INHERITANCE OF ISOZYME PHENOTYPES OF *PINUS MERKUSII*

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Received March 1994

CHANGTRAGOON, S. & FINKELDEY, R. 1995. Inheritance of isozyme phenotypes of *Pinus merkusii*. Isozyme analysis is a powerful tool for studying the population genetics of forest trees. The identification of isozyme marker gene loci is based on a genetic analysis, i.e. the proof of the genetic control and a determination of the mode of inheritance of trait expressions (isozyme phenotypes). The observation of segregation ratios within the haploid endosperms of putatively heterozygous seed trees and their comparison to the expected 1:1 ratio of the two trait expressions is a simple and straightforward method of genetic analysis for most conifer species. Using this approach, 11 polymorphic isozyme gene loci were identified for *Pinus merkusii*, a Southeast Asian pine. Another 7 putative gene loci were found to be completely or nearly completely monomorphic for all populations investigated, rendering a genetic analysis impracticable.

Key words: *Pinus merkusii* - isozyme - genetic analysis - endosperm

CHANGTRAGOON, S. & FINKELDEY, R. 1995. Pewarisan fenotip isozim bagi *Pinus merkusii*. Analisis isozim merupakan satu alat berkesan untuk mengkaji genetik populasi pokok-pokok hutan. Pengecaman lokus-lokus gen penanda isozim adalah berdasarkan analisis genetik, iaitu bukti kawalan genetik dan penentuan mod pewarisan ciri-ciri ekspresi (fenotip isozim). Pemerhatian nisbah-nisbah pengasingan dalam endosperma haploid bagi pokok-pokok berbiji heterozigus dan putatif serta perbandingan dengan nisbah jangkaan 1:1 bagi kedua-dua ciri ekspresi merupakan satu cara yang mudah untuk menjalankan analisis genetik bagi kebanyakan spesies konifer. Dengan menggunakan cara ini, 11 lokus gen isozim polimorfik telah dikenalpasti bagi *Pinus merkusii*, sejenis pain Asia Tenggara. Tujuh lagi lokus gen putatif didapati monomorfik sepenuhnya atau hampir monomorfik sepenuhnya bagi semua populasi yang dikaji, mengakibatkan analisis genetik tidak praktikal.

Introduction

Our knowledge of genetic variation patterns in tropical tree species is limited even for most tropical trees of proven or potential economic value. This knowledge is

crucial for proper management and conservation of forests. Provenance research and breeding gave some insight into spatial patterns of genetic variation in major plantation species. However, the quantitative traits measured by tree breeders are the outcome of a complex interplay between genetic and environmental factors and hence fail to give reliable estimates of the number of genes involved in an observed trait expression and the number of allelic variants present.

The prerequisite for a more detailed understanding of the genetic system of a species, including its mating system as well as spatial and temporal patterns of genetic variation, is the identification of marker gene loci by means of genetic analysis.

A well-established method of gene locus identification for gymnosperms is the observation of segregation among the endosperms of presumably heterozygous seed trees (Bergmann 1973). The "primary'" endosperm of gymnosperms develops from macrogametophyte tissue and is therefore haploid. A seed tree, heterozygous at a particular gene locus, is expected to transmit each of its two alleles in equal frequencies to its endosperms. Hence, the observation of two trait expressions in roughly equal frequencies in a sample of endosperms of a presumably heterozygous seed tree is the key for the identification of a gene locus. This simple method of genetic analysis was adopted in the present study.

Most studies involving marker gene loci in forest tree species are based on isozyme electrophoresis. Environmental stability, co-dominant trait expression, and clear separability of zones on a gel (each zone attributed to one gene locus) make feasible the formulation of hypotheses on the genetic control of observed isozyme phenotypes. However, environmentally dependent trait expressions have been reported for several enzyme systems (notably peroxidase and esterase); so-called "null-alleles" behave completely recessively, and overlapping of zones is common for many enzyme systems. Further complications may arise from the formation of interlocus heterodimers (e.g. Giannini *et al.* 1991) and double or triple bands coded by a single allele (Finkeldey 1992). Thus, the assignment of controlling gene loci to observed phenotypes bears the risk of misclassification and biased estimates of genetic variation if it is not based on a proper genetic analysis. A test of hypothesis on the control of isozyme variation patterns is highly recommended whenever material suitable for a genetic analysis is available (Hattemer 1991).

Pinus merkusii Jungh. and deVriese is a widely distributed tropical pine of Southeast Asia (Cooling 1968). In Thailand, *P. merkusii* is mostly confined to the northern, central, and northeastern part of the country (Werner 1993). A genetic inventory at isozyme marker loci undertaken by the present authors aims at assesing the spatial patterns of genetic variation of Thai provenances of *P. merkusii*. Patterns of genetic variation within and between populations will be examined (Changtragoon & Finkeldey, in prep.). The results shall form a basis for future management and conservation measures. This first report is confined to an examination of the genetic control of the observed traits (isozyme phenotypes).

Materials and methods

Seeds were harvested from single seed trees, and single tree progeny arrays were kept separately. Seeds from 11 populations were investigated to cover all of the major areas of *P. merkusii* in Thailand. The total number of trees studied was 245. At least 6 seeds were investigated from each tree.

Seeds were immersed overnight in water, dissected, and the embryo carefully separated from the endosperm. The embryo and endosperm were ground in two and three drops of homogenizing buffer (0.08 M Tris-HCl, pH 7.2) respectively. Paper wicks saturated with the homogenate were inserted into starch gels. Extracts from the endosperm and the embryo of the same seed were positioned adjacent to each other.

Horizontal starch gel electrophoresis (12% starch concentration) was performed as described in detail by Feret and Bergmann (1976) and Conkle *et al.* (1982). The buffer systems described in Table 1 were used and 2.3 g sucrose 100 ml^{-1} gel buffer were added to all gels. Staining methods were slightly modified from those described by Conkle *et al.* (1982) and Vallejos (1983). Staining for FDH was similar to that for GDH: 2 g formic acid were added to 50 ml staining solution, instead of glutamic acid.

Table 1. Running buffers and conditions

No.	Electrode buffer	Gel buffer	Running conditions	Enzyme system
I	0.06 M NaOH 0.3 M boric acid pH 8.2	0.075 M Tris 0.01 M citric acid pH 8.7	approx. 30 V cm^{-1} for 4 1/2 h max. 80 mA	GOT
II	0.025 M LiOH 0.2 M boric acid pH 8.1	0.05 M Tris 0.01 M citric acid 5% (v/v) electr. buffer pH 8.1	same as I	LAP GDH
III	0.135 M Tris 0.043 M citric acid pH 7.3	0.042 M Tris 0.013 M citric acid pH 7.3	approx. 20 V cm^{-1} for 5 1/2 h max. 130 mA	SKDH IDH 6-PGDH
IV	same as III, but pH 7.0	same as III, but pH 7.0	same as III	PGM MDH
V	same as III, but pH 6.7	0.06 M Tris 0.036 M histidine-HCI 0.04% (w/v) EDTA pH 6.7	same as III	G-6-PDH DIA (+NDH) FDH

Hypotheses on the genetic control of observed phenotypes for 12 enzyme systems, which had clear and reproducible zymograms, were formulated based on experience from other conifer species. Both embryo and endosperm zymogram patterns were scored to formulate plausible hypotheses on their respective genetic

control. The most anodal zone corresponding to a putative gene locus was named A, the next B, and so on. Zones which appeared on a gel were not checked for variation, if the specific substrate of an enzyme was not added to the staining solution, because these zones were unlikely to result from activity of the respective enzyme. No letters were assigned to these zones. Within a zone that supposedly corresponded to alleles of a gene locus, bands were denoted with Arabic numerals according to their relative mobilities in the gel, starting with the most anodal, i.e. the fastest band. The absence of bands in the zymograms of haploid endosperms within a zone was thought to be caused by a "null-allele" (or "silent allele").

Variation in banding patterns of endosperms from the same seed tree indicates heterozygosity of the seed tree at a putative gene locus. Whenever a previously unobserved phenotype in a putatively heterozygous seed tree was detected, at least 20 endosperms of the tree were investigated and the hypothesis on the genetic control and mode of inheritance of that phenotype tested. A log likelihood ratio test (*G*-test) with Williams' correction (Sokal & Rohlf 1981) was performed to check the observed segregation within the endosperms for significant deviations from the expected 1:1 ratio. Probabilities p were calculated to obtain the observed segregation ratios, based on sample size, binomial distribution, and expected 1:1 ratio. This "two-tailed exact test" is recommended for small sample sizes (n<25; Sokal & Rohlf 1981). Non-significant deviation between observation and expectation at 5% level of confidence, i.e. $G < 3.841$ ($K_{0.05;1df} = 3.841$) and $p > 0.05$, indicated the identification of a polymorphic gene locus or the confirmation of the allelic status of a phenotype to an already identified gene locus.

Rare variants which were observed only in embryo zymograms were integrated. However, the hypotheses on the genetic control of these phenotypes could not be tested since no analogous variation was observed within the endosperms of any tree.

Results

Clear and reproducible zymograms of 12 enzyme systems were obtained (Table 2). All observed zymogram patterns are illustrated in Figure 1; phenotypes of heterozygous individuals, whose existence could be expected but were not observed are not included in the figure. Segregation frequencies within endosperms of putatively heterozygous seed trees and results of statistical tests (*G*-test and exact test) are given in Table 3.

Leucine-aminopeptidase

Two widely separated zones of intense staining were observed. Genetic analysis revealed that each zone is controlled by one gene locus. Two active alleles at the LAP-B locus were identified by observing segregation within a single seed tree. A "null-allele" was identified at the LAP-A zone, but only one active allele could be detected. Two occasional bands of much weaker stain were believed to

result from the activity of other aminopeptidases, as the substrate specificity of aminopeptidases is generally low.

Table 2. Investigated enzyme systems, their abbreviations, E.C. numbers, controlling gene loci and numbers of identified alleles

Enzyme system	Abbrev.	E.C. No.	Gene loci	Alleles
Leucine-aminopeptidase	LAP	3.4.11.1	-A	2[5]
			-B	2
Glutamate-oxaloacetate-transaminase	GOT	2.6.1.1	-A[1]	1
			-B[1]	1
			-C	3
Glutamate-dehydrogenase	GDH	1.4.1.3	-A	3[4]
Formate-dehydrogenase	FDH	1.2.1.2	-A	3[4]
Malate-dehydrogenase	MDH	1.1.1.37	-A	3
			-B[2]	2[5]
			-C[3]	2[4]
Shikimate-dehydrogenase	SKDH	1.1.1.25	-A	4[5]
6-phosphogluconate-dehydrogenase	6-PGDH	1.1.1.44	-A	2
			-B	3
Phosphoglucomutase	PGM	2.7.5.1	-A	2
Glucose-6-phosphate-dehydrogenase	G-6-PDH	1.1.1.49	-B	2
Isocitrate-dehydrogenase	IDH	1.1.1.42	-A[1]	1
NAD-dehydrogenase	NDH	1.6.99.3	-A[1]	1
Diaphorase	DIA	1.6.4.3	-A[1]	1

[1]Declaration as a locus was tentative, as no variation was observed;
[2]Declaration as a locus was tentative, as genetic analysis was based on a very small sample size;
[3]Declaration as a locus was tentative, as variation was only observed in embryos;
[4]One of the alleles was only observed in putative heterozygous embryos;
[5]One of the alleles was a "null-allele".

Glutamate-oxaloacetate-transaminase

Four zones of activity were observed in GOT-zymograms. Two upper zones were invariant, indicating that each of these zones could be controlled by a single gene locus. Both zones may result from GOT organelles; isozymes of the plastids are mainly controlled by nuclear genes and show low levels of genetic variation. Three active alleles were identified at the GOT-C locus, which is assumed to be cytosolic GOT, by observing segregation in two seed trees.

In many conifers including pines, the GOT-C locus codes are two widely separated, always co-migrating bands (Kim & Hong 1982, El-Kassaby *et al.* 1987). One of these bands is often moving towards the cathode. However, no activity in the cathodal strip of the gel was observed in this study, but rather an invariant zone of intense staining next to the origin in the anodal part of the gel. This zone was excluded from the genetic interpretation and is believed to be related to the

GOT-C locus. Its constancy in spite of clear variation at the GOT-C zone may be due to running conditions during electrophoresis.

Glutamate-dehydrogenase

Only one zone appeared on gels stained for GDH. Segregation ratios in one seed tree showed no significant deviation from the expected 1:1 ratio for a tree putatively heterozygous A_1A_3. A rare phenotype, observed only in four embryos, suggested the existence of a third allele A_2. In most plant species GDH is a multimeric enzyme tetra- or octomer (Wickneswari 1989). The observed phenotype (Figure 1) fits well to the hypothesis of a genotype heterozygous (A_2A_3) at the gene locus GDH-A, which codes for a tetra- or a hexamer. Unfortunately, no seed tree was found to possess this rare variant. Hence, the assignment of the phenotype to a heterozygous genotype is tentative, as a genetic analysis could not be performed.

Formate-dehydrogenase

Similar to GDH, the single zone of FDH, a multimeric enzyme system, was found to be controlled by one gene locus. In a single embryo, a broad band above the position of the A_2 allele was observed. This embryo was interpreted as heterozygous $A_1 A_2$, but this hypothesis could not be tested.

Malate-dehydrogenase

The simple genetic control of zone A by a gene locus with three active alleles identified so far is confirmed by the observed segregation ratios within the endosperms of two seed trees (Table 3). Interpretation of the other zones was largely based on experience from other conifer species, which proved that an overlapping of zones and the formation of interlocus heterodimers are very common in MDH (El-Kassaby 1981). The observed patterns show a striking similarity to zymograms of malate-dehydrogenase of *Pinus sylvestris* (Thormann & Stephan 1993). Variation within zones MDH-B and MDH-C should be reflected by the supposed interlocus heterodimers between the two zones. Variation within the B-zone was found only in the endosperms of two seed trees presumably heterozygous for a "null-allele" at the B-locus. Unfortunately, only six healthy seeds each could be investigated for both trees (Table 3). Hence, the sample size was too small for a reliable genetic analysis to be made. Variation within the C-zone was confined to three embryos, which appeared to be heterozygous C_1C_2 at the putative MDH-C gene locus (Figure 1).

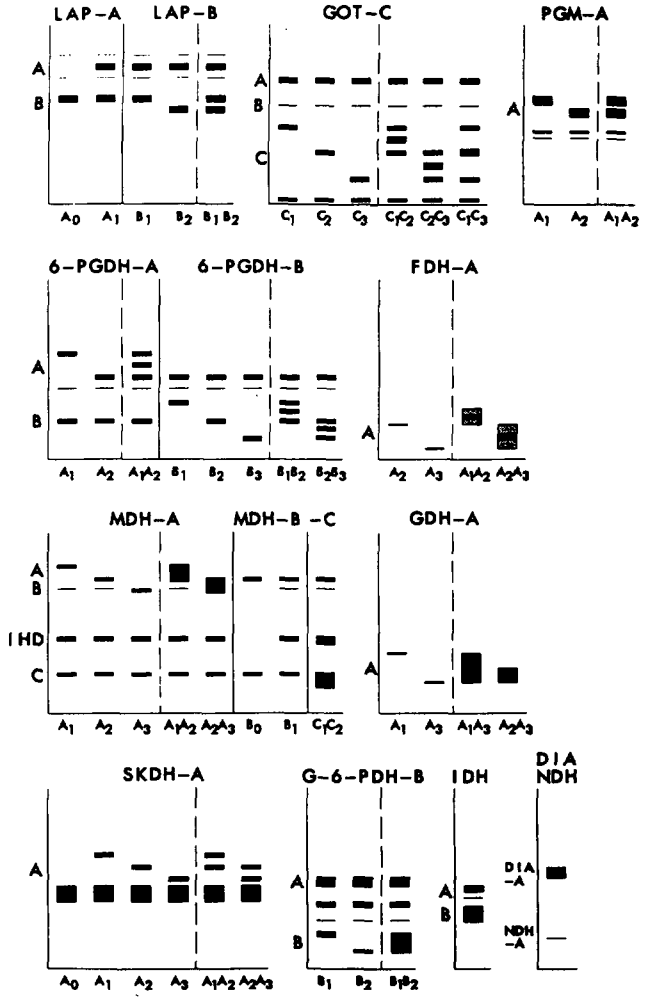

Figure 1. Illustrations of observed isozyme phenotypes. Variants of each zone are shown in combination with the most frequent types of other zones. To the left of the dashed line: phenotypes of haploid endosperms and homozygous embryos; to the right of the dashed line: phenotypes of heterozygous embryos. "IHD" = interlocus heterodimer

Shikimate-dehydrogenase

The upper zone SKDH-A is controlled by a gene locus with three active alleles and one "null-allele". The "null-allele" and the allele A_1 were observed in only one seed tree. The lower zone was diffuse and pale and partially overlapping with a zone not caused by the activity of SKDH (see discussion). Hence, it was omitted from analysis.

Table 3. Observed segregation within endosperms of putatively heterozygous seed trees, and results of tests for statistical significance of deviations from the expected 1:1 ratio (G - test, $K_{\alpha=0.05;\,1df} = 3.841$ and exact probabilities p to obtain deviation from the 1:1 ratio equal to or greater than the observed ratios)

Gene locus	Genotype of seed tree	Sample size	Observed	Segregation	G	p
LAP-A	A_0A_1	135	A_0:64	A_1:71	0.36	0.61
LAP-B	B_1B_2	20	B_1:11	B_2: 9	0.20	0.82
GOT-C	C_1C_2	20	C_1: 8	C_2:12	0.79	0.50
GOT-C	C_1C_3	23	C_1:12	C_3:11	0.04	1.00
GDH-A	A_1A_3	20	A_1: 7	A_3:13	1.78	0.26
FDH-A	A_2A_3	20	A_2: 9	A_3:11	0.20	0.82
MDH-A	A_1A_2	21	A_1: 9	A_2:12	0.42	0.66
MDH-A	A_2A_3	24	A_2:11	A_3:13	0.16	0.84
MDH-B	B_0B_1	6	B_0: 1	B_1: 5	2.69	0.22
MDH-B	B_0B_1	6	B_0: 2	B_1: 4	0.63	0.68
SKDH-A	A_0A_1	135	A_0:69	A_1:66	0.07	0.86
SKDH-A	$A_1A_2$2	24	A_1:11	A_2:13	0.16	0.84
SKDH-A	A_2A_3	20	A_2:10	A_3:10	0.00	1.00
6-PGDH-A	A_1A_2	20	A_1: 8	A_2:12	0.79	0.50
6-PGDH-B	B_1B_2	23	B_1:13	B_2:10	0.38	0.68
6-PGDH-B	B_2B_3	26	B_2:11	B_3:15	0.62	0.56
PGM-A	A_1A_2	38	A_1:13	A_2:25	3.81	0.07
G-6-PDH-B	B_1B_2	24	B_1:10	B_2:14	0.66	0.54

6-Phosphogluconate-dehydrogenase

Two polymorphic gene loci, each controlling one zone, were identified for 6-PGDH. No interlocus heterodimers were formed. However, there was a weak band between both zones, even in the absence of 6-phosphogluconic acid. No genetic interpretation was proposed for this zone.

Phosphoglucomutase

An upper zone of very intense colour was observed in gels stained for PGM. One of the seed trees showed variation in its endosperms. The observed segregation in the endosperms of that seed tree deviated from the expected 1:1 ratio, but the deviation was not significant at the 5% level (Table 3). The lower zone was not caused by the activity of PGM and thus was not interpreted.

Glucose-6-phosphate-dehydrogenase

G-6-PDH is a multimeric enzyme system. The upper zone G-6-PDH-A did not vary in appearances. However, the bands were very broad and therefore the zone was omitted from interpretation. The next two zones, the upper of very intense colour and the lower much weaker, did not show any variation. They were also not

interpreted since they appeared on the gel in the absence of glucose-6-phosphate, the substrate for G-6-PDH. Interpretation was confined to the most cathodal zone G-6-PDH-B. Segregation analysis in one single tree revealed that this zone was controlled by a single gene locus.

Isocitrate-dehydrogenase

IDH isozyme phenotypes were uniform. A band of strong staining intensity which is always accompanied by a weaker band was believed to be an artefact controlled by the monomorphic gene locus IDH-A. The lower zone IDH-B showed only weak and diffuse staining without clear variation. This zone was omitted from analysis as a reliable assessment of phenotypes was impossible.

NAD-dehydrogenase and Diaphorase

Both enzyme systems were stained on the same gel. A single, fine band appeared on the lower part of the gel, in the absence of the staining dye 2-6-dichlorophenol-indophenol (DICIP). This band was interpreted to be coded by a monomorphic NDH-A gene locus. An additional broad band of intense blue colour appeared when DICIP was added to the solution. This zone was believed to be controlled by a monomorphic DIA-A gene locus.

Discussion

A genetic analysis ought to be based on segregation patterns within the offspring of phenotypically known parents or, in the case of haploid endosperms, a phenotypically known seed tree. However, no vegetative diploid tissue (e.g. buds, leaves) of the seed trees was available for this study. The putative genotypes of seed trees from an investigation of a sample of their endosperms were therefore used, without the direct observation of their phenotypes. Although this could be a theoretical drawback, the risk of an incorrect gene locus identification was low in view of the feasibility of observing phenotypes of putatively heterozygous individuals by inspection of embryos. Since this study was confined only to seed tissues, the stability of phenotypes during ontogenetic development was not investigated, although all investigated enzymes were known to show a high developmental stability.

Hypotheses on the genetic control of observed phenotypes were based on three lines of evidence:

- Most important is the comparison of observed patterns with those of other closely related species for which a genetic analysis of the traits has been performed (e.g. MDH).
- The phenotype of putatively heterozygous individuals depends largely on quaternary enzyme structure, which determines the occurrence and number of heteromers. The quaternary structure of a specific enzyme system varies only rarely throughout the plant kingdom. For example, LAP and PGM are

usually monomers, GOT and 6-PGDH are dimers, and GDH, G-6-PDH as well as FDH are multimeric. Hence, the assignment of a phenotype which was observed only in embryos, but never in endosperms, to the heterozygous status at a controlling gene locus could be supported by the (expected) quaternary enzyme structure. In this study the investigation of embryo tissue helped in the formulation of hypotheses on the genetic control of isozyme phenotypes (e.g. for GDH and FDH) but was not used to test these hypotheses.

- Simple tests of enzyme specificity by means of comparisons of zymograms with and without addition of the substrate of the respective enzyme to the staining solution helped to ascertain the link between a zone on the gel to the activity of the enzyme in question. At least one invariant zone was observed for all dehydrogenases which depend on NADP (i.e. SKDH, 6-PGDH, G-6-PDH, IDH, PGM) if the respective substrates were not added to the staining solutions. Banding patterns and positions on the gel indicated that this zone might always result from activity of the same enzyme, most probably isocitrate-dehydrogenase (IDH). All chemicals required for IDH staining were added to the staining solutions of all of these enzymes except the substrate (isocitric acid), but citric acid was a part of the utilized buffer systems (Table 1). Assignment of a monomorphic gene locus for each enzyme system would substantially but erroneously increase the number of monomorphic gene loci.

The genetic analysis was based on statistical comparisons between observed and expected segregation ratios in endosperms of single seed trees. The observed segregation never differed significantly (5% level of significance) from the expected 1:1 ratio. Eleven polymorphic gene loci coding for a total of 29 alleles (including 3 "null alleles") were identified. A reliable genetic analysis could not be performed for two putative MDH gene loci, which showed very low levels of variation.

Hypotheses on the genetic control of observed phenotypes could not be tested for five invariant zones (GOT-A, GOT-B, IDH-A, DIA-A, NDH-A). The assignment of a monomorphic gene locus to each of these zones was only tentative. These putative monomorphic gene loci will be included in a proposed genetic inventory, as the bias caused by an exclusion of these zones from a genetic interpretation is thought to be more severe than that caused by a possible misclassification of a locus. In particular, meaningful comparisons of levels of genetic variation between species require the inclusion of monomorphic gene loci (cf. Hamrick & Godt 1990).

Acknowledgements

Experiments were conducted at the DNA and Isozyme Laboratory, Silvicultural Research Division, Royal Forest Department (RFD) of Thailand in Bangkok. We thank the authorities of the RFD for their support of the work; the staff of RFD

for useful suggestions and help during seed collections; colleagues from the Abteilung für Forstgenetik, Göttingen; Alfred Szmidt, Swedish University of Agricultural Sciences, Umeå; and two anonymous reviewers for their helpful comments on the manuscript. The second author is indebted to Walter Neugebauer and the forest tree nursery "Pein und Pein". A travelling scholarship incidental to the "Klaus-Stern-Award in Forest Genetics" sponsored by the "Pein und Pein" nursery enabled him to become familiar with *P. merkusii* in Thailand. This research was financially supported by the Volkswagen-Stiftung.

References

BERGMANN, F. 1973. Genetische Untersuchungen bei *Picea abies* mit Hilfe der Isoenzym-Identifizierung. II.Genetische Kontrolle von Esterase- und Leucinaminopeptidase-Isoenzymen im haploiden Endosperm ruhender Samen. *Theoretical and Applied Genetics* 43 : 222 - 225.

CONKLE, M.T., HODSKISS, P.D., NUNALLY, L.B. & HUNTER, S.C. 1982. *Starch Gel Electrophoresis of Conifer Seeds: A Laboratory Manual.* General Technical Report PSW-64. Pacific Southwest Forest and Range Experiment Station, Berkeley, California. 18 pp.

COOLING, E.N.G. 1968. *Pinus merkusii. Fast Growing Timber Trees of the Lowland Tropics No.4.* Commonwealth Forestry Institute, Oxford. 169 pp.

EL-KASSABY, Y.A. 1981. Genetic interpretation of malate dehydrogenase isozymes in some conifer species. *The Journal of Heredity* 72 : 451 - 452.

EL-KASSABY, Y.A., MEAGHER, M.D., PARKINSON, J. & PORTLOCK, F.T. 1987. Allozyme inheritance, heterozygosity and outcrossing rate among *Pinus monticola* near Ladysmith, British Columbia. *Heredity* 58 : 173 - 181.

FERET, P.P. & BERGMANN, F. 1976. Gel electrophoresis of proteins and enzymes. Pp. 49-77 in Miksche, J.P. (Ed.) *Modern Methods in Forest Genetics.* Springer, Berlin, Heidelberg, etc.

FINKELDEY, R. 1992. Inheritance of isozyme phenotypes of the native *Paulownia* spp. in Taiwan. The *Journal of Heredity* 83 : 140 - 143.

GIANNINI, R., MORGANTE, M. & VENDRAMIN, G.G. 1991. A putative gene duplication in Norway spruce for 6PGD and its phylogenetic implications. Pp. 23-29 in Fineschi, S., Malvolti, M.E., Cannata, F. & Hattemer, H.H. (Eds.) *Biochemical Markers in the Population Genetics of Forest Trees.* SPB Academic Publishing, The Hague.

HAMRICK, J.L. & GODT, M.J. 1990. Allozyme diversity in plant species. Pp. 43-63 in Brown, A.H.D., Clegg, M.T., Kahler, A.L. & Weir, B.S. (Eds.) *Plant Population Genetics, Breeding, and Genetic Resources.* Sinauer, Sunderland.

HATTEMER, H.H. 1991. Genetic analysis and population genetics. Pp. 5-22 in Fineschi, S., Malvolti, M.E., Cannata, F. & Hattemer, H.H. (Eds.) *Biochemical Markers in the Population Genetics of Forest Trees.* SPB Academic Publishing, The Hague.

KIM, Z.S. & HONG, Y.P. 1982. Genetic analysis of some polymorphic isozymes in *Pinus densiflora* (I). *Journal of Korean Forestry Society* 58 : 1 - 7.

SOKAL, R.R. & ROHLF, F.J. 1981. *Biometry.* Second edition. W.H. Freeman, San Francisco. 859 pp.

THORMANN, R. & STEPHAN, B.R. 1993. Interpretation of isozyme patterns of malate dehydrogenase in Scots pine using two different staining methods. *Silvae Genetica* 42 : 5 - 8.

VALLEJOS, C.E. 1983. Enzyme activity staining. Pp. 469-516 in Tanksley, S.D. & Orton, T.J. (Eds.). *Isozymes in Plant Genetics and Breeding. Part A.* Elsevier, Amsterdam.

WERNER, W.L. 1993. *Pinus in Thailand.* Geoecological Research Vol. 7. Franz Steiner Verlag, Stuttgart. 286 pp.

WICKNESWARI, R. 1989. Use of isozyme analysis in a proposed *Acacia mangium* × *Acacia auriculiformis* hybrid seed production orchard. *Journal of Tropical Forest Science* 2 : 157 - 164.