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# Genetic analysis of shikimate dehydrogenase allozymes in *Trifolium repens* L.

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**Abstract** A genetic analysis was carried out on progeny families from pair crosses among plants polymorphic for shikimate dehydrogenase (SDH) isozymes in white clover (*Trifolium repens* L.). SDH was controlled by two independently assorting disomic loci. This result is consistent with the presence of a single gene (*Sdh*) represented in this putative allotetraploid by one locus in each of the genomes. One of the *Sdh* loci is linked ($6.0 \pm 2.0$ cM) to the linamarase (*Li*) locus. There was no evidence for differentiation of the duplicate *Sdh* loci (both carry common alleles). White clover behaves genetically as a diploidised allotetraploid but the possibility of a low frequency of multivalent formation and homoeologous pairing has not been ruled out. The SDH locus is likely to be useful for the marker-assisted selection of agronomic traits.

**Key words** Shikimate dehydrogenase · Isozymes · *Trifolium repens* · Linamarase

## Introduction

White clover (*Trifolium repens* L.) is a legume species with a probable allotetraploid origin (Williams 1987). Although it is regarded as one of the world's most important pasture legume species, it has received little attention from geneticists. Consequently, as plant breeding moves toward the use of marker-assisted and map-based selection methods, there is only a poor informational base on which to develop such programmes in white clover.

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Genetic markers in white clover are few, the major known loci being *V* (allelic variation for shape and position of white leaf marks, Corkill 1971), *R* (alleles vary for shape and position of purple anthocyanin leaf marks, Corkill 1971), *S* (self-incompatibility), *Li* (the dominant *Li* allele conditions linamarase activity while *li*,*li* individuals lack linamarase activity, Corkill 1942; Hughes 1991), *Ac* (the dominant *Ac* allele conditions biosynthesis of the cyanogenic glucosides linamarin and lotaustralin while *ac*,*ac* individuals lack cyanogenic glucosides, Corkill 1942) and the isozyme locus *Pgi-2* (phosphoglucoisomerase, Michaelson-Yeates 1986). To-date no linkages have been confirmed among these loci (Corkill 1971; Williams 1987).

White clover shows regular chromosome pairing (Atwood and Hill 1940), giving rise almost exclusively to bivalents, although a rare mutivalent has been detected at meiosis (Chen and Gibson 1970). It is often assumed that the same chromosomes always pair (exclusive disomic behaviour). However, this is a deduction made from analyses of the inheritance of the genetic loci mentioned above, all of which (except *Pgi-2*) appear to be present as two alleles in each plant and hence occur in only one of the two putative genomes of white clover. The one exception, *Pgi-2*, which is present as four alleles, is generally fixed for two alleles but segregates regularly for the other two alleles – a result consistent with one genome being fixed and the other variable at this locus. A detailed study of the Michaelson-Yeates (1986) results reveals, however, that the possibility of some tetrasomic chromosome pairing (i.e. pairing of chromosomes irrespective of genome) cannot be ruled out and that patterns other than regular disomic pairing may occur.

Further analysis of this question requires more loci which occur with four, rather than two, alleles. To-date, none has been available, but we have found that the locus for shikimate dehydrogenase allozymes fits this criterion. Shikimate dehydrogenase (EC 1.1.1.25; SDH) catalyses the reduction of 3-dehydroshikimic acid by

NADPH to shikimic acid, an intermediate in the biosynthesis of aromatic amino acids. It is alternatively abbreviated as SKD, SKDH, ShDH and SAD.

Inheritance studies in several species have shown SDH to be monomeric (e.g. *Lycopersicon*, Tanksley and Rick 1980; *Phaseolus*, Garvin et al. 1989). In *Trifolium subterraneum* L., *Zea mays*, and several other plants, SDH is monomeric but each allele is associated with co-segregating bands known as 'conformers' (Collins et al. 1984; Wendel et al. 1988). In several species, including pea, *Pisum sativum* L., and carnation, *Dianthus caryophyllus* L., SDH isozymes are associated with the plastids, although coded by a nuclear gene locus (Weeden and Gottlieb 1980; Messeguer and Arus 1996).

In pea and lentil (*Lens culinaris* Medik.), a genetic locus controlling SDH allozymes is located in a region of the genome showing similar linkages in both species (Weeden et al. 1993; Simon et al. 1993). This region includes loci controlling a restriction fragment length polymorphism for nodule-specific glutamine synthase, and isozyme loci controlling esterase-1 and beta-galactosidase-2. In pea, other loci closely linked to SDH involve seed coat colour, chlorophyll a/b-binding protein, internode length and leaf-waxiness traits. In lentil the SDH locus (*Skdh*) has been shown to be a potential marker for a quantitative trait locus controlling seed weight (Tahir et al. 1994). SDH allozymes have been used as markers for high seed-protein content in rice (Shenoy et al. 1990), fruit colour in strawberry (Williamson et al. 1995) and as a biochemical marker for group-5 chromosomes in triticoid grasses (Koebner and Shepherd 1982; William and Mujeeb-Kazi 1992). Thus, given its value, especially in the other legumes, SDH is of possible potential use as a marker for both mapping and selection purposes in white clover.

The objectives of the present study were to determine the mode of inheritance of SDH in white clover (disomic vs tetrasomic inheritance) and to examine whether there was any linkage between SDH and the other traits under investigation.

## Materials and methods

The seed used in this study was obtained from the Margot Forde Forage Germplasm Centre (New Zealand). The genetic analysis was carried out on progeny families from pair crosses among eight plants selected for a range of SDH phenotypes and marker genotypes (see Table 1). The eight parent plants differed in genotype at one or more of four other marker loci (see Table 1), used to verify the accuracy of crossing and to test for linkage. These genotypes were confirmed from progeny tests and by crosses to more than one test plant.

Crosses were carried out by hand pollination of florets in a pollinator-free glasshouse according to the method of Williams (1954). Each parent was tested for self-fertility by rubbing inflorescences (Atwood 1941), and all were confirmed to be self-incompatible. Each parent plant and all progeny plants were grown in 15-cm-diameter plastic pots containing a peat-based potting mix and were maintained in a vegetative state by regular trimming. Plants were maintained for at least 2 weeks prior to sampling in a temperature range of 15–25°C.

For SDH allozyme analyses, several unfolded young leaflets were picked from each mature plant into plastic bags on ice. Leaf tissue (0.15 g) was weighed into a 1.5-ml Eppendorf tube on ice and ground *in situ* using a drill press fitted with a conical steel tip shaped to fit the inside of the tube. Extraction buffer (0.5 ml) consisting of 0.1 M Tris pH 9, 10% w/v sucrose, 0.1% w/v ascorbic acid and 0.8% v/v 2-mercaptoethanol was added and the tube vibrated briefly to mix the components. Samples were centrifuged at 13 000 rpm at 4°C and immediately cooled to − 20°C.

Allozymes of SDH were separated using non-denaturing, discontinuous, polyacrylamide-gel electrophoresis (gels 170 mm wide and 160 mm × 1 mm thick). The running gels (6% acrylamide), stacking gels (3.4% acrylamide) and tank buffer were according to Sambrook et al. (1989). Nine or eleven lanes of test samples and two each of the parent plant extracts were electrophoresed per gel, using 20 µl of the thawed extract. Electrophoresis was conducted at 4°C for 1 h at 50 mA followed by 5 h at 20 W constant power. Staining was according to Tanksley and Rick (1980). Gels were dried overnight and used as records for the interpretation of data. Duplicate evaluations were employed for all the materials tested. Bands were scored for presence or absence using the parental lanes as controls. For linkage analysis, band intensities were also used to determine the dosage of a specific allele in evaluating the plant genotype.

Similar electrophoresis conditions were used for the separation of allozymes of PGI and the staining procedures were according to Tanksley (1980). In some cases, native polyacrylamide gels with a 5–10% gradient were used to obtain better separation of alleles that migrate close to each other.

A scaled-down version of the picrate paper method of Corkill (1940) was used for cyanogenesis testing. One young leaflet and 10 µl of toluene were placed in the bottom of a 1.5-ml Eppendorf tube with a small square of filter paper, soaked in picric acid solution, fitted below the lid. Tubes were incubated at 37°C overnight and then the filter paper read for change of colour. Where appropriate, plants testing negative were further analysed for presence of linamarin/lotaustralin by the addition of 20 µl of 0.5% linamarase and for the presence of linamarase by the addition of 20 µl of 0.5% linamarin. The latter test was also conducted by replacing the linamarin with a leaflet from a plant with a *Acli* (linamarin positive, linamarase negative) phenotype.

White V-markings were visually scored on an intensity scale of 0–3 with 0 denoting absence and 3 an intense white mark. Red flecking (*Rf*) was studied for presence or absence during winter when the expression of anthocyanin pigmentation was greatest.

Goodness of fit to the tested genetic models was carried out using methods described by Mather (1951). Standard $\chi^2$ tests were employed and, where a cross and its reciprocal were analysed, the data were combined for analysis provided that a test of the two data sets indicated an absence of significant heterogeneity. Linkage distances and variances were also calculated using the methods of Mather (1951).

## Results

### Parental phenotypes

The phenotypes of the analysed plants consisted of various combinations of four major band positions in one zone of activity (Fig. 1). These bands were designated A–D in order from the anode. Each allele produced a multi-banded phenotype consisting of a dark- staining band with light bands (conformers) on either side. The dark bands were usually easily distinguished and any uncertain phenotypes were resolved by assessing duplicate gels.

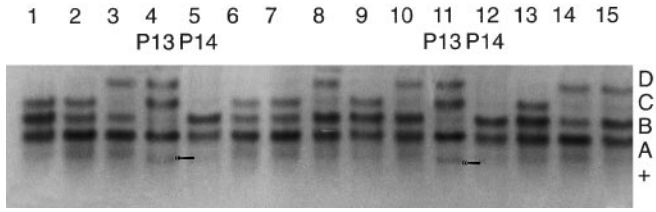


**Fig. 1** SDH gel for an analysis of the cross of plant 13 (A,A;C,D *tracks 4, 11*) with plant 14 (A,B;B,B *tracks 5, 12*). *Tracks 1–3, 6–10, 13–15* show progeny plants. Band *B* is present in single dose in *tracks 2–3, 6–7,* and *14* and two doses in *1, 8–10, 13* and *15* while band *A* is complementary. Bands *C* and *D* from plant 13 are segregating and independently assorting. Conformer bands are apparent, especially below band *A* (*arrows*)

The SDH phenotypes of the eight parent plants used in controlled crosses are summarised in Table 1. The terminology adopted for genotypes is to separate the two duplicate loci with a semicolon and alleles at each locus with a comma. Also in Table 1 are the genotypes derived from progenies of the parent plants. These progeny tests are summarised below.

## Disomic versus tetrasomic inheritance

In a tetraploid, alleles can be arranged at a locus in six different configurations (excluding complete homozygosity). Two alleles can be arranged in three ways: (1) A,A;A,B (2) A,B;A,B (3) A,A;B,B. Three alleles can be arranged in two ways: (4) A,B;C,C (5) A,C;B,C; and four alleles in one way, (6) A,B;C,D. Gamete analysis of each of these combinations shows that with configuration (1), disomic and tetrasomic inheritance patterns are identical and such plants can be used as testers. For configurations (2) and (5), disomic and tetrasomic behaviours require very large populations to distinguish them. For combinations (3), (4) and (6), tetrasomic inheritance gives rise to unique genotypes, thus enabling the two types to be easily distinguished. The population sizes needed to detect at least one unique genotype at the expected frequencies of 0.33 or 0.17 with a probability of 0.99 are 12 and 30 plants respectively (Mather 1951; Sedcole 1977). Only progeny families larger than these numbers were used, as appropriate, in tests of disomic versus tetrasomic inheritance.

## SDH inheritance in controlled crosses

Table 2 provides progeny test data confirming that plants 1, 7, 8 and 14 had configuration (1) so that these plants could be used as testers for disomic vs tetrasomic inheritance. Plants 1 and 14 carried genotype A,B;B,B while plant 7 was C,C;C,D and plant 8 was B,C;C,C. Reciprocal pair crosses among these plants were analysed except for $14 \times 8$ (where the cross was not made). Only in the case of the crosses $1 \times 14$ and $14 \times 1$ was heterogeneity between reciprocal crosses detected. In these crosses, one contaminant was also detected and eliminated on the basis of the presence of a non-parental allele. As shown in Table 2, the segregation data from bulked families fitted very closely to the expected 1:3 ratio (12:33), but $1 \times 14$ showed a ratio of 3:22 and $14 \times 1$ a ratio of 9:11. When these families were further checked for other markers (PGI, leaf marks) for which the parents differed (Table 1), no irregularities were found and so the apparent heterogeneity is attributed to a sampling effect arising from the small populations tested. These plants were therefore used as testers in crosses with plants with configurations that could distinguish between disomic and tetrasomic inheritance.

Table 3 presents the results of crosses involving plant 13 which carried three alleles, one of which was putatively homozygous in one genome (A,A;C,D). Plant 13 is potentially most instructive for the analysis of disomic versus tetrasomic inheritance as the expectations for the two outcomes are quite different. All three progenies showed significant deviations from tetrasomic inheritance and conformed statistically to the

**Table 1** Phenotypes of the parent plants for SDH, PGI and leaf marks and genotypes for SDH, and cyanogenesis (HCN) derived from progeny tests in this study. Accession numbers are those of the Margot Forde Forage Germplasm Centre, Palmerston North, New Zealand

| Plants | Accession No. | SDH phenotypes | *Sdh* genotypes | PGI phenotypes | Leaf mark phenotypes[a] | HCN genotypes |
|---|---|---|---|---|---|---|
| 1 | C 5897-1 | AB | A,B;B,B | C,D | Intense Vi | *ac ac li li* |
| 6 | C 7544-1 | BCD | B,C;C,D | C | Vl | *Ac Ac Li li* |
| 7 | C 7544-2 | CD | C,C;C,D | C | Light double V | *Ac ac li li* |
| 8 | C 7544-3 | BC | B,C;C,C | C | Vi | *Ac ac Li li* |
| 11 | C 5897-9 | BCD | B,C;B,D | C,K,B | v | *ac ac li li* |
| 13 | C 13338-3 | ACD | A,A;C,D | C,M | v | *ac ac li li* |
| 14 | C 13338-4 | AB | A,B;B,B | C | v | *ac ac li li* |
| 15 | C 13338-5 | BD | B,D;B,D | C,K | Vl; Rf | *ac ac li li* |

[a] Vi and Vl are white V markings in intermediate and low positions on the leaflet, respectively, whereas v leaves are unmarked. Rf is an anthocyanin leaf flecking. *Ac* designates the production of the cyanogenic glucosides, linamarin and lotaustralin, and *Li* the $\beta$-glucosidase, linamarin

**Table 2** Observed (Obs) and expected (Exp) phenotype frequencies in progenies of crosses between plants carrying only two SDH alleles, one allele present in three doses, configuration (1). $\chi^2$ analyses tested for departures of observed from expected frequencies. Reciprocal crosses were bulked for analysis. Heterogeneity $\chi^2$ tests indicate whether the results were consistent for both directions of each cross

| Cross | | Phenotype frequencies | | | | | | | | | | $\chi^2$ | Signif. | Het $\chi^2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CD | BCD | ABCD | ABD | C | BC | ABC | B | AB | Total | | | |
| 1 × 7 | Obs | | 11 | 7 | | | 13 | 11 | | | 42 | 1.810 | ns | ns |
| A,B;B,B × C,C;C,D | Exp | | 10.5 | 10.5 | | | 10.5 | 10.5 | | | 42.0 | | | |
| 1 × 8 | Obs | | | | | | 33 | 20 | | | 53 | 3.180 | ns | ns |
| A,B;B,B × B,C;C,C | Exp | | | | | | 26.5 | 26.5 | | | 53.0 | | | |
| 1 × 14 | Obs | | | | 1[a] | | | | 12 | 33 | 45[a] | 0.052 | ns | * |
| A,B;B,B × A,B;B,B | Exp | | | | 0 | | | | 11.25 | 33.75 | 45.00 | | | |
| 7 × 8 | Obs | 12 | 14 | | | 9 | 9 | | | | 44 | 1.637 | ns | ns |
| C,C;C,D × B,C;C,C | Exp | 11 | 11 | | | 11 | 11 | | | | 44 | | | |
| 7 × 14 | Obs | | 6 | 7 | | | 7 | 8 | | | 28 | 0.286 | ns | ns |
| C,C;C,D × A,B;B,B | Exp | | 7 | 7 | | | 7 | 7 | | | 28 | | | |
| 8 × 14 | Obs | | | | | | 11 | 7 | | | 18 | 0.888 | ns | na |
| B,C;C,C × A,B;B,B | Exp | | | | | | 9 | 9 | | | 18 | | | |

* Significant difference ($P < 0.05$)
[a] Unexpected ABD plant treated as a contaminant and omitted
ns – no significant difference
na – not applicable, reciprocal crosses were not analysed

**Table 3** Observed (Obs) and expected (Exp) phenotype frequencies in progenies of crosses involving plant 13 (three alleles, one homozygous). Expectations for disomic (2n) and tetrasomic (4n) inheritance are given separately. Following heterogeneity tests, reciprocal crosses were bulked for analysis

| Cross | | Phenotype frequencies | | | | | | | | | $\chi^2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CD | BCD | ACD | ABCD | ABD | AD | ABC | AB | Total | |
| 1 × 13 | Obs | | 0 | | 0 | 25 | | 18 | 1 | 44 | |
| A,B;B,B × A,A;C,D | Exp 2n | | 0 | | 0 | 21.5 | | 21.5 | 0 | 43.0[a] | 1.140 |
| | Exp 4n | | 3.67 | | 3.67 | 14.67 | | 14.67 | 7.33 | 44.01 | 19.170** |
| 13 × 14 | Obs | | 0 | | 0 | 17 | | 26 | 0 | 43 | |
| A,A;C,D × A,B;B,B | Exp 2n | | 0 | | 0 | 21.5 | | 21.5 | 0 | 43.0 | 1.884 |
| | Exp 4n | | 3.58 | | 3.58 | 14.33 | | 14.33 | 7.17 | 42.99 | 24.326*** |
| 13 × 15 | Obs | 0 | 0 | 3 | 12 | 15 | 6 | 8 | 0 | 44 | |
| A,A;C,D × B,D;B,D | Exp 2n | 0 | 0 | 5.5 | 11 | 16.5 | 5.5 | 5.5 | 0 | 44.0 | 2.544 |
| | Exp 4n | 1.22 | 6.11 | 2.44 | 9.78 | 17.11 | 3.67 | 2.44 | 1.22 | 43.99 | 23.561*** |

**·*** Significant deviation, $P < 0.01$, 0.005, respectively
[a] Exceptional AB individual omitted from the disomic $\chi^2$ test

expectation for disomic inheritance. The progeny from the cross 1 × 13 (A,B;B,B × A,A;C,D) showed a single exceptional plant with a phenotype (AB) that is consistent with the tetrasomic inheritance model. The genetic markers have eliminated the possibility that this plant resulted from selfing and are fully consistent with the correct parentage.

The cross 13 × 14 shown in Fig. 1 provides a good demonstration of the monomeric enzyme structure. Hybrid bands expected from a dimeric enzyme are absent and the progeny phenotypes are fully consistent with a monomeric enzyme to the extent that band intensities read both visually and with image analysis, are in proportion to expected allele doses. The segregation and independent assortment of bands C and D was also consistent with disomic inheritance.

The results of crosses involving plant 6 (B,C;C,D) and plant 11 (B,C;B,D), both of which carried three alleles with no homozygosity (configuration 5), are given in Table 4. All four crosses analysed conformed to disomic inheritance and two showed significant deviations from tetrasomic inheritance. The inferences from the other two crosses are inconclusive, probably because of the small populations analysed. The cross 6 × 8 was omitted from this data set because tests on the associated leaf mark and cyanogenic loci revealed significant deviations indicative of an error during crossing.

**Table 4** Observed (Obs) and expected (Exp) phenotype frequencies in progenies of crosses involving plants 6 and 11 (three alleles, none homozygous). Expectations for disomic (2n) and tetrasomic (4n) inheritance are given separately. Following heterogeneity tests, reciprocal crosses were bulked for analysis

| Cross | | Phenotype frequencies | | | | | | | | | | | $\chi^2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CD | BCD | ABCD | BD | ABD | C | BC | ABC | B | AB | Total | |
| 6 × 7 | Obs | 16 | 12 | | | | 5 | 6 | | | | 39 | |
| B,C;C,D × C,C;C,D | Exp 2n | 14.63 | 14.63 | | | | 4.88 | 4.88 | | | | 39.00 | 0.863 |
| | Exp 4n | 16.25 | 13 | | | | 3.25 | 6.5 | | | | 39.00 | 1.061 |
| 8 × 6 | Obs | 4 | 6 | | | | 4 | 6 | | | | 20 | |
| B,C;C,C × B,C;C,D | Exp 2n | 2.5 | 7.5 | | | | 2.5 | 7.5 | | | | 20.0 | 2.400 |
| | Exp 4n | 3.33 | 6.67 | | | | 1.67 | 8.33 | | | | 20.00 | 4.118 |
| 1 × 11 | Obs | | 3 | 9 | 3 | 11 | | 3 | 4 | 4 | 5 | 42 | |
| A,B;B,B × B,C;B,D | Exp 2n | | 5.25 | 5.25 | 5.25 | 5.25 | | 5.25 | 5.25 | 5.25 | 5.25 | 42.00 | 12.10 |
| | Exp 4n | | 3.5 | 3.5 | 7 | 7 | | 7 | 7 | 3.5 | 3.5 | 42.0 | 17.57* |
| 11 × 14 | Obs | | 10 | 3 | 3 | 4 | | 7 | 6 | 3 | 7 | 43 | |
| B,C;B,D × A,B;B,B | Exp 2n | | 5.38 | 5.38 | 5.38 | 5.38 | | 5.38 | 5.38 | 5.38 | 5.38 | 43.00 | 8.543 |
| | Exp 4n | | 3.58 | 3.58 | 7.17 | 7.17 | | 7.17 | 7.17 | 3.58 | 3.58 | 43.00 | 18.95** |

**·* Significant deviation ($P < 0.01$, 0.05, respectively)

### Linkages of SDH with other markers

Two plants (6 and 8) were used to test for linkage of SDH with *Li*. The results of crosses of plant 8 (B,C;C,C *Li*,*li*) with plants 1, 7 and 14 (all *li*,*li*) showed that *Li* and the B allele of *Sdh* are linked in this plant, with an estimated recombination frequency of 6.19% (7/113, Table 5). Data from crosses involving plant 6 (B,C;C,D *Li*,*li*) which confirms linkage between *Li* and the *Sdh* allele D with a recombination frequency of 4.76% (1/21) are presented in Table 6. This table also shows that *Sdh* allele B in plant 6 assorted independently of *Li*, as expected from the evidence for independent assortment of the duplicate *Sdh* loci. Crosses between plant 8 and plant 6, both *Li*,*li*, further supported the linkage of *Li* with *Sdh* ($\chi^2$ test for independent assortment, 11 $df = 29.600$, $P < 0.005$). The combined estimate of linkage distance between *Sdh* and *Li* was $6.0 \pm 2.0$ centimorgans (cM).

Plants 7 and 8 each segregated for *Ac*/*ac* and for one *Sdh* allele; and in all relevant crosses involving these plants no significant departures from independent assortment were found (Table 7 a).

Linkage between *Sdh* allele B and the *Rf* allele (red fleck) was tested in the cross between plants 13 (lacking red fleck; A,A;C,D) and 15 (*Rf*; B,D;B,D). These loci were found to assort independently (Table 7b).

The results of several crosses indicated independent assortment of the *V* locus and *Sdh*. The crosses 13 × 15 and 14 × 15 effectively tested the linkage to *V* of both *Sdh* loci carried by parent 15, with negative indications in both cases (Table 7 c).

Plant 11 (*Sdh* B,C;B,D; *Pgi-2* C,C;K,B) provided a good test of linkage of *Pgi-2* with *Sdh* as it carried identifiable *Sdh* alleles in both genomes and two distinctive *Pgi-2* alleles in one genome. In crosses to plant 14 (*Sdh* A,B;B,B; *Pgi-2* C,C;C,C), which tested linkages of *Sdh* alleles C and D to *Pgi-2* alleles B and K, progeny genotypes were consistent with independent assortment of the *Pgi-2* alleles from *Sdh* alleles in both genomes (Table 7 d).

## Discussion

Progeny test evidence was consistent with the hypothesis that SDH was inherited as a monomeric enzyme controlled by duplicate, independently assorting, loci. The results were consistent with the presence of an SDH locus in each of the two genomes in this putative allotetraploid species and the inheritance pattern indicated that each locus was disomic. The same, or similar, alleles occurred in both genomes.

The presence of multiple-banding phenotypes due to 'conformer' or 'ghost' bands which co-segregate and give each SDH allele a multibanded phenotype has probably caused confusion in the past. Such conformers were regularly, but not always, seen in the present study and have been noted in several other legumes including *Trifolium* (Collins et al. 1984), *Phaseolus* (Garvin et al. 1989), *Vigna* (Jaaska and Jaaska 1989) and *Glycine* (Yu and Kiang 1993). Co-segregating double-banded phenotypes were also reported for other plants including maize, incense cedar and *Capsicum* (Wendel et al. 1988).

### Disomic inheritance

Three crosses involving plant 13 would have detected tetrasomic inheritance of SDH with near certainty, if it

**Table 5** Segregation analyses involving plant 8 to detect linkage between *Sdh* B′ and *Li*. Plant 8, carrying Sdh B and Li, each in a single dose, was crossed with three tester plants. The *Sdh* B allele carried by plant 8 is designated B′ to distinguish it from similar alleles carried by other plants. Plant 8 gametes carrying B′ also carried one C allele while gametes lacking B′ were CC. In crosses with plants 1 and 14, band intensities were used to identify progeny plants carrying one C allele and hence also B′. $\chi^2$ values were calculated for departure from expectations based on the independent assortment of *Sdh* B′ and *Li*

| Cross | | Genotype frequencies | | | | | $\chi^2$ |
|---|---|---|---|---|---|---|---|
| | | Parental | | Recombinant | | | |
| | | Li/B′ | -/- | Li/- | -/B′ | Total | |
| 8 × 14 | Observed | 10 | 7 | 0 | 0 | 17 | |
| B′,C;C,C Li,li × A,B;B,B li,li | Expected | 4.25 | 4.25 | 4.25 | 4.25 | 17.0 | 18.058*** |
| 8 × 7 and reciprocal | Observed | 21 | 19 | 1 | 2 | 43 | |
| B′,C;C,C Li,li × C,C;C,D li,li | Expected | 10.75 | 10.75 | 10.75 | 10.75 | 43.00 | 32.069*** |
| 8 × 1 and reciprocal | Observed | 30 | 19 | 3 | 1 | 53 | |
| B′,C;C,C Li,li × A,B;B,B li,li | Expected | 13.25 | 13.25 | 13.25 | 13.25 | 53.00 | 42.923*** |

***$P < 0.005$

**Table 6** Segregation analyses involving plant 6 to test for linkage of *Sdh* and *Li*

**(a)** Test for linkage of *Sdh* D′ with *Li*. Plant 6, carrying *Sdh* D and *Li*, both in a single dose, was crossed with plant 7 (C,C;C,D li, li). The *Sdh* D allele in parent 6 (designated D′ to distinguish it from the allele in parent 7) could be traced in 50% of the progeny by counting all plants with no D band and all with two copies of D, as indicated by band intensities. Expected frequencies were calculated assuming disomic inheritance and independent assortment of the loci, and tested for goodness of fit by $\chi^2$

| Cross | | Genotype frequencies | | | | | $\chi^2$ |
|---|---|---|---|---|---|---|---|
| | | Parental | | Recombinant | | Total | |
| | | *Li*/D′ | -/- | *Li*/- | -/D′ | | |
| 6 × 7 and reciprocal | Obs | 11 | 9 | 1 | 0 | 21 | |
| B,C;C,D′ *Li*,*li* × C,C;C,D *li*,*li* | Exp | 5.25 | 5.25 | 5.25 | 5.25 | 21.0 | 17.667*** |

**(b)** Test for linkage of *Sdh* B with *Li*. In the same cross as (a) the *Sdh* B and *Li* alleles were unique to plant 6. Progeny plants were classified for presence or absence of B and *Li*. Expected frequencies were calculated assuming disomic inheritance and goodness of fit was tested by $\chi^2$

| Cross | | Genotype frequencies | | | | | $\chi^2$ |
|---|---|---|---|---|---|---|---|
| | | Parental | | Recombinant | | Total | |
| | | *Li*/B′ | -/- | *Li*/- | -/B′ | | |
| 6 × 7 and reciprocal | Obs | 10 | 7 | 8 | 13 | 38 | |
| B,C;C,D′ *Li*,*li* × C,C;C,D *li*,*li* | Exp | 9.5 | 9.5 | 9.5 | 9.5 | 38.0 | 2.210 |

had occurred. In no case among these crosses was a tetrasomic inheritance pattern for SDH found to occur (Table 3). In two other families, segregation ratios also departed significantly from tetrasomic expectations while conforming to the expected outcomes for disomic inheritance (Table 4). All families analysed fitted a disomic inheritance pattern and it is apparent from these results that SDH is controlled in white clover by duplicate loci with each of the duplicates behaving disomically and assorting independently. The results are consistent with these duplicates being homoeoloci, i.e. a single locus represented once in each ancestral genome of an allotetraploid, although other interpretations cannot be eliminated at this stage. A homoeologous arrangement would be consistent with findings in other allopolyploid species, e.g. wheat, where homoeoloci have been mapped in the A, B, and D genomes (Hart et al. 1993; Nelson et al. 1995). If, as in the cereals, these homoeoloci extend to related genera and species, then mapping of such loci could clarify the ancestry of white clover.

In the absence of any evidence for distinguishing two loci, the gene is tentatively designated *Sdh*. This interpretation is consistent with the inheritance pattern of phosphoglucoisomerase (PGI) allozymes in white clover, demonstrated by Michaelson-Yeates (1986), in which disomic inheritance occurred at the duplicate *Pgi-2* locus. This pattern of SDH inheritance is highly consistent with an allotetraploid genetic structure with regular pairing of homologous chromosomes and no homoeologous pairing (with one possible exception to be discussed further). Such a result is also expected

**Table 7** Segregation data for tests of linkage between *Sdh* and other genetic loci

**(a)** To test for linkage to *Ac* (cyanogenic glucoside), plant 7, carrying *Ac* and *Sdh* D, both in single dose, was crossed with plant 1 which carried neither *Ac* nor *D*. Expected segregation frequencies were calculated for independent assortment of *Ac* and *D*, and goodness of fit was tested by $\chi^2$. Reciprocal crosses were combined for analysis after a heterogeneity test indicated that the data were homogeneous

| Cross | | Genotype frequencies | | | | | $\chi^2$ |
|---|---|---|---|---|---|---|---|
| | | Parental | | Recombinant | | | |
| | | D/*Ac* | -/- | D/- | -/*Ac′* | Total | |
| 1 × 7 and reciprocal | Observed | 9 | 8 | 9 | 16 | 42 | |
| A,B;B,B ac,ac × C,C;C,D *Ac*,*ac* | Expected | 10.5 | 10.5 | 10.5 | 10.5 | 42.0 | 3.904 |

**(b)** To test for linkage of *Rf* (red leaf mark) with *Sdh*, plant 15, with *Sdh* B in both genomes and *Rf* in single dose, was crossed with plant 13, which carried neither B nor *Rf*. Expected segregation frequencies were calculated for independent assortment of *Rf* and B, and goodness of fit was tested by $\chi^2$. Reciprocal crosses were combined for analysis after a heterogeneity test indicated that the data were homogeneous

| Cross | | Genotype frequencies | | | | | $\chi^2$ |
|---|---|---|---|---|---|---|---|
| | | Parental | | Recombinant | | | |
| | | B/*Rf* | − / − | B/ − | − /*Rf* | Total | |
| 13 × 15 and reciprocal | Observed | 14 | 4 | 20 | 5 | 43 | |
| A,A;C,D *rf*,*rf* × B,D;B,D *Rf*,*rf* | Expected | 16.125 | 5.375 | 16.125 | 5.375 | 43.0 | 1.589 |

**(c)** To test for linkage of *V* (white leaf mark) with *Sdh*, plant 15, with *V* in single dose and doubly heterozygous for *Sdh* B and D, was crossed with plants 13 and 14, both vv and carrying alternative *Sdh* alleles. A similar cross was carried out between plants 1 (*V*, light *V*, *Sdh* A) and 11 (*vv*, no A). Observed segregation frequencies were compared with expected frequencies calculated on the basis of independent assortment, and goodness of fit tested by $\chi^2$. Reciprocal crosses were combined for analysis after a heterogeneity test indicated that the data were homogeneous

| Cross | | Genotype frequencies | | | | | $\chi^2$ |
|---|---|---|---|---|---|---|---|
| | | Parental | | Recombinant | | | |
| | | B/*V* | -/- | B/- | -/*V* | Total | |
| 13 × 15 and reciprocal | Observed | 17 | 2 | 15 | 7 | 41 | |
| A,A;C,D *v*,*v* × B,D;B,D *V*,*v* | Expected | 15.375 | 5.125 | 15.375 | 5.125 | 41.000 | 2.772 |
| | | D/V | -/- | D/- | -/V | | |
| 14 × 15 | Observed | 16 | 4 | 14 | 2 | 36 | |
| A,B;B,B *v*,*v* × B,D;B,D *V*,*v* | Expected | 13.5 | 4.5 | 13.5 | 4.5 | 36.0 | 1.927 |
| | | A/V | -/- | A/- | -/V | | |
| 1 × 11 | Observed | 14 | 7 | 6 | 13 | 40 | |
| A,B;B,B *V*,*V light* × B,C;B,D *v*,*v* | Expected | 10 | 10 | 10 | 10 | 40 | 5.000 |

**(d)** A test for linkage between *Sdh* and *Pgi-2* involved the cross of plant 11 with plant 14. This cross enabled *Sdh* alleles C and D (one in each genome) and *Pgi* alleles K and B (both in one genome) to be analysed in the progeny, thus testing simultaneously for linkage in both genomes. Expected frequencies were calculated assuming disomic inheritance and independent assortment of the two loci. Reciprocal crosses were combined for analysis after a heterogeneity test indicated that the data were homogeneous. The phenotypic classes are given as *Sdh*/*Pgi*

| Cross | | Genotype frequencies | | | | | | | | Total | $\chi^2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | D/K | D/B | C/K | C/B | C,D/K | C,D/B | -/K | -/B | | |
| 11 × 14 and reciprocal | Observed | 4 | 3 | 7 | 6 | 7 | 5 | 5 | 5 | 42 | |
| *Sdh* B,C;B,D × A,B;B,B | Expected | 5.25 | 5.25 | 5.25 | 5.25 | 5.25 | 5.25 | 5.25 | 5.25 | 42.00 | 2.571 |
| *Pgi* C,C;K,B × C,C;C,C | | | | | | | | | | | |

from the inheritance patterns of other (single) disomic loci in white clover: *V* (white leaf markings), *R* (anthocyanin leaf markings), *Li* (linamarase), *Ac* (cyanogenic glucosides) and *S* (gametophytic incompatibility) (Corkill 1971). In all of these cases only one locus with two alleles is present in the tetraploid plant and the absence of homoeologous pairing is indicated from the regular disomic inheritance patterns shown by these genetic traits. The regularity of homologous chromosome pairing is also suggested by the very regular bivalent formation at meiosis (Atwood and Hill 1940; Chen and Gibson 1970) and the effectiveness of the gametophytic incompatibility system which would fail if a significant amount of homoeologous chromosome pairing occurred. Nevertheless, one apparent multivalent was observed by Chen and Gibson (1970) and so occasional homoeologous pairing may be possible. If occasional homoeologous pairing can occur then the genetic implications could be considerable (Kakes and Hakvoort 1994). In the present study, one individual (Table 3) may have had such an origin but further research is needed to eliminate other possibilities. Similarly, Michaelson-Yeates (1986), studying PGI inheritance, also found progeny that could be explained by homoeologous pairing but, in that case also, alternative explanations could not be eliminated.

Plants like plant 13, with three alleles, one homozygous, are especially useful in such studies of tetrasomic inheritance, and/or occasional homoeologous pairing, since they generate progeny which cannot be produced by disomic inheritance. Such genotypes require only minimum population sizes to detect tetrasomic inheritance (fewer than 12 in the present study; Mather 1951) and can also distinguish between complete tetrasomy and occasional homoeologous pairing. Other genotypes either fail to separate disomy and tetrasomy (e.g. those with two alleles, one present in three doses, Table 2) or do so only for larger populations (e.g. those with three alleles, none of which are homozygous, Table 4).

## Linkage analysis

Linkage analyses involving plants 6 and 8 indicated relatively close linkage between *Li* and *Sdh* in one of the white clover genomes. Larger families are being subjected to further analysis to determine a more accurate genetic distance than the combined estimate of $6.0 \pm 2.0$ cM from the four small families evaluated in this study. The results of one cross ($6 \times 7$, Table 6 b) also showed that the *Sdh* locus in the alternative genome assorted independently of *Li*, as expected from the evidence for independent assortment of the duplicate *Sdh* loci. The *Sdh-Li* linkage was established without use of band intensities ($8 \times 7$, Table 5). However, in some crosses, band intensities were used to provide supporting data. This was possible because plants of known genotype were used and the relative intensities of complementary bands could always be used to provide a double check and give reliable results. For example, Fig. 1 shows band B segregating as either B or BB and simultaneously band A is complementary as AA or A, respectively. Thus, although band B is weaker than A at the same dosage, the single-dose B is clearly readable where it is less intense relative to adjacent BB plants and simultaneously markedly less intense than the AA band in the same plant. Similar complementary effects were used for some of the linkage analyses shown in Tables 5 and 6 a.

*Sdh* also assorted independently of the other markers tested (*Pgi-2*, *V*, *Ac*, *R*). The linkage of *Sdh* and *Li* is one of the first recorded for white clover and is one of immediate potential use. First, it could serve as a flanking marker for recombination studies in the vicinity of *Li* where some duplication has been identified (Oxtoby et al. 1991). Second, the *Sdh-Li* region is a potential marker region for agronomic performance in white clover. There is a body of evidence for ecological correlations between cyanogenesis and adaptation to environmental variables. In Europe there is a strong latitudinal and altitudinal clinal effect towards acyanogenic populations at cooler winter temperatures (Daday 1954a, b 1958) and this effect has been confirmed experimentally (Daday 1965). The last study also indicated that both *Ac* and *Li* in the absence of the other (i.e. *Ac*,*li* and *ac*,*Li*) showed a growth advantage over *ac*,*li* at high temperatures. The regression coefficients of *Ac* and *Li* frequencies with winter temperatures in Europe were significantly different, as were the calculated selection responses for *Ac* and *Li* derived from controlled-environment observations. It therefore appears that natural selection may act independently on *Ac* and *Li*. Morley (1959) and Daday (1965) have indicated that these apparent selection effects may be indirect rather than depending directly on the cyanogenic potential per se, i.e. there are probably polygenes enhancing fitness at high or low temperatures linked to the dominant and recessive alleles of both loci. Ennos (1981a, b) found that individuals carrying the *Li* allele had larger leaves than those lacking *Li* and that the *Li* phenotype was associated with total yield in competition experiments. Dommee et al. (1980) found that plants carrying *Li* grew longer roots than *li* plants while the *Ac* locus showed no association with root growth. Foulds and Grime (1972a, b) invoked the linkage of *Ac* with genes concerned with a response to the environment to explain an apparent association of *Ac* with drought susceptibility in natural populations in Staffordshire, U.K. In New Zealand, an association between positive cyanogenesis and high vegetative yields was exploited for many years by the use of the picrate paper method as a quality assurance test for certified New Zealand Mother Seed (Foy and Hyde 1937). It is thus likely that the presence or absence of *Li* (and/or *Ac*) could be a useful marker system for

important genes associated with plant performance in response to environmental factors. The finding that *Sdh* is closely linked to *Li* should provide a useful additional tool to aid in the unravelling of these potentially very significant effects.

Linkage analysis in white clover, as shown in Table 7, presents some problems. For example, the data shown in Table 7 a test for linkage in only one of the two genomes. Relatively special genotypes are needed to simultaneously test both genomes (e.g. Table 7 d). This study exemplifies the difficulties associated with linkage and mapping studies in polyploid species where the existence of identical alleles at loci in both genomes makes it difficult to establish which of the two loci is linked to another marker (Sorrells 1992). In this study unequivocal linkage was established only where an allele of each locus under study was present in single-dose in one parent plant. The method used here thus paralleled the use of single-dose restriction fragments which have been shown to facilitate the detection of linkage and the mapping of polyploid species (Sorrells 1992; Wu et al. 1992). Genetic mapping in white clover will require further use of this method.

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