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Enzyme variation and inheritance in *Glechoma hederacea* (Lamiaceae), a diploidized tetraploid

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The chromosome number of the polyploid species *Glechoma hederacea* was found to be 2n = 36 in a sample of 93 ramets derived from 27 sites in N and C Europe. Variation in 10 enzymes was surveyed in material from S Sweden and S Czech Republic. The genetic control of variation was investigated using segregating progeny from crosses and self-fertilized heterozygous plants. The genetic analysis comprised 30 of 32 putative alleles detected in the geographical survey. Five loci (*Aat-2*, *Tpi-1*, *Tpi-2*, *Pgd-2* and *Mnr*) behaved as isoloci with one copy of a locus being monomorphic for a common allele, the other di-allelic for a common allele and a variant allele. In four isoloci (*Pgd-1*, *Pgi-2*, *Mdh-2* and *Adh*), both copies of the duplicated locus were polymorphic, with one allele common to both copies and with another allele unique for each copy except for *Pgd-1* where both copies were tri-allelic. Three loci, *Pgm-3*, *Skd-1* and *Skd-2* were regarded as being non-duplicated. Segregation ratios for all enzyme loci were in close agreement with expectations based on disomic inheritance. Our data suggest that the tetraploid *G. hederaca* is a diploidized autotetraploid.

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Duplicated enzyme loci are common in plants and animals, either as a result of polyploidy or as a consequence of chromosomal rearrangement involving the duplication of segments of the genome. The estimation of basic population genetic parameters, such as allelic frequencies assayed by starch-gel electrophoresis, is complicated in duplicated loci if these loci share the same codominant allelic variants. Even if the relative staining intensity of the electromorphs can be used to estimate the doses of variant alleles, it is often impossible to tell which copy of a locus is segregating. Maximum likelihood approaches can be used to estimate allele frequencies in natural populations of polyploids (WAPLES 1988), particularly if information on the type of polyploidy (auto- or allopolyploidy) support the interpretation of allele frequencies.

Two types of evidence are used to distinguish autopolyploids from allopolyploids: cytogenetic analysis (e.g., JACKSON 1982) and enzyme electrophoresis (e.g., SOLTIS and RIESEBERG 1986). Traditional cytogenetics predicts that the four homologous chromosomes in a newly arisen auto- or allopolyploid derived from closely related species form multivalents during meiosis (STEBBINS 1947). Thus, species that produce large numbers of multivalents are assumed to be autopolyploids, while those that produce only bivalents are assumed to be allopolyploids. However, this method of distinguishing between auto- and allopolyploidy has proved to be unreliable since many factors are known to control bivalent formation (WALL et al. 1971). There are documented cases of autopolyploids that exhibit only bivalents in meiosis (SOLTIS and RIESEBERG 1986), and some allopolyploids have been found to form multivalents (WALL et al. 1971).

Genetic analysis of allozyme variants is an alternative method for distinguishing between auto- and allotetraploidy (QUIROS 1982; SHORE 1991). Because of preferential pairing of homologous chromosomes, allotetraploids exhibit independent segregation at the duplicated loci (disomic inheritance). Segregation in autotetraploids is tetrasomic with no preferential pairing of chromosomes even though only bivalents are formed (SOLTIS and RIESEBERG 1986). For diagnostic heterozygous genotypes, random association of the four homologues during meiosis yields fewer homozygous gametes than would be produced by an allotetraploid of the same genotype.

Polyploid organisms may undergo a process of "diploidization" during which disomic inheritance is wholly or partially restored (FERRIS and WHITT 1977; LI 1980). This diploidization can proceed through complete silencing of one of the copies of the duplicated gene or through a substantial divergence of gene function. Differences in the rate at which disomy is restored can lead to heterogeneity in the mode of inheritance within a species (SEARS 1969). Of the loci that remain duplicated, some pairs may thus diverge substantially, whereas others, termed

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Table 1. *Expected segregation ratios for the progeny of selfed plants of different parental phenotypes according to the model of tetrasomic and disomic inheritance at isoloci. Ratios for diagnostic phenotypes in bold (see text and Fig. 1)*

Parent phenotype	Tetrasomic inheritance Offspring phenotype					Disomic inheritance Offspring phenotype				
	I	II	III	IV	V	I	II	III	IV	V
I	1					1				
II	1	2	1			1	2	1		
III	**1**	**8**	**18**	**8**	**1**	**1**	**4**	**6**	**4**	**1**
III								**1**		
IV			1	2	1			1	2	1
V					1					1

"isoloci" have gene products with identical electrophoretic mobilities (WAPLES 1988). Thus isoloci typify auto- and allopolyploids derived from ancestral species with considerable genetic similarity. The identification of common and unique alleles at such isoloci can be used to distinguish diploidized autopolyploids from allopolyploids (WERTH 1989). Both can be expected to show disomic inheritance, the former with little divergence of genes on homologous chromosomes and the latter with substantial differentiation at isoloci.

The aims of this study are (1) to survey enzyme variation in populations of *Glechoma hederacea*, a gynodioecous, clonal plant (HUTCHINGS and PRICE 1999), (2) to use segregation data to determine the mode of inheritance of putative loci, and (3) to establish the ploidy-level and the type of polyploidy (auto- or allopolyploidy).

THE MODEL

We assume that we are examining isoloci, i.e. two copies of a locus with allelic variants with the same relative mobility and stainability in enzyme electrophoresis. If we consider the simplest case of a tetraploid with two allelic variants, *a* and *b*, in each genome, there are five potentially different phenotypes for monomeric and dimeric enzymes (Table 1), provided the phenotypes can be differentiated on the basis of the relative number of alleles corresponding to each band (Fig. 1). Segregation in the progeny after selfing plants with the different multi-banded phenotypes can be used to determine whether inheritance is disomic or tetrasomic. In a newly arisen autotetraploid with tetrasomic inheritance, selfing the multi-banded phenotypes will always result in segregation in the progeny with phenotypic proportions reflecting the genotype of the mother plant. When a plant that is of phenotype III (Table 1) is selfed this will give rise to a progeny with phenotypes I to V that segregate as 1:8:18:8:1. Selfed plants of phenotype II will give rise of a progeny segregating the phenotypes I to III in the ratio of 1:2:1, while selfed plants of phenotype IV will give rise to phenotypes III to V in the ratio of 1:2:1.

In allo- and autopolyploids that have undergone diploidization we expect to find disomic inheritance. When phenotypes II and IV are selfed segregation will result in three phenotypes (I, II, III and III, IV, V respectively) in the Mendelian ratio of 1:2:1. Phenotype III may either be homozygous for *a* in one genome and for *b* in the other genome, or heterozygous (*ab*) at both isoloci. In the first case selfing will result in progeny with fixed heterozygosity (phe-

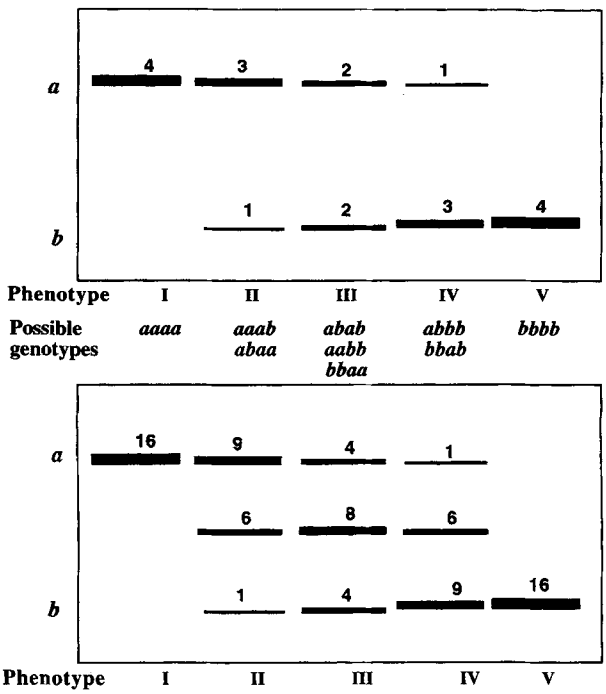

Fig. 1. Expected phenotypes at isoloci for monomeric (above) and dimeric (below) enzymes. Figures above each band refer to relative staining intensity.

Table 2. *Origin of individual plants of G. hederacea used in crossing experiments for the analysis of segregating progeny. S = Sweden, CR = Czech Republic, N = Number of plants in the segregation analyses*

Plant no.	Origin		Type of cross	Paternal plant	N
	Country	Locality			
1	S	Öved	Selfing		47
2	S	Öved	Selfing		25
4	S	Öved	Selfing		59
5	S	Öved	Selfing		60
6	S	Öved	Cross	4	31
6	S	Öved	Selfing		35
7	S	Öved	Selfing		8
20	S	Öved	Selfing		28
8-1	S	Dalby	Selfing		33
8-2	S	Dalby	Selfing		12
15	S	Kullaberg	Cross	13	12
9	CR	Trebon	Selfing		16
11	CR	Trebon	Selfing		33
13	CR	Trebon			

notype III), whereas in the latter case, segregation will result in all five phenotypes in the Mendelian ratio of 1:4:6:4:1 (Table 1). Thus, when phenotype III is selfed, segregation in the progeny can be used to distinguish between disomic and tetrasomic inheritance. In this study, we design a crossing experiment on the basis of plants that are of phenotype III. As a first step, we test whether segregation after selfing conforms to disomic or tetrasomic inheritance after which we investigate whether segregation ratios after selfing phenotypes II and IV conform to the mode of inheritance suggested in the first step.

MATERIAL AND METHODS

Chromosome studies

Samples of ramets derived from Sweden and C Europe were transferred to the Botanical Gardens, Lund University (from 16 sites in Sweden, 8 sites in the Czech Republic, 2 sites in Slovakia and 2 sites in Germany, cf. WIDÉN 1996). When neighbouring sites were sampled, the distance between sites exceeded 200 m. Root tips were fixed in a modification of Navashin's fixative (LÖVE and SARKAR 1956), embedded in paraffin wax, sectioned at 12–14 μ and stained in 1% crystal violet and 1% aniline solution (1:1), and examined under a light microscope.

Enzymes

Geographical survey.—To cover as much of the enzyme variation in *G. hederacea* as possible we screened ramets from seven natural populations in S Sweden (N = 909) and three populations in the Czech Republic (N = 403). Details of the sampling design are given in WIDÉN (1996).

Crosses.—On the basis of variation in putative loci, twenty ramets derived from Sweden and the Czech Republic (Table 2) were selected to represent plants that were heterozygous at separate loci. To initiate flowering, we kept the plants outdoors during the winter and transferred them to a heated greenhouse in early March. Since each crossed flower produces a maximum of four seeds we needed a great number of hand-pollinated flowers to achieve a sufficient number of progeny to test alternative hypotheses for the inheritance of the enzymes. Unfortunately, cultivation conditions were not optimal for flowering and some plants produced few flowers. Seed production in some plants was further reduced by herbivory by the larvae of a moth so that not all zymograms found in the geographical survey could be tested.

In all, eleven selfed hermaphrodite plants produced progeny. We also used out-crossed progeny, one female plant (no 15) and one plant that changed sex (no 6) being used as maternal plants in cross-pollination (Table 2). The plants were grown in isolation in a greenhouse, and flowers were hand-pollinated by brushing the receptive stigmas with dehiscing anthers from the same plant or from a pollen donor. Seeds were germinated on petri dishes and seedlings were transferred to separate pots a few days after germination. After the plants had been growing for about one month, they were used in electrophoresis.

The putative multilocus genotype of individual plants and the number of plants used in the analysis of enzyme inheritance are given in Table 2 and 3.

Electrophoresis.—Young leaf material was soaked in water for 12 hours in darkness and then crushed in an extraction buffer (0.05 M tris/mercaptoethanol) at

pH 7.0. The crude extract from each sample was then absorbed onto 9 × 3 mm Whatman no 3 filter paper wicks and stored at −80°C. Horizontal starch gel (12%) electrophoresis was used to obtain zymograms for ten enzyme systems. Asparate aminotransferase (AAT), menadione reductase (MNR), triose-phosphatate isomerase (TPI) and alcohol dehydrogenase (ADH) were assayed using buffer system no 7 as in SOLTIS et al. (1983). Phosphoglucomutase (PGM), phosphogluconate dehydrogenase (PGD), phosphoglucoisomerase (PGI), malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH) and shikimate dehydrogenase (SKD) were assayed using buffer system no 2 as in WENDEL and WEEDEN (1989).

Interpretation of zymograms.—Previous studies have shown that PGM and SKD are monomeric, that PGI, TPI, AAT, ADH, MDH and PGD are dimeric, and that MNR is tetrameric (see e.g., WEEDEN and GOTTLIEB 1980; GOTTLIEB 1981; PICHERSKY and GOTTLIEB 1983). The putative loci for different enzymes are shown in Fig. 2, so that the locus nearest the anode is called 1. When the banding pattern could be interpreted consistently, the putative alleles (*a* to *e*, with allele *a* denoting the fastest allele) are indicated in Fig. 2. To denote the two copies of a duplicated locus, we use the superscript ′ for the copy having the fastest alleles and the superscript ″ for the copy having the slowest alleles (based on the average migration rate of allozymes that can be designated to a specific locus).

The observed progeny ratios and the results of the genetic analysis for each locus and cross are presented in Appendix A and B (a few crosses with poor resolution have been excluded). The observed ratios were compared with those expected for the presumed type of inheritance by using a log likelihood ratio test (G). The presence of linkage between pairs of loci were also investigated by log likelihood ratio tests (Appendix C). Because of the scarcity of offspring in some crosses we had to pool plants to meet the criterion of at least 5 expected individuals in each category (SOKAL and ROHLF 1995). Since the samples consisted of less than 200 plants in all our crosses, we follow the recommendation of SOKAL and ROHLF (1995) to routinely correct *G* for small sample sizes by the method suggested by WILLIAMS (1976). This correction results in a conservative test that lowers the risk of Type I error.

RESULTS

Chromosome numbers

The chromosome number was determined for 93 ramets from 28 sites in N and C Europe. In 90 ramets, the number 2n = 36 was found; 2n = 42 was found in one ramet derived from Mölle in Skåne (Sweden), and 2n = 54 in two ramets, one from Slovakia (Devinska Kobyla) and the other from a population in Sweden (Öved in Skåne).

Interpretation of enzymes

All consistent zymograms found in the crossing experiments and in the geographical survey are shown in Fig. 2. Combinations of individual zymograms for different enzymes yielded 231 unique multilocus genotypes among the 1312 ramets in the geographical survey. Only one multilocus genotype was found in two populations.

PGM has three regions of activity. There may be two alleles at each of the loci *Pgm-1* and *Pgm-2* (not

Table 3. *Genotypes at three non-duplicated loci and nine duplicated loci of plants used in crossing experiments for the analysis of segregating progeny in G. hederacea. For reference to alleles at individual loci see Fig. 2.*

Plant no.	Locus											
	Skd-1	*Skd-2*	*Pgm-3*	*Aat-2*	*Pgi-2*	*Tpi-1*	*Tpi-2*	*Pgd-1*	*Pgd-2*	*Adh*	*Mdh-2*	*Mnr-1*
1	*ab*	*aa*	*bb*	*aaab*	*aabb*	*aaaa*	*aaaa*	*bccd*	*aaaa*	*abbc*	*bbbb*	*aaab*
2	*bc*	*aa*	*bc*	*aaaa*	*aaab*	*aabb*	*aaab*	*cccd*	*aabb*	*abbc*	*abbb*	*aaaa*
4	*cc*	*bb*	*cc*	*aaab*	*aaaa*	*aaab*	*aaaa*	*bccd*	*aaaa*	*abcc*	*bbbb*	*aaaa*
5	*aa*	*ab*	*cc*	*aaaa*	*aaab*	*aaab*	*aaaa*	*cccd*	*aaaa*	*abbb*	*bbbc*	*aaaa*
6	*ac*	*aa*	*cc*	*aaaa*	*aabc*	*aaab*	*aaab*	*bccc*	*aaaa*	*abbb*	*abbb*	*aaaa*
7	*bc*	*ab*	*cc*	*aaab*	*aaab*	*aaab*	*aaaa*	*bccd*	*aaaa*	*bbcc*	*bbbc*	*aaab*
8-1	*bb*	*bb*	*cc*	*aaab*	*aaab*	*aabb*	*aaab*	*bcce*	*aaab*	*bbbc*	*abbb*	*aaab*
8-2	*ac*	*ab*	*cc*	*aaaa*	*aaab*	*aaab*	*aaab*	*ccde*	*aaaa*	*abbc*	*abbb*	*aaaa*
9	*bc*	*aa*	*bc*	*aabb*	*aaaa*	*aaaa*	*aaaa*	*bbcd*	*aaaa*	*abbc*	*aabb*	*aaaa*
11	*bc*	*aa*	*bb*	*aabb*	*aaab*	*aaaa*	*aabb*	*bcdd*	*aaaa*	*bbbc*	*bbbb*	*aaaa*
13	*ab*	*bb*	*cc*	*aabb*	*aabc*	*aabb*	*aaaa*	–	*aaab*	*bbbb*	*bbbb*	*aaaa*
15	*ac*	*aa*	*bc*	*aabb*	*aaac*	*aaab*	*aaaa*	*ccdd*	*aaaa*	*bbbb*	*bbbb*	*aaaa*
20	*ab*	*ab*	*cc*	*aaab*	*aaab*	*aaab*	*aaab*	*bbcc*	*aaab*	*abbb*	*aabb*	*aaaa*

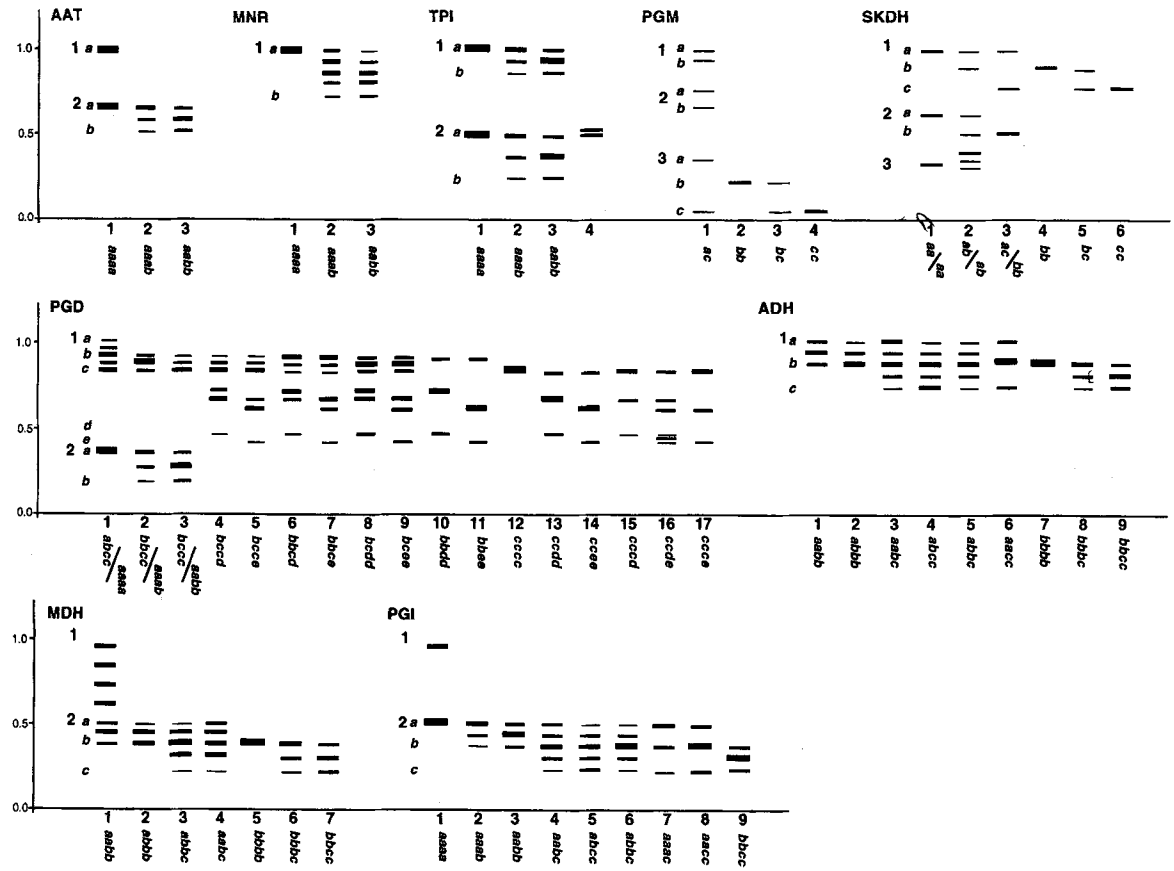

Fig. 2. Diagrammatic representation of all zymograms for putative, interpretable loci found in *G. hederacea*.

considered further). Four phenotypes were found for *Pgm-3*, suggesting three alleles; viz. *a*, *b* and *c*. Phenotype 1 was rare, occurring only in one population in the Czech Republic. In good gels phenotypes 2 and 4 showed a faint band in the position of allozymes b and c respectively. Most plants belonged to phenotype 2 or 4.

SKD has three regions of activity. *Skd-1* showed six phenotypes. There may, accordingly, be all possible combinations of three alleles (*a*, *b* and *c*) in a monomeric enzyme locus. The banding pattern for *Skd-2* showed three phenotypes, due to two alleles, *a* and *b*. *Skd-3* showed two consistent banding patterns with phenotype 2 found only rarely. No allele model could be fitted to this variation.

AAT has two zones of activity. *Aat-1* is monomorphic, whereas *Aat-2* is polymorphic and is expressed as three phenotypes in most populations. The banding pattern is best understood if we assume that there are two alleles, and that the three phenotypes represent the genotypes *aaaa*, *aaab* and *aabb*.

TPI has two zones of activity. Both *Tpi-1* and *Tpi-2* were polymorphic with three and four phenotypes respectively. Phenotype 4 for *Tpi-2* is rare and was only found once in a single population (Silvåkra). Two alleles (*a* and *b*) code for both *Tpi-1* and *Tpi-2*. Phenotype 4 for *Tpi-2* can best be explained if we assume a null allele at one of the copies of this locus.

IDH is monomorphic with three bands, and no genetic model is suggested here.

MNR has one zone of activity, either as one-banded (1) or five-banded phenotypes (2 and 3). Since MNR is a tetrameric enzyme, the simplest explanation is that there are two alleles (*a* and *b*) the one-banded phenotypes being *aaaa* (phenotype 1), the five-banded phenotypes being either *aaab* (phenotype 2) or *aabb* (phenotype 3). The five-banded phenotypes are difficult to separate since the staining intensity of the band nearest the cathode is often indistinguishable from the other four bands in both phenotype 2 and 3. The gels have to be scored very soon after beginning the staining procedure because of the risk of over-staining.

PGD has two regions of activity. In the geographical survey the region closest to the anode, *Pgd-1*, displayed twelve banding patterns and in the segregation study five additional phenotypes, whereas that nearest the cathode, *Pgd-2*, showed three banding patterns. We suggest that there are five alleles (*a* to *e*)

in *Pgd-1*, four of which were represented in the segregation study (*b* to *e*). Variation in *Pgd-2* can best be understood as the result of the segregation of two alleles, (*a* and *b*).

MDH has two zones of activity, one of which had numerous bands that proved impossible to interpret (*Mdh-1*). Seven phenotypes were distinguished for *Mdh-2*, and a model with 3 alleles (*a*, *b* and *c*), agreed with the segregation patterns (see below). PGI has two regions of activity. The faster of them, *Pgi-1*, is monomorphic, while the slower, *Pgi-2*, is polymorphic. In the geographical survey the banding pattern of *Pgi-2* revealed 6 phenotypes that were consistent among screening runs and three additional phenotypes in the segregation study. Phenotypes 4–6 were rare in the geographical survey. Three alleles (*a*, *b* and *c*) are suggested for *Pgi-2*.

ADH has one anodal region of activity. Three phenotypes were found in the geographical survey, but because of technical difficulties only part of the material was scored. The progeny analyses showed 9 phenotypes, suggesting the presence of three alleles (*a*, *b* and *c*).

Segregation patterns

Most plants showed one-banded phenotypes for several loci (Table 3). The progeny of selfed plants with one-banded phenotypes never segregated, nor did the progeny after crosses between plants with the same one-banded phenotype.

Monomeric loci.—Segregation ratios in selfed plants with two-banded phenotypes conformed with a one-locus three-allele model for *Skd-1* and a one-locus two-allele model for *Skd-2* and *Pgm-3*. Crosses between plants with different phenotypes gave rise to progeny that segregated according to expectation (Appendix A). Taken together, the results suggest that there is only one locus in *Pgm-3*, *Skd-1* and *Skd-2*, respectively.

Dimeric and tetrameric isoloci.—Five isoloci showed three phenotypes (*Pgd-2*, *Aat-2*, *Tpi-1*, *Tpi-2* and *Mnr*) with two putative alleles each. The progeny of selfed plants of phenotype III in our model (Table 1) with the putative genotype *aabb* did not segregate indicating homozygozity for *a* and *b*, respectively, in the two gene copies (Appendix A). Thus the fixed heterozygosity in the progeny of *aabb* plants suggests a disomic inheritance at these loci. In offspring from selfed plants that have phenotype II (*aaab*), no significant deviation from a two-allele model was detected (except for *Tpi-2* in plant 8-2 which produced a small number of offspring after selfing), indicating homozygozity (*aa*) at one copy and heterozygosity (*ab*) at the other copy of individual isoloci. The cross *aaaa* x *aaab* for *Aat-2* and *Tpi-1* produced progeny that segregated in two phenotypes in the expected ratio of 1:1. The same cross for *Pgd-2*, however, gave progeny that deviated from the expected ratio of 1:1, although the number of progeny was few. The lack of more than three phenotypes in the geographical survey supports a model with one monomorphic copy (*Pgd-2'*, *Aat-2'*, *Tpi-1'*, *Tpi-2'* and *Mnr'*, respectively) and one two-allele copy (*Pgd-2''*, *Aat-2''*, *Tpi-1''*, *Tpi-2''* and *Mnr''*, respectively) of these isoloci in *G. hederacea*.

Four dimeric isoloci showed more than three phenotypes (*Mdh-2*, *Pgi-2* and *Adh*, *Pgd-1*). The progeny of selfed plant with the three-banded phenotype III in our model (Table 1) did not segregate at these four isoloci (Appendix B), indicating disomic inheritance for each isoloci with one copy homozygous for one allele (for instance, *a* in *Mdh-2*, *Pgi-2* and *b* in *Adh* and *Pgd-1*) and the other copy homozygous for another allele (for instance, *b* in *Mdh-2*, *Pgi-2* and *c* in *Adh* and *Pgd-1*). After selfing plants with the three-banded phenotypes II and IV in our model, the progeny segregated in the expected disomic Mendelian ratio (with one exception; *Pgd-1* for plant 5), suggesting that the maternal plant was homozygous for one copy of the isolocus and heterozygous for the other copy (Appendix B). The progeny of selfed plants with phenotypes comprising more than three bands was often too small to allow attempts to test the model of two independently segregating copies of an isolocus (pooling the progeny of three plants, however, supported the model for *Pgd-1*). The progeny of crosses between plants with different phenotypes usually segregated as expected (for exceptions see below).

Mdh-2, *Pgi-2* and *Pgd-1*. Our results suggest that *Mdh-2* and *Pgi-2* consist of two copies each with one shared allele (*b*) and one allele (*a*) confined to one locus (*Mdh-2'* and *Pgi-2'*, respectively) and another allele (*c*) confined to the other locus (*Mdh-2''* and *Pgi-2''*, respectively). We suggest that both copies of *Pgd-1* have one allele in common (*c*), that one of the loci (*Pgd-1'*) has one variant allele (*b*) and that the other locus (*Pgd-1''*) has two variant alleles (*d* and *e*).

Adh. Patterns of segregation in the progeny of plant 20 (phenotype 2, *abbb*) did not correspond with the expected 1:2:1 ratio (data not shown). This family appeared to be fixed for a genotype corresponding to phenotype 7 (*bbbb*), presumably because the *b* allele stained strongly and the bands corresponding to the *a* allele and the heterodimer band between *a* and *b* were very faint. Four plants (1, 2, 8-2 and 9) represented phenotype 5 (*abbc*). On the basis of a model of two independently segregating copies that are het-

erozygous (*ab* and *bc*, respectively) we expected segregation into nine phenotypes after selfing. However, none of the progenies included all expected phenotypes and the pooled progeny deviated significantly from the expected ratio. Given the scarcity of phenotypes representing the *a* allele, we probably underestimated the frequency of heterozygotes at one of the copies of this isolocus, presumably because activity was low at this locus. This interpretation is supported by the progeny of the cross between plant 6 (*abbb*) and plant 4 (*abcc*): all seedlings showed the bands corresponding to the alleles *b* and *c* (including the heterodimer band), whereas the bands corresponding to *a* and the heterodimer band between *a* and *b* were faint and could not be scored (data not shown).

Taken together, the geographical survey and the segregation found in the progeny support a model of two independently segregating loci that have one shared allele (*b*) and have one unique allele at each locus (*a* at *Adh'* and *c* at *Adh''*). The *Adh'* locus displayed low activity and was rarely resolved in the geographical survey.

Independent segregation and linkage

The expected numbers of the offspring phenotypes were too small to allow a valid test for linkage disequilibrium. Instead, we pooled the progeny of selfed plants that had the same genotypes at pairwise loci (Appendix C). For two isoloci, *Adh* and *Pgd-1*, it was possible to test for co-segregation of putative alleles at the two copies of the same isolocus. For *Pgd-1'* and *Pgd-1''*, there was no indication of linkage and no support for a deviation from Mendelian behaviour of the putative alleles, *b* and *c* at *Pgd-1'* and *c* and *d* at *Pgd-1''*. For *Adh*, however, the segregation pattern did not conform to predictions based on independent segregation of the putative alleles at the two loci (see above). No evidence of linkage could be detected in the segregation pattern for the genotypes *bc* at *Pgd-1'* and *ab* at *Aat-2''*, or for the genotypes *bc* at *Pgd-1'* and *ab* at *Pgi-2'*. However, the segregation ratio for *Pgi-2'* (*ab*) and *Tpi-1''* (*ab*) did deviate from the expected ratio of two independently segregating loci.

DISCUSSION

Chromosome numbers

The ploidy level in *G. hederacea* has been discussed by many authors (e.g., SUGIURA 1939; HARA et al. 1954) most of whom agree that the basic number is $x = 9$ and that the species is a tetraploid with $2n = 36$, although HARA et al. (1954) suggested $x = 6$ on the basis of a report of $2n = 24$ by LÖVE and LÖVE (1942). This report, however, is indeterminate since the origin of the plants was not given, in either LÖVE and LÖVE (1942 or 1961). ZUKOWSKI and SLOWINSKA (1979) reported $2n = 24$ from two localities in Poland, and suggested that there were two chromosome races of *G. hederacea*, one inhabiting forests ($2n = 24$) and one meadow race ($2n = 36$). No such races have been found in other studies. Several authors have reported $2n = 18$ (Scherer in TISCHLER 1940; SUGIURA 1939 and 1940; SUZUKA and KORIBA 1952; SORSA 1963; GILL 1979; LAANE 1971; SEDLAKOVA 1981; MAJOVSKY et al. 1987), most of them representing scattered occurrences. For instance, the only chromosome counts from Norway and Finland suggest $2n = 18$, but these reports represent only one count from each country. In our extensive material, which comprised 93 putative clones derived from 27 localities in N and C Europe, we were unable to detect a single plant with $2n = 18$ or $2n = 24$, the overwhelming majority having $2n = 36$: we found three exceptions only, viz. one plant with $2n = 42$ and two plants with $2n = 54$. In our opinion plants with $2n = 42$ and $2n = 54$ are rare abberants of little significance in natural populations, $2n = 36$ agreeing with the majority of reports of chromosome numbers in *G. hederacea* (HARA et al. 1954; SKALINSKA et al. 1959; MULLIGAN 1961; MAJOVSKY et al. 1987; GILL 1979; ZUKOWSKI and SLOWINSKA 1979; SEDLAKOVA 1981; MESICEK and JAROLIMOVA 1992; Lövkvist and Hultgård, in prep.). In particular, Lövkvist and Hultgård (in prep.) reported $2n = 36$ for 19 clones representing 15 sites mainly in S Sweden. One of our abberants ($2n = 54$ from Devinska Kobyla in Slovakia) did not set seeds after selfing (M. Widén, personal observation), indicating that the plant was sterile. Scherer in TISCHLER (1940) found that plants with $2n = 18$ had low pollen fertility. In a clonal plant such as *G. hederacea*, chromosomal aberrants may arise by somatic mutations and be preserved in a population for a long time by vegetative growth.

Genetic analysis

Segregation ratios at 12 loci were in close agreement with a genetic model based on disomic inheritance. We performed 63 independent segregation tests and found that the pattern of segregation deviated significantly from Mendelian expectation only in four cases. One of the significant deviations (*Adh*) can be attributed to poor banding pattern: the strong staining intensity of two allozymes interfered with the weak staining intensity of a third allozyme and distorted the interpretation of the banding pattern. Two other deviations from expectation (*Tpi-2* and *Pgd-2*) concerned only twelve plants. Moreover, in an analysis comprising 62 tests (excluding the deviant *Adh*), we

can expect to find by chance at least three deviations significant at the 5% level so that we cannot reject the hypothesis that the observed enzyme patterns and segregation ratios in *G. hederacea* reflect normal Mendelian behaviour of putative alleles at each of the loci investigated.

Nine loci behaved as isoloci (*Aat-2*, *Tpi-1*, *Tpi-2*, *Pgd-1*, *Pgd-2*, *Mdh-2*, *Mnr*, *Pgi-2* and *Adh*). In five loci (*Aat-2*, *Tpi-1*, *Tpi-2*, *Pgd-2* and *Mnr*), one of the copies of the duplicated locus was monomorphic and the other polymorphic. In *Pgi-2*, *Pgd-1*, *Mdh-2* and *Adh*, both copies of the duplicated locus were polymorphic, one allele being common to both copies and the other alleles being unique to each copy. Three loci, *Pgm-3*, *Skd-1* and *Skd-2*, behaved as non-duplicated loci, although *Pgm-3* sometimes showed extra bands of low activity which may correspond to one of the copies of a duplicated locus. However, these bands did not interfere with the staining intensity of other bands at *Pgm-3*, which supports our decision to score this putative locus as non-duplicated. In diploid plants, the number of enzyme loci is highly conserved (GOTTLIEB 1981, 1982). In general 1-2 and 2 enzyme loci code for *Skd* and *Pgm*. The fact that we found 3 loci for each enzyme suggests that both loci have undergone duplication, with a subsequent differentiation of the two copies of each isolocus, which could readily have been tested if our search for diploid plants (2n = 18) had been successful.

We have analysed 30 of the 32 putative alleles in *G. hederacea*. Two further bands, the *a* bands at *Pgm-3* and *Pgd-1* are probably also allozymes. We have some preliminary results suggesting that the *a* allele of *Pgd-1* belongs to *Pgd-1'* (unpublished).

In our geographical survey, we found that one of the loci representing *Aat-2*, *Tpi-1*, *Tpi-2*, *Pgd-2*, and *Mnr* was monomorphic, since we did not encounter all the phenotypes expected when both copies segregate (cf. Fig. 1). This perhaps may never happen if an allele has a low frequency in one of the copies (WAPLES 1988). It is only possible to detect low-level variation at one locus if the sample is extremely large. We sampled about 1,300 ramets representing more than 200 genets in 10 natural populations from an extensive area (N and C Europe). Even though the possibility of low-level variation at the second locus in individual populations cannot be ruled out, the number of populations we sampled without finding any "missing" phenotypes is sufficient to support adopting the working hypothesis that the second locus is fixed for *Aat-2*, *Tpi-1*, *Tpi-2*, *Pgd-2*, and *Mnr*. We found no phenotypes in the geographical survey that indicated that the four isoloci (*Pgi-2*, *Pgd-1*, *Mdh-2* and *Adh*) with both copies polymorphic shared more than the common allele. Thus, since the two copies of the locus for *Pgd-1*, *Pgi-2*, *Adh* and Mdh each have just one or three unique variant alleles each we can score and calculate allele frequencies for each copy separately.

Origin of polyploidy

Since we found the chromosome number to be 2n = 36 and because of the behaviour of enzyme loci, we consider *G. hederacea* to be a tetraploid. *Glechoma hederacea* has a close relative in SE Europe, *G. hirsuta* (2n = 36) which differs from *G. hederacea* in being more pubescent and in the size of the calyx and corolla; it occurs allopatrically with *G. hederacea*. Putative hybrids between the two taxa are frequently found (TUTIN et al. 1972) and the two taxa are fully interfertile (M. Widén, unpublished), though no diploid taxa are known that could have been involved in the origin of the tetraploids *G. hederacea* and *G. hirsuta*.

The disomic inheritance demonstrated at the enzyme loci of *G. hederacea* can be interpreted as evidence that the tetraploid *G. hederacea* is of allopolyploid origin. However, recent studies of polyploid taxa have suggested that autopolyploidy is more common than previously thought (THOMPSON and LUMARET 1992; SOLTIS and SOLTIS 1993; RAMSEY and SCHEMSKE 1998). Several lines of evidence point to an autotetraploid origin with subsequent diploidization in *G. hederacea*. First, the allele at the monomorphic copy of the duplicated loci has the same relative mobility as the commonest allele in the polymorphic copy. When both copies are polymorphic, one allele common to both copies usually occurs in high frequency. In an allopolyploid, we expect greater differences between the two ancestral genomes. Second, in the diploidization process, one of the copies of three duplicated loci (*Pgm-3*, *Skd-1* and *Skd-2*) appears to have been subjected to complete gene silencing. An alternative explanation, that *Skd-1* and *Skd-2* represent the two copies of a duplicated locus, fits with a model involving diploidization and subsequent divergence of the gene products of the two copies. Moreover, at another isolocus, *Adh*, one of the copies show much less activity than the other copy, which may indicate a first step in the process of gene silencing or a differentiation of the gene products of the two copies of *Adh*. At *Tpi-2*, there is an indication of a rare null allele (phenotype 4), which could also be the result of the process of gene silencing.

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APPENDIX A: *Observed genotype frequencies, genotype ratios predicted according to different hypotheses, and log likelihood ratio tests (G) comparing observed and predicted frequencies in the progeny of selfed plants and in crosses between different parental genotypes for non-duplicated loci (Skd-1, Skd-2 and Pgm-3) and isoloci with one monomorphic and one polymorphic copy (Aat-2, Tpi-1, Tpi-2, Pgd-2 and Mnr). When there were less than 10 offspring the segregating ratio was not tested.* $G_{adj} = G/q$, *where* $q = 1 + (a^2 - 1)/6\ n\ df$, *a = number of classes, n = sample size, df = degree of freedom. S = selfed,* $*P \leq 0.05$. $**P < 0.01$

Plant/	Parent	Progeny genotypes					
Cross	genotype	*aaaa*	*aaab*	*aabb*	Sum	Expected	G_{adj}	df
Aat-2								
1 S	*aaab*	11	27	9	47	1:2:1	1.240	2
4 S	*aaab*	15	34	10	59	1:2:1	2.375	2
8-1 S	*aaab*	8	15	9	32	1:2:1	0.183	2
20 S	*aaab*	5	16	7	28	1:2:1	0.900	2
Total	*aaab*	39	92	35	166	1:2:1	2.169	2
7 S	*aaab*	4	4	0	8			
6 × 4	*aaaa* × *aaab*	11	14		25	1:1	0.361	1
15 × 13	*aabb* × *aabb*			12	12			
9 S	*aabb*			16	16			
11 S	*aabb*			33	33			
Tpi-1								
4 S	*aaab*	12	33	14	59	1:2:1	0.983	2
5 S	*aaab*	17	33	10	60	1:2:1	2.427	2
6 S	*aaab*	10	16	9	35	1:2:1	0.308	2
8-2 S	*aaab*	4	5	3	12	1:2:1	0.469	2
20 S	*aaab*	9	14	5	28	1:2:1	1.149	2
6 × 4	*aaab* × *aaab*	12	14	5	31	1:2:1	3.235	2
Total	*aaab*	64	115	46	225	1:2:1	3.067	2
7 S	*aaab*	6	2	0	8			
15 × 13	*aaab* × *aabb*		7	5	12	1:1	0.335	1
2 S	*aabb*			25	25			
8-1 S	*aabb*			32	32			
Tpi-2								
2 S	*aaab*	5	11	9	25	1:2:1	1.505	2
6 S	*aaab*	10	20	5	35	1:2:1	2.399	2
8-1 S	*aaab*	4	19	9	32	1:2:1	3.081	2
8-2 S	*aaab*	1	4	7	12	1:2:1	6.290*	2
20 S	*aaab*	6	12	10	28	1:2:1	1.570	2
Total	*aaab*	26	67	40	133	1:2:1	2.994	2
6 × 4	*aaab* × *aaaa*	18	13		31	1:1	1.208	1
11 S	*aabb*			33	33			
Pgd-2								
8-1 S	*aaab*	7	12	13	32	1:2:1	3.820	2
20 S	*aaab*	6	14	8	28	1:2:1	0.284	2
Total	*aaab*	13	26	21	60	1:2:1	2.958	2
15 × 13	*aaaa* × *aaab*	1	11		12	1:1	9.751**	1
2 S	*aabb*			25	25			
Mnr								
1 S	*aaab*	14	23	10	47	1:2:1	0.687	2
8-1 S	*aaab*	10	15	7	32	1:2:1	0.652	2
Total	*aaab*	24	38	17	79	1:2:1	1.311	2
7 S	*aaab*	7	1	0	8			

Plant/	Parent	Progeny genotypes								
Cross	genotype	*aa*	*ab*	*ac*	*bb*	*bc*	*cc*	Sum	Expected	G_{adj}	df
Skd-1											
1 S	*ab*	7	28		11			46	1:2:1	3.071	2
20 S	*ab*	5	19		4			28	1:2:1	3.730	2
Total	*ab*	12	47		15			74	1:2:1	5.788	2

Plant/	Parent	Progeny genotypes									
Cross	genotype	*aa*	*ab*	*ac*	*bb*	*bc*	*cc*	Sum	Expected	G_{adj}	df
6 S	*ac*	7		19			9	35	1:2:1	0.505	2
8 S	*ac*	4		5			3	12	1:2:1	0.469	2
Total	*ac*	11		24			13	48	1:2:1	0.166	2
2 S	*bc*				5	12	8	25	1:2:1	0.731	2
11 S	*bc*				6	17	10	33	1:2:1	1.033	2
9 S	*bc*				1	11	4	16	1:2:1	4.168	2
Total	*bc*				12	40	22	74	1:2:1	3.461	2
7 S	*bc*				3		5	8	1:2:1		
6×4	*ac*×*cc*			14			17	31	1:1	0.291	1
15	*ac*×*ab*	1	2	4		5		12	1:1:1:1	1.550	3

Plant/	Parent	Progeny genotypes					
Cross	genotype	*aa*	*ab*	*bb*	Sum	Expected	G_{adj}	df
Skd-2								
5 S	*ab*	5	26	15	46	1:2:1	5.980	2
8-2 S	*ab*	4	3	4	11	1:2:1	2.308	2
20 S	*ab*	6	16	6	28	1:2:1	0.568	2
Total	*ab*	15	45	25	85	1:2:1	2.813	2
7 S	*ab*	0	8	0	8			
15×13	*aa*×*bb*		12		12			
6×4	*aa*×*bb*		31		31			

Plant/	Parent	Progeny genotypes					
Cross	genotype	*bb*	*bc*	*cc*	Sum	Expected	G_{adj}	df
Pgm-3								
2 S	*bc*	9	7	9	25	1:2:1	4.960	2
9 S	*bc*	5	11	0	16		–	2
Total	*bc*	14	18	9	31	1:2:1	1.697	2
15×13	*bc*×*cc*		10	2	12	1:1	5.822	1

APPENDIX B: *Observed genotype frequencies, genotype ratios predicted according to different hypotheses, and log likelihood ratio tests (G) comparing observed and predicted frequencies in self progenies and in crosses between parental genotypes for isoloci with both copies polymorphic (Pgi-2, Pgd-1, Mdh-2 and Adh). When the number of offspring was less than 10, the segregating ratio was not tested.* $G_{adj} = G/q$, *where* $q = 1 + (a^2 - 1)/6\,n\,df$, *a = number of classes, n = sample size, df = degree of freedom. S = selfed,* ***$P < 0.001$

Plant/	Parent	Progeny genotypes											
Cross	genotype	*aaaa*	*aaab*	*aabb*	*aabc*	*abbc*	*abcc*	*aaac*	*aacc*	*bbcc*	Sum	Expected	G_{adj}	df
Pgi-2														
2 S	*aaab*	8	14	2							24	1:2:1	4.478	2
5 S	*aaab*	20	29	11							60	1:2:1	2.706	2
8-1 S	*aaab*	6	18	8							32	1:2:1	0.782	2
8-2 S	*aaab*	2	8	2							12	1:2:1	1.332	2
11 S	*aaab*	9	16	8							33	1:2:1	0.088	2
20 S	*aaab*	9	16	3							28	1:2:1	3.680	2
Total	*aaab*	52	101	34							187	1:2:1	4.993	2
7 S	*aaab*	0	4	4							8	1:2:1		
6 S	*aabc*	2	4	2	13	0	9	2	1	2	35	1:2:1:4:2:2:2:1:1		
6×4	*aabc*×*aaaa*	10	9		6			6			31	1:1:1:1	1.208	3
15×13	*aaac*×*aabc*	0	4		6		1	1			12	1:1:2:1:3		
1 S	*aabb*			47							47			

Plant/	Parent	Progeny genotypes															
Cross	genotype	*bbcc*	*bbcd*	*bbdd*	*bbee*	*bccc*	*bccd*	*bcdd*	*bcee*	*cccc*	*cccd*	*ccdd*	*ccde*	*ccee*	Sum	Expected	G_{adj}	df
Pgd-1																		
2 S	*cccd*									5	11	9			25	1:2:1	1.505	2
5 S	*cccd*									6	22	32			60	1:2:1	23.75**	2
Total	*cccd*									11	33	41			85	1:2:1	22.64**	2
6 S	*bccc*	9				15				11					35	1:2:1	0.911	2
9 S	*bbcd*	4	9	3											16	1:2:1	0.388	2
8-1 S	*bcee*				8				14					10	32	1:2:1	0.718	2
8 S	*ccde*											1	8	3	12	1:2:1	2.357	2
11 S	*bcdd*			10				12				11			33	1:2:1	2.515	2
1 S	*bccd*	2	6	0		3	12	7		1	12	4			47			
4 S	*bccd*	4	4	2		8	12	7		4	12	2			55			
7 S	*bccd*			1		1	4				2				8			
Total	*bccd*	6	10	3		12	28	14		5	26	6			110	1:2:1:2:4:2:1:2:1	13.37	8
20 S	*bbcc*	28																

Plant/	Parent	Progeny genotypes							
Cross	genotype	*aabb*	*abbb*	*bbbb*	*bbbc*	*bbcc*	Sum	Expected	G_{adj}	df
Mdh-2										
2 S	*abbb*	6	9	10			25	1:2:1	2.968	2
6 S	*abbb*	7	20	8			35	1:2:1	0.778	2
8-1 S	*abbb*	3	7	4			14	1:2:1	0.141	2
Total	*abbb*	16	37	22			75	1:2:1	0.962	2
5 S	*bbbc*			17	26	17	60	1:2:1	1.065	2
7 S	*bbbc*			1	7	0	8	1:2:1		
6×4	*abbb*×*bbbb*		14	17			31	1:1	0.291	1
20 S	*aabb*	28					28			
9 S	*aabb*	16					16			

Plant/	Parent	Progeny genotypes												
Cross	genotype	*aabb*	*aabc*	*aacc*	*abbb*	*abbc*	*abcc*	*bbbb*	*bbbc*	*bbcc*	Sum	Expected	G_{adj}	df
Adh														
1 S	*abbc*	1	3	0	12	14	0	2	13	2	47			
2 S	*abbc*	0	0	2	5	8	0	0	4	6	25			
8 S	*abbc*	0	0	0	5	0	0	6	1	0	12			
9 S	*abbc*	0	0	0	0	2	1	3	6	4	16			
Total	*abbc*	1	3	2	22	24	1	11	24	12	100	1:2:1:2:4:2:1:2:1	60.872***	8
5 S	*abbb*	7			14			7			28	1:2:1	0.000	2
6 S	*abbb*	6			18			9			33	1:2:1	0.877	2
Total	*abbb*	13			32			16			61	1:2:1	0.459	2
11 S	*bbbc*							11	17	5	33	1:2:1	2.336	2
8-1 S	*bbbc*							10	11	11	32	1:2:1	3.226	2
Total	*bbbc*							21	28	16	65	1:2:1	1.928	2
4 S	*abcc*			11			26			21	58	1:2:1	3.800	2
7 S	*bbcc*									8	8			

APPENDIX C

*Observed genotype frequencies and log likelihood ratio tests for segregation at pairwise loci after pooling selfed plants of G. hederacea with the same genotype. The expected segregation ratio was 1:2:1:2:4:2:1:2:1. ns = non significant, * = $P < 0.05$.*

Aat-2'' and *Pgd-1'*, genotypes *ab/bc*, plants 1, 4, 7 and 8-1. N = 143

	Genotypes at *Pgd-1'*		
Genotypes at *Aat-2''*	*bb*	*bc*	*cc*
aa	7	16	13
ab	15	39	25
bb	5	14	9

$G_8 = 8.6$ns

Pgi-2' and *Pgd-1'*, genotypes *ab/bc*, plants 6, 7, 8-1 and 11. N = 108

	Genotypes at *Pgd-1'*		
Genotypes at *Pgi-2'*	*bb*	*bc*	*cc*
aa	4	9	8
ab	20	25	19
bb	4	12	7

$G_8 = 9.6$ns

Pgi-2' and *Tpi-1''*, genotypes *ab/ab*, plants 5, 6, 8 and 20. N = 135

	Genotypes at *Tpi-1''*		
Genotypes at *Pgi-2'*	*ab*	*bb*	*bc*
aa	5	25	6
ab	27	35	16
bb	8	8	5

$G_8 = 18.5$*