GENETIC STUDIES IN A TROPICAL PINE - *PINUS KESIYA* I. INHERITANCE OF SOME ISOENZYMES AND LINKAGE AMONG ALLOZYME LOCI

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BOYLE, T.J.B., LIENGSIRI, C. & PIEWLUANG, C. 1990. Genetic studies in a tropical pine - *Pinus kesiya* I. Inheritance of some isoenzymes and linkage among allozyme loci. Up to 15 embryo-gametophyte pairs from each of 38 parent trees of *Pinus kesiya* Royle ex Gordon were assayed using starch gel electrophoresis and the staining pattern observed for 18 isoenzyme loci. For 14 of these loci, haploid megagametophyte genotypes were recorded allowing an analysis of single and multilocus segregation ratios. Results indicated that there were no significant deviations from the expected 1:1 ratio of haploid genotypes from heterozygotic parents at any locus. Thus, simple Mendelian inheritance can be assumed. Deviation from a 1:1:1:1 two-locus ratio of doubly heterozygous parents, indicating linkage, was detected for the locus pair 6PG-1 : 6PG-2.

Key words: Isoenzymes - inheritance - linkage - tropical pines - *Pinus kesiya*

Introduction

Studies of the population genetics of commercially important species can be used to enhance their management and accelerate progress from tree improvement programmes. Over the past several decades, the development of biochemical methods of genetic analysis, such as that using isoenzymes, has allowed considerable progress to be made in population genetics studies (Lewontin & Hubby 1966). The advantages of isoenzymes for such studies include their codominant expression, freedom from environmental influences, and the ease with which a large number of individuals can be simultaneously evaluated over many loci (Lewontin 1974).

Before isoenzyme loci can be used for genetics studies, however, the genetic basis of observed variation must be established. This requires confirmation that staining patterns are repeatable, such that a given sample produces a consistent pattern, and the development of a genetic model to explain the observed variation. In coniferous species, the presence of haploid gametophytic tissue in the mature seed greatly facilitates this process. Confirmation of a 1:1 segregation ratio of haploid genotypes among gametophytes of apprarently heterozygous parents is generally taken to confirm the genetic basis of the variation.

Several population genetics analyses require an assumption of independence of loci. It is, therefore, important to investigate the presence of strong

linkage among isoenzyme loci that are to be used in population genetics analyses. A 1:1:1:1 segregation ratio of megagametophytes from trees heterozygous at two loci is evidence for jointly independent inheritance, or absence of linkage.

Pinus kesiya Royle ex Gordon is a medium elevation (1000 - 2000 *m*) tropical pine with a wide but discontinuous range in the Philippines, India, Burma, Thailand, and Vietnam (Critchfield & Little 1966). In Thailand, it is confined to the mountain ranges of the north and northwest, with some isolated outliers in the northeast (Turnbull *et al.* 1980). *P. kesiya* has been widely planted as an exotic conifer in parts of Africa (Armitage 1980, Pousujja 1986) and, because of its desirable fibre characteristics, has recently gained prominence within ASEAN (the Association of South East Asian Nations). Planting programmes are already underway in Thailand and the Philippines. The species suffers from several defects, including lack of stem straightness, coarse branching and volume of juvenile wood (Guldager *et al.* 1980) but, as these traits are amenable to genetic improvement, breeding programmes have been initiated in several countries.

In support of existing and proposed genetic management programmes an investigation of genetic variation among some Thai populations of *P. kesiya*, by means of isoenzyme analysis, was undertaken by the ASEAN-Canada Forest Tree Seed Centre. This paper reports on the inheritance and linkage relationships of nine enzyme systems.

Materials and method

Seeds from a total of 38 trees, representing four populations, were provided by the seed bank of the Royal Forest Department Pine Improvement Centre, Huay Kaew Arboretum, Chiang Mai, Thailand. The seed was germinated for a period of about four days, at which time the megagametophyte and germinating embryo were separated. For each parent tree, 15 embryo-gametophyte pairs were assayed, following procedures outlined by Liengsiri *et al.* (1990). Haploid genotypes were recorded for 18 loci, representing ten enzyme systems (Table 1). However, for MDH, the embryonic tissues did not stain sufficiently strongly at any of the four loci to permit scoring. Individual gametophyte genotypes were therefore not recorded, so inheritance and linkage analyses were not possible for MDH loci.

Loci and alleles were designated according to their mobility. The most mobile (anodal) locus and allele were assigned the number "1", the next in order of mobility was designated "2", and so on.

In order to efficiently detect deviations from expected segregation ratios a large number of gametophytes should be observed from trees known to be heterozygous. However, the use of relatively few gametophytes from a larger number of trees can provide valid, though less precise, information on inheritance and linkage relationships (Boyle & Morgenstern 1985). A log-likelihood G-test (Sokal & Rohlf 1969) is used to detect deviations from a 1:1 ratio. A pooled G value tests for the overall deviation from a 1:1 ratio, and

can be compared with a χ^2 distribution with one degree of freedom. When more than one tree is found to be heterozygous at any locus, a heterogeneity G value can be calculated and compared with a χ^2 distribution, with degrees of freedom equal to the number of heterozygous trees less one. This heterogeneity G-test examines whether the segregation ratios are consistent for all heterozygous parents.

Table 1. Enzyme systems assayed

Enzyme system	EC code	Abbreviation	Number of loci
Aspartate aminotransferase	2.6.1.1	AAT	1
Glucose-6-phosphate dehydrogenase	1.1.1.49	G6P	1
Isocitrate dehydrogenase	1.1.1.42	IDH	1
Leucine aminopeptidase	3.4.11.1	LAP	1
Malate dehydrogenase	1.1.1.37	MDH	4
Menadione reductase	1.8.1.4	MR	2
Phosphoglucomutase	2.7.5.1	PGM	1
6-phosphogluconic dehydrogenase	1.1.1.44	6PG	3
Phosphoglucose isomerase	5.3.1.9	PGI	2
Shikimic acid dehydrogenase	1.1.1.25	SDH	2

A similar three-way test (Sokal & Rohlf 1969) can be used to detect linkage. Recombination fractions (r) are calculated by the binomial estimator: r = k/n, where k is the number of observations in the smaller class of the two possible two-locus haploid genotype pairs (*i.e.* frequency of AB + ab *versus* frequency of Ab + aB), and n is the total number of gametophytes observed. The standard error of this estimate is simply the square root of the binomial variance (*i.e.* r(1-r)/n). However, as Geburek and Von Wuehlisch (1989) discuss, the correct identification of gametes in the coupling or repulsion phase requires knowledge of whether or not the particular parent is a recombinant. In the absence of such information, estimates of the recombination fraction will be biased, especially for small sample sizes and/or weak linkage. They, therefore, recommend a maximum likelihood estimator:

$$L(r/n_i, k_i; i = 1,...,N)$$

where, n is the number of gametes analysed for the i double heterozygote, k is the number of gametes in the smaller class, and N is the number of double heterozygotes.

For each individual double heterozygote, this estimator is given as:

$$L(r_i/n_i, k_i) = \binom{n_i}{k_i} (r_i^{k_i} (1-r_i)^{[n_i k_i]} + r_i^{[n_i k_i]} (1-r_i)^{k_i})$$

and the overall likelihood is calculated as the product of individual tree likelihoods. In view of the small sample size in this study, maximum likelihood estimates of the recombination fraction were calculated in this way, using an iterative procedure to maximize overall probability; 95% confidence

intervals were estimated in a similar fashion, using the formulae given by Geburek and Von Wuehlisch (1989).

Results

Four of the 18 loci (IDH, MDH-2, SDH-2, and PGI-2) were monomorphic. Banding patterns observed at the 14 polymorphic loci, including the patterns for heterozygous diploid samples, are shown in Figure 1 and summarised below.

Aspartate aminotransferase (AAT)

Although two bands of activity were apparent for AAT, the more cathodal band was very indistinct and considered unsuitable for analysis. Two alleles were recorded at the more anodal band and four trees were heterozygous.

Glucose-6-phosphate dehydrogenase (G6P)

A single band of activity was evident for G6P, with two alleles and four heterozygous parent trees. These trees produced a heterogeneity G-value that was significant at the 5% level, but the pooled G-value indicated that over all heterozygous trees there was no deviation from a pooled 1:1 ratio (Table 2).

Table 2. Pooled and heterogeneity G-tests for a 1:1 segregation ratio among gametophytes of heterozygous trees

Locus	Genotype	Pooled G	Heterogeneity G	df
AAT-1	12	0.00	1.34	3
G6P	12	0.00	8.00*	3
LAP-2	12	2.09	11.03	6
MR-1	10	3.40	-	-
MR-2	10	0.74	10.10	10
	12	0.31	0.95	1
6PG-1	12	2.22	29.13*	16
6PG-2	12	2.81	13.43	20
6PG-3	12	1.58	28.62	18
PGI-2	12	1.21	2.26	1
PGM	12	2.66	7.70	5
	23	3.40	-	-
SDH-1	12	3.29	3.21	4

Note: Significance level: * = 5%

Leucine aminopeptidase (LAP)

As for AAT, two zones of activity were evident with one being too indistinct to score. In the case of LAP, however, the more cathodal zone was scored and was found to have two alleles, with seven trees being heterozygous.

Menadione reductase (MR)

Two zones of activity were scored for MR. At the more anodal locus a single parent was found to be heterozygous for a null allele. At the cathodal locus a null allele was much more common, producing 11 heterozygotes and one homozygote. Two other alleles were also present, with two heterozygotes detected.

6-Phosphogluconic dehydrogenase (6PG)

Three loci stained strongly for 6PG and all three were highly polymorphic, with two common alleles at each locus. At 6PG-2, a third allele was found as the pollen contribution to a single embryo. At 6PG-1 there was a low level of heterogeneity among heterozygous parents in their segregation ratios ($P<0.05$) (Table 2).

Phosphoglucose isomerase (PGI)

Two zones of activity were detected, with the anodal locus proving to be monomorphic among both the parents and embryos. At PGI-2, only two trees were found to be heterozygous.

Phosphoglucomutase (PGM)

One weakly staining zone could be scored for PGM. Three alleles were found in the parents, with six trees being "12" heterozygotes and a single tree being "23". A fourth allele was found among the embryos.

Shikimic acid dehydrogenase (SDH)

Two loci were apparent for SDH, the more cathodal locus being monomorphic for both the parents and embryos. At SDH-1, two alleles were detected among the parents, with five trees being heterozygous. A third allele was found among the embryos.

Malate dehydrogenase (MDH)

Four zones of activity stained for the haploid material, but for the diploid tissue the staining was too weak to be scored. At MDH-1, two alleles were recorded; MDH-2 was monomorphic; MDH-3 yielded two alleles; and, MDH-4 proved to be the most variable locus, with five alleles being detected.

None of the putative loci for which heterozygous parents were detected yielded segregation ratios that deviated significantly from the expected 1:1 ratio (Table 2). The two cases noted above for which there was significant heterogeneity in segregation ratios were G6P and 6PG-1.

Of the 55 possible two-locus combinations among the 11 polymorphic loci for which individual haploid genotypes were recorded, doubly heterozygous parents were recorded for 23 cases. Of these, six yielded segregation ratios differing significantly from the expected 1:1:1:1 ratio (Table 3). In only two cases, however, was the estimated recombination fraction < 0.5 by more than two standard errors (6PG-1 : 6PG-2; r= 0.098; and MR-1 : MR-2; r = 0.200). No heterogeneity was apparent for any of these locus pairs.

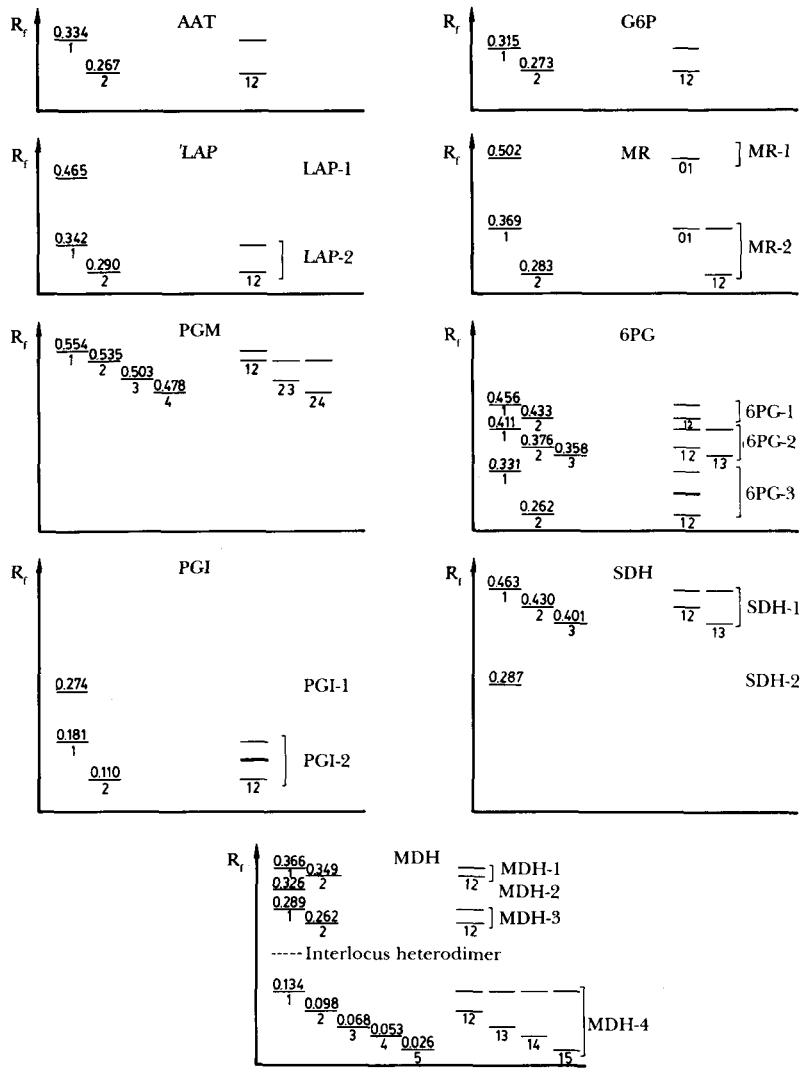

Figure 1. Banding patterns for nine enzyme systems, representing 14 polymorphic loci; patterns observed for heterozygous diploid tissue are also presented except for MDH loci, for which no diploid staining was observed

Table 3. Loci pairs for which significant deviations from a 1:1:1:1 segregation ratio for doubly heterozygous trees could be detected, or for which the maximum likelihood (ML) recombination fraction was less than 0.500

Locus pair	Pooled G	Heterogeneity df	Heterogeneity G	Heterogeneity df	Recombination fraction (SE)	ML estimate (95% CI)
AAT : G6P	1.81	1	-	-	0.333 (0.122)	0.347
G6P : 6PG-2	15.59*	7	1.05	2	0.477 (0.075)	0.500
G6P : 6PG-3	18.54**	7	2.25	2	0.400 (0.073)	0.303
LAP-2 : 6PG-210.96*		4	3.24	1	0.389 (0.114)	0.500
LAP-2 : SDH-1	1.14	1	-	-	0.333 (0.122)	0.497
MR-1 : MR-2	7.81	1	-	-	0.200 (0.103)	0.200 (0.450-0.054)
MR-1 : 6PG-2	1.45	1	-	-	0.357 (0.124)	0.415
MR-1 : SDH-1	0.66	1	-	-	0.333 (0.122)	0.347
MR-2 : 6PG-1	21.73	16	6.53	5	0.429 (0.052)	0.369
MR-2 : 6PG-2	23.77	22	5.44	7	0.474 (0.046)	0.378
MR-2 : 6PG-3	28.15	19	3.74	6	0.475 (0.049)	0.366
MR-2 : PGM	4.27	4	0.14	1	0.433 (0.090)	0.350
6PG-1 : 6PG-2	201.12**	40	17.06	13	0.098 (0.023)	0.098 (0.147-0.060)
6PG-1 : 6PG-3	38.17	22	8.81	7	0.340 (0.043)	0.353
6PG-1 : PGM	9.14	4	1.66	1	0.318 (0.085)	0.343
6PG-2 : PGM	10.64	10	5.17	3	0.444 (0.064)	0.267
6PG-3 : SDH-1	9.75*	4	2.28	1	0.467 (0.073)	0.500

Notes: Significance level: * = 5%; ** = 1%; 95% confidence intervals (CI) for ML estimates are given only for those loci for which the interval did not straddle 0.500

The effect of the bias introduced by a small sample size can be seen by comparing estimates of recombination fractions with those estimated from the maximum likelihood procedure. Whilst the estimates of strong linkage are identical for both methods, for loci at which weak linkage was detected the estimates differ (Table 3). However, in almost every case, loci pairs for which the 95% maximum likelihood confidence intervals straddled 0.5 also had simple recombination estimates that were within two standard errors of 0.5.

Discussion

The genetic origin of all the zones of staining which could be scored and for which putative heterozygote parents were recorded can be assumed due to the absence of any significant deviations in the segregation ratios. As noted earlier, the analysis of a large number of gametophytes from known heterozygous parents is more efficient than using small numbers of gametophytes from more parents. The low level of heterogeneity detected for two loci is probably the result of the comparatively small number of gametophytes analysed for each parent.

Two loci, 6PG-1 and 6PG-2, are clearly tightly linked, with an estimated recombination fraction of 0.098. The only other locus pair which produced a maximum likelihood recombination estimate < 0.5 at the 95% confidence level was MR-1 and MR-2, and only one tree was doubly heterozygous for this pair. The other four locus pairs for which significant segregation deviations were detected were also only represented by two or three trees in each case. With so

few observations, and given the large recombination fractions estimated, linkage of these pairs cannot be considered to have been conclusively demonstrated.

For most enzyme systems assayed, the genetic model derived is similar to those postulated for many other conifer species (Rudin & Ekberg 1978, Guries & Ledig 1978, Adams & Joly 1980, Conkle 1981, El-Kassaby 1981, King & Dancik 1983, Danzmann & Buchert 1983, Boyle & Morgenstern 1985). Among the pines, two loci have been reported for AAT and LAP (Guries & Ledig 1978, O'Malley *et al.* 1979, Adams & Joly 1980), and PGI (Guries & Ledig 1978) and PGM (Guries & Ledig 1978), Adams & Joly 1980). However, for jack pine (*P. banksiana* Lamb.), Danzmann and Buchert (1983) reported four loci for AAT and only one for LAP. Single loci are usually reported for G6P and IDH, though Danzmann and Buchert (1983) found two IDH loci in jack pine.

The interpretation of two loci, 6PG and MDH, has tended to vary widely from species to species. This is particularly true for MDH (El-Kassaby 1981), for which there have been reports of two loci in loblolly pine (*P. taeda* L.) (Adams & Joly 1980) and pitch pine (*P. rigida* Mill.) (Guries & Ledig 1978), four loci in jack pine (Danzmann l& Buchert 1983), and three or four loci in other species (King & Dancik 1983, Boyle & Morgenstern 1985). Two loci have usually been reported for 6PG, with the exception of Scots pine (*P. sylvestris* L.), for which Krzakova and Szweykowski (1979) recorded three loci, and loblolly pine, for which Adams and Joly (1980) found only one.

Conclusions

The absence of significant deviations from the expected segregation ratios at 11 of a postulated 18 loci from ten enzyme systems provides confirmation of the genetic origin of the observed variation. The consistent staining of the monomorphic zones also indicates that these represent genetic loci. All 18 are therefore suitable for use in population genetics studies, with the exception of the MDH loci for which analyses involving gametophytes only are possible.

Based on the small sample sizes available only one locus pair can be considered to be definitely linked, but the linkage between this pair (6PG-1 : 6PG-2) is very tight. Only one of these loci should, therefore, be used for analyses requiring an assumption of independence. In addition, MR-1 and MR-2 may be linked, but the small sample size does not allow confirmation of this.

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