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Inheritance and Linkage of Some Polymorphic Isozymes in *Ginkgo biloba* L.[1]

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은행나무의 몇가지 多形的 同位酵素의 遺傳樣式 및 連關[1]

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ABSTRACT

Isozyme variants of 15 enzyme systems were analyzed in megagametophytes of *Ginkgo biloba* L. Five enzyme systems (ADH, G6PD, IDH, MPI, and UGPP) appeared to be monomorphic. Only 11 isozyme zones observed in 10 enzyme systems were polymorphic : ACON-A, FST-B, GDH-A, GOT-B, MDH-B, MDH-C, MNR-A, PGI-B, PGM-A, 6PGD-B and SKDH-B. The segregation ratio and heterogeneity at most polymorphic zones suggested that each isozyme zone was controlled by a single locus with codominant alleles, but significant deviation from 1 : 1 segregation was observed at MDH-B in pooled data. Three pairs of isozyme loci (ACON-A : MDH-B, GOT-B : PGI-B, and MNR-A : SKDH-B) were found to be weakly linked. Recombination frequencies between them ranged from 0.38 to 0.40 ($p<0.05$).

要 約

감자전분젤을 매개로 한 수평식 전기영동장치를 이용하여 은행나무(*Ginkgo biloba* L.)의 megagametophyte로부터 15개 동위효소의 변이가 분석되었다. 분석된 효소 가운데서 ADH, G6PD, IDH, MPI, UGPP의 5개 효소에서는 변이가 나타나지 않았으며, 나머지 10개 효소의 11개 동위효소 구역(ACON-A, FST-B, GDH-A, GOT-B, MDH-B, MDH-C, MNR-A, PGI-B, PGM-A, 6PGD-B, SKDH-B)에서 다형성이 관찰되었다. 이중MDH-B를 제외한 모든 구역에서 관찰된 동위효소 변이들이 1 : 1의 독립적 분리비를 보임으로써, 이들이 단일 유전자좌에 의해 조절되는 공우성 대립유전자임을 추정할 수 있었다. 한편, 동위효소 유전자좌의 3가지 조합 (ACON-A : MDH-B, GOT-B : PGI-B, MNR-A : SKDH-B)에서 약한 연관관계가 관찰되었으며, 이들의 재조합 비율은 0.38-0.40로 계산되었다 ($p<0.05$).

Key Word : *isozyme, inheritance, linkage*, Ginkgo biloba L., *megagametophyte 동위효소, 유전양식, 연관, 은행나무, megagametophyte*

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INTRODUCTION

Ginkgo biloba L. is one of the most ancient gymnosperms in the world. Out of ten species came under this genus were widely distributed in the world during the Jurassic period, but most of them were extincted at glacial epochs of the Tertiary period. At present, ginkgo trees, represented as living descendant, are native to southern China, and this species is known to be introduced into Korea at very early date (Del Tredici, 1993). A large number of ginkgo trees aged over several hundred years have been grown near temples and palaces in Korea and most of them are designated as natural monuments or protected trees. Traditionally, this species has been planted mainly as ornamental trees. Recently, different kinds of medicinal substances, such as ginkgo-flavone glycosides, ginkgolides and bilobalide, have been identified and extracted from leaves. They are known to be very effective in improving insufficient cerebral or peripheral blood flows and inhibiting platelet-activating factor (PAF) (Van Beek and Lelyveld, 1991).

Verification of inheritance mode of a genetic marker is prerequisite for further genetic studies. So far, only a few studies have reported the inheritance mode of some isozymes as well as some molecular markers (Tsumura *et al.*, 1987, 1992). In this study, we analyzed 15 isoenzyme systems and verified the inheritance modes of 11 polymorphic loci. Linkage analysis among 11 Mendelian loci was performed.

MATERIALS AND METHODS

In 1995-1997, open pollinated flesh-coated seeds of ginkgo trees were collected from 160 maternal trees throughout the South Korea. After ripening in outdoors for 2 months, collected seeds were peeled off and stored at 4℃ until needed. A small portion of megagametophyte was homogenized in a few drops of extraction buffer [0.2M Phosphate buffer, pH 7.5] and the extracts were subjected to horizontal starch gel (12.5%) electrophoresis using two buffer systems. System I was that reported by Poulik (1957) with slight modifications : an electrode buffer of 0.063M Sodium hydroxide and 0.299M boric acid titrated to pH 8.25 and a gel buffer of 0.076M Tris and 0.0068M citric acid titrated to pH 8.75. System II was composed of an electrode buffer of 0.07M Tris titrated to pH 7.0 with 0.02M citric acid and a gel buffer of 1/10 dilution of the electrode buffer. The names of isozymes assayed were shown in Table 1. The staining procedures of enzyme were followed the recipes of Conkle *et al.*'s (1982) and Kim *et al.*'s (1994b) with some modifications. When an enzyme was separated into two or more zones, they were designated as alphabetical orders (i.e., A, B, C, etc.) from anode to cathode based on their migration rate. Likewise, in each zone, the isozyme phenotypes were numbered from anode to cathode.

To select putatively heterozygous maternal trees, six megagametophytes per tree were analyzed. The probability of mis-identifying a heterozygous tree as homozygous tree is $(1/2)^5$, approximately 3%. With the assumption of random distribution of gametes after meiosis, Mendelian loci were verified by χ^2-test for a 1 : 1 segregation of isozyme variants in same zone. Due to the limited number of seeds, only 40 of 160 maternal trees were finally used for further experiments. A number of twenty-six to one hundred megagametophytes per mother tree were analyzed for each segregation and linkage analyses.

Statistical evaluation on the agreement between observed and expected number of allozymes was obtained from the χ^2-test for "goodness of fit" for 1 df. When the mother trees showed several specific allelic combinations in their isozyme phenotypes, χ^2-tests were also used to test heterogeneity among these trees and linkage relationship for pairs of their segregating loci. Recombination ratios were estimated for all pairs of loci with joint segregation significantly different from expectation ($p<0.05$).

Table 1. Isozymes assayed, acronyms, and applied gel and tray buffer systems.

Isozyme systems	Acronyms	E.C. No	Buffer system
Aconitase	ACON	4.2.1.3	Ⅱ
Alcohol dehydrogenase	ADH	1.1.1.1	I
Fluorescent esterase	FST	3.1.1.1	Ⅱ
Glucose-6-phosphate dehydrogenase	G6PD	1.1.1.49	I
Glutamate dehydrogenase	GDH	1.4.1.3	I
Glutamate-oxaloacetate transaminase	GOT	2.6.1.1	I
Isocitric dehydrogenase	IDH	1.1.1.42	Ⅱ
Malate dehydrogenase	MDH	1.1.1.37	Ⅱ
Mannose phosphate isomerase	MPI	5.3.1.8	I
Menadion reductase	MNR	1.6.99.2	Ⅱ
6-phosphogluconate dehydrogenase	6PGD	1.1.1.44	Ⅱ
Phosphoglucose isomerase	PGI	5.3.1.9	I
Phosphoglucomutase	PGM	2.7.5.2	Ⅱ
Shikimate dehydrogenase	SKDH	1.1.1.25	Ⅱ
UDP-glucose pyrophosphorylase	UGPP	2.7.7.9	I

RESULTS & DISCUSSION

1. *Enzyme Phenotypes and Segregation*

1) Monomorphic enzymes

Alcohol dehydrogenase (ADH) : Although two single-banded zones were observed (Fig. 1), the resolution of this enzyme was not good enough to be scored clearly. Tsumura *et al.* (1992) also reported that ADH showed 3 invariable zones in *Ginkgo biloba*. Rudin and Ekberg (1978) reported two invariable zones in *Pinus sylvestris*, whereas Kim and Hong (1985) identified two loci of an invariable (A) and a variable (B) locus with three alleles in *Pinus densiflora*. In *Thuja orientalis* (Xie *et al.*, 1991) and *Abies pinsapo* (Pascual *et al.*, 1993), three single-banded monomorphic loci were observed in megagametophytes, respectively.

Glucose-6-phosphate dehydrogenase (G6PD) : Two monomorphic zones were observed. The G6PD-A was observed as triple-banded phenotype (Fig. 1). On the contrary, in *Larix laricina, Pinus attenuata,* and *Abies pinsapo*, one zone with some variants was observed in this enzyme (Cheliak and Pitel, 1985; Strauss and Conkle, 1986; Morgante *et al.*, 1993; Pascual *et al.*, 1993).

Isocitric dehydrogenase (IDH) : One monomorphic zone of a triple-banded phenotype was observed (Fig. 1). It is generally known that this enzyme is controlled by two loci in conifers (Xie *et al.*, 1991; Morgante *et al.*, 1993; Pascual *et al.*, 1993; Hussendörfer *et al.*, 1995).

Mannose phosphate isomerase (MPI) : Two invariable zones were shown on gels (Fig. 1). The MPI-B showed a faint double-banded phenotype (Fig. 1). Whereas MPI-A zone revealed two variants in *Pinus densiflora* and no varinat in *Pinus thunbergii*, MPI-B zone revealed four variants in both species. In *Pinus koraiensis*, a single locus with 3 alleles was verified in MPI (Kim *et al.*, 1994a, b).

Udp-glucose pyrophosphorylase (UGPP) : An invariable zone of activity was observed (Fig. 1). In some *Pinus* spp., 2 isozyme loci were verified in UGPP (Kim *et al.*, 1994a).

2) Polymorphic enzymes

Aconitase (ACON) : One zone with two double-banded variants was observed (Fig. 1). The segregation ratio for ACON variants did fit the expected 1 : 1 ratio, supporting the hypothesis of a single locus with two alleles (Table 2). Inheritance mode of a single zone of activity for ACON has been also reported in many conifers (Strauss and Conkle, 1986; Adams *et al.*, 1990; Fady and Conkle, 1992;

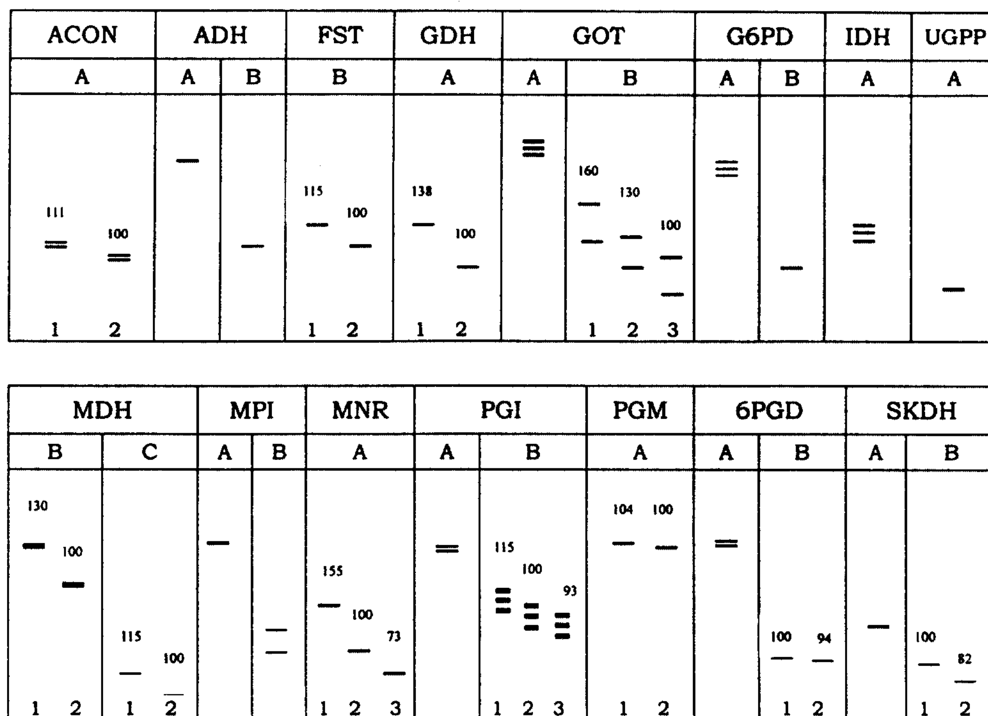

Fig. 1. Schematic illustrations of allozymes at 22 loci observed in megagametophytes in *Ginkgo biloba*. Electrophoretical migration was expressed relative to the most common variant, set to be 100.

Table 2. Observed single locus segregation of allozymes from heterozygous mother trees : Chi-square tests for heterogeneity of mother trees and goodness of fit to the 1 : 1 ratio.

Enzyme locus	Pair	Allele 1 (*F*)	Allele 2 (*S*)	Total	Heterogeneity		Segregation	
					χ^2(df)	*P*	χ^2(df 1)	*P*
ACON-A	1 : 2	316	328	644	6.826 (11)	0.813	0.224	0.636
FST-B	1 : 2	28	28	56	0.644 (1)	0.725	0.000	1.000
GDH-A	1 : 2	36	44	80	-	-	0.800	0.371
GOT-B	1 : 2	100	93	193	3.551 (3)	0.314	0.254	0.614
GOT-B	1 : 3	358	396	754	4.488 (11)	0.985	1.915	0.166
GOT-B	2 : 3	86	72	158	1.842 (3)	0.606	1.241	0.265
MDH-B	1 : 2	177	248	425	7.000 (7)	0.501	11.861	0.001*
MDH-C	1 : 2	43	44	87	0.389 (1)	0.533	0.011	0.915
MNR-A	1 : 2	310	276	586	6.195 (9)	0.799	1.973	0.160
MNR-A	1 : 3	40	46	86	1.564 (2)	0.457	0.419	0.518
PGI-B	1 : 2	155	166	321	1.116 (5)	0.981	0.377	0.539
PGI-B	1 : 3	77	89	166	3.171 (1)	0.075	0.867	0.352
PGI-B	2 : 3	52	64	116	1.330 (2)	0.249	1.241	0.265
PGM-A	1 : 2	323	364	687	6.508 (11)	0.837	2.447	0.118
6PGD-B	1 : 2	52	57	109	2.672 (2)	0.263	0.229	0.632
SKDH-B	1 : 2	146	126	272	7.486 (4)	0.112	1.471	0.225

Morgante *et al.*, 1993; Pascual *et al.*, 1993).

Fluorescent esterase (FST) : Two single-banded zones of activity were observed (Fig. 1, 2). The bands in FST-A zone were not clear enough to be scored, while two variants were scored in FST-B zone (Fig. 1, 2). On the basis of χ^2-test for 1 : 1 segregation of the variants, they were verified as 2 alleles in a locus of FST-B (Table 2).

Glutamate dehydrogenase (GDH) : One zone of activity with two variants was observed (Fig. 1). Since anodal phenotype (A1) was very rare in frequency, the segregation test for the observed variants was performed with only one mother tree, which turned out to be 2 alleles in a single locus (Table 2).

Glutamate-oxaloacetate transaminase (GOT) : Gels stained for GOT showed three zones of enzyme activity (Fig. 1). The GOT-A zone has a triple-banded phenotype with no variation. Both in GOT-B and in GOT-C zones, 3 single-banded variants were observed. Interestingly, the allozymes observed in GOT-B and GOT-C were comigrated (Fig. 3). From this result, we could hypothesize that single gene or two tightly linked genes may control these two enzyme zones. Tsumura *et al.* (1992) also reported the same result for *Ginkgo biloba* in this enzyme system. The segregation and heterogeneity tests for GOT-B revealed that this zone is controlled by a single locus with 3 codominant alleles (Table 2).

Malate dehydrogenase (MDH) : Three zones of activity were observed on MDH gels (Fig. 1). Since the bands in MDH-A zone were faint and smeared, they were not scored. Two variants were observed in MDH-B and MDH-C, respectively (Fig. 1). From 2 of eight heterozygous trees and pooled data, significant deviation from 1 : 1 segregation ratio of double-banded phenotypes was observed in MDH-B zone (i.e., excess of B2 over B1). No significant deviation was observed in MDH-C zone with two single-banded phenotypes (Table 2), which suggested that MDH-C be controlled by one independent locus with two alleles.

Menadion reductase (MNR) : One zone of activity with three phenotypes was observed (Fig. 1). Tsumura *et al.* (1992) also reported the same results for DIA in *G. biloba*, which is considered to be the same enzyme as MNR (Yi and Kim, 1994). Segregation tests for pairwise combinations of the observed variants revealed that there were non-significant deviations from expected 1 : 1 ratio in pooled data (Table 2).

Phosphoglucose isomerase (PGI) : Gels stained for phosphoglucose isomerase showed two zones of activity (Fig. 1, 4). Only the slow migrating PGI-B zone revealed three different variants with triple-banded phenotype, which was in good agreement with the previous report by Tsumura *et al.* (1992). Although slightly significant deviation from the expected Mendelian segregation ratio was observed in 1 of the 10 trees (p=0.049) which

Fig. 2. The zymogram of fluorescent esterase (FST) in megagametophytes of *Ginkgo biloba* observed under UV light.

Fig. 3. The zymogram of Glutamate-oxaloacetate transaminase (GOT) in megagametophytes of *Ginkgo biloba*. The enzyme phenotypes observed in GOT-B and GOT-C were comigrated.

showed polymorphism in PGI-B zone, no significant deviation was observed for any of the pairwise combinations of 3 variants both in single tree data and in pooled data (Table 2). These observations suggested that PGI-B zone is controlled by a single locus with three codominant alleles. Generally, PGI has been reported to be controlled by 2 loci in plants, where slow migrating one is highly polymorphic (Adams and Joly, 1980; Cheliak and Pitel, 1985; Fady and Conkle, 1986; Adams *et al.*, 1990; Xie *et al.*, 1991; Morgante *et al.*, 1993; Pascual *et al.*, 1993; Hussendörfer *et al.*, 1995).

Phosphoglucomutase (PGM) : Two zones of activity were observed in PGM, but the PGM-B zone with triple-banded phenotype was too faint to be scored reliably. PGM-A zone was polymorphic with two variants (Fig. 1, 5). The segregation tests for 2 variants in PGM-A zone showed no deviation from the expected 1 : 1 ratio (Table 2).

6-phosphogluconate dehydrogenase (6PGD) : Two zones of activity were observed (Fig. 1, 5). 6PGD-A zone showed monomorphic double-banded phenotype. Two variants of single-banded phenotype were observed in 6PGD-B zone (Fig. 1, 6). Segregation tests for 2 variants in 6PGD-B zone supported that it is controlled by one gene with 2 alleles (Table 2). Generally, in conifers, 6PGD has been known to be controlled by 2 loci (Cheliak and Pitel, 1985; Adams *et al.*, 1990; Xie *et al.*, 1991; Morgante *et al.*, 1993; Pascual *et al.*, 1993; Hussendörfer *et al.*, 1995) and 3 loci (Strauss and Conkle, 1986).

Shikimate dehydrogenase (SKDH) : Two zones of activity were observed (Fig. 1). Whereas SKDH-A zone was monomorphic, SKDH-B zone was polymorphic with 2 variants (Fig. 1). In SKDH-B zone, although 1 of the 5 heterozygous trees showed slight excess of B1, no deviations from 1 : 1 segregation ratio were observed in the remaining single trees and pooled data (Table 2).

Our observation generally agreed well with the results of Tsumura *et al.* (1987, 1992) with some minor discrepancies. For example, whereas 2 loci were verified in MDH and 6PGD in this study, only 1 locus was verified in both isozymes by Tsumura *et al.* (1992). Similarly, whereas only 1 polymorphic locus was verified in PGI and PGM in this study, 2 polymorphic loci were verified in both isozymes by Tsumura *et al.* (1992). This discrepancy might be resulted from the differences in experimental conditions and sampling schemes.

Fig. 5. The zymogram of Phosphoglucomutase (PGM) enzyme system in megagametophytes of *Ginkgo biloba.*

Fig. 4. The zymogram of Phosphoglucose isomerase (PGI) enzyme system in megagametophytes of *Ginkgo biloba.*

Fig. 6. The zymogram of 6-phosphogluconate dehydrogenase (6PGD) enzyme system in megagametophytes of *Ginkgo biloba.*

Three loci (MDH-B, PGI-B, and SKDH-B : data not shown) revealed significant segregation distortion from Mendelian expectations in 4 individual trees. However, all pooled data were in good agreement with the Mendelian segregation ratio with the exception of MDH-B locus, which suggested that the significance of segregation distrotion in PGI-B and SKDH-B loci, estimated with single tree, would be caused by sampling error or limited number of their megagametophytes. For MDH-B locus, the significant distortion in segregation of alleles was also observed even when the segregation ratio was tested with the pooled data of nine heterozygous trees. Two individual trees showing significant segregation distortion might be responsible for the segregation distortion for the pooled data although the remaining 6 heterozygous trees showed the same tendency of slight excess of B2.

In conifers, it has been occasionally reported that some enzymes are in condition of distortions from the Mendelian segregation. Strauss and Conkle (1986) proposed several factors contribution to these distortions. For example, when gel or enzyme systems have difficulties in distinguishing different alleles, there may be a tendency to score the common allele most often. The presence of heterodimeric bands between MDH loci could complicate scoring this loci in diploid tissues (Strauss and Conkle, 1986; Pascual *et al.*, 1993). However, we can not be sure that it can fully account for the segregation distortions observed in this study where megagametophyte tissues of the haploid genome were subjected to isozyme analysis. Further intensive studies of segregation in those loci should be followed to ascertain the causes for the segregation distortion.

2. *Linkage Analysis*

For the 11 polymorphic loci verified in this study, 38 of the possible pairs of loci were represented by at least one mother tree which were double heterozygous. The number of trees used for linkage analysis and the pairwise combinations of loci tested were shown in Table 3.

Three locus pairs (ACON-A : MDH-B, GOT-B : PGI-B, and MNR-A : SKDH-B) revealed linkage disequilibrium statistically, but their estimated rate of recombination indicated only weak linkage between loci : a pair of ACON-A and MDH-B loci showed recombination frequency (r) of 0.38 and a pair of MNR-A and SKDH-B loci showed r of 0.39, from each pooled data(Table 4). Although a pair of GOT-B and PGI-B loci showed r of 0.40 from pooled data, linkage disequilibrium was observed

Table 3. Number of trees analyzed for linkage (upper right half) and results of statistical testing (lower half) for each pair of allozyme loci in *Ginkgo biloba.*

	1	2	3	4	5	6	7	8	9	10	11
1. ACON-A		-	1	10	3	1	6	2	4	2	2
2. FST-B	-		-	2	-	1	2	-	-	-	-
3. GDH-A	n.s.	-		1	-	-	1	1	1	1	-
4. GOT-B	n.s.	n.s.	n.s.		4	2	10	5	8	2	3
5. MDH-B	*	-	-	n.s.		-	-	2	3	1	2
6. MDH-C	n.s.	n.s.	-	n.s.	-		1	-	-	-	-
7. MNR-A	n.s.	n.s.	n.s.	n.s.	-	n.s.		2	4	2	1
8. PGI-B	n.s.	-	n.s.	*	n.s.	-	n.s.		5	2	1
9. PGM-A	n.s.	-	*[1)]	n.s.	n.s.	-	n.s.	n.s.		2	2
10. 6PGD-B	n.s.	-	n.s.	n.s.	*[1)]	-	n.s.	n.s.	n.s.		-
11. SKDH-B	n.s.	-	-	n.s.	n.s.	-	*	n.s.	n.s.	-	

Significant levels : * 5% ([1)] : result based on only one tree)

Table 4. Test for linkage and recombination frequencies among pairs of potential linked loci in *Ginkgo biloba*.

Enzyme locus	Individual No.	Allelic combination				Total	Equality of complementary gametes		Test of independent assortment		Recombination frequency (r)
							χ^2 (df 2)	P	χ^2 (df 1)	P	
		A_1/B_1	A_1/B_2	A_2/B_1	A_2/B_2						
Acon-A * Mdh-B	AN 5	21	13	13	24	71	0.200	0.905	5.085	0.024	0.37
	NS C	11	9	9	11	40	0.000	1.000	0.400	0.527	-
	CHN 9730	10	5	5	12	32	0.182	0.913	4.500	0.034	0.31
	Pooled	**42**	**27**	**27**	**47**	**143**	**0.281**	**0.869**	**8.566**	**0.003**	**0.38**
		B_1/B_1	B_1/B_2	B_2/B_1	B_2/B_2						
Got-B * Pgi-B	SL 7	9	5	4	9	27	0.111	0.946	3.000	0.083	-
	YM 1	30	19	20	31	100	0.042	0.979	4.840	0.028	0.39
	NS 11	23	11	13	28	75	0.657	0.720	9.720	0.002	0.32
	YN 3	18	22	21	22	83	0.423	0.809	0.108	0.742	-
	KB 6	12	8	7	12	39	0.067	0.967	2.077	0.150	-
	Pooled	**92**	**65**	**65**	**102**	**324**	**0.515**	**0.773**	**12.642**	**0.000**	**0.40**
		A_1/B_2	A_1/B_2	A_2/B_1	A_2/B_2						
Mnr-A * Skdh-B	MD 1	15	24	20	12	71	0.697	0.706	4.070	0.044	0.38
	HN 1	16	26	24	16	82	0.080	0.961	3.951	0.047	0.39
	Pooled	**31**	**50**	**44**	**28**	**153**	**0.536**	**0.765**	**8.007**	**0.005**	**0.39**
		A_1/A_1	A_1/A_2	A_2/A_1	A_2/A_2						
Gdh-A * Pgm-A	YN 3	21	18	13	29	81	2.086	0.352	4.457	0.035	0.38
		B_1/B_1	B_1/B_2	B_2/B_1	B_2/B_2						
Mdh-B * 6pgd-B	YM 1	25	16	15	24	80	0.053	0.974	4.050	0.044	0.39

in only 2 of the five individual trees which were double heterozygous in both loci (Table 4). Two pairs of GDH-A : PGM-A and MDH-B : 6PGD-B also showed r of 0.38 and 0.39, respectively, only for a single tree (Table 4). Therefore, until additional evidence of linkage is confirmed in more number of trees, linkage disequilibrium for these pairs of loci observed in this study could not be confident.

Because overall degree of linkages observed in this study was weak, the possibility of statistical type I error must be considered. Further linkage analysis with large sample of seeds from more trees should be needed to confirm their linkage relationships. On the other hand, whether these linkage groups locate on the same chromosome remains obscure due to the lack of the number of linkage groups observed. For this issue, more number of isozyme loci should be included in linkage analysis.

In conclusion, we expected that these results could contribute to an understanding of the genetic controls of isozyme systems in *Ginkgo biloba* and provide genetic information for future studies on investigating the genetic structure of populations of *Ginkgo biloba*.

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