

## Short Communication: Variation in isozymic pattern of germplasm from three ginger (*Zingiber officinale*) varieties

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**Abstract.** Setyawan AD, Wiryanto, Suranto, Bermawie N. 2014. Variation in isozymic pattern of germplasm from three of ginger (*Zingiber officinale*) varieties. *Nusantara Bioscience* 6: 86-93. Ginger (*Zingiber officinale* Rosc.) has long been as spices, flavoring agent and raw material for herbal medicines. In Indonesia, there were three varieties based on color and size of the rhizome, i.e. gajah (big-white ginger), merah (red ginger), and emprit (small-white ginger). This research was conducted to find out: (i) isozymic pattern of three ginger varieties, and (ii) phylogenetic relationship of those three varieties. The plant materials were gathered from Wonogiri, Surakarta and Kulonprogo, Yogyakarta. Two enzyme systems, namely esterase (EST) and peroxidase (PER, PRX) were used in this study. The relationship among ginger varieties was determined by UPGMA. The result indicated that EST showed two bands (i.e. Rf 0.04 and 0.10), and PRX showed six bands (i.e. Rf 0.04, 0.05, 0.09, 0.10, 0.11, and 0.15). Peroxidase produce more numerous and more diverse isozymic bands than esterase, resulting in a more complex relationship. The data used to compile dendrogram affect the grouping; the more data used, the more obvious clustering of accessions in a population. Dendrogram generated from esterase and peroxidase banding patterns produced distinct clusters based on varieties and location.

**Key words:** Ginger, isozyme, varieties, *Zingiber officinale*, Zingiberaceae

### INTRODUCTION

Ginger (*Zingiber officinale* Rosc.), is one of the most important and oldest spices a well-known plant in the Nusantara archipelago, consumed as a delicacy, food preservation, medicine, or spice (de Padua et al. 1999). Its highest value is given by the pungent and aromatic essential oils that produced throughout the plant, especially the rhizomes. The essential oils are responsible for the aroma while the non-volatile components are responsible for the pungency with gingerol the most pungent component in fresh ginger (Juliani et al. 2007). This herbaceous rhizomatous perennial reed-like plant with annual leafy stems is most useful Zingiberaceae in the tropics, as well as turmeric, cardamom and galangal (Poth and Sauer 2000).

The Zingiberaceae consist of approximately 47 genera and 1400 species, that are found in all tropical regions, but are mostly concentrated in Southeast Asia (Purseglove 1972; Kress 1990; de Padua et al. 1999). This spice is produced from the rhizome (underground stem) of the plant (Purseglove 1981). Distribution and use of ginger in Indonesia are very widespread (Burkill 1935; Heyne 1950). The aromatic and spicy rhizome is used as a spice, seasoning, and medicine (Heyne 1950; Holttum 1950), and the less spicy rhizome was used for food and beverages.

*Zingiber officinale* has three varieties based on size and color of rhizome, i.e. *Z. officinale* var. *officinale* (big white ginger or giant ginger, *badak* or *gajah*), *Z. officinale* var. *amarum* (small white ginger, *emprit*), and *Z. officinale* var. *rubrum* (small red ginger, *merah* or *berem*) (Ochse 1931;

Burkill 1935; Heyne 1950). These three varieties may partly be deferred from their essential oil contents and are used for different purposes. The essential oil content of the big white ginger is the lowest compared with the other varieties (Setyawan 2002). The big white ginger is usually used for fresh (green) ginger products, food and beverages, while the other gingers are mostly used for medicinal purposes.

Ginger produces clusters of white and pink flower buds that bloom into yellow flowers (Watt and Breyer-Brandwijk 1962). There are no differences in flower characters between varieties of gingers; therefore it is necessary to use other characters. In agriculture, the proper identification and characterization of varieties of the cultivated plant is needed to obtain the right herbal material used to develop products of high economic value. It is also necessary for plant breeding as marker assisted selection. Genetic markers are needed to trace the genetic nature of the parent and its descendants.

The assessment of genetic variation may be done by using various techniques such as isozymes or DNA analysis (Mondini et al. 2009). Isozymic pattern is a highly effective genetic marker that can be obtained through the quick and easy laboratory process, and less expensive than the DNA sequences. It can be used to determine genetic variation of populations, even to accession genetic differences (Crawford 1990). Isozyme can be used as a genetic trait to study the diversity of accessions in a population, classification of plant species, varieties and identifying of hybrid (Beer et al. 1993; Murphy and

Phillips 1993), as well as markers of plant resistance to certain diseases (Alcazar et al. 1995).

Isozyme is a codominant character; heterozygous accessions can be distinguished with homozygous (McDonald and McDermont 1993). However, the isozyme patterns may be affected by different environments and the stage of plant development. Moreover, only limited numbers of isozymes loci are available for certain taxa (McDonald and McDermont 1993; Mangolin et al. 1997; Garkava et al. 2000). Isozyme variations only reflect difference in protein-coding sequences or intron (Adam 1999; Sharma and Jana 2002).

Research on ginger diversity by using isozymic pattern has been done by Shamita (1997) irrespective of varieties, while the same research on the other Zingiberaceae family has been conducted on *Curcuma* (Ibrahim et al. 1991; Apavatjirut et al. 1999; Chokthaweeapanich and Paisooksantivatana 2003; Tang et al. 2008; Deng et al. 2011), *Curcuma alismatifolia* (Paisooksantivatana 2001), *Curcuma longa* (Shamina et al. 1998), *Curcuma xanthorrhiza* (Azizah 2011), *Amomum* (Shanmugapriya and Prabha 2012), *Boesenbergia* (Vanijajiva et al. 2003), *Hedychium spicatum* (Jugran et al. 2011) and several species of Zingiberaceae (Ibrahim et al. 1996).

The aim of this study was to determine (i) the variation of isozymic pattern of ginger (*Zingiber officinale* Rosc.) from three different varieties from two different populations, Wonogiri and Kulonprogo, Indonesia, differentiated by color of rhizomal peels and size (small red, small white, and big white) and (ii) the genetic relationship between populations based isozymic pattern.

## Materials and methods

**Plants material.** Three different varieties of ginger (*Zingiber officinale* Rosc.), i.e. big white, small white, and small red gingers (Figure 1), were collected from the dry fields in Eromoko, Wonogiri, Central Java and Kokap, Kulonprogo, Yogyakarta, Indonesia, each location with three varieties and for each variety three accessions were collected. The rhizome was harvested at the end of the dry season at the age of about 9-10 months and stored for 2-3 months before investigated. The plant materials were authenticated at the Department of Biology, Sebelas Maret University, Surakarta, Indonesia. Enzyme dye systems

were esterase (EST) and peroxidase (PER, PRX), and separated on a polyacrylamide gel.

**Procedures.** The electrophoresis procedure refers to Crawford (1990) and Weeden and Wendel (1989), and modified by Suranto (1991).

**Planting of rhizomes.** The rhizome was placed in a plastic tray lined with wet paper or cloth to keep moisture, until the leaves grow along 2-5 mm. Shoots were cut and immediately used for examining, or could be stored in a refrigerator at 4°C for maximum 14 days, but effectively used within seven days after cutting. Leaf extracts that stored in a refrigerator at 4°C can survive for 30 days.

**Buffer.** Tank buffer was made by dissolving 14.4 g of boric acid and 31.5 g of borax (sodium borate), in distilled water to a volume of 2 L. Extraction buffer was made by dissolving 0.018 g of cysteine, 0.021 g of ascorbic acid, and 5 g of sucrose (PA) in 20 mL of borax buffer pH 8.4. Running buffer was TAE (Tris-Acetic Acid-EDTA) 50x diluted to a concentration of 1x.

**Preparation of gel.** *First stock solution:* 27.2 g Tris and 0.6 g sodium dodecyl sulfate (SDS) dissolved in 120 mL of distilled water, adjusted to pH 8.8 by adding HCl, then added distilled water up to 150 mL. *Second stock solution:* 9.08 g Tris and 0.6 g SDS dissolved in 140 mL of distilled water, adjusted to pH 6.8 to 7.0 by adding HCl, then added distilled water up to 150 mL. *Third stock solution:* 175.2 g of acrylamide and 4.8 g bis-acrylamide dissolved in 400 mL of distilled water and then make up to 600 mL. *Loading dye:* 250 uL of glycerol and 50 uL bromphenol blue (BPB) dissolved in 200 uL of distilled water. *Separating gel:* 3.15 mL of the 1st stock solution and 5.25 mL of the 2nd stock solution, added to 4.15 mL of distilled water, 5 uL of TEMED, and 10 uL of APS 10% (new). The mixed solution was poured into the mold, then added with saturated isobutanol. When the gel was formed (~45 minutes), saturated isobutanol was absorbed by blotting paper. *Stacking gel:* 1.9 mL of the 2nd stock solution and 1.15 mL of the 3rd stock solution, added to 4.5 mL of distilled water, 5 uL TEMED, and 10 uL APS 10% (new). Stacking gel was poured above the separation gel, fitted with a comb to make wells that released after gel formation. The formed gel was transferred into the clamping frame and put in a buffer tank, then filled with running buffer until submerged.



**Figure 1.** Rhizome of ginger varieties. A. Small red ginger, B. Small white ginger, C. Big white ginger. (photo: Anna Frodesiak)

**Extraction.** Fresh leaf tissue was put in the extraction buffer, with a ratio of 1:4 (w/v), i.e. 68 ug (0.068 g) of leaf samples were pulverized in 272 uL (0.272 mL) of extraction buffer. Crushed in a porcelain dish that was placed above ice crystals, to keep cold (4°C). Samples were centrifuged at 8500rpm for 20 minutes at 4°C, then soaked in ice crystals. Supernatant was put in the wells of the gel.

**Electrophoresis.** 3.5 uL supernatant was pipetted with a micro pipette, added with loading dye and sample loading guide, and then placed in the wells. The electrophoresis were undertaken at 200 volts, 60 mA for 5 minutes to reach the separating gel, and followed by further electrophoresis at 150V, 400 mA, for 60 minutes, until the loading dye reaches ~56 mm from the wells toward anode. The gel was transferred into a plastic tray and colored with enzyme dyes.

**Staining.** *Peroxidase:* 0.0125g of O-Dianisidine put into erlenmeyer and dissolved in 2.5 mL of acetone, and then added to 50 mL of 0.2M acetate buffer pH 4.5 and two drops of H<sub>2</sub>O<sub>2</sub>. *Extras:* 0.0125g of  $\alpha$ -Naphthyl acetate put into erlenmeyer and dissolved in 2.5 mL of acetone, and then added to 50 mL of 0.2M phosphate buffer pH 6.5 and 0.0125 g Fast Blue BB Salt. Separately, the gel was soaked into that solution for 10 minutes and shaken gently every 2 minutes. Once the banding pattern appears, the enzyme dye was disposed and rinsed with distilled water, then recorded by a camera or scanner.

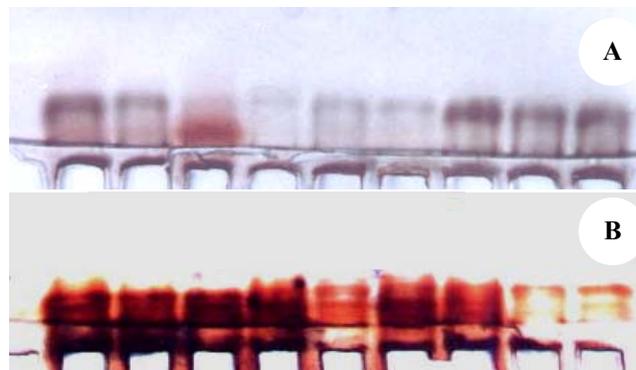
**Data analysis.** Data were analyzed qualitatively based on the presence or absence of bands. Only clear, unambiguous and reproducible bands were considered for data analysis. Rf (*retardation factor*) value was calculated based on the relative movement of isozyme and loading dye. The present bands were given a value of 1, while those absence bands were given a value of 0. Data were entered in a spreadsheet to create a binary matrix. The genetic similarity and genetic distance among the accessions was calculated according to Jaccard coefficients (Jaccard 1908). The matrices were computed and the corresponding dendrograms of genetic relatedness were constructed by applying the unweighted pair group method with arithmetic mean (UPGMA) clustering algorithm (Sneath and Sokal 1973), using the Dendro-UPGMA program (Garcia-Vallvé and Puigbo 1999; Garcia-Vallve et al. 2002).

## Results and discussion

### Variation of isozymic pattern

Ginger samples used in this study had a relatively uniform condition; they were all harvested at maturity (9-10 months), stored for 2-3 months, and then planted to get the shoot tip. Uniformity stage of plant development is very important to ensure similar types of enzymes produced. Physiological developmental stages of an accession can produce different types of enzymes, as observed in the stages of embryogenesis (Bapat et al. 1992; Rout and Das 1995; Dodeman and Ducreux 1996), fruit development (Loveless 1992; Sadka et al. 2000), and colorizing plants (Barrett and Shore 1989). Isozyme pattern may also change in response to environmental condition, which followed by genetic material changes (Kahler et al. 1980; Mastenbroek et al. 1984; Guse et al. 1988; El-Bendary et al. 1998; Li Z and Rutger 2000; Gämperle and Schneller 2002; Oja 2002;

Staszak et al. 2007; Zhang et al. 2009; Dasgupta et al. 2010; Shah and Nahakpam 2012). Figure 2 showed examples of zimogram that demonstrate the diversity of ginger by esterase and peroxidase banding pattern.



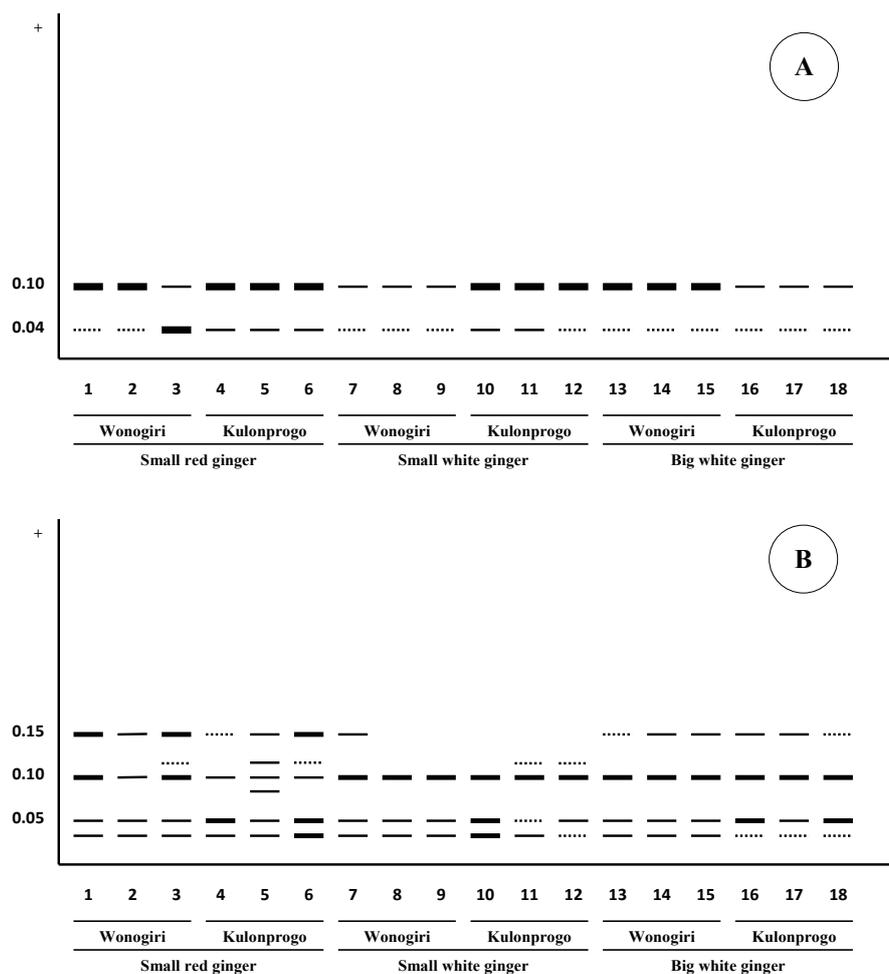
**Figure 2.** Zimogram of ginger using enzyme staining systems of esterase (A) and peroxidase (B).

**Esterase (EST).** Esterases (EC no. 3.1.x) are hydrolyzing enzymes which catalyze the addition or removal of water in biological reactions by splitting esters into acids and alcohols in a chemical reaction with water that called hydrolysis. Variation in esterases banding patterns exists due to differences in substrate specificity, protein structure and biological function. Wide variation in esterases are reported in nature and occurs frequently in plants (IUBMB 1992).

In this observation, there were not many variations of esterase banding pattern among different ginger cultivars. Esterase isozyme indicated only two bands EST-1 located at Rf 0.10 and EST-2 located at Rf 0.04 from anodal zone, with purple-blue to red color (Figure 3.A). Some bands are fairly thick, but there are also thin bands or very thin, thus overlooked in the research. The difference of isozyme banding thickness is probably due to the differences in the copy number of the gene. On the other hand, a thick band may also be caused by two bands coincide, which indicates heterozygote for two alleles of the monomer, and a thin band indicating homozygote, however, esterase generally have thick bands.

The combination of the presence esterase isozymic banding produced four variations of the 18th accessions tested. In accession 3, a small red ginger of Wonogiri, a band of EST-1 at Rf 0.04 was very thick, while in the other accessions was very thin or absent. The existence of unique band was very valuable as a marker for identification of the accession and useful in conservation biology. Perhaps the difference in staining intensity may be related to certain agronomic important traits, but this must be confirmed.

On the other ginger family, esterases are also generating a little variation in isozymic banding patterns. Application of esterase on some species of *Curcuma* only produced 1-4 bands depending on the species (Apavatjirut et al. 1999), while in some species of *Amomum* this enzyme raised only one band, and could not show the variation among species (Shanmugapriya and Prabha 2012). The limited number of banding patterns caused low variation among populations.



**Figure 3.** Isozymic banding pattern of ginger varieties. A. Esterase, B. Peroxidase. Note: 1,2,3. Small red ginger of Wonogiri; 4,5,6. Small red ginger of Kulonprogo; 7,8,9 Small white ginger of Wonogiri; 10,11,12. Small red ginger of Kulonprogo; 13,14,15. Big white ginger of Wonogiri; 16,17,18. Big white ginger of Kulonprogo.

The results showed that among the three accessions in one population (the same varieties and locations), two or all three have in common esterase isozymic pattern, but the same pattern can also be found precisely in accessions of different varieties and locations. It was shown that there was ginger uniting each other based on the location, i.e. Wonogiri and Kulonprogo or on the variety, i.e. big white ginger, small white ginger, and small red ginger. But this tendency was disguised by grouping of different location and a variety. A small number of band pattern emerged and it supported this complication. This is most likely because it was taken from the same location, and ginger is always propagated vegetatively thus genetically similar. It might be caused by the history of evolution of the three gingers. They were made up divergently, thus they changed each other genetic code, or the emerging morphological difference might be being coded by genetic material, which has no relation with esterase enzyme.

**Peroxidase (PER, PRX).** Peroxidases (EC no. 1.11.1.x) are a large family of enzymes that typically catalyze a reaction of  $ROOR' + \text{electron donor} (2 e^-) + 2H^+$

$\rightarrow ROH + R'OH$ . For many of these enzymes the optimal substrate is hydrogen peroxide, but others are more active with organic hydroperoxides such as lipid peroxides. Peroxidases can contain a heme cofactor in their active sites, or alternately redox-active cysteine or seleno-cysteine residues (IUBMB 1992).

In this study, peroxidase indicated six isozymic bands, PRX-1 at Rf 0.04, PRX-2 at Rf 0.05, PRX-3 at Rf 0.09, PRX-4 at 0.10, PRX-5 at Rf 0.11, and PRX-6 at Rf 0.15 with red color (Figure 3.B). Of the 18 accessions tested, as many as 8 variations of the isozyme banding pattern appear; hence the character is very valuable as distinguishing evidence. Two unique bands were found in accession number 5 (small red ginger of Kulonprogo) at Rf 0.09, and 0.11. The band at Rf 0.9 was only found in accession number 5, while the band at Rf 0.11 was also detected in accession number 3, 6 (small red ginger), 11 and 12 (small white ginger). These bands were not detected in the other accessions. The band at Rf 0.9 can be used as genetic markers for accession 3. Peroxidase created many variations of isozymic banding patterns. In higher plants, peroxidase has a wide distribution and exhibit broad substrate specificity. On

other ginger family, namely *Amomum*, peroxides are generating four banding patterns. It is much more than esterase that creating one band only (Shanmugapriya and Prabha 2012).

The presence of many peroxides patterns caused more varied relationship. The isozyme can be used to test genetic variability between accessions within a species. It caused accession of the same population, i.e. similar in variety and planting location, able to have a different isozymic pattern, thus the relationship would be in a different situation, even though there could be a tendency that the same accessions of a same population tend to have a closer relationship than accession of different population.

#### Phylogenetic relationship

**Esterase (EST).** The esterase only generated two isozymic bands at Rf 0.04 and 0.10. Variations between populations are very little, hanya terdapat dua cluster. In dendrogram, many accessions join at the similarity index up to 100%, although different varieties and locations of

origin. Except for accession 3, all accessions of ginger of Wonogiri, both small red ginger (1,2), small white ginger (7, 8, 9), and big white ginger (13,14,15), and also one accession of small white ginger of Kulonprogo (12) and big white ginger of Kulonprogo (16, 17, 18) join with similarity index up to 100%, although these populations are from different varieties and locations of origin.

Other accession joins a cluster with a similarity index of up to 100%, i.e. one small red ginger of Wonogiri (3), all accessions of small red ginger of Wonogiri (4,5,6) and two small white ginger of Kulonprogo (10, 11). In this cluster, some varieties the same accessions join, but a different site of origin, i.e. small red ginger of Wonogiri (3) and of Kulonprogo (4,5,6). There is also the accession of the same origin, but different varieties, i.e. all small red ginger (4,5,6) and two small white ginger (10,11) of Kulonprogo. However, ginger that derived from the same varieties and location generally have a very strong relationship with similarity index up to 100%, for example, all populations of the small red ginger of Kulonprogo (4, 5, 6), big white ginger of Wonogiri (13, 14, 15), small white ginger of Wonogiri (7, 8, 9), and big white ginger of Kulonprogo (16,17,18) (Table 1A; Figure 4.A).

The dendrogram showed that among the three accessions of ginger in one variety and one location, the two or all of them have a same esterase banding pattern up to 100%, but a same pattern could be found precisely with similarity index up to 100% on accession from different variety and location (Figure 4.A). Since, esterase only generate two isozymic banding patterns, many different varieties and location of ginger joint together in a cluster, that less reflects the grouping based on variety and location.

**Peroxidase (PER, PRX).** The peroxidase isozyme generated six bands with Rf value of 0.04, 0.05, 0.09, 0.10, 0.11, and 0.15. Peroxidase has much more variation of isozymic pattern. Relationship dendrogram shows that there are only two independent ginger accessions, where the similarity index with the other accession less than 100%, i.e. the accession small red ginger of Kulonprogo (5) and the accession small white ginger of Kulonprogo (11). Meanwhile, other accessions have in similarity index up to 100% with one or more other accessions. There is a large cluster with similarity index 100% consisting of various varieties of ginger, i.e. three accessions small red ginger of Wonogiri (1, 2, 3) and one of Kulonprogo (6), one accessions small red ginger of Wonogiri (7) and two accessions small red ginger of Kulonprogo (14, 15).

There is also a cluster with % similarity index 100 that contains one accession small red ginger of Kulonprogo (4), two accessions small white ginger of Wonogiri (8, 9), one accession small red ginger of Kulonprogo (10), and one accession big accession white ginger of Wonogiri (13). Meanwhile, two ginger accessions of Kulonprogo from different varieties have up to similarity index 100%, i.e. one accession small white ginger (12) and one accession of big white ginger (18). On the other hand, two big white ginger accessions of Kulonprogo (16, 17) form a separate cluster.

Those accessions are generally still joined in one group, although one of them is a separate accession. Population ginger that still collects all members in the group with

similarity index 100% are the small red ginger of Wonogiri (1,2,3). Meanwhile, on the other population, there are only two accessions clustered together with similarity index 100%, while the others are separated, for example, small red ginger of Wonogiri (1,2, 3), small white ginger of Wonogiri (8,9), the big white ginger of Wonogiri (14,15), and big white ginger of Kulonprogo (16,17) (Table 1.B; Figure 4.B).

Grouping generally occurs in accessions that have same varieties and locations of origin, but it may also occur in accessions that have different varieties and locations of origin. This because, ginger generally propagated vegetatively and have close relationships, although differ on varieties or locations of origin. Meanwhile, the existence of stand alone accessions, i.e. the accession small red ginger of Kulonprogo (5) and the accession small white ginger of Kulonprogo (11), shows the existence of evolutionary process, i.e. changes in the genetic composition of vegetative propagation of ginger. This mutation is important for survival against environmental changes.

**Combination of esterase and peroxidase.** The dendrogram, which based on the combination of esterase and peroxidase markers, is clearer in showing the grouping based on its variety, locations of origin or both. Grouping based on location can be found either in ginger population of Wonogiri or Kulonprogo. In Wonogiri, two accessions red gingers (1, 2), one accession small white gingers clustered with two accessions big white gingers (14, 15) with similarity index 100%. Meanwhile, two small red ginger accessions from different locations, i.e. Wonogiri (3) and Kulonprogo (6) is also clustered with the similarity index 100%. Finally, the two groups joined.

In Kulonprogo, the accession small white ginger (12) and big white ginger (18) which has similarity index 100%, and the two accessions big white ginger (16, 17) which also has similarity index 100% joined in one cluster. On the other hand, two accessions of different variety are also clustered with the similarity index 100%, i.e. a small red ginger (4) and a small white ginger (10). In Wonogiri, two accessions small white ginger (8, 9) and one accession big white ginger (13) also integrates with similarity index 100%. Finally, the two groups joined (Table 2; Figure 4.C).

Relationship dendrogram shows that the more data that is used as a distinguishing character, the clearer the grouping based on the variety and growing location. On esterase-based dendrogram, it appears that the groupings based on population is less reflected, while on peroxidase-based dendrogram, the presence of grouping in population is more obvious. In the dendrogram based on the combined data of esterase and peroxidase, the population with which the members fall into one group is increasing.

Meanwhile, as in peroxidase-based dendrogram, it is shown that in the population, the two members congregate but the other one apart. In the combined dendrogram accession 5 and 11, each of them tends to stand alone. The existence of such accessions is very valuable for plant breeding, because of these differences reflect a typical mutation, making it very useful in adapting to the climate and environment changes, such as global warming and land use change (Table 2).

**Table 1.** Matrix of similarity computed with Jaccard coefficient on three varieties of ginger from Wonogiri and Kulonprogo, Indonesia based on esterase and peroxidase isozymic pattern.

																			A. Similarity matrix of esterase banding pattern																		
																			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
1	1	1.000	0.500	0.500	0.500	0.500	1.000	1.000	1.000	0.500	0.500	1.000	1.000	1.000	1.000	1.000	1.000	1.000																			
2	1.000	1	0.500	0.500	0.500	0.500	1.000	1.000	1.000	0.500	0.500	1.000	1.000	1.000	1.000	1.000	1.000	1.000																			
3	1.000	1.000	1	1.000	1.000	1.000	0.500	0.500	0.500	1.000	1.000	0.500	0.500	0.500	0.500	0.500	0.500	0.500																			
4	0.750	0.750	0.750	1	1.000	1.000	0.500	0.500	0.500	1.000	1.000	0.500	0.500	0.500	0.500	0.500	0.500	0.500																			
5	0.667	0.667	0.667	0.500	1	1.000	0.500	0.500	0.500	1.000	1.000	0.500	0.500	0.500	0.500	0.500	0.500	0.500																			
6	1.000	1.000	1.000	0.750	0.667	1	0.500	0.500	0.500	1.000	1.000	0.500	0.500	0.500	0.500	0.500	0.500	0.500																			
7	1.000	1.000	1.000	0.750	0.667	1.000	1	1.000	1.000	0.500	0.500	1.000	1.000	1.000	1.000	1.000	1.000	1.000																			
8	0.750	0.750	0.750	1.000	0.500	0.750	0.750	1	1.000	0.500	0.500	1.000	1.000	1.000	1.000	1.000	1.000	1.000																			
9	0.750	0.750	0.750	1.000	0.500	0.750	0.750	1.000	1	0.500	0.500	1.000	1.000	1.000	1.000	1.000	1.000	1.000																			
10	0.750	0.750	0.750	1.000	0.500	0.750	0.750	1.000	1.000	1	1.000	0.500	0.500	0.500	0.500	0.500	0.500	0.500																			
11	0.500	0.500	0.500	0.667	0.333	0.500	0.500	0.667	0.667	0.667	1	0.500	0.500	0.500	0.500	0.500	0.500	0.500																			
12	0.500	0.500	0.500	0.667	0.333	0.500	0.500	0.667	0.667	0.333	1	1.000	1.000	1.000	1.000	1.000	1.000	1.000																			
13	0.750	0.750	0.750	1.000	0.500	0.750	0.750	1.000	1.000	1.000	0.667	0.667	1	1.000	1.000	1.000	1.000	1.000																			
14	1.000	1.000	1.000	0.750	0.667	1.000	1.000	0.750	0.750	0.750	0.500	0.500	0.750	1	1.000	1.000	1.000	1.000																			
15	1.000	1.000	1.000	0.750	0.667	1.000	1.000	0.750	0.750	0.750	0.500	0.500	0.750	1.000	1	1.000	1.000	1.000																			
16	0.750	0.750	0.750	0.500	0.500	0.750	0.750	0.500	0.500	0.500	0.250	0.667	0.500	0.750	0.750	1	1.000	1.000																			
17	0.750	0.750	0.750	0.500	0.500	0.750	0.750	0.500	0.500	0.500	0.250	0.667	0.500	0.750	0.750	1.000	1	1.000																			
18	0.500	0.500	0.500	0.667	0.333	0.500	0.667	0.667	0.667	0.333	1.000	0.667	0.500	0.667	0.500	0.667	0.667	1																			

B. Similarity matrix of peroxidase banding pattern

Note: 1,2,3. Small red ginger of Wonogiri; 4,5,6. Small red ginger of Kulonprogo; 7,8,9 Small white ginger of Wonogiri; 10,11,12. Small white ginger of Kulonprogo; 13,14,15. Big white ginger of Wonogiri; 16,17,18. Big white ginger of Kulonprogo.

**Table 2.** Matrix of similarity and distance computed with Jaccard coefficient on three varieties of ginger from Wonogiri and Kulonprogo, Indonesia based on a combination of isozymic pattern of esterase and peroxidase.

																			Similarity matrix computed with Jaccard coefficient																		
																			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	
1		1.000	0.833	0.667	0.625	0.833	1.000	0.800	0.800	0.667	0.500	0.600	0.800	1.000	1.000	0.800	0.800	0.600																			
2	0.000		0.833	0.667	0.625	0.833	1.000	0.800	0.800	0.667	0.500	0.600	0.800	1.000	1.000	0.800	0.800	0.600																			
3	0.167	0.167		0.833	0.750	1.000	0.833	0.667	0.667	0.833	0.667	0.500	0.667	0.833	0.833	0.667	0.667	0.500																			
4	0.333	0.333	0.167		0.625	0.833	0.667	0.800	0.800	1.000	0.800	0.600	0.800	0.667	0.667	0.500	0.500	0.600																			
5	0.375	0.375	0.250	0.375		0.750	0.625	0.500	0.500	0.625	0.500	0.375	0.500	0.625	0.625	0.500	0.500	0.375																			
6	0.167	0.167	0.000	0.167	0.250		0.833	0.667	0.667	0.833	0.667	0.500	0.667	0.833	0.833	0.667	0.667	0.500																			
7	0.000	0.000	0.167	0.333	0.375	0.167		0.800	0.800	0.667	0.500	0.600	0.800	1.000	1.000	0.800	0.800	0.600																			
8	0.200	0.200	0.333	0.200	0.500	0.333	0.200		1.000	0.800	0.600	0.750	1.000	0.800	0.800	0.600	0.600	0.750																			
9	0.200	0.200	0.333	0.200	0.500	0.333	0.200	0.000		0.800	0.600	0.750	1.000	0.800	0.800	0.600	0.600	0.750																			
10	0.333	0.333	0.167	0.000	0.375	0.167	0.333	0.200	0.200		0.800	0.600	0.800	0.667	0.667	0.500	0.500	0.600																			
11	0.500	0.500	0.333	0.200	0.500	0.333	0.500	0.400	0.400	0.200		0.400	0.600	0.500	0.500	0.333	0.333	0.400																			
12	0.400	0.400	0.500	0.400	0.625	0.500	0.400	0.250	0.250	0.400	0.600		0.750	0.600	0.600	0.750	0.750	1.000																			
13	0.200	0.200	0.333	0.200	0.500	0.333	0.200	0.000	0.000	0.200	0.400	0.250		0.800	0.800	0.600	0.600	0.750																			
14	0.000	0.000	0.167	0.333	0.375	0.167	0.000	0.200	0.200	0.333	0.500	0.400	0.200		1.000	1.000	0.800	0.800																			
15	0.000	0.000	0.167	0.333	0.375	0.167	0.000	0.200	0.200	0.333	0.500	0.400	0.200	0.000		0.800	0.800	0.600																			
16	0.200	0.200	0.333	0.500	0.500	0.333	0.200	0.400	0.400	0.500	0.667	0.250	0.400	0.200	0.200		1.000	0.750																			
17	0.200	0.200	0.333	0.500	0.500	0.333	0.200	0.400	0.400	0.500	0.667	0.250	0.400	0.200	0.200	0.000		0.750																			
18	0.400	0.400	0.500	0.400	0.625	0.500	0.400	0.250	0.250	0.400	0.600	0.000	0.250	0.400	0.400	0.250	0.250																				

Distance matrix based on Jaccard coefficient

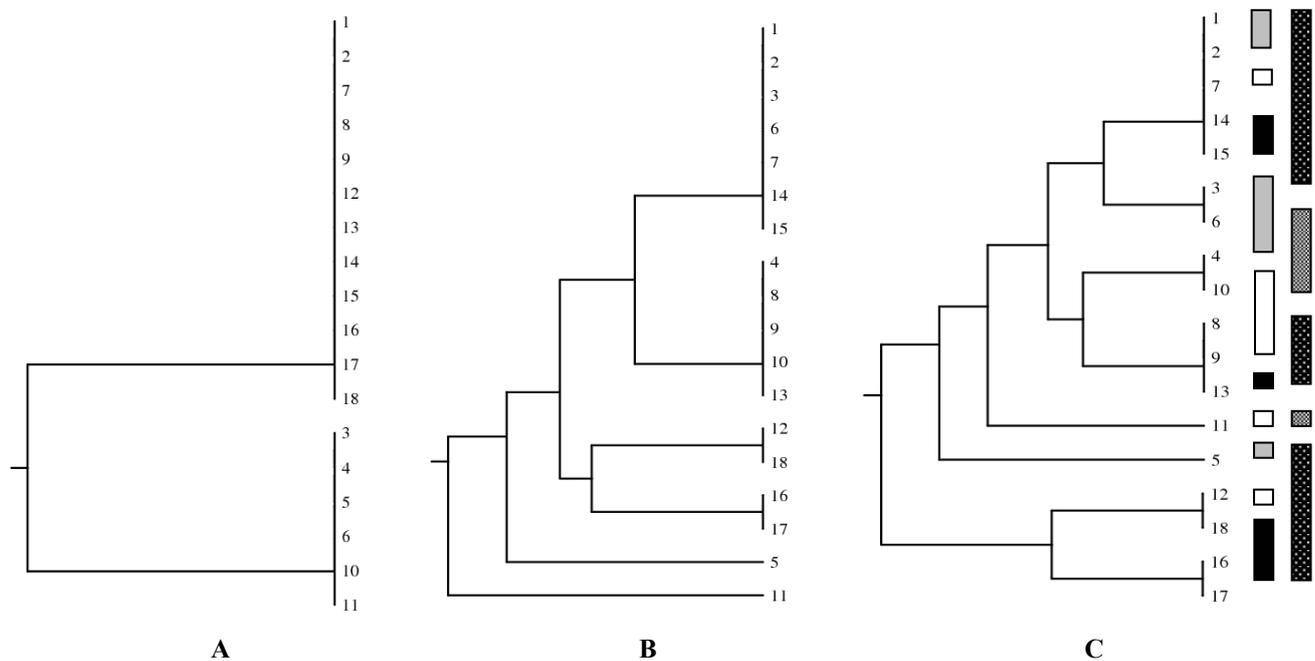
Note: 1,2,3. Small red ginger of Wonogiri; 4,5,6. Small red ginger of Kulonprogo; 7,8,9 Small white ginger of Wonogiri; 10,11,12. Small white ginger of Kulonprogo; 13,14,15. Big white ginger of Wonogiri; 16,17,18. Big white ginger of Kulonprogo.

This study still requires further study to obtain a more stable marker accuracy, but the identification result has been added the information on genetic diversity of ginger for the selection and for the improvement of varieties. Further research with other chromosomal data, such as karyotype and molecular cytogenetic will greatly assist the identification of ginger diversity, especially when they are combined with DNA sequence data.

Isozymes were only able to detect the genetic diversity in the introns, which was then translated into protein material. This led to the ability of isozyme, as

distinguishing characteristics are relatively limited, given the parts of introns that are not translated are undetectable for its diversity. Some researchers began to leave isozymes as genetic markers and test its genetic diversity right on the DNA.

Results showed that ginger undergoes genetic variation due to a wide range of ecological conditions. This investigation was an understanding of genetic variation within the accessions. It will also provide an important input into determining resourceful management strategies and help to breeders for the ginger improvement program.



**Figure 4.** The dendrogram relationship of ginger varieties based on isozymic banding patterns of esterase (A), peroxidase (B) and combination of esterase and peroxidase (C). Note: 1,2,3. Small red ginger of Wonogiri; 4,5,6. Small red ginger of Kulonprogo; 7,8,9 Small white ginger of Wonogiri; 10,11,12. Small white ginger of Kulonprogo; 13,14,15. Big white ginger of Wonogiri; 16,17,18. Big white ginger of Kulonprogo. Note: ■ small red ginger, □ small white ginger, ■ big white ginger; ▨ Kulonprogo ginger, ▩ Wonogiri ginger.

In conclusion, variation was observed in esterases and peroxidases in the three varieties of ginger. Esterase generated two isozyme bands, at the Rf value of 0.04 and 0.10, while peroxidase generated six isozyme bands, at the Rf value of 0.04, 0.05, 0.09, 0.10, 0.11, and 0.15. Accessions 3 has specific isozyme banding, on the other hand, accession 5 has the most specific peroxidase banding. Dendrogram on esterase generated 4 groups of 18 accessions; while based on peroxidase, there were 15 groups. Peroxidase produces more numerous and more diverse isozymic bands than esterase, resulting in a more complex relationship. The amount of data that is used to compile dendrogram affects the grouping, the more data used, the more obvious clustering of accessions in a population.

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#### REFERENCES

- Adam RP. 1999. Systematics of multi-seeded eastern hemisphere *Juniperus* based on leaf essential oils and RAPD fingerprinting. *Biochem Syst Ecol* 27: 709-725.
- Alcazar MD, C Egea, A Espin, E Cadela. 1995. Peroxidase isozymes in the defense response of *Capsicum annuum* to *Phytophthora capsici*. *Physiol Pl* 94: 736-742.
- Apavattjirut P, Anuntalabhochai S, Sirirungsa P, Alisi C. 1999. Molecular markers in the identification of some early flowering *Curcuma* L. (*Zingiberaceae*) species. *Annual of Botany* 84: 529-534.
- Ashraf K, Ahmad A, Chaudhary A, Mujeeb M, Ahmad S, Amir M, Mallick N. 2014. Genetic diversity analysis of *Zingiber officinale* Roscoe by RAPD collected from subcontinent of India. *Saudi J Biol Sci* 21 (2): 159-165.
- Azizah N. 2011. Identification of genetic diversity on *Curcuma xanthorrhiza* collection clones in Java and Madura. The 2nd International Symposium on Temulawak in IPB International Convention Center 26-27 May 2011
- Bapat SA, Rawal SK, Mascarenhas AF. 1992. Isozyme profiles during ontogeny of somatic embryos in wheat (*Triticum aestivum* L.). *Plant Sci* 82 (2): 235-242
- Barrett RCH, Shore JS. 1989. Isozyme variation in colonizing plants. In: Soltis DE, Soltis PS (eds). *Isozymes in Plant Biology*. Dioscorides Press, New York.
- Beer SC, Grofeda J, Philips TD, Murphy JP, Sorrels ME. 1993. Assesment of genetic variation in *Avena sterilis* using morphological traits, isozyme and RFLPs. *Crop Sci* 33: 1386-1393.
- Burkill IH. 1935. *A Dictionary of the Economic Products of the Malay Peninsula*. Vol. I. Governments of the Straits Settlements and Federated Malay States by the Crown Agents for the Colonies, London.
- Chokthaweepanich H, Paisooksantivatana Y. 2003. Classification of the genus *Curcuma* L. (*Zingiberaceae*) based on morphological characters

- and isozyme pattern. In: Chantaranothai P (ed). Proceeding of the 3rd Symposium on the Family Zingiberaceae, Khon Kaen, Thailand.
- Crawford DJ. 1990. Plant Molecular Systematics, Macromolecular Approaches. John Wiley and Sons, New York.
- Dasgupta N, Nandy-Datta P, Tiwari C, Das S. 2010. Salinity-imposed changes of some isozymes and total leaf protein expression in five mangroves from two different habitats. J Pl Interact 5 (3): 211-221.
- de Padua LS, Bunyaprapatsara N, Lemmens RHMJ (eds). 1999. Plant resources of South-East Asia No. 12 (1): Medicinal and poisonous plants 1. PROSEA Foundation, Bogor.
- Deng JB, CB Ding, L Zhang, YH Zhou, Yang RW. 2011. Relationships among six herbal species (*Curcuma*) assessed by four isozymes. Pyton 80: 181-188
- Dodeman VL, Ducreux G. 1996. Isozyme patterns in zygotic and somatic embryogenesis of carrot. Plant Cell Reports 16 (1-2): 101-105
- El-Bendary AA, Mabrouk Y, El-Metainy A. 1998. Peroxidase isozyme variants as genetic markers for early evaluation of Fe-efficiency and Fe-nutritional status in maize lines. Field Crops Res 59 (3): 181-185
- Gämperle E, Schneller JJ. 2002. Phenotypic and isozyme variation in *Cystopteris fragilis* (Pteridophyta) along an altitudinal gradient in Switzerland. Flora 197 (3): 203-213
- Garcia-Vallve S, Palau J, Romeu A. 1999. Horizontal gene transfer in glycosyl hydrolases inferred from codon usage in *Escherichia coli* and *Bacillus subtilis*. Mol Biol Evol 9: 1125-1134.
- Garcia-Vallvé S, Puigbo P. 2002. DendroUPGMA: A dendrogram construction utility. Biochemistry and Biotechnology Department. Universitat Rovira i Virgili (URV). Tarragona, Spain. <http://genomes.urv.cat/UPGMA/>
- Garkava PA, Rumpunen K, Bartish IV. 2000. Genetic relationships in Chaenomeles (Rosaceae) revealed by isozyme analysis. Sci Hortic 85: 21-35.
- Ghosh S, Mandi SS. 2011. Study of genetic variation among some wild landraces of *Zingiber officinale* Roscoe correlated with their antioxidant potential status. J Mol Biol Res 1 (1): 77-87
- Guse RA, Coors JG, Drolsom PN, Tracy WF. 1988. Isozyme marker loci associated with cold tolerance and maturity in maize. Theor Appl Genet 76 (3): 398-404
- Heyne K. 1950. De Nuttige Planten van Indonesie. Deel I. W. van Hoeve, 's-Gravenhage.
- Holtum RE. 1950. The Zingiberaceae of the Malay Peninsula. Gard Bull Sing 13 (1): 1-249.
- Ibrahim H, Hussin KH, Sanusi A. 1991. Taxonomic implications of isozyme and anatomical studies on *Cucurma* species. Botany 2000 Asia. Zingiberaceae workshop, Prince of Songkla University, Hat Yai, Thailand, 15-18 October 1991.
- Ibrahim H. 1996. Isozyme variations in selected Zingiberaceae spp. In: Wu TL et al. (eds). Proceedings of the 2nd Symposium on the Family Zingiberaceae. Zhongshan University Press, Guangzhou, China.
- IUBMB [International Union of Biochemistry and Molecular Biology]. 1992. Enzyme Nomenclature 1992, Academic Press, San Diego.
- Jaccard P. 1908. Nouvelles recherches sur la distribution florale. Bull Soc Vard Sci Nat 44: 223-270
- Jugran A, Bhatt ID, Rawat S, Giri L, Rawal RS, Dhar U. 2011. Genetic diversity and differentiation in *Hedychium spicatum* Buch. Ham. ex. D. Don - a high value medicinal plant of Indian Himalaya. Biochem Genet (DOI 10.1007/s10528-011-9451-7)
- Juliani HR, Koroch AR, Simon JE, Asante-Dartey J, Acquaye D. 2007. Chemistry and quality of fresh ginger varieties (*Zingiber officinale*) from Ghana. Acta Hort (ISHS) 756: 399-406.
- Kahler AL, Allard RW, Krzakowa M, Wehrhahn CF, Nevo E. 1980. Associations between isozyme phenotypes and environment in the slender wild oat (*Avena barbata*) in Israel. Theor Appl Genet 56 (1-2): 31-47
- Kress J. 1990. The diversity and distribution of *Heliconia* (Heliconiaceae) in Brazil. Acta Bot Bras 4 (1): 159-167.
- Li Z, Rutger JN. 2000. Geographic distribution and multilocus organization of isozyme variation of rice (*Oryza sativa* L.). Theor Appl Genet 101 (3): 379-387
- Loveless MD. 1992. Isozyme variation in tropical trees: patterns of genetic organization. New Forests 6 (1-4): 67-94
- Mangolin CA, Prioli AJ, Machado MFPS. 1997. Isozyme variability in plants regenerated from calli of *Cereus peruvianus* (Cactaceae). Biochem Genet 35: 189-204.
- Mastenbroek O, Hogeweg P, Heringa J, Niemann GJ, van Nigtevecht G, van Brederode J. 1984. Isozyme variation in *Silene pratensis*: a response to different environments. Biochem Syst Ecol 12 (1): 29-36
- McDonald BA, McDermond JM. 1993. Population Genetics of Plant Pathogenic Fungi. BioScience 43 (5): 311-319.
- Mondini L, Noorani A, Pagnotta MA. 2009. Assessing plant genetic diversity by molecular tools. Diversity 19: 35.
- Murphy J, Philips TD. 1993. Isozyme variation in cultivated oats and its progenitor species *Avena sterilis*. Crop Sci 33: 1366-1372.
- Ochse JJ. 1931. Vegetables of The Dutch East Indies. Archipel Drukkerij, Buitenzorg.
- Oja T. 2002. Genetic divergence and interspecific differentiation in the *Bromus madritensis* complex (Poaceae) based on isozyme data. Biochem Syst Ecol 30 (5): 433-449
- Paisooksantivatana Y, Kako S, Seko H. 2001. Genetic diversity of *Curcuma alismatifolia* Gagnep. (Zingiberaceae) in Thailand as revealed by allozyme polymorphism. Genet Res Crop Evol 48 (5): 459-465.
- Poth S, Sauer G. 2000. The Spice Lilies: Eastern Secrets to Healing with Ginger, Turmeric, Cardamom. Healing Art Press, Vermont, Canada.
- Purseglove JW, Brown EG, Green CL, Robbins SRJ. 1981. Spices, Volume 2. Longman Tropical Agriculture Series, London.
- Purseglove JW. 1972. Tropical Crops Monocotyledones. Longman, London.
- Rout GR, Das P. 1995. Isoenzyme profile during somatic embryogenesis and *in vitro* flowering of *Bambusa vulgaris*. J Plant Biochem Biotech 4 (1): 43-45
- Sadka A, Dahan E, Or E, Cohen L. 2000. NADP+-isocitrate dehydrogenase gene expression and isozyme activity during citrus fruit development. Plant Sci 158 (1-2): 173-181
- Setyawan AD. 2002. Variations on ginger (*Zingiber officinale* Rosc.) varieties based on the chemical constituent of volatile oils. BioSMART 4 (2): 48-54. [Indonesian]
- Shah K, Nahakpam S. 2012. Heat exposure alters the expression of SOD, POD, APX and CAT isozymes and mitigates low cadmium toxicity in seedlings of sensitive and tolerant rice cultivars. Plant Physiol Biochem 57: 106-113
- Shamina A, Zacharias JT, Sasikumar B, George JK. 1998. Biochemical variation in turmeric (*Curcuma longa* Linn.) accessions based on isozyme polymorphism. J Horticult Sci Biotechnol 73 (4): 479-483.
- Shamita A, Zachariah TJ, Sasikumar B, George JK. 1997. Biochemical variability in selected ginger (*Zingiber officinale* Rosc.) germplasm accessions. J Spic Arom Crops 6 (2): 119-127.
- Shanmugapriya M, Prabha ML. 2012. Isoenzyme analysis of *Amomum* species. Intl J Pharm Biol Arch 3 (5): 1091-1094
- Sharma TR, Jana S. 2002. Species relationships in *Fagopyrum* revealed by PCR-based DNA fingerprinting. Theor Appl Genet 105: 306-312.
- Sneath PHA, Sokal RR. 1973. Numerical Taxonomy. W.H. Freeman, Co., San Francisco.
- Staszak J, Grulke NE, Marrett MJ, Prus-Glowacki W. 2007. Isozyme markers associated with O3 tolerance indicate shift in genetic structure of ponderosa and Jeffrey pine in Sequoia National Park, California. Environmental Pollution 149 (3): 366-375
- Suranto. 1991. Studies of Population Variations in Species of Ranunculus. [M.Sc.-Thesis]. Department of Plant Science, University of Tasmania, Hobart.
- Tang JY, Li QM, Yang RW, Liao JQ, Zhou YH. 2008. Study on isozymes in six species of *Curcuma*. China J Chin Mat Med 33 (12): 1381-1386.
- Vanijajiva O, Suvachittanont W, Sirirugs P. 2003. Isozyme analysis of relationships among *Boesenbergia* (Zingiberaceae) and related genera in Southern Thailand. Biochem Syst Ecol 31 (5): 499-511.
- Watt JM, Breyer-Brandwijk MG. 1962. The medicinal and poisonous plants of southern and eastern Africa: being an account of their medicinal and other uses, chemical composition, pharmacological effects and toxicology in man and animal. E. and S. Livingstone, Edinburgh.
- Weeden VF, Wendel JF. 1989. Visualization and interpretation of plant isozymes. In: Soltish DE, Soltish PS (eds.) Isozyme in Plant Biology. Dioscorides Press Portland, Oregon.
- Zhang S, Lu S, Xu X, Korpelainen H, Li C. 2009. Changes in antioxidant enzyme activities and isozyme profiles in leaves of male and female *Populus cathayana* infected with *Melampsora larici-populina*. Tree Physiol 30 (1): 116-128.