Indonesian Journal of Biotechnology, December, 2012 Vol. 17, No. 1, pp.154-162

Paternity Analysis of Tea (*Camellia sinensis* L. Kuntz) Hybrids Using Isozyme Marker

Titin Setyorini[1], Taryono[1], Suyadi[1], and Sapto Indrioko[2]

[1] Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia
[2] Faculty of Forestry, Universitas Gadjah Mada, Yogyakarta, Indonesia

Abstract

Tea plant has been categorized as self-incompatible crop. This is the reason behind the high genetic diversity. Natural pollination is possible to occur and the male parent is usually unknown, therefore, there is a need of method to identify male parent of hybrids through paternity analysis. Isozyme markers have been successfully used for paternity analysis due to their co-dominant polymorphism. This research aimed to predict male parents of hybrids by figuring out the mating system through isozyme banding patterns. In this experiment, seven enzyme systems were evaluated, of which only two of the enzyme systems i.e. esterase and shikimate dehydrogenase showing clear band pattern of *Est-1, Est-2*, and *Shd-1* loci. The mating system of tea could be categorized as a mixed mating model, with high estimated out-crossing rate of 98.6 %. The pollen contributors were not always originated from the vicinity of the female parents.

Key words: isozyme markers, paternity analysis, tea

Introduction

Tea plant is a self-incompatible crop and as a result, high yielding clones could probably be selected from natural pollination. In natural pollination, pollen source or male parent is generally unknown; therefore it requires a method to identify male parents of crossing products through paternity analysis.

Paternity analysis or parentage analysis could be a great help to check the cross compatibility, to study factors that affecting the success of cross pollination, to discriminate between seeds produced by hand pollination and those coming from fertilization by undesirable pollen (Diaz *et al*., 2007). Because paternity reflected the availability and the origin of pollen and also could measured the success pollination via male function (Bernasconi, 2003), paternity analysis technique has often been used to know the contribution of male parent to the formation of seed, and the progeny of crossing product could be genetically identified. Molecular markers have been wide applied in assessing genetic parentage and mating systems (Avise, 2000), because the usage of molecular markers have been realized to be effective and reliable (Mookerje, 2004). Ashley (2010) even highlighted that molecular markers provide a precise result in assigning parentage to seed and seedling, though Butcher *et al*. (2002) added some characteristics of ideal molecular markers for parentage analysis such as polymorphic, co-dominant, uniformly distributed throughout the genome, easily differentiated in genetically similar individuals and easily generated. Electrophoration as a technique to produce molecular markers will continue to be valuable tool for estimating out-crossing rate, paternity and gene flow within and between population (Brown, 1989), and isozyme

***Corresponding author :**
Taryono,
Department of Plant Breeding, Faculty of Agriculture, Universitas Gadjah Mada
Jl. Flora, Bulaksumur, Yogyakarta, Indonesia, 55281
E-mail: taryono@faperta.ugm.ac.id, tariono60@gmail.com

marker would prove to be useful for the early identification of hybrid seedling from open pollinating orchards (Wichneswari, 1989). The advantage of the isozyme technique was due to the fact that enzymes are the executive tools of the genes. It offered opportunity to acquire information from the genome that was very close to the source of information. Rajora (1986) reported that isozyme could be used to demonstrate parental effect in *Pinus* sp., and Cremer *et al*. (2003) could use such marker for seed source identification in Silver Fir.

This research was therefore conducted to identify the banding pattern of isozyme markers in tea and to predict the male origin of F1 tea seedling from polyclonal seed orchards by using parentage analysis.

Materials and Methods

Research material

Young leaves of seven tea clones i.e. Malabar 2 (Mal 2), Suka Ati 40 (SA 40), Tea Research Institute 2025 (TRI 2025), Pasir Sarongge I (PS I), Kiara 8, Cinyuruan 143 (CIN 143), andSuka Maju 118 (SKM 118). F1 of half sib seedlings were taken from polyclonal tea seed garden and F1 plant nursery of Pagilaran Tea Plantation, located in more 800 m attitude of Batang district in Central Java Province respectively. Isozyme analysis was then carried out at the laboratory of Forest Breeding and Genetics, Faculty of Forestry, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Isozyme analysis

There were several steps to carry out isozyme analysis included enzyme extraction, electrophoration and staining. Enzyme was extracted based on modified technique of Magoma *et al*. (2003). Fresh young tea leaves (120–150 mg) in the extraction buffer of 1 M Tris-HCl pH 7,5; Glycerol; Tween 80; Ditiothretol and Polyvinyl-polypyrolidone were crushed with mortar and pestle. After extraction, crude protein product was separated using *polyacrylamide vertical slab* gel electrophoresis in Tris-HCl buffer with pH of 7.5 for 3–4 h with 100 mA of electric current. Electrophorated gel was then incubated in the staining buffers related to the enzyme systems. In this experiment, 7 enzyme systems i.e. *Esterase* (EST; E.C.3.1.1), *Phospho-gluco isomerase* (PGI; E.C.5.3.1.9), *Shikimate dehydrogenase* (SHD; E.C.1.1.1.25), *Phosphoglucomutase* (PGM; E.C.2.7.5.1), 6-*Phosphogluconate dehydrogenase* (6PG; E.C.1.1.1.44), *Isocitrate dehydrogenase* (IDH; E.C.1.1.1.41), and *Glutamate oxaloacetate transaminase* (GOT; E.C.1.11.1.7) which were based on the result of Mondal *et al*. (2000), Magoma *et al*. (2003), and Chen *et al.* (2005) were used to check the expression of the related genes.

Data analysis

Every enzyme system in each gel was observed to determine the related allele based on the banding pattern and to calculate the relative value to the bromophenol blue front (Rf value).

All biochemical data collected from the banding pattern were used for paternity analysis.

1. Banding pattern

Variables collected from banding pattern were locus and alles number from each clones and F1 seedling based on polymorphic enzyme banding (Liengsiri *et al.*, 1990).

2. Paternity analysis

Paternity analysis was done to predict tea mating system using MlTR program (Ritland, 2004). Based on allele owned by each individual plant of F1 population, some mating type parameters were estimated.

a. t_m: multilocus population outcrossing rate
b. t_s : minimum variance of single locus population outcrossing rate
c. F_m: minimum variance of single inbreeding coefficient of maternal parents
d. r_p: correlation of paternity (fraction of sibling showing the same father)
e. r_s: the correlation of selfing among families (normalized variance of selfing)

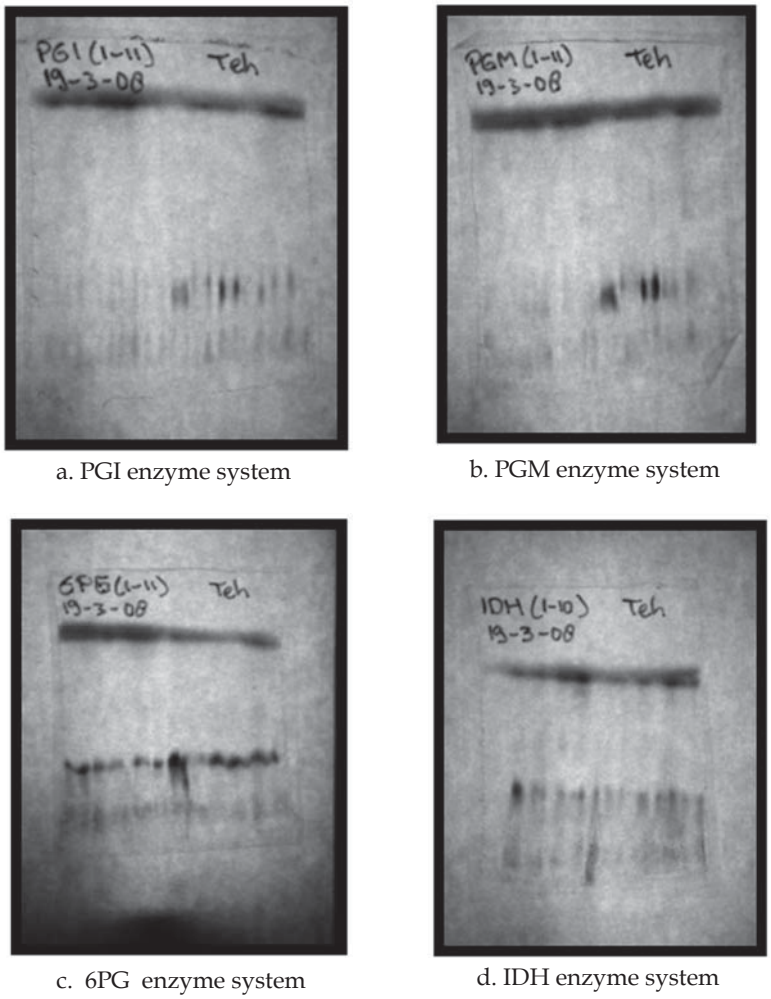
Result and Discussion

Enzyme system screening

Research result showed that GOT enzyme system could not produce good banding pattern, whereas PGI, PGM, 6PG and IDH enzyme systems could visualize better banding patterns, however the patterns were not repeatable (Figure 1). Two other enzyme systems i.e. EST and SHD produced consistent banding pattern (Figure 2).

Wendel and Weeden (1989) reported that the absence of isozyme banding pattern could be caused by the activity of secary metabolite such as phenol and tannin, also enzyme phenoloxidase. Beside that, the absence of banding pattern could be the result of the presence of damaging enzyme during extraction and electrophoration. The chemical substrate was probably damaged.

Inconsistent enzyme banding patterns could be the result of the presence of contaminant or the production of another enzyme banding pattern due to similar pH requirement and the characteristics of staining solution. Banding pattern could be considered as visually consistent when banding pattern is clearly visualized in electrophoretic gel. With clear banding pattern, the pattern could be observed easily and they could be characterized either as monomorphic or polymorphic types. In this experiment, EST and SHD enzyme systems showed polymorphic banding pattern. Similar results were reported by Magoma *et al*. (2003), Mondal *et al*. (2004), and Chen *et al*. (2005).



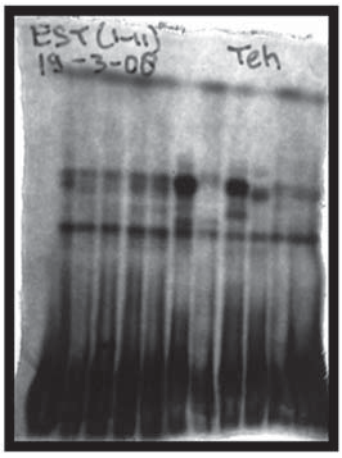
a. PGI enzyme system

b. PGM enzyme system

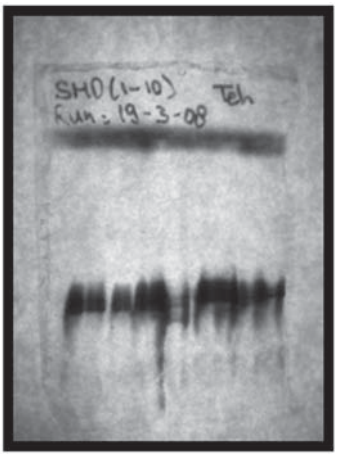
c. 6PG enzyme system

d. IDH enzyme system

Figure 1. Inconsistent isozyme banding pattern



a. EST enzyme system



b. SHD enzyme system

Figure 2. Consistent isozyme banding pattern

Table 1. Allele number and Rf of each loci

Enzyme system	Loci	Allele numbers	Rf (%)				
			a	b	c	d	E
EST	*Est-1*	3	65	69	73	-	-
	Est-2	5	82	85	89	92	96
SHD	*Shd-1*	3	43-45	50-52	52-54	-	-

The result of EST and SHD enzyme systems screening showed that there were only 2 clones i.e. SA40 and TRI2025 that could be used as mother plants because they had different banding pattern compared to other 5 clones. These clones were therefore exploited as mother plants to know the tea mating system and paternity analysis. Based on the genotypic analysis to all F1 progeny, the allele numbers and Rf position for each loci (Table 1) could be estimated.

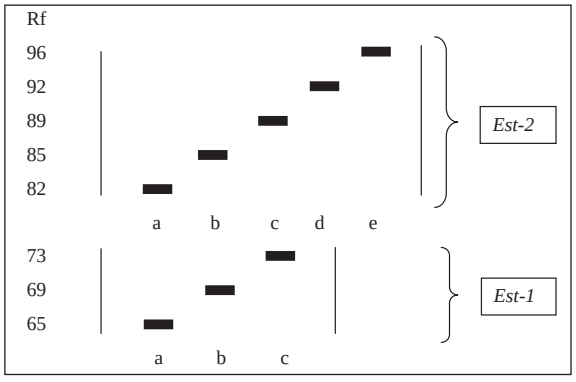


Figure 3. Zymogram of EST enzyme system banding pattern

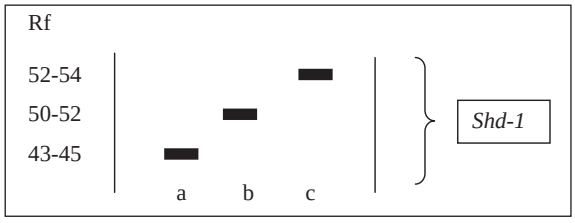


Figure 4. Zymogram of SHD enzyme system banding pattern

The position of each allele at every locus could be seen at zymogram (Figure 3 and 4). *Est-1* and *Est-2* loci could be considered as dimers. Magoma *et al*. (2003) also reported that EST system enzyme could produce 2 loci and showed dimer characteristics. Different result was observed for *Shd-1* locus due to tetramer characteristic. This result was different from the report of Magoma *et al*. (2003) and Ozaki *et al*. (2003). In these report, SHD enzyme system was controlled by 2 loci i.e. *Shd-1* and *Shd-2*, with each locus consisted of 3 alleles (Magoma *et al*., 2003). SHD enzyme system was only controlled by 1 locus i.e *Shd-1* with monomorphic characteristic (Ozaki *et al*., 2003).

Mating system

Crossing level estimated based on multi loci (t_m) was 0.986 or 98.6% (Table 2). The high value of t_m indicated that tea tended to have cross mating system. The estimation of average crossing level based on multi loci seemed more appropriate than that of single locus due to the fact that multi loci provided more informations (Muluvi *et al*., 2004). Higher value of t_m compared to t_s (t_m-t_s=0,021) indirectly could be intrepeted as few possibilities of bi-parental inbreeding (Table 2). This result was supported by low proportional value of progeny originated from similar parents ($r_{p(m)}$), which was 0,019 (Table 2). The minimum variance of single locus inbreeding coefficient of maternal parents (F) was 0 (Table 2) indicating that the progeny population is the result of random mating.

Number of effective pollen for fertilization (N_{ep}) was 52.632 (Table 2). Monocious plant theoretically had a high opportunity of selfing compared to diocious one. Tea has hermaprodite monocious flower. However, in this experiment, it showed a very low selfing rate which was approximately 1.4 % (Table 2).

Crossing could be caused by several factors. Kittelson and Maron (2000) clarified that selfing proportion of population was dependent upon self incompatibility mechanism, flower development and pollinator agent behavior.

Tea plant showed self incompatibility mechanism because the flower had a heritable capability to reject pollen originated from its own (Ozaki *et al*., 2003). Ozaki *et al*. (2003) reported that tea shows gametophytic self-incompatibility system through selfing or crossing. Incompatibility characteristic was controlled by a single locus. Ozaki *et al*. (2003) mentioned that rejection event involving the introduction of similar gene product between pollen and style, and the time of the gene action event was similar to the one of anthesis.

Plant natural pollination was depended upon the involvement of suitable pollinator agent to influence pollen transfer. Pollinator agent had to provide enough benefit from the flower, so that the visit of the pollinator would act as a part of its life activity. Several pollinator agents which were able to pollinate tea were including fly (*Diptera spp*) and bee (Clowes, 2008). Pollinator agents could transfer pollen so that they could improve crossing possibility.

Table 2. Mating system parameter estimation using MlTR

Parameter	Estimation value
Family	2
Progeni	105
t_m (SD)	0.986 (0.007)
t_s (SD)	0.965 (0.009)
t_m-t_s (SD)	0.021 (0.005)
$r_{p(m)}$ (SD)	0.019 (0.006)
F_m	0.000
N_{ep} ($1/r_p$)	52.632
r_s($1 - t_m$)	0.014

Other important parameters produced by MlTR software analysis were allele frequency of pollen and ovule (Table 3). The contribution of similar pollen and ovule was one of the assumption for mixed mating model (Ritland, 2004). Chi-square (χ^2) analysis result showed that the contibution of pollen and ovule deviatedfrom such assumption. There were several factors affecting the deviation such as limited samples, different contibution of pollen and ovule among individual plants

Table 3. Pollen and ovule allele frequecy evaluated using chi-square (χ^2) test

Loci	Allele	Pollen	Ovule	χ^2
Est-1	a	0.905	0.500	83.106**
	b	0.085	0.500	
	c	0.010	0.000	
Est-2	a	0.541	1.000	145.608**
	b	0.068	0.000	
	c	0.127	0.000	
	d	0.205	0.000	
	e	0.059	0.000	
Shd-1	a	0.314	0.000	28.820**
	b	0.203	0.500	
	c	0.483	0.500	

Note: ** there is significant difference at 1% significant level

in the population, pollen originating from outside the population, and assortive mating during crossing event.

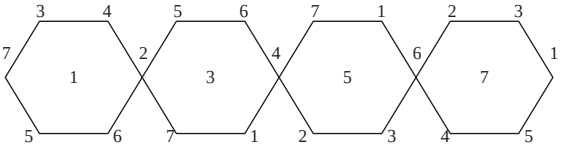
Paternity analysis

The mating system analysis result showed that tea was a cross-breeding plant, therefore pollen source could be originated from several other plants surrounding the mother plant. Polyclonal seed garden of Pagilaran plantation consisted on 7 clones which mated freely and the F1 seedlings produced were the result of random mating between clones in the seed garden.

One constraint of random mating was that pollen source could not be determined properly. Paternity analysis had therefore be done to search the origin of pollen source through the observation of genetic constitution of the progeny (Nason *et al*., 1996 and Bernasconi, 2003). Paternity analysis could be done after the genetic constitution of the parent and the progeny was known.

There were different genetic constitutions among clones at the polyclonal seed garden which were observed based on 3 loci i.e. *Est-1, Est-2*, and *Shd-1* (Table 4). There were only 2 clones showed relatively similar genetic constitution from 7 clones at the seed garden, and these clones were PS I and Kiara 8.

Table 4 showed that there was probably different genetic constitution among individual plants in similar clone. This phenomenon was theoretically impossible atvegetatively propagated plants such as tea. Mangoendidjojo (2003) informed that crop propagated vegetatively through cutting had



Note : 1. Mal 2 clone 4. PS I clone 7. SKM 118 clone
2. SA 40 clone 5. Kiara 8 clone
3. TRI 2025 clone 6. CIN 143 clone

Figure 5. Double triangle cultivation system

to be genetically uniform, because the clone was generally originated from similar mother plant. However, the clone was sometime developed using mass selection technique and the uniformity during selection was normally only based on morphological characteristics. Observed polymorphic loci at individual plant in similar clone depicted different genetic constitution. Such phenomenon was also supported by tea's natural self incompatible characteristic.

Table 5 showed the percentage of F1 individual plants receving pollen from its different putative male plant in every block for each mother parent. The result of paternity analysis using isozyme marker in this experiment might be different from morphological markers which were normally used by the plantation. Paternity analysis based on morphological marker was conducted using phenotypic data with assumption based on double triangle cultivation system with 6 x 6 x 6 m spacing (Figure 5). Pollen was originated from male parent surrounding the female plant, so that only 6 clones would be considered as a putative male parent for every female clone.

Table 4. Genetic constitution of 7 clones grown at Pagilaran polyclonal seed garden

Clones	1			2			3			4			5		
	Est-1	*Est-2*	*Shd-1*	*Est-1*	*Est-2*	*Shd-1*	*Est-1*	*Est-2*	*Shd-1*	*Est-1*	*Est-2*	*Shd-1*	*Est-1*	*Est-2*	*Shd-1*
Mal 2	aa	aa	bc	ab	aa	bc	-	-	-	-	-	-	-	-	-
SA 40	ac	aa	bc	aa	aa	cc	aa	Aa	cc	-	-	-	-	-	-
TRI 2025	ab	ac	ac	ab	aa	ac	ab	Aa	ac	aa	aa	ac	-	-	-
PS I	aa	Ab	bb	aa	ab	bb	aa	Ab	bb	aa	ab	bb	aa	ab	-
Kiara 8	aa	aa	-	aa	aa	cc	aa	Aa	cc	aa	aa	cc	-	-	-
CIN 143	aa	Ad	cc	aa	ad	cc	-	-	-	-	-	-	-	-	-
SKM118	aa	aa	-	aa	aa	cc	aa	Aa	bc	-	-	bc	-	-	-

Table 5. Paternity analysis of SA40 and TRI2025 as mother plant

Mother plant genotype			Putative male parent genotype			Paternity analysis result (%) F1 seedling at block						
Est-1	*Est-2*	*Shd-1*	*Est-1*	*Est-2*	*Shd-1*	1	2	3	4	5	Total	Average**
ac	Aa	Bc	aa	aa	bc	55*	20	0	27	0	102	28
SA 40 clone			Mal 2 clone									
			aa	aa	cc	27	20*	0	18	0	65	
			Kiara 8 clone									
			ab	Aa	ac	36	20	18*	55	70	199	
			TRI 2025 clone									
			aa	Ad	cc	36	20	0	27*	0	83	
			CIN 143 clone									
			aa	aa	cc	27	20	0	18	0*	65	
			SKM 118 clone									
ab	Aa	Ac	ab	aa	bc	67*	60	38	50	-	215	
TRI 2025 clone			Mal 2 clone									
			ab	aa	bc	67	60*	38	50	-	215	
			SA 40 clone									
			aa	aa	cc	25	33	31*	7	-	96	
			Kiara 8 clone									
			aa	aa	cc	25	33	31	7*	-	96	
			SKM 118 clone									
					Total						1136 %	

** Average = Total/n = 1136/41 = 28

* Identification result based on morphological markers

Based on morphological marker, Mal2 (block 1), Kiara 8 (block 2), TRI2025 (block 3), CIN 143 (block 4) and SKM116 (block 5) were identified as male parents of SA40 mother, whereas Mal 2 (block 1), SA 40 (block 2), Kiara 8 (block 3), and SKM 118 (block 4) were for TRI2025.

The percentage of F1 individual plants that were pollinated by putative male parent were very variable and the value tended to be low with an average of approximately 28%. The low value identifying F1 individual plants might be due to the limited number of isozyme markers that could be used to analyze. Another reason for this was related to sampling technique. Leaf samples for isozyme analysis were only taken from male parent located around the mother plant due to the belief that pollens are originated from plants that located only in the vicinity of the mother plant such as that for the morphological marker. This result showed that such believe could not be trusted. The result of paternity analysis showed that random mating had been occured among 7 clones at Pagilaran seed garden. Consequently, pollen could be originated randomly from each individual plant of the male parent surrounding the mother plant.

Tea polymorphic banding patterns based on isozym analysis with EST and SHD staining were considered as *Est-1, Est-2*, and *Shd-1* loci. Tea mating system at Pagilaran polyclonal seed garden was random mating, with the tendency of very high outcrossing rate reaching 98.6%. Paternity analysis based on isozyme marker could show the average percentage of F1 individual plant with the true predicted pollen source from putative male parent reach to 28%.

Acknowledgement

We are very thankful to all staff members of Pagilaran tea plantation management for providing all facilities.

References

Ashley, M. V. 2010. Plant parentage, pollination, and dispersal: how DNA microsatellite have altered the landscape. *Crit. Rev. Plant Sci.*, **29**, 148–161

Avise, J. C. 2000. Phylogeography: the History anf Formation of Species. Harvard University Press. Cambridge. Massachussets

Bernasconi, G. 2003. Seed Paternity in Flowering Plants: An Evolutionary Perspective. http://www.urbanfischer.de/journals/ppees

Brown, A. H. D. 1989. Genetics characterization of plant mating systems. In: Population Genetics, Breeding, and Germplasm Resource (Brown *et al*.,eds.). Sinauer, Sunderland

Butcher, M. I., Laboute, D.R., Jarret, R.L., and Macchiavelli, R.E. 2002. Microsatellite based paternity analysis in polyploid sweetpotato. *J. Am. Soc. Hort. Sci.*, **127**, 392 - 396

Chen, J., Wang, P., Xia,Y., Xu, M.,and Pei, S. 2005. Genetic Diversity and Differentiation of *Camellia sinensis* L. (Cultivated Tea) and Its Wild Relatives in Yunnan Province of China, Revealed by Morphology, Biochemistry and Allozyme Studies. *Gen. Res. Crop Evol*., **52**, 41 – 52

Cremer, E.,Liepert, S., Ziegenhagen, B., and Hussendorfer, E. 2003. Microsatellite and isozyme markers for seed source identification of Silver Fir. *Forest Gen*., **10**, 165 – 170

Clowes, M. 2008. Handbook of Flowering Vol. VI. http://www.sandiesfloralgallery.com

Diaz, A., Martin, A., Rallo, P., and De la Roza, R. 2007. Cross-compatibility of the parents as the parents as the main factors for successfull olive breeding. *J. Am. Soci.Hort. Sci.*, **132**, 830 – 835

Kittelson, M.P and Maron, J.L. 2000. Outcrossing rate and inbreeding depression in the perennial yellow bush lupine, *Lupinus arboreus (Fabaceae). Am. J. Botany.* **87**, 652-660

Liengsiri, C., Piewluang, C., and Boyle, T.J.B. 1990. Starch Gel Electrophoresis of Tropical Trees. A Manual. ASEAN-Canada Forest Tree Seed Centre, Muak Lek, Saraburi 18180. Thailand dan Petawawa National Forestry Institute, Chalk River, Ontario K0J1J0

Magoma, G.N.,Wachira, F.N., Imbuga, M.O. and Agung, S.G. 2003. Biochemical Differentiation in *Camellia sinensis* and its wild relatives as revealed by isozyme and catechin patterns. *Biochem. Sys. Ecol.*, **31**, 995-1010.

Mangoendidjojo, W. 2003. Dasar-dasar Pemuliaan Tanaman. Kanisius, Yogyakarta.

Mondal, T. K., Bhattacharya, A., Laxsmikumaran, M., and Ahuja, P. S. 2004. Recent advances of tea (*Camellia sinensis*) biotechnology. *Plant Cell, Tissue and Organ Culture.76*, 195-254

Mookerjee, S., Guerin, J., Collins, G., Ford, C., and Sedsky, M. 2005. Paternity analysis using microsatellite marker to identify pollen donors in an olive grove. *Theo.App. Gen.*, **111**, 1174 – 1182

Muluvi, G. M., Sprent, J.I., Odee, D.,and Powell, W. 2004. Estimates of outcrossing rates in *Moriga oleifera* using amplified fragment length polymorphism (AFLP). *Afr. J. Biotech*., **3**, 146-151

Nason, J.D., Herre, E.A. and Hamrick, J.L. 1996. Paternity analysis of the breeding structure of strangler fig populations: Evidence for substantial long-distance wasp dispersal. *J. Biogeography.* **23**, 501-512

Ozaki, Y., Kanda, M., Miyajima, I., and Okubo, H. 2003. Variation of self-incompatibility in *Camellia japonica* L. cultivars. *Journal of Faculty of Agriculture, Kyusu University* **47**, 251-255

Rajora, O.P. 1986. Mating systems and fertilizing pollen gene pools of *Populus altoides* Marsh and *P. nigra* L. Clones located in different compatible species neighborhoods as inferted from isozyme analysis. In: Proceeding of the 20th

Canadian Tree Improvement Association 1985 Part 2. Canadian Forest Service. Ottawa

Ritland, K. 2004. Multilocus Mating System Program MlTR Version 3.0. http://genetics.forestry.ubc.ca/ritland/programs

Wendel, J. F. and Weeden, N.F. 1989. Introduction of Isozymes in Plant Biology. In Isozymes in Plant Biology (Soltis and Soltis, eds.). Chapman and Hall, London

Wickneswari, R. 1989. Use of isozyme analyses in a proposed *Acacia mangium* and *A. Auriculiformis* hybrid seed production orchards. *J. Trop. Forest Sci.*, **2**, 157 -164