TISSUE CULTURE OF PILI NUT (*CANARIUM OVATUM*) AND ISOZYME ELECTROPHORESIS OF SOME RELATED *CANARIUM* SPECIES

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

HORTICULTURE

DECEMBER 1995

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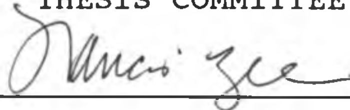

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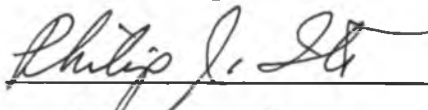

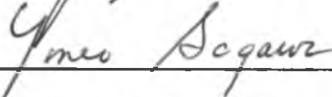

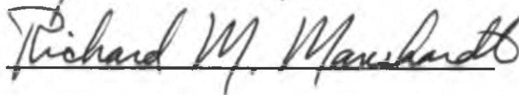

ACKNOWLEDGEMENTS

I would like to express my gratitude to Dr. Zee for serving as my advisor. His guidance and encouragement during this study was greatly appreciated. I am very grateful to Dr. Manshardt for his warm help and suggestions in all aspects of electrophoresis. My sincere thanks and appreciation to Dr. Sagawa for the generous use of his laboratory facilities and all his helpful suggestions regarding to tissue culture work. Thanks to Dr. Ito for serving on my thesis committee.

Thanks are due to Dr. Malli Aradhya for his valuable assistance in electrophoresis; John Kunisaki for his helpful suggestions in tissue culture; personel in USDA-ARS, NCGR, Hilo, especially Claire Arakawa and Mark Munekata who provided assistance during my time in Hilo.

Finally, a special thanks goes to my husband, Xiangjia Min for his continuous support, help and encouragement, and my parents for their love and support.

ABSTRACT

Studies were conducted for the *in vitro* culture of pili nut. Different explant sources, such as petiole, leaf, mature cotyledon, immature cotyledon and embryo axis were used. Horizontally placed petioles produced vigorous callus growth in half and full strength WPM with combinations of 2,4-D and Kinetin. The highest callus fresh weight was obtained in the medium with 1 mg/l 2,4-D, or 1 mg/l 2,4-D plus 1 mg/l Kinetin. Embryogenesis or shoot inductions were not successful from the petiole callus. Leaf and cotyledon explants produced limited callus using the same medium and growth regulator treatments, and immature embryo axes produced no callus. Shoot tips harvested from *in vitro* seedlings showed shoot and new leaf growth in WPM with a combination of BA and Kinetin, at 1 or 2 mg/l, but no root induction occurred when NAA and IAA were used. Shoot tips and nodal cuttings harvested from greenhouse grown seedlings were difficult to disinfect and did not perform well in *in vitro* culture.

In this study, isozyme phenotypes (fingerprinting) of seven *Canarium* species were also developed using six enzyme systems (LAP, MDH, PGI, PGM, TPI and UGPP) utilizing a histidine - citric acid (pH 6.5) buffer system. Fifty-two plant samples of different *Canarium* species (twenty-two *C.*

ovatum, five *C. album*, four *C. megalanthum*, six *C. harveyi*, eight *C. mehenbethene*, two *C. odontophyllum*, three *C. indicum* and two unknown) and one *Dacroydes rostrata* plant were surveyed. Ninety-seven different phenotypes of the six enzyme systems were obtained. All six enzymes showed high polymorphism. Phenotypic polymorphism (P_j) ranged from 0.93 (MDH) to 0.75 (TPI). The average P_j was 0.86 and the weighted polymorphism (P_w) was 0.84 among the accessions tested. All seven species were polymorphic, the P_j ranged from 0.53 for *C. ovatum* to 0.18 for *C. mehenbethene*. The pattern from cluster analysis agreed with most of the accession identities. Accessions of the same species formed a distinct cluster from other species. Some unique banding in different species have been tentatively identified and could be useful for the classification of the *Canarium* species.

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LIST OF ABBREVIATIONS

ABA = Abscisic Acid

BA = 6-Benzylaminopurine

2,4-D = 2,4-Dichlorophenoxyacetic Acid

IAA = Indole-3-acetic Acid

IBA = Indole-3-butyric Acid

2-iP = 2-Isopentenyladenine

KT = Kinetin

MS = Murashige and Skoog Media (1962)

NAA = Naphthaleneacetic Acid

TDZ = 1-phenyl-3-(1,2,3-thiadiazol-5-yl)urea

WPM = McCown's Woody Plant Basal Salt Mixture (1981)

PART I

TISSUE CULTURE OF PILI NUT (*CANARIUM OVATUM*)

INTRODUCTION

Canarium ovatum Engl, commonly known as pili nut or Philippine nut, belongs to Burseraceae (Coronel, 1983). The large tropical trees are common in Malaysia, the Philippines, and are also found in Africa and northern Australia (Steffey, 1986; Smith, 1985).

Pili nut is one of the most important nut-bearing trees in the genus, it is the No. 1 resource of fat and protein in the diet of the far Pacific residents (Menninger, 1977). Pili nut is native to the Philippines and was first introduced to Hawaii from Los Banos, Philippines, in 1922 (Wanitprapha et al. 1992).

Pili nut is largely propagated by seed, but it is dioecious, seedlings show diverse characteristics (Coronel, 1983). Pili nut was reported to be propagated vegetatively by budding, grafting, marcotting and inarching, but not at commercial efficiency (Coronel, 1983). In general, seedling pili nut bears fruits in five to six years, with economic yields in the 10th year. For marcotted and grafted trees, production starts from the second or third year and economic yields in the fifth or sixth year (Wanitprapha et al., 1992).

The lack of an efficient propagation method for pili nut is the major obstacle of developing a pili nut industry. The purpose of this study was to develop an *in vitro* propagation protocol for pili nut.

LITERATURE REVIEW

Pili Nut

Pili nut is a tropical plant, it likes hot, humid climates and does not withstand frost. It grows well on deep, fertile and well-drained soil (Coronel, 1983; Howes, 1953; Martin, 1985). A mature tree is about 20 meters in height, has alternate compound leaves with 5-7 leaflets and terminal inflorescence. Fruits are ellipsoid to oblong and each hard-shell seed has a single kernel. The kernel is the main product of pili nut, which is considered superior to almond (Howes, 1953). Pili nut is a good source of fat (74%), protein (12%) and starch (12%). The resin from the tree is traded as Manila elemi. The wood is good for firewood (Coronel, 1983; Rosengarten, 1984).

The Philippines is the only known commercial producer and processor of pili nuts in the world (Coronel, 1983). Most of the production in the Philippines is from wild trees. Early in the twentieth century, pili nuts were exported on a fairly large scale from the Philippines to the United States, France, the United Kingdom and Japan (Coronel, 1983; Rosengarten, 1984), however, this trade has fallen off due to limited local supply, poor pulp removal, lack of effective cracking devices, and poor quality controls. In 1991, a unit retail price of in-shell pili nuts in metro Manila was about $0.67. A 1.8-oz package of sugar-coated pili nuts was sold for $0.27 (Wanitprapha et

al., 1992). Pili nut is a minor nut crop with promising potential, especially if easy-to-crack thin-shelled varieties could be selected and vegetatively propagated to establish future orchards.

Plant Tissue Culture

Concept and potential: The term plant tissue culture broadly refers to the cultivation *in vitro* of all plant parts - single cells, tissues, and organs (Torres, 1988). Tissue culture has four potential applications: 1) production of natural products, 2) genetic improvement of crops and germplasm storage, 3) production of disease-free plants, and 4) rapid multiplication. The last application offers the greatest significance to commercial propagators (Zimmerman, 1984).

Factors effecting tissue culture of woody plants: Nutrient composition in a medium is an important factor in cell and tissue culture, different plants may require different nutrients (Stafford and Warren, 1991). Auxin and cytokinin are the most common growth regulators added to the nutrient medium to control growth and differentiation. The type of morphogenesis that occurs in a plant tissue culture is largely dependant on the ratio and concentrations of auxin and cytokinin (Bonga and Aderkas, 1992). Another important aspect of micropropagation of trees is the choice of

explant, usually more juvenile and actively growing parts, such as shoot tips, are preferred (Hartmann et al., 1990; Dirr and Heuser, 1987). For example, shoot tips of black walnut were successfully cultured and proliferated on solid WPM with 2 mg/l and 5 mg/l BA, but rooting was difficult (Heile et al., 1984). Plantlets were regenerated from shoot tip of mature chestnut on half MS with 0.2 mg/l BA (Vieitez et al., 1983). Single node explants were also used successfully as *in vitro* explants, *Quercus shumardii* single node explants showed optimal shoot proliferation and elongation in liquid WPM with 2 mg/l BA, rooting was obtained with 2 mg/l IBA (Bennett and Davis, 1984). Reed and Abdelnour-Esquivel reported (1991) shoot growth on modified WPM with 4 mg/l Zeatin with a wide range of *Vaccinium*. Rathore et al., (1992) reported that *Maytenus emarginata* (Willd) produced multiple shoots on MS medium containing 0.1 mg/l IAA and 2.5 mg/l BA.

Other tissues such as leaves, petioles, cotyledons and embryos are used in *in vitro* culture. Morteza and Sink (1980) used leaf explants to induce callus of *Rosa manetti*, but didn't get shoots; Leege and Tripepi (1993) obtained shoots from leaf explants of European birch (*Betula pendula)* on WPM with 15 μM BA. Somatic embryos were obtained from cotyledons of Eastern black walnut (*Juglans nigra)* on WPM with 0.5 μM TDZ and 1 μM 2,4-D (Neuman et al., 1993), and adventitious shoots were induced from juvenile black locust

cotyledons on WPM containing 22.2 μM BA and 0.4 μM 2,4-D (Arrillaga and Merkle, 1993).

Embryo culture of some plants is also reported to be successful. Immature embryos of papaya (*Carica papaya L.*) produced somatic embryos on half MS with 0.1 to 25 mg/l 2,4-D and 6% sucrose (Fitch and Manshardt, 1990). Somatic embryogenesis was also obtained from zygotic embryos of pecan after transferral from WPM medium with 2 mg/l 2,4-D plus 0.25 mg/l BA to a hormone-free WPM (Wetzstein et al., 1989).

Adventitious shoots were induced on petioles from *in vitro* grown shoots of olive, regeneration was achieved in the dark on both MS and modified olive medium containing 5-40 μm TDZ or 10 μm 2iP + 2.2 μm BA. The highest regeneration was achieved from the proximal part of the petiole (Mencuccini and Rugini, 1993). The grape petioles formed shoots in MS medium with 0-0.5 μm TDZ (Reisch and Martens, 1988). Somatic embryos were obtained from petioles of *Juglans regia* on *Juglans* basal salt medium (DKW) with 2 mg/l kinetin and 0.02 mg/l IBA (Liu et al., 1992).

MATERIALS AND METHODS

Explant Types

Different explants from pili nut seedlings were tested for *in vitro* culture, including: 1) Shoot tips and nodal segments from greenhouse grown seedlings; 2) shoot tips and nodal segments from *in vitro* germinated seedlings; 3) petiole cuttings from greenhouse seedlings; 4)leaf cuttings with or without midribs from greenhouse seedlings; 5) mature and immature cotyledons; 6)immature embryo axes, and 7) mature whole kernels.

Procedure of Disinfestation

1. Shoot tips and nodal segments: The shoot tip or nodal segment with a single axillary bud, was excised from a 1.5 year-old pili seedling maintained in a greenhouse, at the Department of Horticulture, University of Hawaii at Manoa. It was trimmed to 1 cm^2 before being washed with mild detergent, rinsed under running tap water for 30 minutes and dipped into 95% alcohol for 1 minute. The segment was surface sterilized in 15% clorox (0.788% sodium hypochlorite) with Tween 20 (3 drops /100 ml) for 15 minutes. Using a dissection microscope, two to three outer layers of the bud sheaths were removed from each bud. The bud explant was then placed in 10% clorox solution for 10 minutes, trimmed in 1% clorox solution to 2-3 mm before being placed in 5% clorox for 5 minutes. After the final

disinfestation in 1% clorox for 60 minutes, each explant was rinsed 3 times in sterile deionized water (Table 1.1) before inoculation onto culture media.

2. Mature seeds: Pulps were physically removed from mature fruits harvested from the University of Hawaii, Waiakea Experiment Station, Hilo, HI. Each seed was cracked with a vise. The kernel was removed, surface sterilized in 10% clorox for 15 minutes and rinsed 3 times with sterile deionized water (Table 1.1) before placing into the media. The seedlings germinated *in vitro* provided the aseptic shoot tips and nodal segments used in the experiment.

3. Immature seeds: Green fruits were obtained from the University of Hawaii, Waiakea Station, Hilo, HI. Pulps were removed and seeds were surface sterilized in 10% clorox for 60 minutes and rinsed 3 times with sterile water (Table 1.1). Under aseptic conditions, the hard shell was cracked with a sterilized vice-grip. Parts of the kernel, such as the cotyledon and embryo axis were placed in different media.

4. Petioles and leaves: Young petioles and leaves collected from 6 month or older seedlings maintained in the greenhouse, Department of Horticulture, University of Hawaii were thoroughly rinsed in running tap water for 30 minutes, surface sterilized with 5% clorox plus Tween 20 (3 drops/100ml) for 10 minutes, and three rinses with sterile water (Table 1.1). Under aseptic conditions,

Table 1.1. The disinfestation procedures for different explants of pili nut

Explant	Agent	Time
shoot tip	running tap water	30 min
	95% alcohol	1 min
	15% clorox	15 min
	10% clorox (dissected)	10 min
	5% clorox (dissected)	5 min
	1% clorox	60 min
nodal segment	same as shoot tip	
mature kernel	10% clorox	15 min
immature seed	10% clorox (shell) cracked under hood	60 min
petiole	running tap water	30 min
	5% clorox	10 min
leaf	same as petiole	

petioles were cut into 0.5 cm pieces, leaves into 0.5 cm^2 segments that contained either the leaf blade or leaf blade with midrib.

Media

Media tested included McCown's Woody Plant (WPM) Media Lloyd and McCown, 1980) and Murashige and Skoog (MS) (Murashige and Skoog, 1962) at full and half strength salts, supplemented with full vitamins (Appendix). Both media were supplemented with 100 ml/l filtered coconut water, 3% sucrose, 0.8% agar (Sigma, type A). Different rates and combinations of plant growth regulators were added to the medium for each explant (Tables 1.2 & 1.3). The pH of all media was adjusted to 5.7 prior to autoclaving at 121°C and 20 PSI for 15 minutes.

Culture Conditions

Cultures were maintained at 25^0C and 16 hr photoperiod under a white fluorescent light (photosynthetic photon flux of 30-50 μmol $s^{-2}m^{-1}$). Additional modifications were used during the initiation period of the cultures:

1. Shoot tips were kept in the dark (wrapped in aluminum foil) for one week before transferral into 16 hr photoperiod. Shoot tips were transferred into fresh medium every two days during the first week and every 4 weeks thereafter.

2. Mature kernels were cultured on solid WPM with no growth regulators, and maintained at 25^0C and 16 hr photoperiod.

3. Immature embryo axes and cotyledons were maintained in the dark at 25^0C. The cultures were transferred to new medium every two weeks for a month, and every 4 weeks thereafter.

4. Half of the petiole cultures (about 4-6 cultures for each treatment) (Table 1.5) and leaf explants were maintained in the dark for 4 weeks at 25^0C before transferral to 16 hr photoperiod. The other half of the petiole explants were maintained in the dark throughout this study. The leaf and petiole explants were transferred to fresh medium every two weeks during the first month, and once every 4 weeks thereafter.

Table 1.2. The combinations of plant growth regulators (mg/l) and basic growth media[a] applied on the *in vitro* culture of several different explants of pili nut (*Canarium ovatum*) .

Plant growth regulator					Explant[b]				
2,4-D	IAA	NAA	BA	KT	sh+ns1	sh+ns2	em+co	pe	le
0	0	0.1	1	1	wmh	w			
0	0	1	0	1	w				
0	0	0	1	1	wmh	w			
0	0	0	2	2	w	w			
0	0	0	1	0	wmh	w			
0	1	0	1	1		w			
0	0	0	0	0			wmh	w	
0	0	1	0	0			w		
1	0	0	0	0			wmh	wmh	w
1	0	0	1	0			w	w	w
1	0	0	2	0				w	
0	0	1	0	1			w		
5	0	0	0	0			w		
1	0	0	0	1			w	w	w
1	0	0	0	2				w	w
1.5	0	0	0	0				w	
2	0	0	0	0				w	
1.5	0	0	0	1				w	
1.5	0	0	0	2				w	
2	0	0	0	1				w	w
2	0	0	0	2				w	

a. wmh: the explants were cultured on different basic media under same combinations of plant growth regulators. w:WPM; m:MS; h:half MS.

b. sh+ns1: shoot tip and nodal segment from greenhouse; sh+ns2: shoot tip and nodal segment from *in vitro*; em+co: immature embryo axis and cotyledon; pe: petiole: le: leaf.

Table 1.3. Different plant growth regulator combinations for tentative somatic embryogenesis of petiole callus of pili nut on WPM

PGR[a]	2,4-D	NAA	BA	KT	ABA	N	Result
	0.1	0	0	0.1	0	6	0
(mg/l)	0	0.1	0.1	0	0.1	6	0
	0.1	0	0	0	0	6	0
	0	0	0	0	0	6	0

a PGR: Plant growth regulator.

RESULTS

Petioles

Petiole cuttings were the best explant source that showed positive response to *in vitro* culture. The average percentage of disinfestation was about 78% (Table 1.4). Visible swellings on the cut ends of the petioles were observed one week after initiation, with creamy-white callus growth at two weeks. Later, the bark in each explant split and was followed by callus growth over the entire petiole.

The placement of explants on medium appeared to be critical in callus formation. Petiole explants placed horizontally developed more callus at 4 weeks after initiation than those that were placed vertically or completely buried in the medium. Explants that were emerged in the medium died soon after. The vertically placed explants showed slight callus growth on the exposed surface, but were dead 4 weeks after initiation.

Comparing WPM, full and half-strength MS each incorporated with 1 mg/l 2,4-D, it appeared that WPM was the best medium for callus induction and growth in pili leaf petiole cuttings. About 85% of the petiole pieces on WPM developed callus, as compared to 30% of those on MS or half MS. The callus in WPM appeared to be more vigorous and the medium showed less discoloration or browning.

No difference was observed in callus growth between full and half WPM. However, full or half WPM were observed

to produce more vigorous callus growth than 1/4 or 1/8 WPM. In general, the callus in the 1/4 and 1/8 WPM gradually lost vigor and died following 2 to 3 subcultures.

Factorial combinations between growth regulators 2,4-D (1, 1.5 and 2 mg/l) and kinetin (0, 1 and 2 mg/l) induced callus growth on petiole cuttings. The callusing rate showed no difference among these treatments (Table 1.5). However, the highest callus weight was obtained in petioles cultured in WPM with 1 mg/l 2,4-D, or 1 mg/l 2,4-D plus 1 mg/l kinetin (Table 1.5).

Explants cultured in medium with BA (1 or 2 mg/l) combined with 1 mg/l 2,4-D also produced callus, but the callusing rate was about 50%, lower than that for kinetin with 2,4-D (about 80%) and size of callus were visually smaller (fresh weight was not measured).

At 6 to 8 weeks after initiation, two of the thirteen petiole callus in WPM in 1.5 mg/l 2,4-D plus 2 mg/l kinetin, and four of the ten callus in WPM with 2 mg/l 2,4-D plus 2 mg/l kinetin developed adventitious roots. Three to five roots were observed on each of these callus (Table 1.5).

The callus was transferred to WPM without any growth regulators, or reduced concentration of 2,4-D and kinetin to 0.1 mg/l, or replaced 2,4-D with NAA plus ABA. All these attempts to induce embryogenesis failed (Table 1.3). The callus remained alive on the medium but no embryo development was observed.

Table 1.4. The frequency of different explant disinfestation procedures of pili nut.

Explant	Total[a]	Contamination[b]	Disinfestation[c]
shoot tip	10	5-8	30.0%
mature embryo	40	26	35.0%
immature embryo	10	0-1	97.5%
petiole	15	2-6	78.0%
leaf	10	1-5	80.0%

a. The Number of explants in each treatment.

b. Range of explant numbers with contamination in different treatments.

c. The average disinfestation percentage. % of disinfestation = No. of clean cultures / No. of the original explants.

Table 1.5. Factorial combinations of 2,4-D (1, 1.5 and 2 mg/l) and kinetin (0,1,2 mg/l) effects on pili nuit callus formation of petiole on WPM

2,4-D (mg/l)	kinetin (mg/l)	explant[a] No.	callus No.	callusing[b] (%)	callus[c] weight(g)	callus w/root	root No/ explant
1	0	8	7	87.5	72.1a	0	0
1	1	12	10	83.3	69.2a	0	0
1	2	10	8	80.0	45.6c	0	0
1.5	0	12	10	83.3	42.2c	0	0
1.5	1	11	11	100	49.7bc	0	0
1.5	2	13	12	92.3	51.3b	2	3-5
2	0	8	7	87.5	45.4b	0	0
2	1	7	6	78.1	41.6d	0	0
2	2	10	8	80.0	43.2c	4	3-5
average				85.8	52.0		

a. Total clean explants without contamination (initial total number is 15)

b. Callusing rate = No. of explants producing callus / No. of explants.

c. LSD test, the column with same letter means no significant difference between different treatments, P=0.05.

Other Explants

1. Shoot tips and nodal segments from the greenhouse grown seedlings were difficult to clean. The disinfestation percentage of the shoot tips was about 30% (Table 1.4) and less than 10% for nodal segments. The shoot tips on WPM swelled and greened up slightly, but not those on MS. In WPM, less phenolic browning of the medium was observed when liquid medium was used. The various combinations of growth regulators in WPM induced only limited swelling of the leaves. Nodal segments showed no response to all treatments (Table 1.2).

2. Shoot tips and nodal segments from *in vitro* germinated seedlings: approximately 35% of the mature seeds were successfully disinfested using the described process (Tables 1.1 & 1.4). Shoot tips harvested from these seedlings were grown in WPM with BA and kinetin at combinations of 1 or 2 mg/l for each of these chemicals. The addition of 0.1 mg/l NAA and 1 mg/l IAA into media did not induce root formation. The nodal explants from *in vitro* seedlings showed limited initial swelling under these treatments.

3. Immature embryo axes and cotyledons: The disinfestation procedure for immature seed yielded 97.5% clean culture (Table 1.4). A small amount of callus formation was observed with 3 out of the 10 cotyledons in WPM or WPM plus 1 mg/l 2,4-D. Callus only formed in older cotyledons. The callus, however, turned brown after two transfers. No

growth or callus formation was observed on embryo axes on all treatments (Table 1.2).

4. Mature Cotyledons: Greenish colored callus was observed on cut surfaces of green mature cotyledons when cultured in WPM plus 1 mg/l 2,4-D. Three of eight cultures produced callus, but lost after 2-3 transfers.

5. Leaves: About 80.0% of the leaf explants were cleaned using the disinfestation procedures (Table 1.1 and 1.4). About 18% of the leaf explants with midribs showed callus formation at 6-8 weeks after initiation (Table 1.6). The callus growth was observed on the cut surface of the midribs, they were slow growing and turned brown after 3-4 months. No callus were produced on leaf segment without midribs.

Table 1.6. The frequencies of callus formation of leaf explants on WPM with different combinations of plant growth regulators (mg/l).

2,4-D	BA	KT	explant	callus	callusing (%)
1	0	0	5	1	20.0
1	1	0	9	1	11.1
1	0	1	8	2	25.0
1	0	2	9	2	22.2
2	0	1	7	1	14.3
average					18.5

SUMMARY

Some steps towards a complete *in vitro* protocol for pili nut have been identified by this study.

Petiole segments harvested from greenhouse-grown pili nut plants are the best explant source because they are readily available, easy to disinfect and responsive to callus induction.

Vigorous callus growth was obtained from petiole explants within two weeks after being placed horizontally on WPM medium with 1 mg/l 2,4-D or WPM medium with 1 mg/l 2,4-D and 1 mg/l Kinetin.

Shoots obtained from *in vitro* germinated seedlings can be grown in WPM with combinations of BA and Kinetin at 1 or 2 mg/l.

There was no successful root induction with shoot tips harvested from in vitro germinated seedlings at low concentration of NAA (0.1 mg/l) and IAA (1 mg/l). Rooting occurred on petiole callus in WPM with a combination of 2,4-D (1.5 or 2 mg/l) and Kinetin (2 mg/l).

The missing link to complete a protocol for pili tissue culture is to develop a medium that will induce embryogenesis and shoot growth from the petiole callus.

APPENDIX

Inorganic components and vitamins of McCown's Woody Plant Media (WPM)[a] and Murashige Skoog (MS)[b] plant tissue culture media.

Components	WPM	MS
NH_4NO_3	400	1650
KNO_3		1900
$Ca(NO_3)_2.4H_2O$	556	
$MgSO_4.7H_2O$	370	370
$CaCl_2.2H_2O$	96	440
KH_2PO_4	170	170
K_2SO_4	990	
$FeSO_4.7H_2O$	27.8	27.8
Na_2EDTA	37.3	37.3
$MnSO_4.H_2O$	22.3	22.3
$ZnSO_4.7H_2O$	8.6	8.6
H_3BO_3	6.2	6.2
KI		0.83
$Na_2MoO_4.2H_2O$	0.25	0.25
$CuSO_4.5H_2O$	0.25	0.025
$CoCl.6H_2O$		0.025
Myo-inositol	100	100
Nicotinic acid	0.5	0.5
Pyridoxine HCl	0.5	0.5
Thiamine HCl	0.1	0.1
Glycine	2.0	2.0

a. WPM, Lloyd and McCown, 1980
b. MS, Murashige and Skoog, 1962

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PART II

ISOZYME ELECTROPHORESIS OF SOME RELATED *CANARIUM* SPECIES

INTRODUCTION

Canarium is a large genus in Burseraceae with about 100 species. Trees of *Canarium* are valued for their fragrant resins and lumber. Both the fruit pulp and kernel of some species are edible. Pili nut, *Canarium ovatum*, and Chinese olives, *Canarium album* and *C. pimela*, are the only *Canarium* fruits to reach world markets (Coronel, 1983; Steffey, 1986).

The dioecious trees are distributed throughout tropical Africa, Indian Ocean islands, southern China, northeastern Australia, Micronesia, Solomon Islands, Tonga and Samoa (Smith, 1985; Steffey, 1986; Evans, 1992). Pope (193-) reported on about 80 species of *Canarium* of which 10 produced fruits (edible?). Coronel (1983) stated that there were about 100 species in the tropical forests of Asia and Malaysia, of which 35 were found in the Philippines.

Twenty-one species of *Canarium* from Polynesia, Micronesia and east Melanesia, northern New Guinea and adjacent islands were described by Leenhouts (1959), using morphological markers, such as the presence or absence of stipules and stipule scars, and the position of stipules on the leaf petioles; however, the taxonomy of *Canarium* species remained confusing (Leenhouts, 1959). For example, pili nut is the common name of *C. ovatum*, but it is also used as the common name for *C. luzonicum* in the Philippines, *C. vulgare* in Fiji, and sometimes for any *Canarium* species that yields

large kernels (Howes, 1953). Nomenclature confusion occurred between *C. indicum* and *C. commune*, which were later determined to be synonymous (Leenhouts, 1959; Smith, 1985).

In addition to the morphological characters, isozyme markers from electrophoresis may be used as a valuable tool in the classification of *Canarium* species. The objectives of this study are: 1) to develop a reliable isozyme electrophoresis protocol for the *Canarium* species, 2) to assess the isozyme polymorphism of the *Canarium* germplasm collection maintained in Hawaii, 3) to identify and distinguish *Canarium* species (fingerprinting), and 4) to determine how the different *Canarium* species are genetically related.

LITERATURE REVIEW

Isozyme Electrophoresis

Isozymes are multiple molecular forms of enzymes with identical or similar functions (Markert and Moller, 1959). The molecular variation in isozymes is revealed by the combination of starch gel electrophoresis and histochemical staining (Hunter and Markert, 1957) using their catalytic properties. This technique has been successfully used in plant and animal population genetics, cytogenetics, developmental biology, taxonomy, evolution and breeding (Shaw and Prasad, 1970; Buth, 1984; Soltis and Soltis, 1989).

Electrophoresis is the movement of enzymes in a gel or other medium under the influence of an electric current (Pasteur et al., 1988). Most practical methods of electrophoresis involve a gel onto which samples are loaded, with the two ends of the gel in contact with a buffer solution containing the electrodes. Hydrolyzed potato starch is a common gel medium for isozyme electrophoresis due to its relatively low cost, non-toxicity and relative ease of preparation and operation as compared to agarose, cellulose acetate or polyacrylamide (Conkle et al., 1982; Harris and Hopkinson, 1976; Moss, 1979). Various tissue types, including seeds, buds, petioles, leaves and shoots are used for isozyme studies, but leaves are most commonly employed. Proper gel composition, with regard to gel

concentration, buffer type and pH are critical for the best resolution of isozymes during electrophoresis (Soltis and Soltis, 1989).

Electrophoretic evidence has some advantages over morphological traits for taxomomic purposes: 1) The collinearity between protein structures and genes avoids problems of convergence and functional correlation often prevail in morphological characters. 2) The numbers and kinds of enzymes present are precisely and directly quantifiable. 3) Isozymes are genetically independent of environmental influences. 4) Equal weighting or value can be accorded to enzymes in similarity matrices or other methods of evaluating divergence or relationship (Gottlieb, 1977).

Application of Isozyme Electrophoresis in Plant Systematics and Genetic Diversity

Isozyme analysis has become particularly prominent in systematic, evolutionary biology and agronomy to analyze the taxonomic, genetic and evolutionary relationships of plant populations (Tanksly and Orton, 1983). Electrophoresis and its application in plant systematics were reviewed by Gottlieb (1977) and Crawford (1989). Allozymes are inherited as codominants in a simple Mendelian fashion, and this allows one to ascertain the allelic frequencies of the genes and to quantify the similarity between populations,

groups of populations or species (Gottlieb, 1977). For example, starch gel electrophoresis and allozyme analyses were employed to assess the systematic relationships among morphologically similar *Aeschynomene virginica, A. indica* and *A. rudis* (Carulli and Fairbrothers, 1988). It was also used to clarify the relationships among the revised *Lens* taxa and provided insight as to the origin of the cultivated lentil, *L. culinaris ssp. culinaris* (Hoffman et al., 1986). The genetic similarities among seven species of *Prosopis* (Leguminosae: Mimosoideae) were demonstrated by using isozyme analysis (Saidman and Vilardi, 1987); and also the genetic diversity and variability among 20 populations of genus *Elytrigia* collected from different geographical areas (Soler et al., 1993).

Optimal utilization of crop genetic resources requires a knowledge of the range and structure of the variations present in the gene pool of interest, and isozyme analysis is a tool to identify these diversities. The genetic diversity among one hundred and twelve accessions of cultivated cowpea and 43 wild cowpea were revealed using 26 isozyme loci, and the cultivated accessions were identified to have very low genetic diversity (Vaillancourt et al., 1993). The isozyme variation at 17 polymorphic loci within African Manihot germplasm was used to describe the diversity among 365 *Manihot esculenta* cultivars and 109 wild relatives (*M. glaziovii* and spontaneous hybrids). Accessions within

M. esculenta and *M. glaziovii* showed high levels of polymorphism (Lefevre and Charrier, 1993). High levels of genetic diversity were also shown between four morphologically similar species, *Liquidambar formosana*, *L. styraciflua*, *L. orientalis* and *L. acalycina* from different continents (eastern Asia, eastern United States, Central America and southwest Turkey, respectively (Hoey and Parks, 1991).

Enzyme gel electrophoresis is primarily useful for comparing closely related taxa, such as races and species. Some examples are: Five isoenzyme loci (two peroxidase loci and three phosphoglucomutase loci) were used to classify the species and feral types of *Ananas* (Dewald et al.,1992); alcohol dehydrogenase (ADH), phosphoglucose isomerase (PGI), peroxidase(PER) were used to identify 18 cultivars of red maple (Tobolski and Kemery, 1992); phosphoglucomutase (PGM), isocitrate dehydrogenase (IDH) and triose phosphate isomerase (TPI) banding patterns were used for the identification of 55 raspberry accessions (Cousineau and Donnelly, 1992). Fifty-two inbred lines of cultivated sunflower of diverse origins were studied using 8 isozymes (Quillet et al., 1992)

Tropical forests have long been of interest to biologists because of their high species diversity. Data from 97 isozyme studies on the genetics of tropical woody species demonstrated that cultivated taxa maintain higher

percentages of polymorphic loci and higher mean heterozygosities than native tropical species (Loveless, 1992).

Applications of Isozyme Analysis in Fruit and Nut Trees

In fruit and nut crops, the isozyme electrophoresis technique has been used extensively for cultivar identification and hybrid confirmation. Some examples include characterization of cherimoya cultivars (Pascual et al., 1993), walnut cultivars (Aleta et al., 1990), mango (*Mangifera indica L.*) cultivars (Degani et al., 1990), pear (Santamour and Demuth, 1980), apple, sweet cherry and raspberry (Stamper and Smole, 1992), apricot (Byrne and Littleton, 1989), almond (Hauagge et al., 1987), and hybrids between peach and almond (Chaparro et al., 1987), peach and plum (Parfitt et al., 1985), and intraspecific hybrids of grapes (Weeden et al., 1988).

MATERIALS AND METHODS

Sample Preparation

Fully expanded, young leaves were collected from the U. S. Department of Agriculture, Agricultural Research Service (USDA-ARS), National Clonal Germplasm Repository (NCGR) in Hilo, Hawaii. A total of fifty-three plants including 7 *Canarium* species, (*C. megalanthum, C. indicum, C. mehenbethene, C. odontophyllum, C. ovatum, C. harveyi and C. album*), two unknown *Canarium* species and one *Dacroydes rostrata* were used in this study (Table 2.1). Each plant was treated as an operational taxonomic unit (OTU). Leaves were stored at 4^0C from 7 to 10 days.

Gel and Buffer Preparation

A 12% (w/v) starch gel of hydrolyzed potato starch (Sigma Chemical Co.) was used. Forty-four grams of potato starch and 370 ml of gel buffer were heated to 75 to 80^0C with continuous stirring in a 1000 ml vacuum flask. The heated gel was vacuumed for 15-20 seconds to remove air bubbles and poured into a 18 x 13 x 1 cm gel mold. Bubbles that formed in the gel were removed with a glass pipet immediately after pouring. For convenience, the gels were prepared the day before, wrapped with plastic film and stored at room temperature until used. The gels were refrigerated (4^0C) for 1 hour before sample loading.

Table 2.1. Plant Samples of Seven *Canarium* Species Used in the Study

NO.	ID#	NCGR#	SPECIES	ORIGIN
1	T1A	HCAN 6	megalanthum	Malaysia
2	T1B	"	"	"
3	T1C	"	"	"
4	T3A		indicum	Waiakea Exp. St.
5	T3B		"	"
6	T3C		"	"
7	T2		mehenbethene	Waiakea Exp. St.
8	T7		"	"
9	T7A		"	"
10	T17A		"	"
11	T17B		"	"
12	T17C		"	"
13	T17D		"	"
14	T17E		"	"
15	T5	HCAN	odontophyllum	Malaysia
16	T4		ovatum	Waiakea Exp. St.
17	T4A		"	"
18	T4B		"	"
19	T4C		"	"
20	T4D		"	"
21	T6		"	"
22	T9A	N91-26	"	Poamoho Exp. St.
23	T9B	"	"	"
24	T9C	"	"	"
25	T9D	"	"	"
26	T10A	N92-16	"	Philippines
27	T10B	"	"	"
28	T10C	"	"	"
29	T11	N92-17	"	"
30	T12A	N92-12	"	"
31	T12B	"	"	"
32	T12C	"	"	"
33	T12D	"	"	"
34	T13A	N92-14	"	"
35	T13B	"	"	"
36	T13C	"	"	"
37	T13D	"	"	"
38	T14	N92-30	harveyi	Poamoho Exp. St.
39	T15	N91-22	"	Fiji
40	T18A	HCAN 2	album	China
41	T18B	"	"	"
42	T18C	"	"	"
43	T18D	"	"	"
44	T18E	"	"	"
45	R11T1		harveyi v. harveyi	
46	R11T5		"	
47	R11T13		"	
48	T20A	N94-49	"	Waiakea Exp. St.
49	R11T11		odontophyllum	
50	No. 6		megalanthum	
51	R11T3		Dacroydes rostrata	
52	2A		unidentified	
53	2B		unidentified	

Three buffer systems were tested: Histidine/citrate, pH 6.5; tris/citrate, pH 7.5 and morpholine/citrate, pH 6.1 (Table 2.2). Tray buffers were stored at (4^0C) before use.

Enzyme Extraction and Gel Loading

About 25 mg of fresh leaves from each sample was ground in a frozen grinding block with 4 to 5 drops of extraction buffer on ice (Table 2.3). Each extract was loaded onto paper wicks (10 x 2 mm, Whatman NO.3) and inserted into the gels. Thirty individual wicks were loaded per gel.

Electrophoresis

Electrophoresis was conducted at 4^0C. For histidine/citrate (HC) and morpholine/citrate (MC) buffer systems, the voltage was kept constant at 200v; for tris/citrate (TC), the current was kept constant at 65 mA. After the initial running of 30 minutes, the wicks were removed from the gels. The average time per electrophoresis was about 5.5 to 6 hours.

Enzyme Staining and Fixing

After electrophoresis, each gel was sliced horizontally into six thin slices for staining. Fourteen selected enzyme systems were assayed (Shaw and Prasad, 1970; Soltis et al, 1983; Arulsekar and Parfitt, 1986). The staining solutions were made fresh every time. The enzyme systems LAP, EST and PER were stained at room temperature, and the other enzymes

were stained at 37^{0}C (Appendix A). The average staining time was between 45 to 60 minutes. When the bands were well-resolved, the staining solution was discarded, and the gel was rinsed in deionized water and fixed in a mixture of methanol, deionized water, and glacial acetic acid (5:5:1 v/v/v) overnight.

The banding patterns on each gel were recorded. The most anodic band in each enzyme system was identified as band 1. Gels were wrapped with plastic film and refrigerated for preservation.

Data Analysis

Numerical analysis: The gels were scored for presence (1) or absence (0) of isozyme bands. Jaccard's similarity coefficients (S_j) were generated based on a pairwise comparison of the presence/absence patterns in individuals (Sneath and Sokal, 1973), and coefficient values ranged from 0.0 to 1.0. A cluster analysis was obtained by means of unweighted pair-group method using arithmetic averages (UPGMA) (Sneath and Sokal, 1973).

All numerical analyses were made with programs contained in the computer statistical package NTSYS (Exeter Software Co., New York).

Phenotypic Polymorphism: phenotypic polymorphism for each enzyme and species (P_j) was estimated by

$$P_j = \sum_{i=1}^{n} P_i(1-P_i) = 1 - \sum_{i=1}^{n} P_i^2$$

where P_i is the frequency of the ith phenotype and n is the number of phenotypes observed per enzyme and species. The weighted average amount of phenotypic polymorphism P over all observed enzymes is given by

$$P = \sum_{j=1}^{k} (1/N_j)P_j \ / \ \sum_{j=1}^{k} (1/N_j)$$

where N_j is the total number of phenotypes observed (collection wide) per jth enzyme for k enzymes (Kahler et al., 1980).

Table 2.2. Buffer systems used in electrophoretic analysis of *Canarium*.

Chemical	Electrode	Gel	Reference
L-histidine (free base)	0.065	0.016M	Cardy et al.
Citric acid (anhydrous)	0.007M	0.002M	1981
pH	6.5	6.5	
Tris	0.223M	3.5% dilution	Shaw and Prasad
Citric acid (anhydrous)	0.085M	of electrode buffer	1970
pH	7.5	7.5	
Citric acid (anhydrous)	0.040M	1:20 dilution of	
morpholine	adjust pH with -(3-aminopropyl) -morpholine	electrode buffer	Soltis et al., 1983
pH	6.1	6.1	

Table 2.3. Recipe for the extraction buffer (Aradhya, 1992)

Concentration	Chemical
0.10 M	Tris (hydroxymethyl)
0.20 M	Sucrose
0.50 mM	Ethylenediaminetetraacetic acid (EDTA,disodium)
0.005 M	Dithiothreitol (DTT)
0.012 M	Cysteine-HCl
0.025 M	Ascorbic acid
1 %	Polyethylene glycol (PEG)
10 %	Dimethyl sulfoxide (DMSO)
2 %	Tween 80
pH was adjusted to 7.5 with NaOH, then the following components were added	
0.1 %	Bovine serum albumin (BSA)
0.02 M	Sodium metabisulfite
0.005 M	Diethyldithiocarbomate (DIECA)
0.30 mM	Nicotinamide adenine dinucleotide phosphate (NADP)
0.40 mM	Nicotinamide adenine dinucleotide (NAD)
10 %	β-mercaptoethanol
10 %	Polyvinyl polypyrollidone (PVPP)

β-mercaptoethanol and PVPP are added just before use.

RESULTS

Selection of Buffers and Enzyme Systems

Fourteen enzymes (Appendix A) and three buffer systems (Table 2.2) were tested on seven species of *Canarium*.

No activity was detected in esterase (EST), peroxidase (PER), glucose 6-phosphate dehydrogenase (G-6PDH) or aconitase (ACO) in any buffer systems. Alcohol dehydrogenase (ADH), isocitrate dehydrogenase (IDH), and shikimate dehydrogenase (SKDH) showed no activity on morpholine - citric acid (MC). Poorly resolved bands were observed on histidine-citric acid (HC) buffer and Tris-citric acid (TC) buffer only in certain samples of *C. ovatum*. Bandings of 6-phosphogluconate dehydrogenase (6PGD) were poor and showed no variation between samples in either HC or TC buffer.

High resolution was obtained from leucine aminopeptidase (LAP), phosphoglucomutase (PGM), and uridine diphosphoglucode pyrophosphorylase (UGPP) in HC and TC buffer, but the bands of these enzymes were more consistent and well separated in HC buffer. Malate dehydrogenase (MDH), phosphoglucose isomerase (PGI), triose-phosphate isomerase (TPI) gave good resolution only in HC buffers (Table 2.4). Only data from LAP, MDH, PGI, PGM, TPI and UGPP on HC buffer (pH 6.5) were used in the analysis.

Table 2.4. Activity[a] of 14 different enzyme systems on 3 different buffer systems of *Canarium*

Isozyme	Buffer system[b]		
	HC	TC	MC
ACO	no	no	no
ADH	poor	poor	no
EST	no	no	no
G6PDH	no	no	no
IDH	poor	poor	no
LAP	good	good	poor
MDH	good	poor	poor
PER	no	no	no
6PGD	poor	poor	poor
PGI	good	poor	poor
PGM	good	good	poor
SKDH	poor	poor	no
TPI	good	poor	poor
UGPP	good	good	poor

a: no: no activity.
poor: weak banding.
good: clear banding.

b: HC: histidine-citric acid, pH 6.5
TC: Tris-citric acid, pH 7.5
MC: morpholine-citric acid, pH 6.1.

Isozyme Polymorphism

Phenotypic polymorphism for six enzyme systems

A total of 70 different bands was found in the 53 accessions. Some of the bands are unique to some specific species. MDH had the largest number of bands, with 18 bands, followed by PGI, UGPP, TPI, PGM and LAP with 12, 12, 11, 9 and 8 bands, respectively (Table 2.5). The number of bands in different species and different phenotypes varied dramatically in MDH, for example: there were 11 different bands in the phenotype A of *C. megalanthum*, but only 3 bands showed in the D phenotype of *C. odontophyllum* (Figure 2.1). No large difference of band numbers between different species and different phenotypes was observed in the other five enzyme systems (Figure 2.2 to 2.6). Observed different band frequencies of six enzymes were listed in Appendix B.

Altogether, ninety-seven different phenotypes (zymograms) were observed, 17 for LAP, 16 for UGPP, 15 for PGI, 12 for TPI and 11 for PGM (Table 2.6). Two major isozyme phenomenon were observed: First, each species had its own array of isozyme phenotypes for each enzyme with some overlapping in different species, e.g. Accession 2A had the same phenotype as some of the *C. indicum* in PGI, while 2B had the same phenotype with *C. mehenbethene* (Figure 2.2); *C. album* and *C. ovatum* had the common phenotype E and F in PGM (Figure 2.3); one of *C. harveyi* zymograms (F) was the same as *C. mehenbethene,* and one *C. indicum* was the same as

C. ovatum in LAP (Figure 2.4); *C. indicum* had identical isozyme phenotypes as *C. ovatum* in TPI (Figure 2.5); Accessions 2A and 2B had the same phenotypes as *C. megalanthum* accessions in UGPP (Figure 2.6). Second, all seven species are polymorphic due to the coexistence in each species of more than one phenotype (Figure 2.1-2.6, Appendix C).

Genetic interpretation of polymorphism was not possible due to insufficient samples for some species and lack of segeration progenies from known parents. The magnitude of variation in isozyme phenotypes was expressed by the estimation of phenotypic polymorphism (P_j) and weighted polymorphism (P_w). When the phenotypic polymorphism of different enzymes was calculated over all samples, MDH was the highest (P_j=0.93), TPI was the lowest (P_j=0.75), and P_j for the other four enzymes PGI, PGM, LAP, and UGPP were 0.87, 0.82, 0.89 and 0.88, seperately. The average P_j was 0.86, the weighted P was 0.84. When the mean P_j was calculated based on different species, LAP (P_j = 0.44) was the most polymorphic enzyme and TPI (P_j = 0.11), was the least, with MDH, PGI, PGM and UGPP were in the intermediate with the P_j values of 0.29, 0.39, 0.22 and 0.28 respectively (Table 2.7). The average P_j over the six enzymes and all the samples was 0.29 and the weighted P was 0.26. The comparisons of phenotypic polymorphism between different species were inconclusive due to the difference in sample

numbers between species.

Cluster Analysis

A cluster dendrogram (Figure 2.7) was established based on Jaccard's similarity matrix with the unweighted pair group method using arithmetic means (UPGMA). The cophenetic correlation coefficient between the phenogram and similarity matrix was 0.96739, which indicated that the phenogram structure was a good representation of the actual individual and individual plant relationships. The results demonstrated that there were agreement between accession labels (collection labels) and the allozymic patterns of variation. Plants that were identified as the same species formed easily distinguishable groups in the dendrogram. One exception was observed with sample T15 (#39), labeled as *C. harveyi*, it was shown to have more affinity to *C. indicum* (0.55) than other *C. harveyi* samples. Cluster analysis on the allozyme data revealed 7 groups at 0.5 or greater in genetic similarity, including the *C. ovatum* group at 0.5, *C. megalanthum* at 0.70, *C. mehenbethene* at 0.8, *C. indicum* at 0.88, *C. album* at 0.87, *C. harveyi* at 0.9. The two unknown samples showed 0.75 similarity and were closest to *C. megalanthum* at 0.4. *Dacroydes rostrata* (#51) was different from all *Canarium* species tested (Figure 2.7).

Table 2.5. Observed number of bands of each enzyme system in seven *Canarium* species, two unknown *Canarium* species and 1 *Dacroydes rostrata*

Species	N	Enzyme						Total
		MDH	PGI	PGM	LAP	TPI	UGPP	
C.album	5	4	4	3	2	6	4	23
C.megalanthum	4	11	5	5	2	3	2	28
C.harveyi	6	7	6	4	2	5	4	28
C.indicum	3	6	5	3	2	3	3	22
C.mehenbethene	8	6	7	3	1	6	2	25
C.odontophyllum	2	3	8	3	2	5	3	24
C.ovatum	22	12	3	3	4	4	5	31
unknown species	2	9	5	6	2	4	2	28
Dacroydes rostrata	1	5	2	1	1	4	1	14
total[a]	53	18	12	9	8	11	12	70

a: Total numbers of different bands in each enzyme system in the seven species. There are overlaps between different species, so the number is much less than the sum of the bands of the seven species.

Table 2.6. Observed number of phenotypes in each enzyme system in seven *Canarium* species, 2 unknown *Canarium* species and 1 *Dacroydes rostrata*.

Species	No.of plants	Enzyme Systems						Total
		MDH	PGI	PGM	LAP	TPI	UGPP	
C. album	5	1	1	2*	2	1	3	10
C. megalanthum	4	2	2	1	2	1	2*	9
C. harveyi	6	3	2	2	3*	2	2	14
C. indicum	3	1	2*	1	3*	1*	1	9
C. mehenbethene	8	1	3*	1	2*	3	1	10
C. odontophyllum	2	1	2	1	1*	1	2	9
C. ovatum	22	14	2	2*	6*	2*	5*	31
unknown species	2	2	2*	2	2*	1	1	10
Dacroydes rostrata	1	1	1	1	1	1	1	6
Total	53	26	17 (15)*	13 (11)*	22 (17)*	13 (12)*	18 (16)*	108 (97)*

* Phenotypes overlap each other. The numbers in () means different phenotypes.

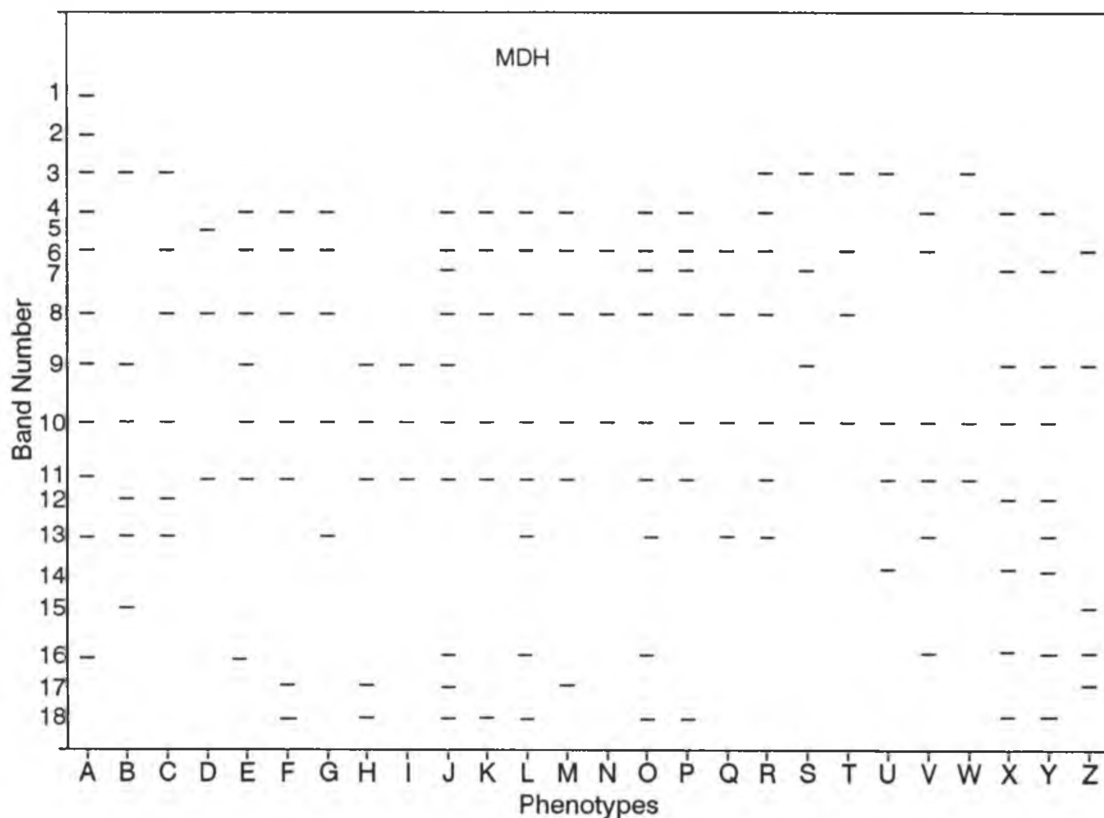

Figure 2.1. Schematic illustration of phenotypes of MDH for all fifty-three accessions. Different letters represent different phenotypes. *C. megalanthum*: **A and V**; *C. indicum*: **C**; *C. mehenbethene*: **B**; *C. odontophyllum:* **D**; *C.ovatum*: **E to R**; *C. harveyi*: **S, T and W**, in which T is the phenotype of T15 (#39); *C.album*: **U**; 2A: **X**; 2B: **Y**; *Dacroydes rostrata*: **Z.**

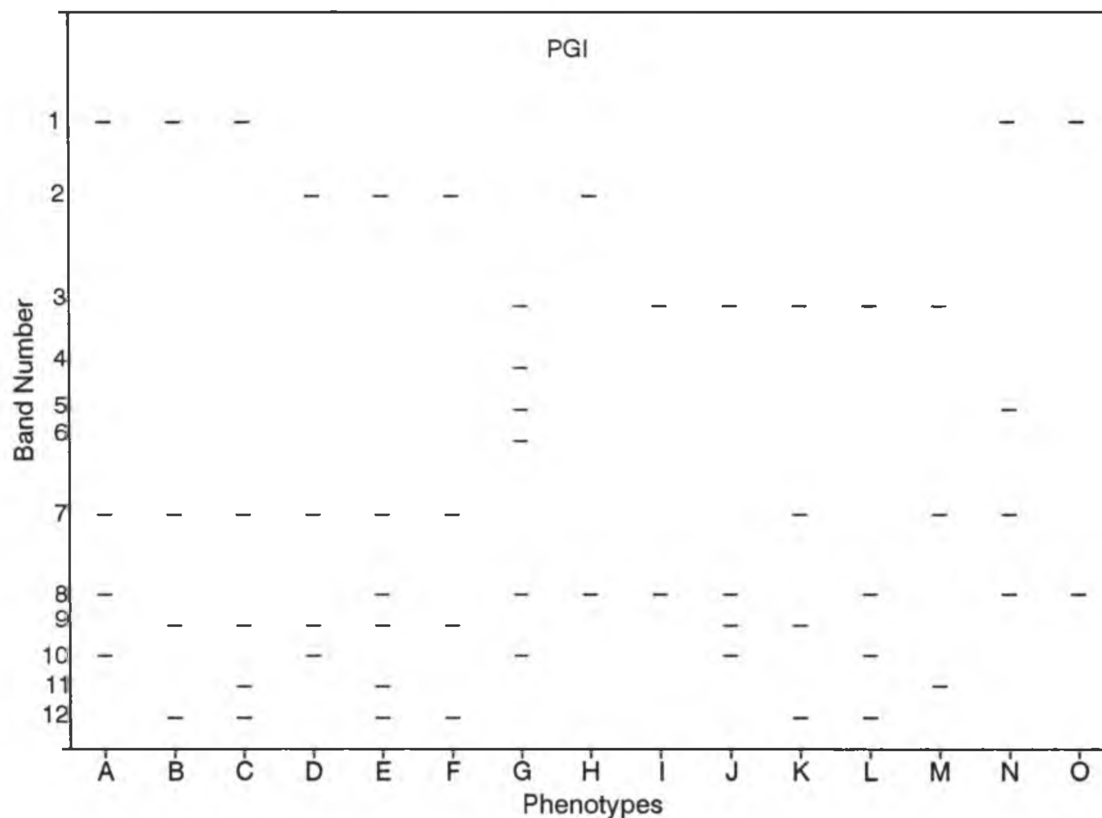

Figure 2.2. Schematic illustration of phenotypes of PGI for all accessions studied. Different letters represent different phenotypes. *C. megalanthum*: **A and N**; *C. indicum*: **B and C**; *C. mehenbethene*: **D, E and F**; *C. odontophyllum*: **G and M**; *C. ovatum*: **H and I**; *C. harveyi*: **J and K**, K is the phenotype of T15; *C. album*: **L**; 2A: **B**; 2B: **D**; *Dacroydes rostrata*: **O.**

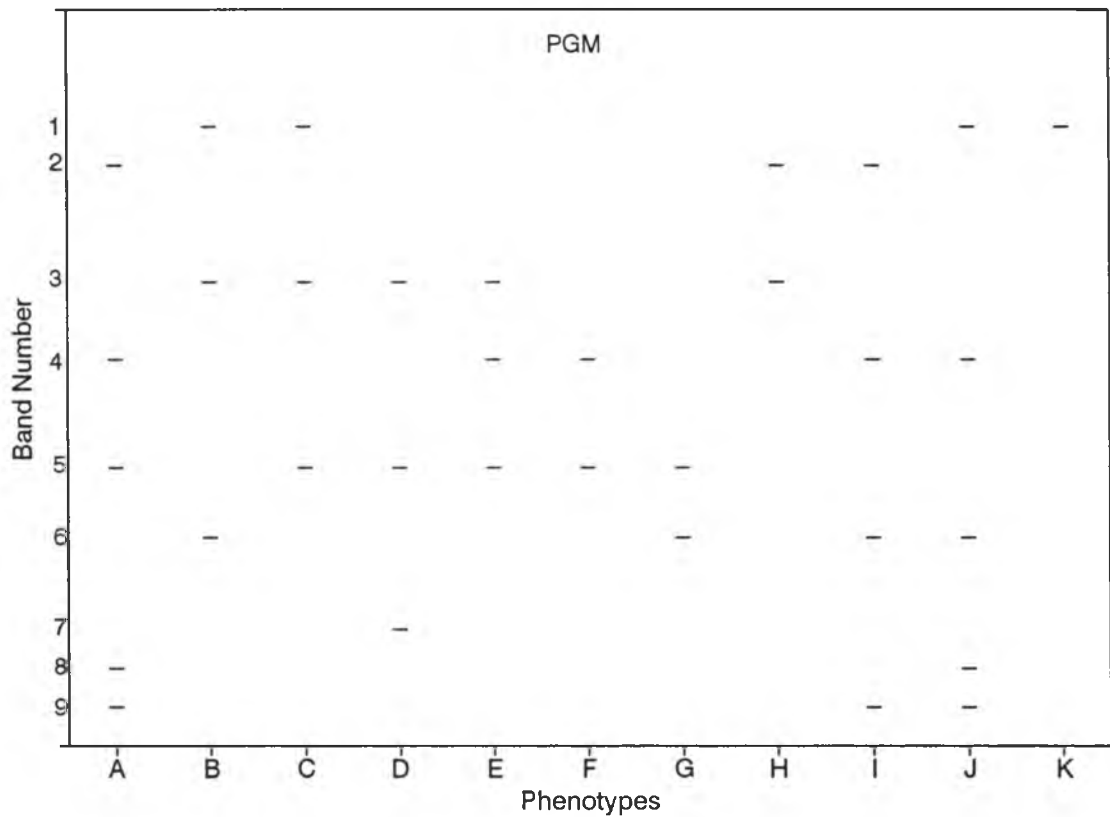

Figure 2.3. Schematic illustration of phenotypes of PGM for all accessions studied. Different letters represent different phenotypes. *C. megalanthum*: **A**; *C. indicum*: **B**; *C. mehenbethene*: **C**; *C. odontophyllum*: **D**; *C. ovatum*: **E and F**; *C. harveyi*: **G and H**; *C. album*: **E and F**; 2A: **I**; 2B: **J**; *Dacroydes rostrata*: **K**.

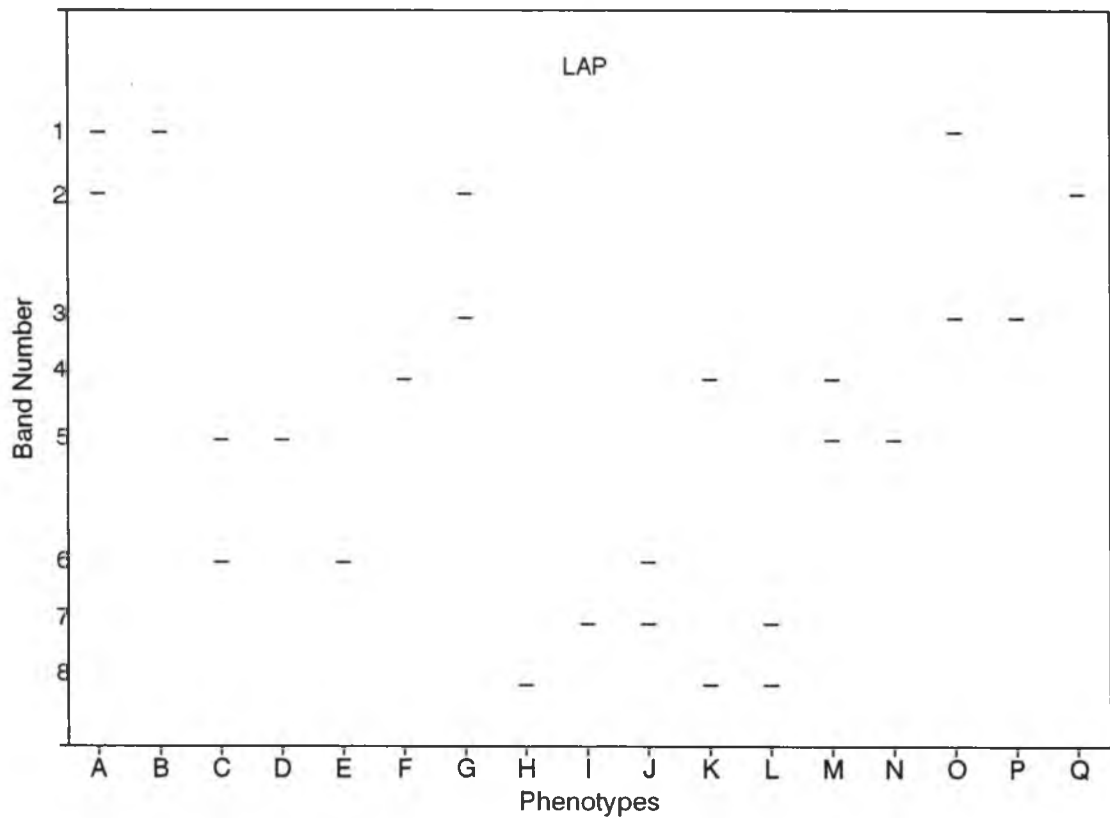

Figure 2.4. Schematic illustration of phenotypes of LAP for all accessions studied. Different letters represent different phenotypes. *C. megalanthum*: **A and B**; *C. indicum*: **C, D and E**; *C. mehenbethene*: **F**; *C. odontophyllum*: **G and Q**; *C. ovatum*: **E, H, I, J, K, L**; *C. harveyi*: **F, M, N**; *C. album*: **O and P**; 2A: **Q**; 2B: **G**; *Dacroydes rostrata*: **Q**.

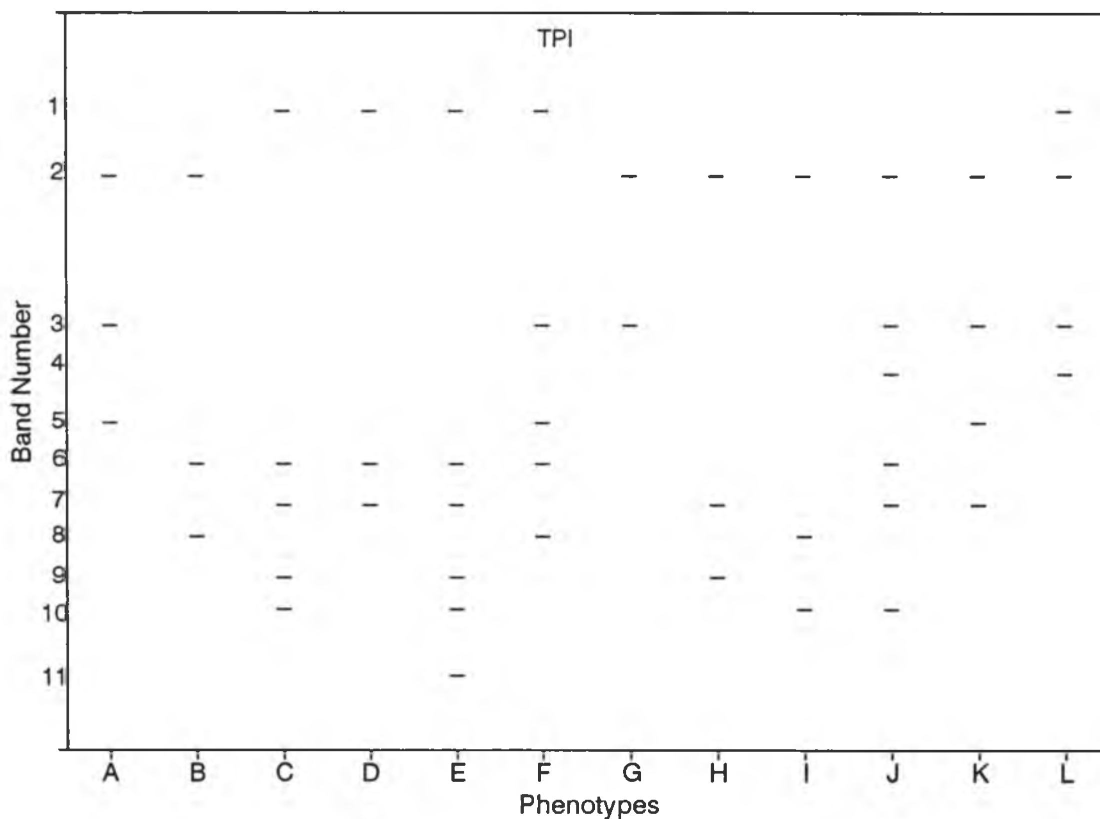

Figure 2.5. Schematic illustration of phenotypes of TPI for all accessions studied. Different letters represent different phenotypes. *C. megalanthum*: **A**; *C. indicum*: **B**; *C. mehenbethene*: **C, D and E**; *C. odontophyllum*: **F**; *C. ovatum*: **B and G**; *C. harveyi*: **H and I**; *C. album*: J; **2A**, 2B: **K**; *Dacroydes rostrata*: **L**.

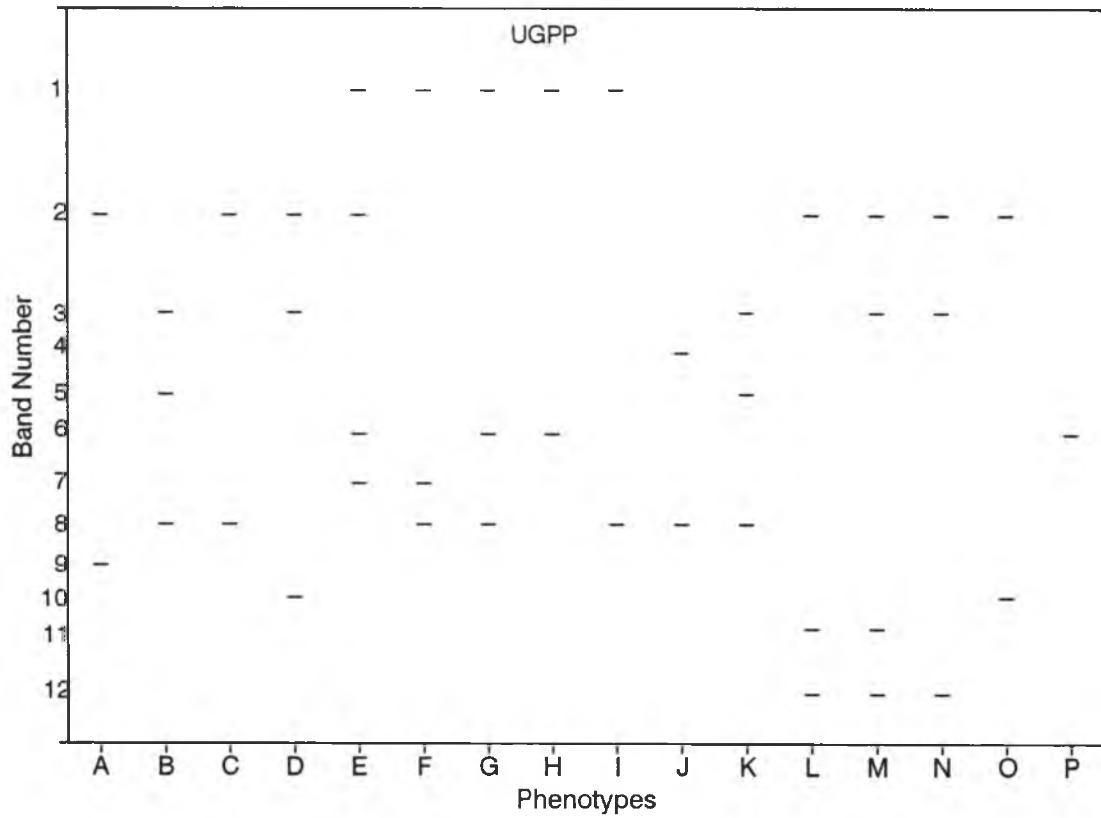

Figure 2.6. Schematic illustration of phenotypes of UGPP for the accessions studied. Different letters represent different phenotypes. *C. megalanthum*: **A**; *C. indicum*: **B**; *C. mehenbethene*: **C**; *C.odontophyllum*: **D and O**; *C. ovatum*: **E, F, G, H, I**; *C. album*: **L, M and N**; *C. harveyi*: **J and K**; 2A, 2B: **A**; *Dacroydes rostrata*: **P.**

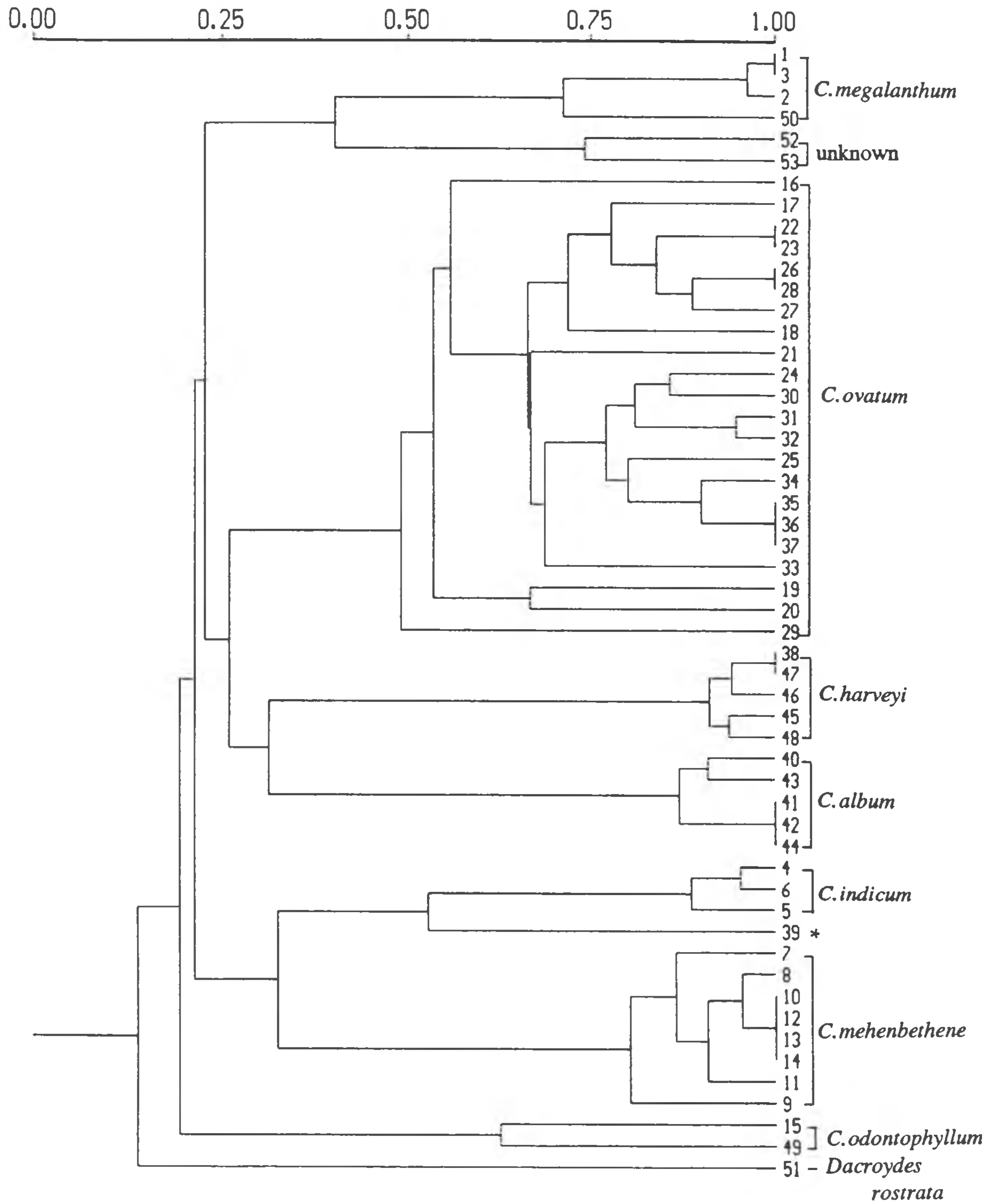

Figure 2.7. Cluster dendrogram with UPGMA among different *Canarium* plants. *: a plant labelled as *C. harveyi*.

Table 2.7. Measures of the amount of phenotypic polymorphism (P_j)[a] over enzyme and species, the average (avg. P_j) and the weighted (P)[a] amount of phenotypic polymorphism over species.

species	N	enzyme							
		MDH	PGI	PGM	LAP	TPI	UGPP	avg.P_j	P
album	5	0	0	0.48	0.48	0	0.56	0.25	0.27
megalanthum	4	0.38	0.38	0	0.38	0	0.38	0.26	0.20
harveyi	6	0.61	0.28	0.28	0.28	0.28	0.28	0.34	0.31
indicum	3	0	0.44	0	0.67	0	0	0.19	0.16
mehenbethene	8	0	0.53	0	0	0.53	0	0.18	0.20
odontophyllum	2	0	0.50	0	0.50	0	0.50	0.25	0.21
ovatum	22	0.88	0.50	0.48	0.69	0.09	0.50	0.53	0.47
unknown-sp	2	0.50	0.50	0.50	0.50	0	0	0.33	0.31
mean		0.29	0.39	0.22	0.44	0.11	0.28	0.29	0.26
%[b]		50	88	50	88	38	63	62	
P_j[c]		0.93	0.87	0.82	0.89	0.75	0.88	0.86	0.84

a formula see text.
b percentage of polymorphic species in one specific enzyme system.
c phenotypic polymorphism over all the samples in each enzyme

Isozyme Polymorphism in *C. ovatum*

Isozyme polymorphism and genetic variation were analyzed between twenty-two accessions of *C. ovatum* (Table 2.8 and Appendix C). A different number was given to each enzyme band. The most anodic band of every enzyme was given 1. With the exception of MDH, the rest of the five enzymes were genetically interpretable.

MDH: This enzyme in pili nut was too complex and variable to be interpreted genetically. There were 14 different phenotypes with 12 bands (Figure 2.8). The numbers of bands in different phenotypes varied from 10 (type 6) to 3 (type 5). A common band (band 7) was present in every phenotype. Band 3 and 5 had the high frequency (90.9%) (Table 2.8).

PGI: There were only two phenotypes in this enzyme with 3 bands (Figure 2.9). One common band was band 3, band 1 and band 2 had a frequency of 45.5 and 54.5% (Table 8). Two putative loci were assumed, one at band 3, and band 1 and band 2 were two alleles of a second locus. About 45% (10) of the accessions in this species had type 1 phenotype, and 55% (12) had type 2.

PGM : There were two phenotypes with 3 different bands (Figure 2.9). The common bands were 2 and 3. Type 1 phenotype had an additional band (band 1) with a frequency of 40.9% (Table 2.8). This enzyme can be putatively divided into 2 loci, band 3 formed 1 locus, band 1 and 2 formed the

other locus. About 41% (9) of plants showed type 1 phenotype, and 59% (11) showed type 2 phenotype.

LAP: This is a diverse enzyme in *C. ovatum* with 6 phenotypes and 4 bands (Figure 2.10). The four bands were at frequencies of 4.55, 40.9, 77.3, 13.6 (Table 2.8). The band number of each phenotype was 1 or 2. This enzyme had the least number of bands among all six enzymes tested. This enzyme was putatively divided into 1 locus with 4 different alleles. Phenotypes 1, 2 and 3 were homozygous, types 4, 5, 6 were heterozygous with two alleles. 13.6% of the plants showed type 1, 45.5% had type 3, 27.3% had type 4, 4.55% for each of type 2, 5 and 6 phenotypes.

UGPP: There were 5 different bands which formed 5 different phenotypes. The five bands were (Figure 2.11) at frequencies of 100, 4.55, 40.9, 9.09 and 81.8 (Table 2.8). The band numbers of different phenotypes varied from 2 to 4. It was divided into 2 loci, one had 3 alleles which were formed by bands 3, 4 and 5, while the second locus was formed by 2 alleles band 1 and 2. The percentages of plant with phenotype were : 4.55% each for 1 and 2, 22.7%, 13.6% and 54.4% for 3, 4, and 5, respectively.

TPI: Except for one plant (#29), all twenty-one samples had the same phenotype (type 2) (Figure 2.9). The two phenotypes shared one common band 1. Band 2 was present in phenotype 1 with the frequency at 4.55%, while band 3 and 4 in phenotype 2 with a frequency of 95.5% (Table 2.8). This

enzyme may be composed of 2 or 3 loci (Figure 2.9). Band 3 and 4 were 2 alleles in a locus, band 2 and 1 were either in 1 locus or 2 loci.

Phenotypic variation in six enzymes

All six enzymes were polymorphic in *C. ovatum* with 31 phenotypes. Among the six enzymes, MDH showed the largest phenotypic polymorphism with P_j value of 0.88 and 14 different phenotypes, followed by LAP (P_j=0.69), UGPP (P_j=0.50), PGI (P_j=0.50), PGM (P_j=0.48) and TPI (P_j=0.09). The average phenotypic polymorphism of the six enzymes in C. ovatum was 0.53, the weighted phenotypic polymorphism for all six enzymes in this species was 0.47 (Table 2.7).

Phenetic analysis

The twenty-two plants in this species formed a cluster at a 0.5 coefficient of similarity and can be divided into 5 subgroups. Group 1 with one plant (#16), group 2 included 7 samples at 0.7, in which #22 and #23, #26 an #28 had coefficient of similarity at 1.0. Group 3 included 11 samples at 0.65, three plants (#35, #36 and #37) in this group showed the 1.0 coefficient of similarity. Group 4 with 2 samples (#20 and #19) at 0.64, and group 5 with (#29) 1 sample (Figure 2.7).

Table 2.8. Numbers of bands and the frequency of each band of the six enzymes in *C. ovatum*

Enzyme	Band Number	No. of plants	Frequency(%)
MDH	1	4	18.2
	2	18	81.8
	3	20	90.9
	4	4	18.2
	5	20	90.9
	6	4	18.2
	7	22	100
	8	18	81.8
	9	8	36.4
	10	4	18.2
	11	4	18.2
	12	12	54.5
PGI	1	10	45.5
	2	12	54.5
	3	22	100
PGM	1	9	40.9
	2	22	100
	3	22	100
TPI	1	22	100
	2	1	4.55
	3	21	95.5
	4	21	95.5
LAP	1	1	4.55
	2	9	40.9
	3	17	77.3
	4	3	13.6
UGPP	1	22	100
	2	1	4.55
	3	9	40.9
	4	2	9.09
	5	18	81.8

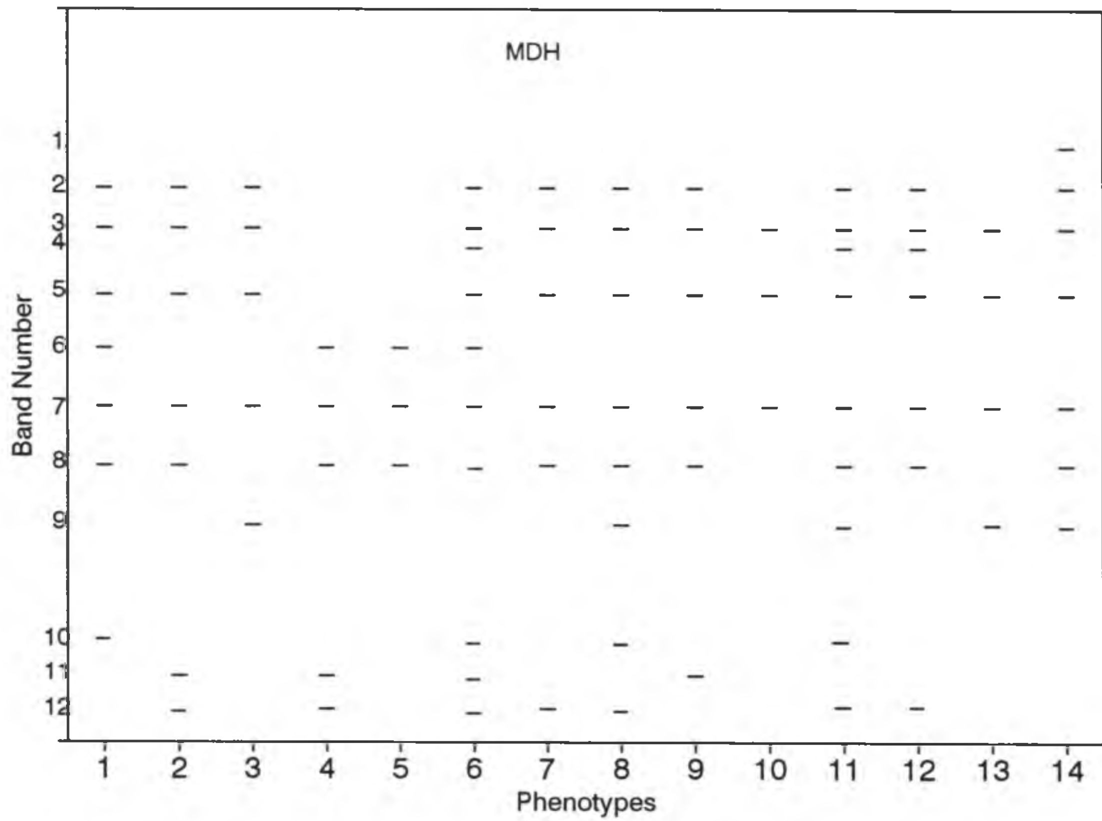

Figure 2.8. Schematic illustration of phenotypes of MDH in *C. ovatum*

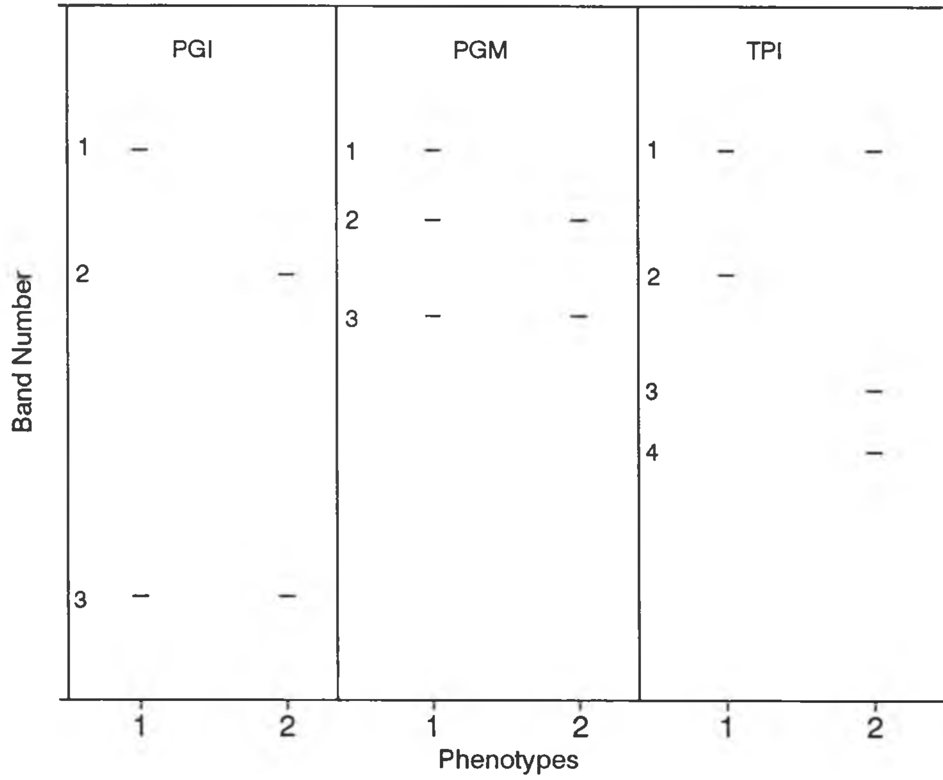

Figure 2.9. Schematic illustration of phenotypes of PGI, PGM and TPI in *C. ovatum*.

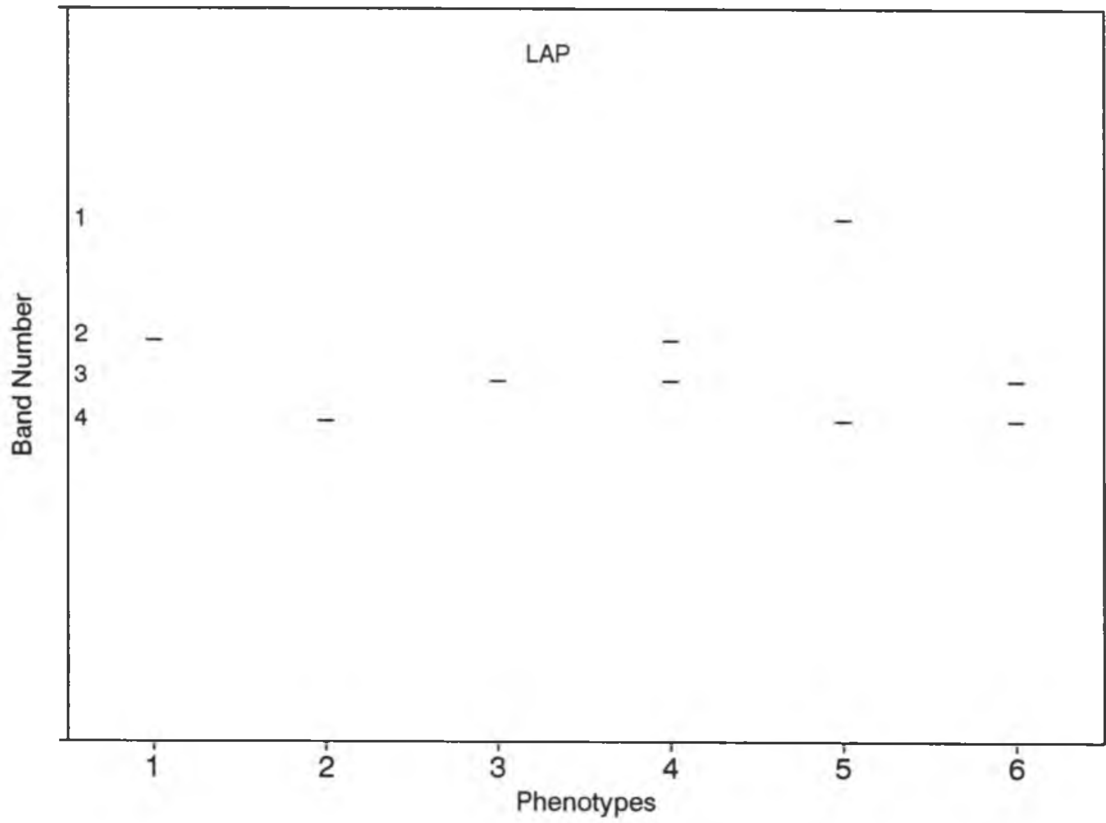

Figure 2.10. Schematic illustration of phenotypes of LAP in *C. ovatum*.

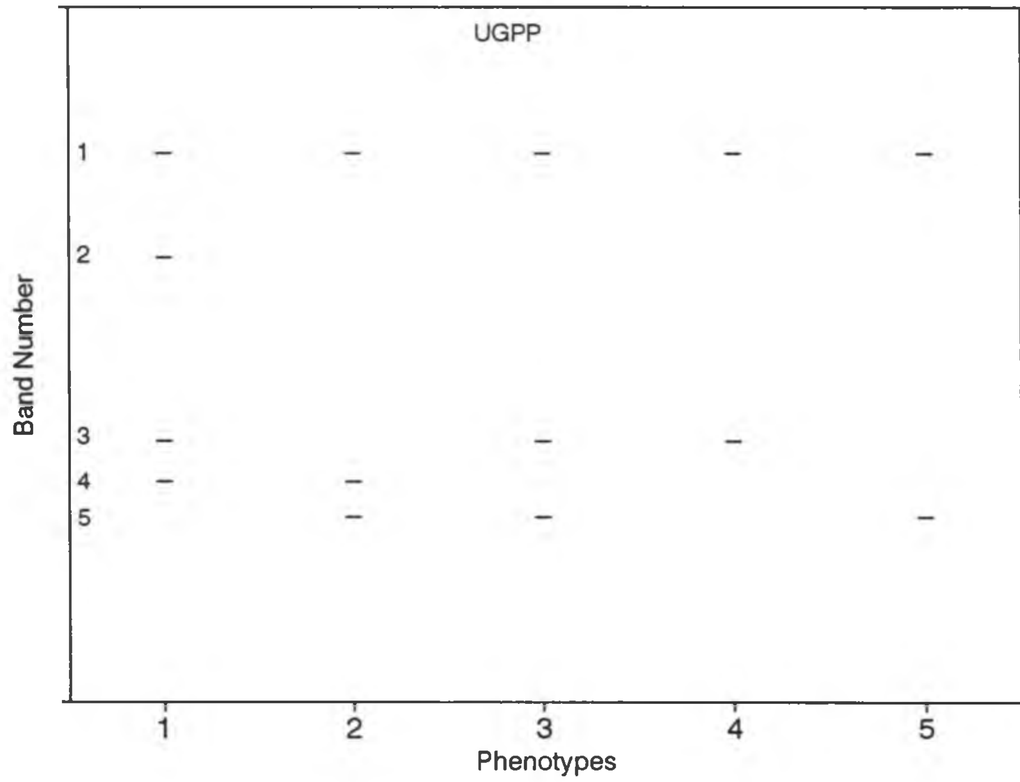

Figure 2.11. Schematic illustration of phenotypes of UGPP in *C. ovatum*.

Other Species

C. album

Five accessions were tested in this species. No polymorphism was observed in MDH, PGI and TPI. All five accessions had the same phenotypes with 4 bands for each MDH and PGI, and 6 bands for TPI. Three enzymes PGM, LAP and UGPP were polymorphic. PGM had 2 different phenotypes with 3 bands, in which bands 2 and 3 were common bands. LAP had 2 phenotypes with 2 different bands, band 2 was present in every plant. UGPP had three different phenotypes with 4 bands, band 1 and 4 were common in 3 phenotypes (Figure 2.12). The enzymes in this species were not genetically interpreterable due to limited plant numbers. Dendrogram cluster showed that T18B(#41), T18C(#42) and T18E(#44) had 1.0 coefficient of similarity.

C. megalanthum

All 4 samples in this species had been identified. PGM, TPI and UGPP were monomorphic with 5, 3 and 2 bands, each of MDH, PGI and LAP had 2 phenotypes with 11, 5 and 2 bands. The band numbers (11) in MDH (type 1) was the largest of all the samples tested, these 2 phenotypes shared 6 common bands (bands 4, 5, 8, 9, 10, 11), while phenotype 1 had an additional 5 bands which were absent in phenotype 2. PGI had three common bands (1,3,4), and each phenotype had its own one additional band. LAP phenotype 1 had two bands,

while type 2 had only one band which was common to type 1 (Figure 2.13). T1A(#1) and T1C(#3) in this species had the coefficient of similarity of 1.0 (Figure 7). This enzyme was not genetically interpretable due to insufficient sample size.

C. harveyi

There were six accessions of this species. Every enzyme showed polymorphism. This was due to sample T15(#39) which always showed different banding patterns from the other five accessions. Except for sample #39, PGI, PGM, TPI and UGPP were monomorphic with 4, 2, 3 and 2 bands among the five accessions, while MDH and LAP have two phenotypes. The 2 phenotypes of MDH had 2 common bands at 1 and 6, while the LAP phenotypes shared one common band at 1 (Figure 2.14). T14(#38) and R11T13(#47) had a coefficient of similarity of 1.0 (Figure 2.7). For accession #39, MDH, PGI, PGM, LAP, TPI and UGPP showed 4, 4, 2, 1, 3 and 3 bands and fell into a cluster away from other *C. harveyi*.

C. indicum

There were three accessions of this species. MDH, PGM, UGPP and TPI were monomorphic with 6, 3, 3, and 3 bands respectively. PGI with 2 phenotypes with 5 bands, and 4 common bands at 1, 2, 3, 5. LAP with 3 phenotypes and 2 bands (Figure 2.15).

C. mehenbethene

Eight accessions of this species were studied. PGI and TPI showed polymorphism, PGI with 3 phenotypes and 7 bands with 3 common bands (band 1,2,4) in every phenotype. TPI with 3 phenotypes and 6 bands and 3 commons bands (1,2,3). The other four enzymes MDH, PGM, LAP, and UGPP were monomorphic, with 6,3,2,2 bands, seperately. MDH, PGI, PGM and UGPP were not genetically interpretable. But, LAP can be putatively divided into one locus with 2 alleles, while UGPP is composed of 2 loci, with one allele in each locus. Four of the samples T17A(#10), T17C(#12), T17D(#13) and T17E(#14) showed exactly the same phenotypes in all six enzymes (coefficient of similarity equals 1.0) (Figure 2.16).

C. odontophyllum

There were two accessions of this species. The two accessions had the same phenotypes of MDH, PGM and TPI, but were different in the other three enzyme systems. The two PGI phenotypes were composed by 6 and 3 bands, in which band 1 was common. LAP had 2 phenotypes and 2 bands. UGPP had three bands, in which bands 1 and 3 were common between the two phenotypes. The genetic interpretation was not possible due to the small number of samples. This species was distinct from other species tested (Figure 2.17).

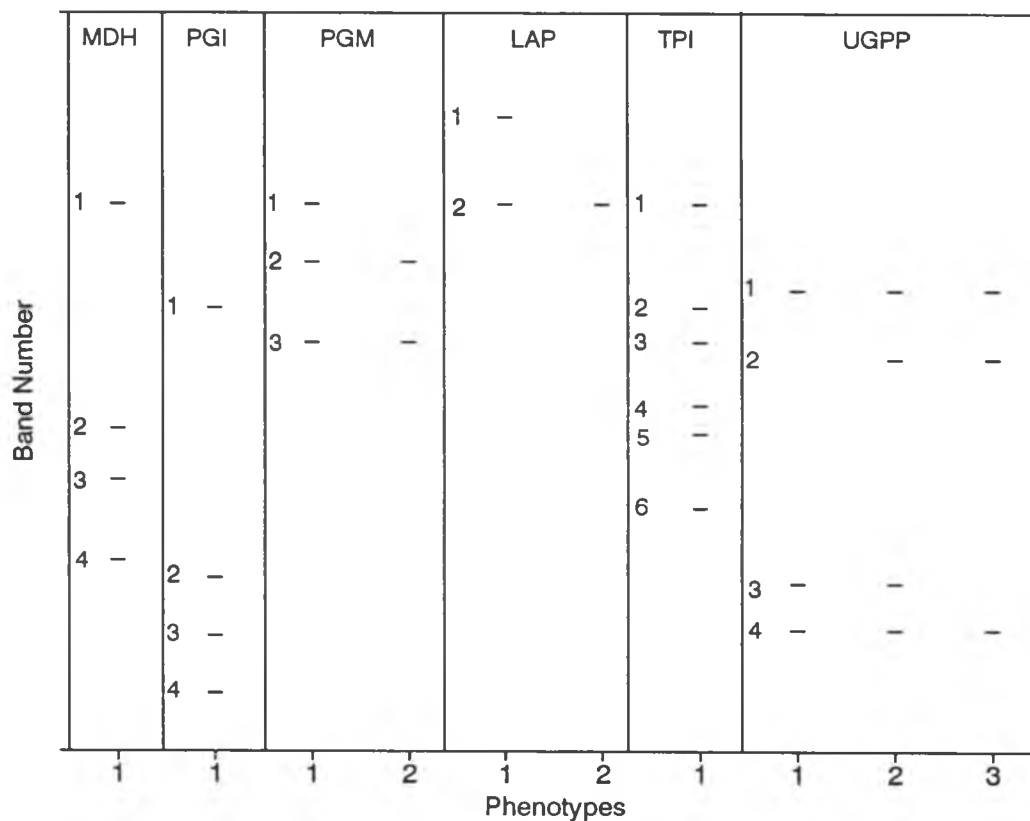

Figure 2.12. Schematic illustration of phenotypes of the Six enzyme studied of *C. album*.

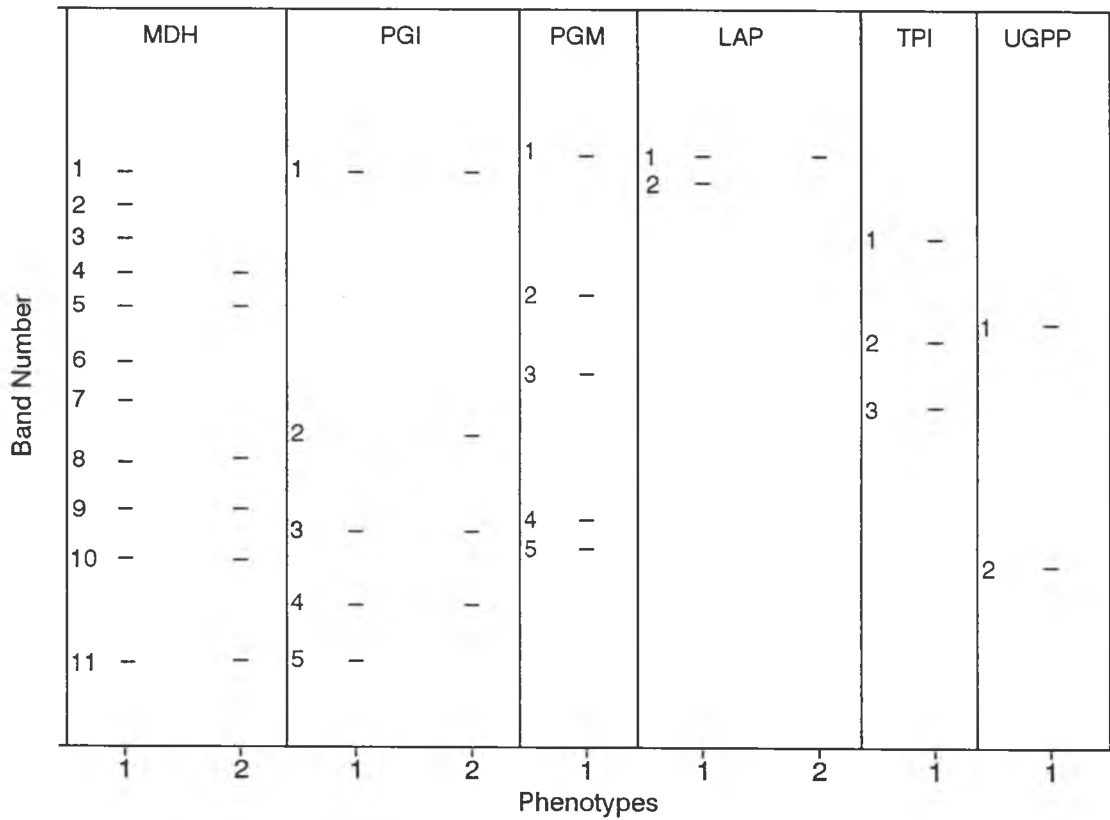

Figure 2.13. Schematic illustration of phenotypes of the six enzymes of *C. megalanthum*.

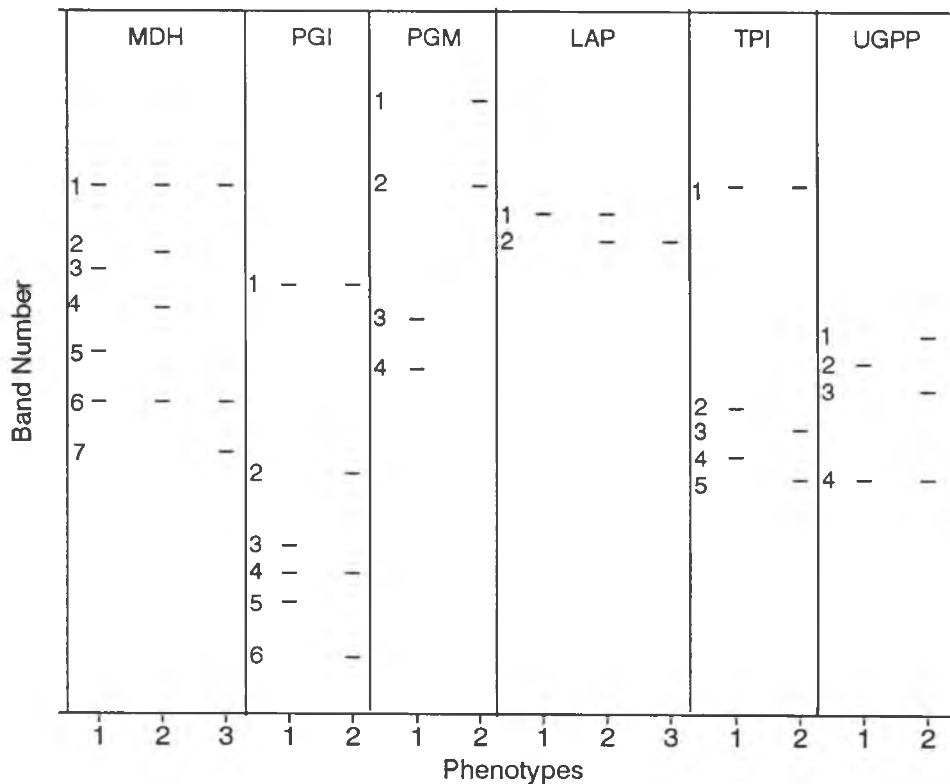

Figure 2.14. Schematic illustration of phenotypes of the six enzymes of *C. harveyi* (2,2,2,3,2,2 in MDH, PGI, PGM, LAP, TPI and UGPP were phenotypes of accession #39).

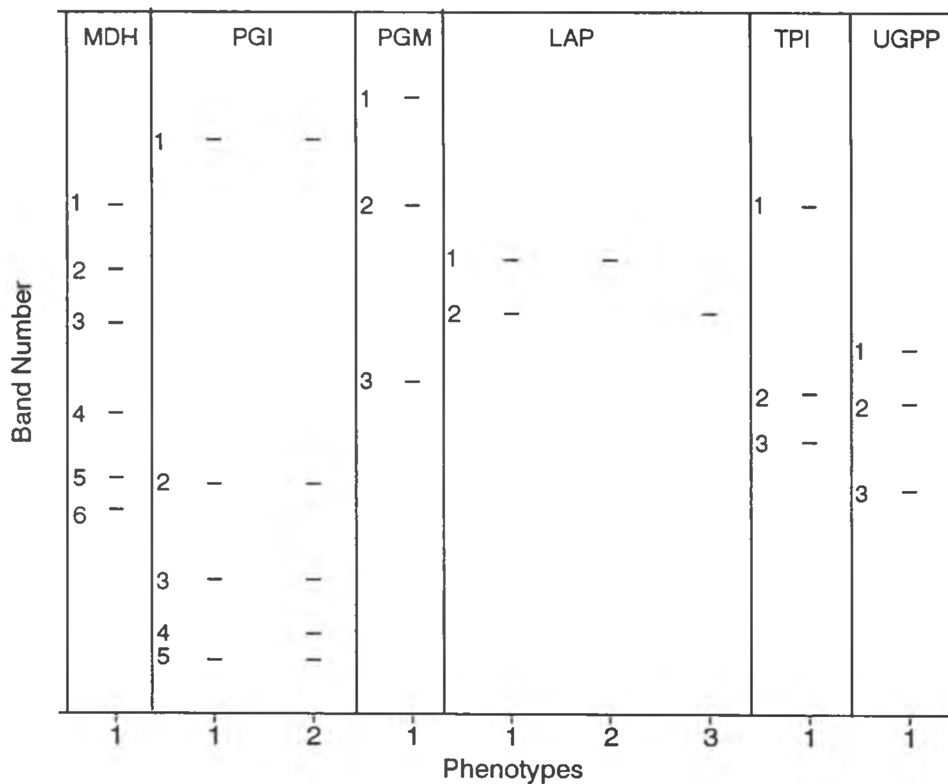

Figure 2.15. Schematic illustration of phenotypes of the six enzymes of *C. indicum*.

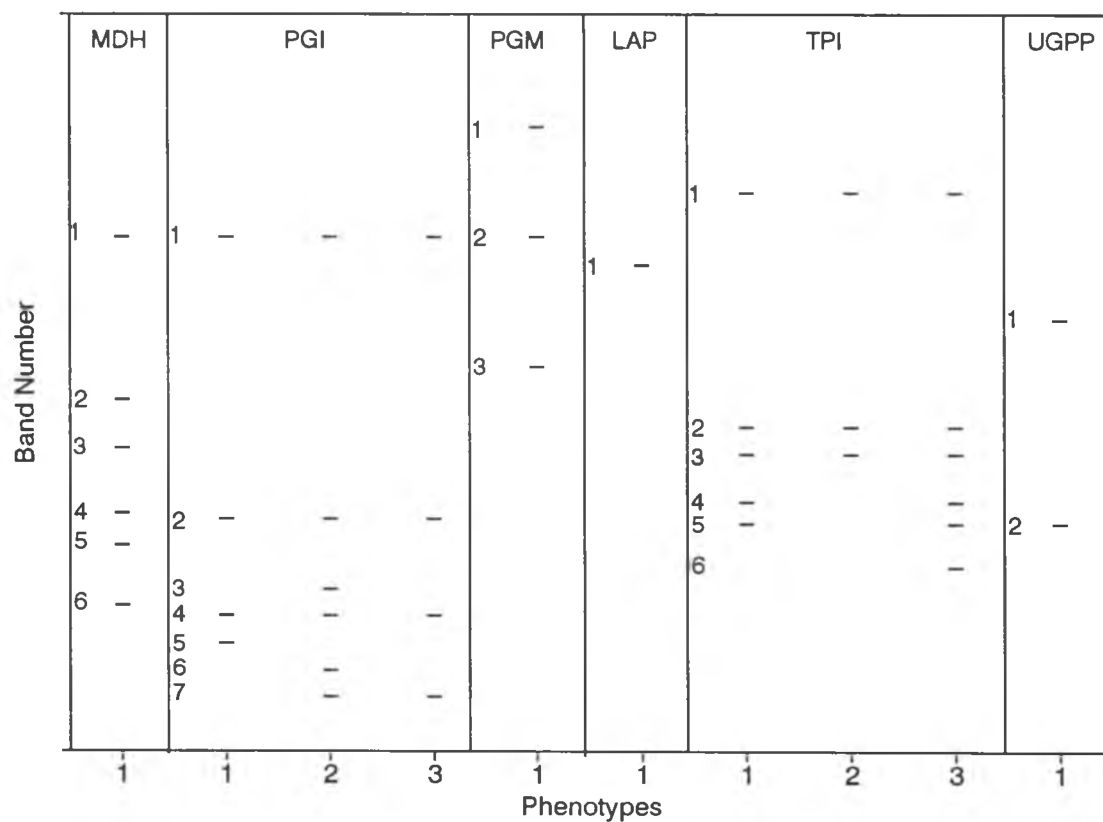

Figure 2.16. Schematic illustration of phenotypes of the six enzymes of *C. mehenbethene*.

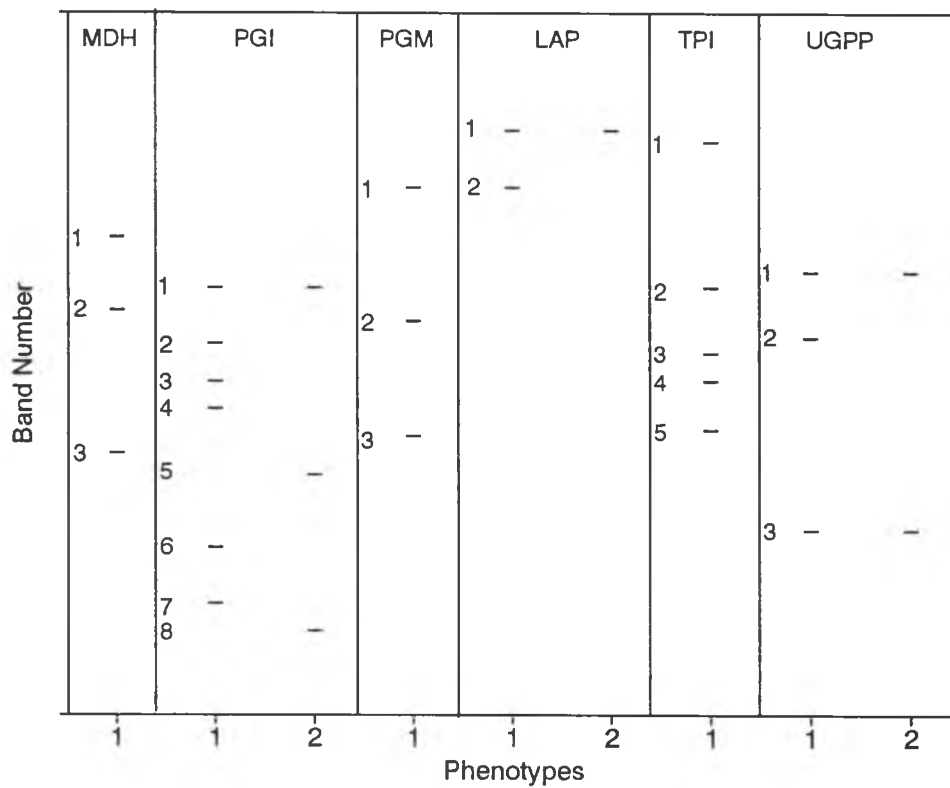

Figure 2.17. Schematic illustration of phenotypes of the six enzymes of *C. odontophyllum*.

DISCUSSION

Agreement Between Isozyme and Accession Records

Characterization of plants based on electrophoretic variations in isozyme has been a powerful technique to separate and classify genotypes in many species (Tanksley and Orton, 1983). Nixon and Taylor (1977) found that morphologically indistinguishable populations of flatworm were genetically quite similar, conversely, Avise and Smith (1977) found that groups of fishes that differed morphologically were genetically indistinguishable. The analysis of *Capsicum* taxa showed agreement between morphological and allozymic patterns in some cases, while in others there was little such agreement (Jensen et al., 1979).

No published report was found on isozyme electrophoresis of *Canarium*. In this study, plants from each species formed distinct group in the cluster analysis based on Jaccard's coefficient of similarity. *C. mehenbethene* formed a cluster with 0.8 similarity, *C. album* cluster with 0.87, *C. harveyi* with 0.9, *C. ovatum* with 0.5, *C. indicum* with 0.88 and *C. megalanthum* with 0.70. Different species groups showed very little similarity to each other, with less than 0.3. Two plants with uncertain introduction records were also identified, they had 0.75 similarity, and most resemble to *C. megalanthum* cluster at about 0.4. From the data we have, we were not able to say

to which species these 2 plants belong to. Plant T15(#39) was labelled as *C. harveyi* but it was not clustered with plants from this species. Sample #39 was more affiliated to the *C. indicum* cluster with about 0.5 similarity, which indicated it was a potentially mislabelled accession. Plant #51 is *Dacroydes rostrata*, it joined the big *Canarium* cluster at 0.15, and suggested this accession is very different from *Canarium* accessions.

Variation of phenotypes has long been used to distinguish different tree species, seedlots and clones (Rothe, 1990). Correlation between isozymic and morphological variations were seldom studied especially in forest tree crops (NRC, 1991). Parallel isozyme and morphological analyses were not conducted in this study, but agreement between variation in isozymic phenotypes and collection classification was confirmed. Isozyme electrophoresis can be used as an additional tool for the classification in *Canarium* species.

Phenotypic Polymorphism and Variation in *Canarium* Species

All six isozymes tested in this study were found to have considerable variation. The P_j over all the samples was very high, from 0.93 (MDH) to 0.75 (TPI), the average P_j was 0.86. It indicated that these six enzymes were very polymorphic and *Canarium* species showed a high genetic variability and diversity. When different species were

considered separately, the mean P_j was low, down to about 0.29. This was probably due to the limited plant sample sizes in some species.

Canarium species were quite diverse isozymically. The phenotypic polymorphism of these species were analyzed. All seven species are polymorphic. The diversity and differences between *Canarium* species were also observed in the isozyme phenotypes. The array of multiple isozyme phenotypes found in each species were distinctly different from other species. Due to limited numbers of samples in some species tested, it is difficult to draw a complete and comprehensive conclusion of the comparison between different species. Isozymic polymorphism not only existed among species, but also within species. The limited number of plants in each species did not allow for a comprehensive estimation of population diversity within species, but it does provide information on diversity within species. Among the 22 accessions in *C. ovatum,* the average P_j was 0.53. The Pj of *C. odontophyllum* and *C. indicum* were 0.25 and 0.19, even though there were only 2 and 3 accessions in these two species.

Some of the species share common phenotypes in certain enzyme systems. *C. ovatum* and *C. album* shared the common enzyme phenotype on PGM, *C. ovatum* and *C. indicum* shared the common phenotype on TPI, *C. harveyi* and *C. mehenbethene* shared the common phenotype on LAP. Some accessions in

certain species showed identical isozyme phenotypes over the six enzyme systems analyzed. These included: Four *C. mehenbethene* (# 10, 12, 13, 14), three *C. album* (#41, 42, 43), seven *C. ovatum* (#22 and 23; #26 and 28; #35, 36 and 37), two *C. harveyi* (#38 and 47) and two *C. megalanthum* (#1 and 3). These accessions were collected from the same source at the same time, and probably from the same mother plant.

Special Band for Identification of Different Species

Electrophoretic data have been a valuable supplement to classical approaches, based on morphological differences in clarifying species relationships, and they have been used in taxonomy in other crop genera including *Capsicum* (Mcleod et al., 1983), *Lycopersicon* (Rick and Fobes, 1975), *Zea* (Doebley et al., 1984) and *Pistacia* (Loukas and Pontikis, 1979).

In this study, intraspecific variation exists in all seven species, and due to the limited sample size, we were unable to generate a specific phenotype for each species. Some unique bands, however, could be used for the identification of certain species. Band 1 in UGPP was presented only in *C. ovatum*, but absent in other species. Other unique UGPP bands observed were: band 4 in *C. harveyi*, band 5 in *C. indicum*, band 9 in *C. megalanthum*, band 10 in *C. odontophyllum,* and band 12 in *C. album* (Figure 2.6). For

MDH, *C. mehenbethene* was the only species with band 15, *C. odontophyllum* with band 5 and the only species in this test that lacked band 10 (Figure 2.1). In PGM, band 7 is only present in *C. odontophyllum* (Figure 2.3). In TPI, bands 1, 6, and 7 were present in every phenotype of *C. mehenbethene* (Figure 2.5). These bands are probably helpful in the evaluation and identification of any future germplasm collected.

SUMMARY AND CONCLUSION

1) Histidine - citric acid (pH 6.5) buffer was the best buffer for isozyme electrophoresis of seven *Canarium* species using young leaf tissue. PGM, PGI, TPI, LAP, UGPP and MDH gave consistent bands with clear resolution. These enzyme systems are useful for the evaluation of phenetic variation of different *Canarium* species.

2) All six enzymes showed polymorphism. MDH gave the highest variation among all Canariums tested, PGM and TPI showed the lowest.

3) Each species has its own distinctive array of phenotypes with each enzyme. Some of the phenotypes in different species overlap. All seven species of *Canarium* showed polymorphism due to the coexistence of more than one phenotype. But the comparison between different species could not be made due to the large difference in sample sizes.

4) A cluster dendrogram was established based on similarity matrix with UPGMA. In most cases there is an agreement between collection identity results. This suggested that isozyme electrophoresis can be used as a reliable method for the classification of *Canarium* species.

5) Twenty-two plants of *Canarium ovatum* were tested and tentative band patterns and loci for this species were established.

6) In the enzyme systems, UGPP, PGI and MDH, there were unique bands associated with specific *Canarium* species, these tentatively identified bands, along with the results from this study, may be useful for the classification of *Canarium* species.

APPENDIX A

Tested isozyme, EC designation, quaternary structure and staining recipes of enzyme systems

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ACO (Aconitase EC 4.2.1.3, monomer)

Tris-HCl 0.1 M pH 8.0	10 ml
Distilled water	10 ml
1.0 M $MgCl_2$	1 ml
cis-aconitic acid	30 mg
Isocitrate dehydrogenase	3-4 units
NADP	1 ml
MTT	1 ml
PMS	1 ml
stain at 37°C in dark	

ADH (Alcohol dehydrogenase EC 1.1.1.1, dimer)

Tris HCl 0.1 M pH 8.0	10 ml
Ethanol 95%	10 ml
NAD	1 ml
MTT	1 ml
PMS	1 ml
stain at 37°C in dark	

EST (esterase, EC 3.1.1.2, monomer)

Na-phosphate 0.1 M pH 6.0	25 ml
@-Naphthyl acetate/acetone	25 mg
Fast blue RR salt	50 mg
stain at room temperature in dark	

G6PDH (Glucose-6-phosphate dehydrogenase 1.1.1.49, dimer)

Tris HCl 0.1 M pH 8.0	10 ml
Distilled Water	10 ml
$MgCl_2$	1 ml
D-glucose-6-phosphate	20 mg
NAD	1 ml
PMS	1 ml
MTT	1 ml
stain at 37°C in dark	

IDH (Isocitrate dehydrogenase EC 1.1.1.42, dimer)

Tris HCl 0.1M pH 8.0	10 ml
Distilled Water	10 ml
$MgCl_2$	1 ml
NADP	1 ml
DL isocitric acid	30 mg
MTT	1 ml
PMS	1 ml
stain at 37°C in dark	

LAP (Leucine aminopeptidase EC 3.4.11.1, monomer)

Tris-Maleate 0.2 M	20 ml
NaOH 0.2 M	8 ml
dH_2O	12 ml
L-leucine-B-naphthylamide(free base)	10 mg
Black K salt	10 mg

stain at room temperature

MDH (Malate dehydrogenase EC 1.1.1.37, dimer)

Tris-HCl 0.1 M pH 8.0	10 ml
dH_2O	10 ml
DL-malic acid 2.0 M pH 7.0	5 ml
NAD	1 ml
MTT	1 ml
PMS	1 ml

Stain at 37°C in dark

PER (peroxidase EC 1.11.1.7, monomer)

Sodium acetate buffer 0.05 M pH 5.0	40 ml
3-amino-9-ethylcarbazole (dissolved in dimethyl formamide)	30 mg
$CaCl_2$	1 ml
3 % H_2O_2	1 ml

stain at room temperature

6PGD (6-phosphogluconic dehydrogenase EC 1.1.1.44, dimer)

Tris-HCl 0.1 M pH 8.0	10 ml
dH_2O	10 ml
$MgCl_2$	1 ml
6-phospholuconic acid	50 mg
NADP	1 ml
PMS	1 ml
MTT	1 ml

stain at 37^0C in dark

PGI (Phosphoglucose isomerase EC 5.3.1.9 dimer)

Tris HCl 0.1 M pH 8.0	10 ml
dH_2O	10 ml
D-fructose-6phosphate, disodium salt	10 mg
$MgCl_2$	1 ml
NADP	1 ml
PMS	1 ml
MTT	1 ml
G6pdh	10 units

stain at 37°C in dark

PGM (phosphoglucomutase EC 2.7.5.1 monomer)

Tris HCl 0.1 M pH 8.0	10 ml
dH_2O	10 ml
D-glucose-1-phosphate, disodium	40 mg
$MgCl_2$	1 ml
NADP	1 ml

PMS	1 ml
MTT	1 ml
G6pdh	10 units
stain at 37°C in dark	

SKDH (shikimic dehydrogenase EC 1.1.1.25, monomer)

Tris HCl 0.1 M pH 8.0	10 ml
dH_2O	10 ml
Shikimic acid	100 mg
NADH	1 ml
PMS	1 ml
MTT	1 ml
stain at 37°C in dark	

TPI (triose-phosphate isomerase EC 5.3.1.1, dimer)

Tris-HCl 0.1 M pH 8.0	10 ml
dH_2O	10 ml
dihydrozyacetone phosphate	10 mg
EDTA	38 mg
Arsenate (sodium salt) 1.0M	1 ml
glyceraldehyde-3-P-dehydrogenase	2 drops
NAD	1 ml
PMS	1 ml
MTT	1 ml
stain at 37°C in dark	

UGPP (uridine diphosphoglucode pyrophosphorylase EC 2.7.7.9, monomer)

Tris-HCl 0.1 M pH 8.0	10 ml
dH_2O	10 ml
$MgCl_2$	1 ml
uridine diphosphoglucose	75 mg
pyriphophate	40 mg
G-1-6-diphosphate	2 grains
phosphoglucomutase (PGM)	2 drops
G6PDH	0.5 ml
NAD	1 ml
PMS	1 ml
MTT	1 ml
stain at 37°C in dark	

Stock and buffer solutions:

	Concentration
NAD (B-nicotinamide adenine dinucleotide)	10 mg/ml
NADP (B-nicotinamide adenine dinucleotide phosphate)	5 mg/ml
PMS (phenazine methosulfate)	1 mg/ml

MTT (3-(4,5,-dimethyl thiazolyl)-2,5-diphenyl tetrazolium bromide)	5 mg/ml water
G6PDH (glucose-6-phosphate dehydrogenase)	50 ug/ml
$MgCl_2$	1 M

Refrigerate all the above solutions except $MgCl_2$.

0.2 M Tris HCL buffer, pH 8.0 for 1 liter

Tris 24.2 g, adjust the pH to 8.0 with concentrated HCl.

Malic acid solution, pH 7.0 for 1 liter

DL-malic acid	134.1g
NaOH	80.0g
Distill water	1.0 liter

APPENDIX B

Observed different band frequencies of six enzymes in seven species

		mega.	indi.	mehe.	odon.	ova.	har.	alb.	total
MDH	1	5.88	0	0	0	0	0	0	5.88
	2	5.88	0	0	0	0	0	0	5.88
	3	5.88	5.88	15.7	0	0	11.8	9.8	49.1
	4	5.88	0	0	0	35.3	0	0	41.2
	5	0	0	0	3.92	0	0	0	3.92
	6	5.88	5.88	0	0	39.2	0	0	51.0
	7	0	0	0	0	7.84	0	0	7.84
	8	5.88	5.88	0	3.92	39.2	7.84	0	62.7
	9	5.88	0	15.7	0	7.84	0	0	29.4
	10	5.88	5.88	15.7	0	43.1	9.8	9.8	90.2
	11	5.88	0	0	3.92	37.3	9.8	9.8	66.7
	12	0	5.88	15.7	0	0	0	0	21.6
	13	5.88	5.88	15.7	0	15.7	0	0	43.2
	14	0	0	0	0	0	0	9.8	9.8
	15	0	0	15.7	0	0	0	0	15.7
	16	5.88	0	0	0	7.84	0	0	13.7
	17	0	0	0	0	7.84	0	0	7.84
	18	0	0	0	0	23.5	0	0	23.5
PGI	1	5.88	5.88	0	0	0	0	0	11.8
	2	0	0	15.7	0	19.6	0	0	35.3
	3	0	0	0	3.92	23.5	9.8	9.8	47.0
	4	0	0	0	1.96	0	0	0	1.96
	5	0	0	0	1.96	0	0	0	1.96
	6	0	0	0	1.96	0	0	0	1.96
	7	5.88	5.88	15.7	1.96	0	0	0	29.4
	8	5.88	0	3.92	1.96	43.1	9.8	9.8	74.5
	9	0	5.88	15.7	0	0	9.8	0	31.4
	10	5.88	0	1.96	1.96	0	9.8	9.8	29.4
	11	0	1.96	3.92	1.96	0	0	0	7.84
	12	0	5.88	13.7	0	0	0	9.8	29.4
PGM	1	0	5.88	15.7	0	0	0	0	21.6
	2	5.88	0	0	0	0	0	0	5.88
	3	0	5.88	15.7	3.92	25.5	0	5.88	56.9
	4	5.88	0	0	0	43.1	0	9.8	58.8
	5	5.88	0	15.7	3.92	43.1	9.8	9.8	88.2
	6	0	5.88	0	0	0	9.8	0	15.7
	7	0	0	0	3.92	0	0	0	3.92
	8	5.88	0	0	0	0	0	0	5.88
	9	5.88	0	0	0	0	0	0	5.88
LAP	1	5.88	0	0	0	0	0	3.92	9.8
	2	3.92	0	0	3.92	0	0	0	7.84
	3	0	0	0	1.96	0	0	9.8	11.8
	4	0	0	15.7	0	1.96	9.8	0	27.5

5	0	3.92	0	0	0	3.92	0	7.84
6	0	3.92	0	0	17.6	0	0	21.5
7	0	0	0	0	33.3	0	0	33.3
8	0	0	0	0	3.92	0	0	3.92
UGPP1	0	0	0	0	43.1	0	0	43.1
2	5.88	0	15.7	3.92	1.96	0	9.8	37.3
3	0	5.88	1.96	0	0	0	7.84	15.7
4	0	0	0	0	0	9.8	0	9.8
5	0	5.88	0	0	0	0	0	5.88
6	0	0	0	0	17.6	0	0	17.6
7	0	0	0	0	3.92	0	0	3.92
8	0	5.88	15.7	0	0	29.4	9.8	60.8
9	5.88	0	0	0	0	0	0	5.88
10	0	0	0	3.92	0	0	0	3.92
11	0	0	0	0	0	0	7.84	7.84
12	0	0	0	0	0	0	9.8	9.8
TPI 1	0	0	15.7	3.92	0	0	0	19.6
2	5.88	5.88	0	0	1.96	9.8	9.8	33.3
3	5.88	0	0	3.92	1.96	0	9.8	21.6
4	0	0	0	0	0	0	9.8	9.8
5	5.88	0	0	3.92	0	0	0	9.8
6	0	3.92	15.7	3.92	0	0	9.8	33.3
7	0	0	15.7	0	0	9.8	9.8	35.3
8	0	5.88	0	3.92	0	0	0	9.8
9	0	0	13.7	0	0	0	9.8	23.5
10	0	0	13.7	0	0	0	9.8	23.5
11	0	0	9.8	0	0	0	0	9.8

APPENDIX C

Observed isozyme phenotypes of the six enzymes for the 53 accessions

No.	ID#	Phenotypes						Species
		MDH	PGI	PGM	LAP	UGPP	TPI	
1	T1A	A	A	A	A	A	A	
2	T1B	A	A	A	B	A	A	C. megalanthum
3	T1C	A	A	A	A	A	A	
50	No.6	V	N	A	A	A	A	
4	T3A	C	B	B	C	B	B	
5	T3B	C	B	B	D	B	B	C. indicum
6	T3C	C	B	B	E	B	B	
7	T2	B	D	C	F	C	C	
8	T7	B	F	C	F	C	C	
9	T7A	B	E	C	F	C	D	
10	T17A	B	F	C	F	C	E	C. mehenbethene
11	T17B	B	E	C	F	C	E	
12	T17C	B	F	C	F	C	E	
13	T17D	B	F	C	F	C	E	
14	T17E	B	F	C	F	C	E	
15	T5	D	G	D	G	D	F	C. odontophyllum
49	R11T11	D	M	D	R	O	F	
16	T4	E	H	E	H	E	B	
17	T4A	F	H	E	I	G	B	
18	T4B	G	H	E	J	H	B	
19	T4C	H	H	F	J	H	B	
20	T4D	I	H	E	J	G	B	
21	T6	J	H	F	K	I	B	
22	T9A	K	I	E	J	G	B	
23	T9B	K	I	E	J	G	B	
24	T9C	L	I	F	J	I	B	
25	T9D	M	I	F	J	I	B	
26	T10A	K	H	F	J	G	B	C. ovatum
27	T10B	K	H	E	J	H	B	
28	T10C	K	H	F	J	G	B	
29	T11	N	H	F	L	I	G	
30	T12A	O	I	F	H	I	B	
31	T12B	P	I	E	K	I	B	
32	T12C	P	I	F	K	I	B	
33	T12D	Q	I	E	H	I	B	
34	T13A	R	I	F	M	I	B	
35	T13B	R	I	F	K	I	B	
36	T13C	R	I	F	K	I	B	
37	T13D	R	I	F	K	I	B	

38	T14	S	J	G	N	J	H	
39	T15	T	K	H	O	K	I	
45	R11T1	W	J	G	N	J	H	C. harveyi
46	R11T5	S	J	G	F	J	H	
47	R11T13	S	J	G	N	J	H	
48	T20A	W	J	G	F	J	H	
40	T18A	U	L	E	P	L	J	
41	T18B	U	L	F	Q	M	J	
42	T18C	U	L	F	Q	M	J	C. album
43	T18D	U	L	F	P	M	J	
44	T18E	U	L	F	Q	M	J	
51	R11T3	Z	O	K	R	P	L	Dacroydes rostrata
52	2A	X	B	I	Q	A	K	unknown
53	2B	Y	D	J	G	A	K	unknown

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