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Absence of association between heterozygosity and biomass production in *Salix exigua* Nutt

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Abstract The relations between heterozygosity derived from a total of 12 variable isoenzyme loci and total above-ground leafless biomass production were examined in four full-sib families of *Salix exigua*, a willow species important in breeding efforts for short-rotation intensive-culture plantations. Relations were investigated by comparing the performance of heterozygotes with that of corresponding homozygotes in locus by locus comparisons, by investigating multiple regression models with individual loci as independent variables, and by employing the adaptive distance model. All these analytical approaches resulted in the manifestation of the absence of any relations between isoenzyme loci and biomass production. Possible reasons that may account for these results are discussed.

Key words Isoenzymes · Heterozygosity · Biomass production · Fitness

Introduction

The significance of positive associations between heterozygosity and characters related to fitness or developmental homeostasis, such as growth, has been debated for decades. Plant and animal breeders while trying to increase yield, have been puzzled by the idea of tracing what makes a specific genotype grow faster. Such vigorous growth has been originally observed in hybrids; in deed, the term "heterosis" was invented in order to describe the phenomenon. Because heterosis was initially defined as a property of hybrids it became closely associated with heterozygosity and synonymous to heterozygote superiority (Zouros and Foltz 1987), thus being used as equivalent to overdominance. There are two alternate hypotheses accounting for the correlation of heterozygosity with growth and yield: (1) the "dominance" (or "inbreeding depression") hypothesis, stating that the loci under investigation may themselves be selectively neutral and that the observed heterozygosity is merely indicative of the degree of genomic heterozygosity; i.e. heterozygosity serves to cover deleterious recessives, and (2) the "overdominance" hypothesis, which states that heterozygosity at the loci examined, or at other loci which are in linkage disequilibrium with the studied loci, leads to increased fitness (Zouros 1993); i.e. heterozygotes are better per se (Smouse 1986). These hypotheses are not necessarily mutually exclusive and heterosis could result from the action of both dominant and overdominant genes (Tsaftaris 1995).

In theoretical terms these hypotheses refer to the relationships of heterozygosity with fitness. It is a well-known fact in biology how notoriously difficult it is to estimate differences in fitness among genotypes in nature (Lewontin 1974). For this reason, the strategy that is being followed is to describe associations between heterozygosity and phenotypic effects that are likely to affect fitness. Heterozygosity is commonly measured in a set of enzyme loci; growth or viability being the most typically used fitness-related characters. Forest trees are good candidates in such studies for several reasons, the most important of them presumably being that genetic diversity is much higher in tree species than other plants, and that trees are among the most polymorphic of organisms (Hamrick et al. 1992).

The present study investigates the associations among isoenzyme heterozygosity and biomass production in *Salix exigua* Nutt. (section *Longifoliae*, subgenus *Salix*), a diploid willow important in breeding programs for short-rotation intensive-culture biomass plantations (Zsuffa and Aravanopoulos 1989). This is a fast-growing species unique for its ability to sprout from root suckers

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and to flower throughout the growing season. The genetic basis of enzymatic variation in *S. exigua* has already been reported (Aravanopoulos et al. 1993). Significant quantitative variation was also detected in this species, indicating its potential for substantial improvement through selection and breeding (Mosseler et al. 1988).

Materials and methods

This study employed the full-sib progenies of a 2 × 2 balanced factorial mating design, involving female clones I61 and I62, and male clones I66 and I293. The progenies were included in a test plot, established as a randomized complete block design with each full-sib family represented by a single, four-tree plot in each of six blocks surrounded by a triple border row, at the Ontario Tree Improvement and Forest Biomass Institute, at Maple, Ontario (44° N, 80° W). A tightly linked locus pair (*Acp-2:Alp-1*) has been shown to exist in *S. exigua* (Aravanopoulos 1998), therefore *Alp-1* was removed from subsequent analysis in order to avoid sampling the same allelic state twice from the same genomic region. Hence, heterozygosity was estimated from 12 segregating loci: *Aco-1, Aco-2, Acp-2, Adh-2, Alp-1, Cto-1, Per-1, Per-3, Pgd-1, Pgi-2, Pgm-1, Ppo-1*, and *Sdh-2*, in ten enzyme systems: acid phosphatase, aconitase, alcohol dehydrogenase, cytochrome oxidase, peroxidase, 6-phosphogluconate dehydrogenase, phosphoglucose isomerase, phosphoglucomutase, polyphenol oxidase and shikimate dehydrogenase. All these loci presented Mendelian inheritance and codominant allelic expression (Aravanopoulos et al. 1993). Seedlings were harvested at $2^1/_2$ years of age (Mosseler et al. 1988) to measure above-ground leafless oven-dry biomass. This is a typical rotation period for a fast-growing species used in short-rotation intensive-culture plantations.

Analysis was conducted using SAS (SAS Institute Inc.). To normalize the data and to eliminate as much environmental variance and block effects as possible, biomass measurements and associated plot means were transformed to the logarithmic scale and all analyses were based on deviations of each tree from its plot mean, which is also a measure of relative-to-plot growth (Strauss 1986).

The advantage of this experimental layout is that the uniform habitat and the randomized design are expected to contribute more accurate measures for biomass, while problems of environmental effects are minimized (Bush et al. 1987).

The null hypothesis of no associations between heterozygosity and biomass production was tested in three different fashions: (1) by comparing the performance of heterozygotes with that of corresponding homozygotes in locus by locus comparisons (Ledig et al. 1983; Wolff and Haeck 1990), (2) by investigating multiple regression models with individual loci as independent variables (Strauss 1986; Strauss and Libby 1987), and (3) by employing the adaptive distance model (Bush et al. 1987; Bush and Smouse 1991a).

In the first approach, for every locus the mean biomass of all homozygotes was subtracted from the mean biomass of all heterozygotes. Under the null hypothesis of no association between single-locus heterozygosity and biomass, the generation of genotypic groups would be a random process with respect to biomass production. The significance of differences in biomass production between homozygotous and heterozygotous individuals, was examined using *t*-test statistics (Sokal and Rohlf 1981). The observed ratio of positive to negative differences was tested against the expected ratio from the null hypothesis of no association (under which positive and negative differences should appear in a 1:1 ratio), with χ^2 tests.

In the second approach, heterozygosity at each locus was encoded as an indicator variable: each locus was defined as either 0 (heterozygous condition), or 1 (homozygous condition). Evaluation of the multiple regression models with the genotypic conditions as dependent variables was performed by investigating the *F*-statistics of the models and by employing the coefficient of determination (R^2) in order to assess the proportion of the variation in biomass production that can be explained by the model (Sokal and Rohlf 1981). If the data show a heterozygote superiority, then it is expected that the regression line will have a negative slope. However, because the response function of the multiple regressions is multi-dimensional, partial regression coefficients were examined. The response function was judged to have a “positive” or “negative” slope when these partial regression coefficients were predominantly positive or negative for a family (Bush et al. 1987). The relative importance and contribution of a locus to growth was also investigated by ranking the loci within and among families. This was studied by ranking the order of inclusion of each locus in a stepwise maximum R^2-improvement regression model. The significance of each locus contribution was determined by the *F*-statistics of the models (Sokal and Rohlf 1981; SAS Institute 1982). Average ranks based on the results of the individual families were calculated. Pooled data were also studied by examining the partial sum of squares (Type-III SS) of the multiple regressions. The Type-III SS indicate the effects of an independent variable separately, holding the effects of the other variables constant, and provide for a given locus a measure of the degree to which heterozygosity at that locus is correlated with biomass production (Sokal and Rohlf 1981; Koehn et al. 1988). Differences in the rankings between average and pooled data were investigated using the Spearman’s rank correlation coefficient (Sokal and Rohlf 1981).

The third approach makes use of a theoretical development, the adaptive distance model (Smouse 1986). According to this model, if selection is acting upon genotypes to produce heterozygote superiority or overdominance, then the fitness of those genotypes can be predicted by their allelic frequencies. Total above-ground leafless biomass was regarded as a first approximation of a fitness surrogate for model purposes. Adaptive distances were calculated from genotype frequencies according to Smouse (1986) and the relationship between log(biomass) and adaptive distances was inferred from multiple regressions. This analysis has been applied in a number of studies on heterozygosity growth relations (Bush and Smouse 1991b). Model evaluation, investigation of heterozygote superiority and contributions of individual loci were tested as in the second approach.

Results

The allelic frequencies of the 11 variable loci in the four full-sib families studied are presented in Table 1. The descriptive statistics of the total above-ground biomass production of the same families are depicted in Table 2. The results of the three different experimental approaches that were employed in order to study heterozygosity biomass relations are presented below. For the first approach, the results of the comparisons between biomass growth of heterozygotes and that of corresponding homozygotes, in locus by locus comparisons, are presented in Table 3. There were 42 possible comparisons. Positive differences slightly outnumbered negative ones and, in general, the results were inconclusive. The numbers of positive and negative differences were almost equal in every family. The overall split was not significantly different from 1:1, in both the total ratio and the ratio of the significant differences only. For the second and third approaches, the results of the multiple regression models investigated are shown in Table 4. Ten models were tested. The employment of the adaptive-distance treatment requires the presence of two allelic classes at a locus (Smouse 1986). Therefore, in the cases where more than

Table 1 Allele frequencies at each of the 11 loci for each of the four full-sib families of *S. exigua*

Family	Allele	*Aco-1*	*Aco-2*	*Acp–2*	*Adh-2*	*Cto-1*	*Per-1*	*Per-3*	*Pgd-1*	*Pgi-2*	*Pgm-1*	*Ppo-1*	*Sdh-2*
I62 × I66	A	0.479	1.000	0.792	0.667	0.000	0.542	1.000	1.000	0.104	0.792	0.000	0.000
	B	0.521	0.000	0.208	0.333	0.646	0.458	0.000	0.000	0.896	0.208	0.812	0.688
	C	0.000	0.000	0.000	0.000	0.354	0.000	0.000	0.000	0.000	0.000	0.188	0.312
I62 × I293	A	0.771	0.667	0.739	0.604	0.000	0.583	1.000	0.500	0.500	0.435	0.000	0.292
	B	0.229	0.333	0.261	0.396	0.458	0.417	0.000	0.500	0.500	0.239	0.667	0.521
	C	0.000	0.000	0.000	0.000	0.542	0.000	0.000	0.000	0.000	0.326	0.333	0.187
I61 × I66	A	0.792	0.896	0.696	0.792	0.229	0.375	0.688	0.625	0.896	1.000	0.217	0.250
	B	0.208	0.104	0.304	0.208	0.583	0.625	0.312	0.375	0.104	0.000	0.566	0.750
	C	0.000	0.000	0.000	0.000	0.187	0.000	0.000	0.000	0.000	0.000	0.217	0.000
I61 × I293	A	1.000	0.457	0.826	0.522	0.348	0.543	0.783	0.239	1.000	0.717	0.348	0.457
	B	0.000	0.543	0.174	0.478	0.435	0.457	0.217	0.761	0.000	0.000	0.522	0.543
	C	0.000	0.000	0.000	0.000	0.217	0.000	0.000	0.000	0.000	0.283	0.130	0.000

Table 2 Descriptive statistics of biomass production in *S. exigua*

Family	Mean	Standard deviation	CV	Range
I6 × I66	0.583	0.231	39.62	0.27–1.18
I62 × I293	0.845	0.336	39.76	0.34–1.46
I61 × I66	0.724	0.369	43.67	0.28–1.60
I61 × I293	0.473	0.260	54.97	0.06–1.29

two alleles were present in a progeny (in loci *Cto-1*, *Pgm-1*, *Ppo-1* and *Sdh-1*), all alleles other than the most common were pooled to an ‘other-alleles’ class.

Results of the separate family models were coincident; statistical significance was attained only in family I61 × I66 (Table 4). There was no marked difference between the results obtained from binary scores and the results obtained from adaptive distances, in terms of *F*-statistics and average R^2 values across families. The latter were not significantly different among the two types of analysis ($t = 1.21$, 6 *df*). A noteworthy result for both types of analysis was that the pooled-data produced models that could not effectively account for any of the variability in biomass production. The pooled data model accounted for 15.6% of the biomass variation, while individual families on the average accounted for 41.4% (binary scores; Table 4). Subsets of the loci employed in the stepwise regression procedure for determining locus ranks, resulted in significant associations between biomass production and heterozygosity in two families: I61 × I66 and I62 × I66 (see Table 6).

The partial regression coefficients of the above models that were employed in order to assess if the data demonstrate heterozygote superiority are presented in Table 5). The results were inconclusive. In two families the response function was ruled to be negative, indicating heterozygote superiority, whilst in another family the opposite result was observed and in the last family there was no association. Pooled data also revealed inconclusive results: the multiple regression function could not be judged, since the positive and negative partial regression coefficients were split in half. Both approaches revealed the same results. The sign and magnitude of the coefficients varied across families for the same locus. The overall split of the four families used did not differ from 1:1 in both applications (binary scores approach, $\chi^2 = 1.600$ $0.20 < P < 0.30$, Table 5).

The relative contributions of individual loci to the overall relationship between heterozygosity and biomass

Table 3 Effects of individual loci in *S. exigua*. Performance for each locus of differences [δ = (mean biomass of heterozygotes)-(biomass of homozygotes] over different families and enzymes (significance levels refer to *t*-tests: *: 0.5, **: 0.01, ***: 0.001; δ values are in “antilog” scale; lns = locus not segregating in that family)

Locus	I62 × I66	I62 × I293	I61 × I66	I61 × I293	δ > 0	δ < 0	Pooled
Aco-1	0.292***	0.037	0.022	lns	3	0	0.351
Aco-2	lns	–0.046	–0.111	0.042	1	2	–0.015
Acp-2	0.048	–0.158	–0.088	0.099	2	2	–0.099
Adh-2	0.232***	–0.160*	0.130**	0.230**	3	1	0.432
Alp-1	lns	lns	–0.163**	0.295*	1	1	0.062
Cto-1	–0.020	0.073	–0.340***	–0.126	1	3	–0.413
Per-1	0.085	–0.109	0.061	0.189*	3	1	0.226
Per-3	lns	lns	0.159**	–0.092	1	1	0.067
Pgd-1	–0.057	0.071	lns	–0.044	1	2	–0.101
Pgi-2	–0.112	lns	-0.194	lns	1	1	0.082
Pgm-1	–0.073	0.093	lns	0.021	3	0	0.187
Ppo-1	–0.280***	–0.042	–0.256***	–0.264*	0	4	–0.862
Sdh-2	–0.015	–0.242**	–0.144*	–0.025	0	4	–0.025
δ > 0	4/10	4/10	4/11	6/11	20	22	7/13
P					0.750		0.209
Sign δ > 0	2/3	0/2	2/6	3/4	7	8	
P					0.775		0.209

Table 4 Regression results in *S. exigua*; multilocus effects. Coefficient of determination and associated *P* for the regression models of log(biomass) on binary scores and adaptive distances (numbers in parentheses are standard deviation values)

Family	Binary scores		Adaptive distances	
	R^2	*P* > F	R^2	*P* > F
I62 × I66	0.544	0.226	0.505	0.312
I62 × I293	0.155	0.877	0.350	0.753
I61 × I66	0.703	0.059	0.743	0.069
I61 × I293	0.257	0.975	0.250	0.977
Average	0.415 (0.253)		0.462 (0.215)	
Pooled	0.156	0.352	0.138	0.482

Table 5 Partial regression coefficients of multiple regression models in *S. exigua* (binary scores used; 0.000 indicates absence of segregation for the locus in question)

Locus	I62 × I66	I62 × I293	I61 × I66	I61 × I293	Total	Pooled
Aco-1	–0.131	–0.020	–0.041	0.000		–0.054
Aco-2		0.058	0.134	–0.014		0.032
Acp-2	–0.010	0.022	0.046	–0.070		–0.005
Adh-2	–0.067	0.065	–0.022	–0.102		–0.044
Cto-1	–0.043	–0.069	0.196	–0.037		0.005
Per-1	–0.040	0.122	–0.050	–0.120		–0.017
Per-3	0.000	0.000	–0.002	–0.014		–0.023
Pgd-1	–0.054	–0.034	0.000	–0.026		–0.012
Pgi-2	0.058	0.000	0.032	0.000		–0.058
Pgm-1	–0.067	–0.002	0.000	–0.105		0.062
Ppo-1	0.087	–0.021	0.080	0.144		0.021
Sdh-2	0.037	0.089	0.045	0.103		0.076
(+) signs	3	5	6	2	16	6
(-) signs	7	5	4	8	24	7

Table 6 Rankings of contribution of each locus to biomass production, based on the order of inclusion in the regression models of individual families and pooled data, in *S. exigua*. Average ranks of the family data are also presented (levels of significance: *: 0.05, **: 0.01, ***: 0.001)

Rank	Pooled	I62 × I66	I62 × I293	I61 × I66	I61 × I293	Avg. rank
1	*Pgd-1*	*Aco-1***	*Sdh-2*	*Cto-1***	*Adh-2*	*Sdh-2* = 2.50
2	*Acp-2*	*Adh-2***	*Per-1*	*Aco-2***	*Sdh-2*	Pgm-1 = 4.33
3	*Per-1*	*Sdh-2***	*Pgm-1*	*Ppo-1****	*Ppo-1*	*Pgi-2* = 4.50
4	*Per-3*	*Pgi-2**	*Aco-2*	*Sdh-2***	*Per-1*	*Aco-2* = 5.00
5	*Pgi-2*	*Pgm-1**	*Pgd-1*	*Pgi-2***	*Pgm-1*	*Adh-2* = 5.00
6	*Ppo-1*	*Per-1*	*Ppo-1*	*Acp-2***	*Acp-2*	*Ppo-1* = 5.25
7	*Pgm-1*	*Pgd-1*	*Aco-1*	*Adh-2***	*Pgd-1*	*Per-1* = 5.25
8	*Cto-1*	*Cto-1*	*Cto-1*	*Per-3***	*Per-3*	*Aco-1* = 6.00
9	*Aco-2*	*Ppo-1*	*Acp-2*	*Per-1***	*Aco-2*	*Pgd-1* = 6.33
10	*Adh-2*	*Acp-2*	*Adh-2*	*Aco-1*	*Cto-1*	*Cto-1* = 6.75
11	*Sdh-2*					*Acp-2* = 7.75
12	*Aco-1*					*Per-3* = 8.00

across families are presented in Tables 6. Ranking results showed different loci to emerge as more important in the different families. SDH was an exception, in being the only locus that achieved a high ranking in every family (average rank 2.50). The results of the relative contributions of loci to the models when data were pooled, were different from the results obtained from individual families and their average ranks (Spearman's coefficient: r_s = 0.483, 0.10 < P < 0.20; Fig. 1).

Discussion

There was a congruence of the results obtained from the three different analytical approaches employed; these revealed essentially the same trends. The results did not indicate any association between enzyme heterozygosity at single loci and biomass production. In almost half of the tests, heterozygotes produced more biomass than homozygotes, and in the rest they produced less. Within-family and pooled-data results did not reveal any trend in favor of either positive or negative heterozygosity growth correlations. The performance of one-locus heterozygotes was not constant either across families or across enzyme systems.

The results using regression of biomass on binary scores and adaptive distances showed high congruence. The amount of biomass variation that was accounted for by these approaches was virtually the same. The use of the adaptive-distances method was advanced in order to investigate if the differential treatment of homozygotes in a model would result in a better explanation of the biomass variability. The failure of the adaptive-distances approach to show this, may indicate that the technique is insensitive to mating schemes other than random, at least under specific circumstances. In particular, adding information

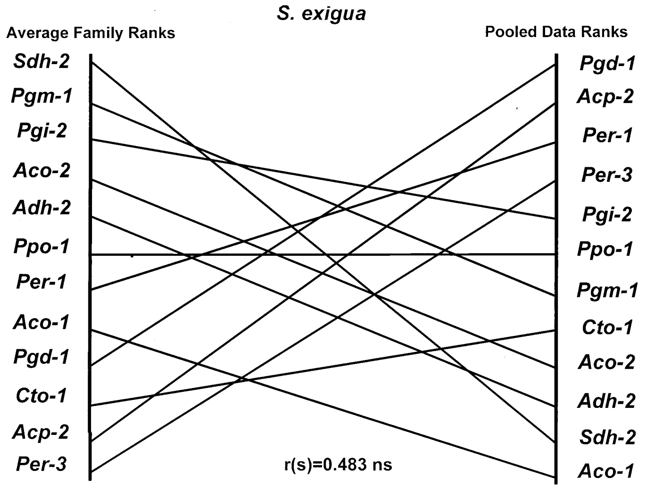

Fig. 1 Variation in the order of importance of individual loci to the overall relationship of heterozygosity and biomass; *lines* connect ranks of each locus

about differences in relative allele frequencies could not be accomplished when the progeny for a single locus segregated in a 1:1 fashion. In these cases adaptive distances and binary scores have an identical performance. The technique provides added information for the other types of genotypic segregation in progenies. These types of segregation existed in about half of the loci studied on average. Clearly, differential treatment of the loci at that level did not result in regression models that explain a higher amount of the variation observed in biomass than the use of binary scores could accomplish. The regression response functions of the binary scores approach did not indicate positive or negative heterozygosity biomass associations, since regression coefficients were not predominantly positive or negative in any of the families studied. Rankings indicated that the relative importance of loci in different families varied; shikimate dehydrogenase was a notable exception. In summary, the analysis of heterozygosity biomass relations revealed that there was no clear (positive or negative) association between heterozygosity and biomass in *S. exigua*. Individual locus contributions in stepwise multiple regressions may have been significant in two families but their contribution was not associated with differences in their homozygosity or heterozygosity status. The high rank of SDH across families is an interesting feature of the results. This enzyme participates in the synthesis of aromatic metabolites. An indirect SDH involvement with growth may have to do with increased lignin production, possible effects of increased disease resistance (through increased facilitation of phenolic compound production by specific SDH genotypes), or alternatively, SDH may be tightly linked to a major gene that affects growth. In a similar analysis in *Salix eriocephala*, SDH also gave rise to a high rank across different families (Aravanopoulos and Zsuffa 1998).

It is interesting to note the conditions under which this study was conducted. Much of the work in this field has been hampered by the limitations of studying natural populations, particularly variable levels of inbreeding, the existence of null alleles, the lack of control over microhabitat differences and variation in both density and age. These confounding factors generate large-scale non-genetic variation in growth (fitness) measurements, cause misinterpretation of allelic variation and may mask true associations or create spurious ones. In addition, the validity of the assumption that electrophoretic heterozygosity is representative of genomic heterozygosity under conditions of random mating has been questioned. Mitton and Pierce (1980) and Chakraborty (1981) have shown that heterozygosity calculated from a small number of randomly selected enzyme loci (the usual electrophoretic sample) is poorly associated with genomic heterozygosity. Smouse (1986), has argued that the measured heterozygosity of a set of sampled loci is not related at all with heterozygosity of the rest of the genome. The present study attempted to overcome these problems. All individuals were of a known breeding history and were planted at the same time and at a regular spacing in a uniform habitat. Total above-ground biomass gave a very accurate estimation of growth. Such conditions lead to the suppression of environmental noise and enhancement of the genetic signal. In progeny individuals, marker heterozygosity is highly associated with genomic heterozygosity (Leary et al. 1987). On the other hand, a study of families has inherent limitations. Genetic variation is inferred only from a limited number of parental genomes. Numbers of progeny individuals are usually small. The range of heterozygosity classes and the combinations of different heterozygous loci are small, compared to natural populations (Foltz and Chatry 1986). There can be no more than four different alleles at a locus present in offspring of a pair cross, and if both parents share one or both alleles, the allelic diversity of offspring is reduced. Growth-affecting alleles that could exist in populations may be absent from controlled crosses (Foltz and Chatry 1986).

Nevertheless, it is very difficult to make unequivocal assertions as far as the underlying mechanisms of the absence of associations between isoenzyme heterozygosity and biomass production are concerned. As Koehn (1990) pointed out, to-date it has been impossible to perform mutually exclusive tests that unequivocally discriminate among the dominance and overdominance hypotheses that may account for heterozygosity fitness associations. Regardless of invoking underlying mechanisms, there could be a simple reason for the absence of heterozygosity biomass associations. Although the prime interest of this study was in the relations of heterozygosity and biomass, at the theoretical level these should reflect heterozygosity fitness relationships; growth is simply regarded as a surrogate of fitness. *S. exigua*, unique among willows for its root suckering ability (the production of new shoots from an extensive lateral root system), has a high occurrence of asexual reproduction and forms uniform populations of highly related individuals (Mosseler 1987). Because the root system of *S. exigua* has an important role in reproduction, this species may accumulate a considerable portion of its total biomass in root growth at its early stages of development. Mosseler et al. (1988) have connected the origins of this species, which are believed to be in Mexico and in the arid zones of the United States (Dorn 1976), with the suggestions that species and provenances within species adapted to drier conditions tend to allocate more of their total biomass to roots that to stems (Cannel et al. 1978; Bongarten and Teskey 1987). It may be questionable whether high growth and biomass production would be significantly associated with fitness, life history characteristics, and the evolutionary stable strategy of resource allocation in this species. As meta-analyses of heterozygosity fitness relations have shown, fitness is not uniformly dependent on isoenzyme variation and may vary with life-history changes or other aspects of a species' biology (Britten 1996). The explanation presented above, may be regarded as a deterministic explanation on the absence of heterozygosity biomass correlations in this species; it does not preclude a priori possible heterozygosity fitness associations.

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