

An increase in isozyme number, however, was observed in *H. multiflora* for [NADP]IDH (Fig. 11) and PGI (Fig. 12), whereas *H. annuus* and *H. bolanderi* possessed the same number of isozymes for these enzymes as found in diploid plants (Fig. 13; Table 1). [NADP]IDH is generally found in plants as two dimeric isozymes, one located in the plastids, and the other in the cytosol (Randall and Givan 1981; Gottlieb 1987), although a single isozyme has been observed in some species (Soltis 1986). Progeny of *H. multiflora* displayed a minimum of four bands of activity and the pattern was true-breeding. The faint IDH-1 isozyme (Fig. 11) was located in the plastid, whereas IDH-2 and IDH-3 (Fig. 11) can be inferred to occur in the cytosol. Individual plants of *H. annuus* and *H. bolanderi*, in contrast, displayed a minimum of two true-breeding bands, suggesting the presence of only two genes encoding [NADP]IDH. Cell fractionation studies demonstrated that IDH-1 was the plastid isozyme and, by inference, that IDH-2 was located in the cytosol.

PGI is typically represented by two dimeric isozymes in diploid plants, one occurring in the plastid and the other in the cytosol (Gottlieb 1982). *Heliomeris multiflora* progeny exhibited a minimum of four enzyme bands (Fig. 12), including the most anodal PGI-1, a fast band encoded by *Pgi-2*, a slow fainter band specified by *Pgi-3*, and an intergenic *Pgi-2/Pgi-3* heterodimeric band of intermediate mobility. Plastid isolations assigned to the chloroplast PGI to *Pgi-1*; thus, PGI-2 and

PGI-3 can be assigned to the cytosol. In contrast, a minimum of two true-breeding bands was observed in *H. annuus* and *H. bolanderi* progeny, indicating that PGI is encoded by two genes in *Helianthus* (Fig. 13).

DISCUSSION

As summarized in Table 1, electrophoretic examination of 13 enzyme systems revealed the presence of nine duplicated isozymes in *H. annuus* and *H. bolanderi* and six duplicated isozymes in *Heliomeris multiflora*. Four of the duplicated isozymes were shared by all taxa. Thus, relative to most diploid angiosperm species surveyed electrophoretically (Gottlieb 1981, 1982), isozyme numbers are high for both *Helianthus* and *Heliomeris*. There is little difference, however, in isozyme number between *Helianthus* and *Heliomeris* suggesting that the use of isozyme number for estimating ploidal level in *Helianthus* and related genera may not be valid.

As mentioned in the introduction, two phenomena can serve to obscure isozyme number differences between closely related species with different ploidal levels: 1) gene duplication not resulting from polyploidy, and 2) gene silencing following polyploidy. Both of these phenomena appear to be operating in *Helianthus* and/or *Heliomeris*.

A number of mechanisms have been given for gene duplications in plants which do not involve polyploidy including unequal crossing over, reciprocal translocations, and/or insertional translocations (Gottlieb and Weeden 1979; Gottlieb 1981, 1983). Nevertheless, the frequency of duplicated genes in diploid seed plants is unclear. Although originally assumed to be rare (Gottlieb and Weeden 1979), duplicated genes have been observed in numerous diploid plant genera such as *Clarkia* (Gottlieb and Weeden 1979), *Lycopersicon* (Tanksley and Kuehn 1985), *Capsicum* (Tanksley 1984), *Sorghum* (Ellstrand, Lee, and Foster 1983), *Layia* (Gottlieb 1987), *Coreopsis* (Crawford and Whitkus 1988), *Allium* (Rieseberg et al. 1987), *Senecio* (Liston, Rieseberg, and Elias unpublished), *Astragalus* (Liston unpublished) and other genera (Gottlieb 1982, 1986; Elisens and Crawford 1988). In most cases, only one or two gene duplications have been reported for a single plant group. Certain groups of plants, however, appear to be unusually susceptible to changes in gene number at the diploid level. As discussed in the introduction, eight duplicated isozymes have been reported in *Clarkia*, and six duplicated isozymes (cytosolic IDH, cytosolic PGI, cytosolic PGM, plastid PGM, ADH, and cytosolic F1,6DP) were observed herein for the diploid species, *H. multiflora*. Furthermore, four of the gene duplications observed in *H. multiflora* were also found in *Helianthus*, indicating that a maximum of five of the nine duplicated isozymes observed in *Helianthus* (Table 1) can be attributed to paleopolyploidy.

Evidence for gene silencing is also apparent in *Helianthus* where a lower percentage of duplicated isozymes was observed than found in the recently derived polyploids, *Tragopogon mirus* Ownbey and *T. miscellus* Ownbey (Roose and Gottlieb 1976). Although the reduced percentage of duplicate loci in *Helianthus* could also reflect high genetic similarity between its diploid progenitors, this explanation is not possible for *AcpH*, *Gdh*, *Idh-1*, *Idh-2*, *Lap-1*, *Lap-2*, *Pgi-2*, and *6-Pgd-3*. These loci are highly variable and an additional isozyme would be quickly detected due to unbalanced staining. Unbalanced staining was observed, however,

for duplicated PGM and 6-PGD (Fig. 4, 6) in *Helianthus*, as well as for duplicated PGM and PGI (Fig. 4, 12) in *Heliomeris multiflora* possibly indicating partial gene silencing.

The actual mode of origin (diploid vs. polyploid) of the duplicated genes observed in *Helianthus* and *Heliomeris* as well as the degree of gene silencing could be further clarified by determining linkage relationships between the duplicated loci and by surveying closely related species and genera with regard to isozyme number. For example, if the loci were tightly linked, this would be regarded as evidence for a tandemly occurring structural gene duplication. If the loci were unlinked, the additional locus could be the result of polyploidy or overlapping reciprocal translocations or insertional translocations (Gottlieb 1983). Knowledge of isozyme number in other species of *Helianthus*, *Heliomeris*, and closely related genera such as *Viguiera* and *Helianthopsis*, however, would be particularly valuable. For example, it is possible that certain of the duplicated loci in *Heliomeris multiflora* are autapomorphic characters such as those found in *Allium constrictum* (Rieseberg et al. 1987). Furthermore, it would be valuable to know whether the same number of isozymes observed in *Helianthus* is found in other presumably paleopolyploid Helianthinae.

Although isozyme number may not be reliable for determining ploidal level in the Helianthinae, the data reported herein do not invalidate the use of isozyme number for estimating ploidal level in other plant groups. In particular, isozyme number may be useful in plant groups where gene duplications not resulting from polyploidy are rare and where gene silencing in polyploid taxa is infrequent.

Finally, the presence of gene duplications for cytosolic IDH, cytosolic PGI, cytosolic PGM, plastid PGM, cytosolic ADH, and cytosolic F1,6DP in *H. multiflora* may provide significant data for phylogenetic reconstructions. It is noteworthy that a cytosolic PGM duplication has also been reported for diploid species throughout the Madiinae (Gottlieb 1987) and is also present in *Coreopsis* (Crawford and Whitkus 1988) of the Coreopsidinae. The possible phylogenetic significance of this duplication has been discussed by Gottlieb (1987) and Crawford and Whitkus (1988). Additionally, Gottlieb (1987) reported a gene duplication for cytosolic IDH in diploid species of *Layia* (Madiinae). Given the difficulties of elucidating intergeneric and subtribal relationships in the Heliantheae (Stuessy 1977), it may be useful to determine the linkage relationships and systematic distribution of these gene duplications.

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