



## **Amylose Content Variation of Indonesian Cassava Genotypes and its Correlation with RAPD and AFLP Markers**

**E. Sudarmonowati, N.S. Hartati, Hartati and L. Sukmarini**

*Research Centre for Biotechnology-LIPI, Jl.  
Raya Bogor Km. 46, Cibinong 16911  
Indonesia*

### **Abstract**

*A total of 20 random decamer primers and 6 primer pairs of RAPD (Random Amplified Polymorphic DNA) and AFLP (Amplified Fragment Length Polymorphism), respectively were screened to analyse over 150 accession numbers of cassava mainly collected in various parts of Indonesia which amylose content ranged between 22.65%-32.53%. Only seven primers (OPE-5, OPE-15, OPF-4, OPF-13, OPH-17, OPE 20, OPB-10) were used for further study and three of them were used to analyse 60-97 genotypes. By correlating the obtained bands with amylose content, five candidate RAPD markers of high amylose i.e. OPE-15 H, OPE-15 I, OPE-15K, OPE-15 L and OPE-15O, and that of high amylopectin i.e. OPE-15-B, OPE-15F and OPE-15I were obtained although further confirmation were needed. Dendrogram constructed based on a combination of three RAPD primers i.e. OPE-15, OPF-4 dan OPF-13 indicated that no distinct pattern between genotypes possessing low amylose with the high amylose one although some genotypes with the same characters were grouped in the same cluster or subclusters. This results were also confirmed with the results obtained using AFLP. These amylose content variations was also compared with the morphological variation. Effort to obtain genotypes with extreme amylose content is underway through genetic engineering and irradiation with or without crossing combination.*

### **Introduction**

In Indonesia, cassava is mostly planted by farmers in a small-scale in marginal lands. The ability of cassava to tolerate drought and low soil fertility facilitates its growth by farmers in dry areas of Indonesia for daily subsistence and small-scale trading. Despite low nutrition content and relatively high cyanide in its tuberous root, and short tuberous root storage, cassava starch industry –as an alternative to potato starch– developed rapidly both nationally and internationally. According to the Indonesian Agrochemical and Forestry Product Directorate General of the Ministry of Industry and Trade, cassava area in 2002 was 1,276,533 ha –a slight decrease from 2001, and could not cope with demand that increased –from 544,439 t in 2001 to 575,433 t in 2002. Ironically, despite the large area and the environment suits to grow cassava, Indonesia became a net importer country for cassava starch –export in 2002 was 20,082 t while the import was 25,754 t. The demand of cassava roots increased 0.9% per year while the production increase was 1.6 % annually due to

2.1 % productivity gains. The growth of plantation area was negative; i.e., -0.5 %, which indicates that the area used for cassava plantation was converted to other crops. Sadly, the production of cassava starch in Indonesia has been decreasing since 1991 despite a high demand of starch.

Starch is the principal carbon-reserve polysaccharide in plants, and the major source of carbohydrates in the diets of both humans and animals. The great economic importance of starches has stimulated much interest in the potential to modify their properties through genetic engineering. This requires a thorough understanding of starch biosynthesis, particularly as it relates to starch granule formation and the formation of amylose and amylopectin, which are key determinants of the industrial properties of starch. Biochemistry, molecular biology and molecular genetics will expand knowledge on cassava starch biosynthesis. Such knowledge will facilitate the use of genetic engineering in the near future to produce novel starches with altered starch properties, which are tailored to specific nutritional, industrial and other end-user requirements. Starch is an important raw material for industrial applications, such as in the paper, textile, plastics, food and pharmaceutical industry. It is currently being used in the production of biodegradable packing materials and in the development of biodegradable plastics, which is becoming an increasingly attractive alternative to petroleum-based products. There is considerable interest from the industry in the prospect of diversifying the structure of starch polymers. Improvement of starch properties for industrial uses can be achieved by chemical or physical modification after isolation, but also through the in planta modification of starch. The amylose-free potato starches can be used in frozen foods to improve the freeze-printing properties. High amylose starches also have numerous industrial applications; e.g. in fried snack products to create crisp, and in the production of gum candies due to their rapid setting properties.

The need of cassava as raw materials in developing countries has been increasing each year by 2.0% for food and 1.6% for feed, while total production based on current production level is projected to reach 168 million t in 2020. Cassava starch has a wide range of applications in both food-related and nonfood-related industries. Cassava starch could be converted to maltotriose, maltose, and glucose and other modified sugar and organic acid. Starch hydrolysate has been widely used as additive compound in food industries (candies, bread, canned food and frozen food). Certain industries require very low amylose for paper filling and other industries require very low amylopectin. In certain countries like Thailand, the majority of cassava starch (~80%) is used in the production of MSG and lysine. Significant quantities are also used to produce sugars (glucose and fructose), sugar alcohol (sorbitol), and citric acid. It is also used as a binding or thickening in various food products, such as ice cream, noodles and puddings. Outside of the food industry, cassava starch is used in the manufacture of paper, textiles and plywood, and more recently in the production of ethanol and biodegradable polymers.

Among starch producing plants, cassava has been considered as the highest producer (25%-40% higher than rice and maize) and as the most efficient (the highest) converter from solar energy to carbohydrate per unit area. Increased demand for cassava starch has spurred interest in the development of new cassava cultivars with greater yields and higher starch contents. Traditionally, cassava varietal improvement has been achieved through conventional breeding. Since most elite cassava cultivars are vegetatively propagated hybrids, a major drawback of this approach is that some of the favorable allelic combinations occurring in the parental generation are lost in the subsequent filial generations. Most of research has focused on starch production in maize, wheat, potato and rice. By contrast, only a handful of starch biosynthetic genes have thus far been characterized in cassava.

Despite several decades of intensive research on the biosynthesis of storage starch, the process is still incompletely understood. However, in the last decade significant progress toward understanding of starch biosynthesis has been made through the use of molecular biology and genetic engineering. Molecular characterization of the genes encoding starch synthetic enzymes has thus far revealed important information on starch biosynthesis in plants. In cassava, however, limited information of starch biosynthesis is currently available and a few numbers of the genes participating in starch biosynthesis of cassava have been characterized. Information ensuing from the isolation and characterization of genes involved in both pathways will lead to a better comprehension of how the root system of cassava generates its starch-filled tuberous roots. Various functional analysis techniques, such as RNA interference (RNAi) and gene over-expression, are also being used to determine the precise effects of individual genes on the properties of starch produced. The knowledge gained from these experiments will greatly facilitate the effort to obtain cassava cultivars with enhanced starch characteristics and yield through such techniques as marker-assisted selection and genetic engineering.

Although Indonesian is not the center of origin of cassava, the crops shows wide variation for leaf, petiole, stem and tuberous root color, as well as tuberous root shape and texture. Genetic marker-aided research on starch composition of the tuberous roots will be of interest and will assist the improvement of this trait by the breeding program.

## Materials and methods

**DNA Extraction** Cassava DNA was extracted using the procedure of Gillies (1997) with a slight modification. The extraction process was either manually or using RETSCH grinder apparatus.

**Primers and RAPD analysis conditions** Twenty primers of kit B, E, F and H were screened which yielded six suitable primers for further analysis. The six primers were OPE-5, OPE-15, OPE-20, OPF-04, OPF-13, OPH-17. PCR conditions were pre-PCR 94°C for 3 min followed by 40 cycles of denaturation at 94°C for 12 min, annealing at 35°C for 24 sec, and extension at 72°C for 2 min, then followed by post-PCR at 72°C for 7 min. DNA fragments yielded from the amplification were separated at 1.5% agarose gel using 1x TAE buffer. A UV trans-illuminator was used for visualization of the amplification products. Documentation was conducted using a camera and Polaroid film.

**Primers and AFLP analysis** Two enzyme restrictions were tried (MseI-PstI and MseI-EcoRI). The analysis was performed following the protocol of Life Technologies Inc. involving pre-amplification and amplification using PCR, which involved the use of restriction enzymes and ligation process.

**Data analysis based on bands visualized on agarose** Data were analyzed using software POPGENE version 1.0 and NTSYS, which are based on the presence and absence of DNA bands on agarose gel. Dendograms were constructed based on each RAPD or AFLP primers and compared with several primer combinations.

**Starch extraction** This process was made manually using grinder and cloth for filtering the grinded roots. Drying was conducted naturally under the sun. Starch content analysis was based on international standard procedure developed by Horwitz (1990).

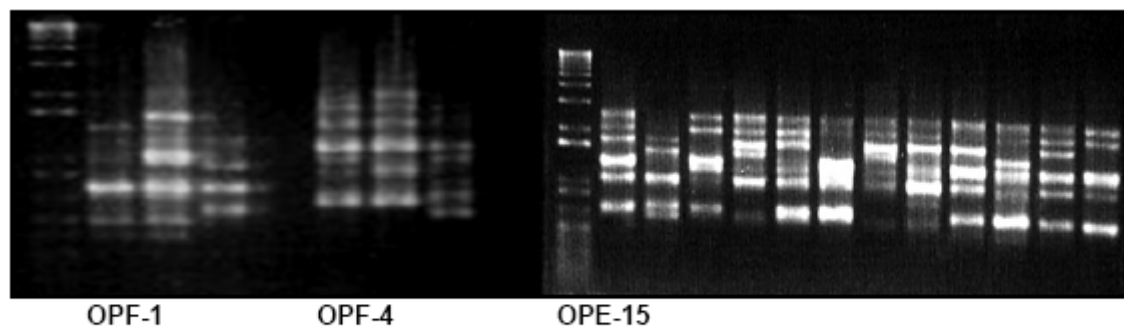
## Results and Discussion

**Amylose content variation** The content of amylose ranged from 22.65% to 32.4% among the 160 cassava genotypes. Groupings based on amylose content were as follows: Group I with very low (22.6%–22.8%), Group II with low (23.8%–24.9%), Group III with medium (25.4%–29.6%) Group IV with high (30%–31.2%), and Group V with very high (32.4%– 32.5%) contents. The lowest amylose content cultivars were genotypes L-89- 26, Selengan (22.6–23.3%) while the highest amylose content cultivar was Iding (32.53%). As the amylose content of the cassava collection was not extremely low or high, efforts to reduce or increase the content were initiated by inducing mutations through  $\gamma$ -irradiation and genetic engineering.

**RAPD analysis** The total number of genotypes that DNA could be amplified by each primer is given in Table 1. Fig. 1 shows DNA bands amplified by OPE-15, OPF- 4 and OPH-17 primers.

**Table 1. RAPD primers and number of genotypes assessed**

| Primers (decamer)     | Number of genotypes |
|-----------------------|---------------------|
| OPE-5                 | 16                  |
| OPE-15                | 96                  |
| OPE-20                | 9                   |
| OPF-4                 | 31                  |
| OPF-13                | 35                  |
| OPH-17                | 35                  |
| OPE-15, OPF-4         | 28                  |
| OPE-15, OPF-13        | 6                   |
| OPE-15, OPH-17        | 8                   |
| OPE-15, OPF-4, OPH-17 | 12                  |
| OPE-15, OPF-4, OPF-13 | 4                   |

**Fig. 1** – Banding pattern of several Indonesian genotypes generated from three RAPD Primers (OPE-15, OPF-1 and OPF-4)

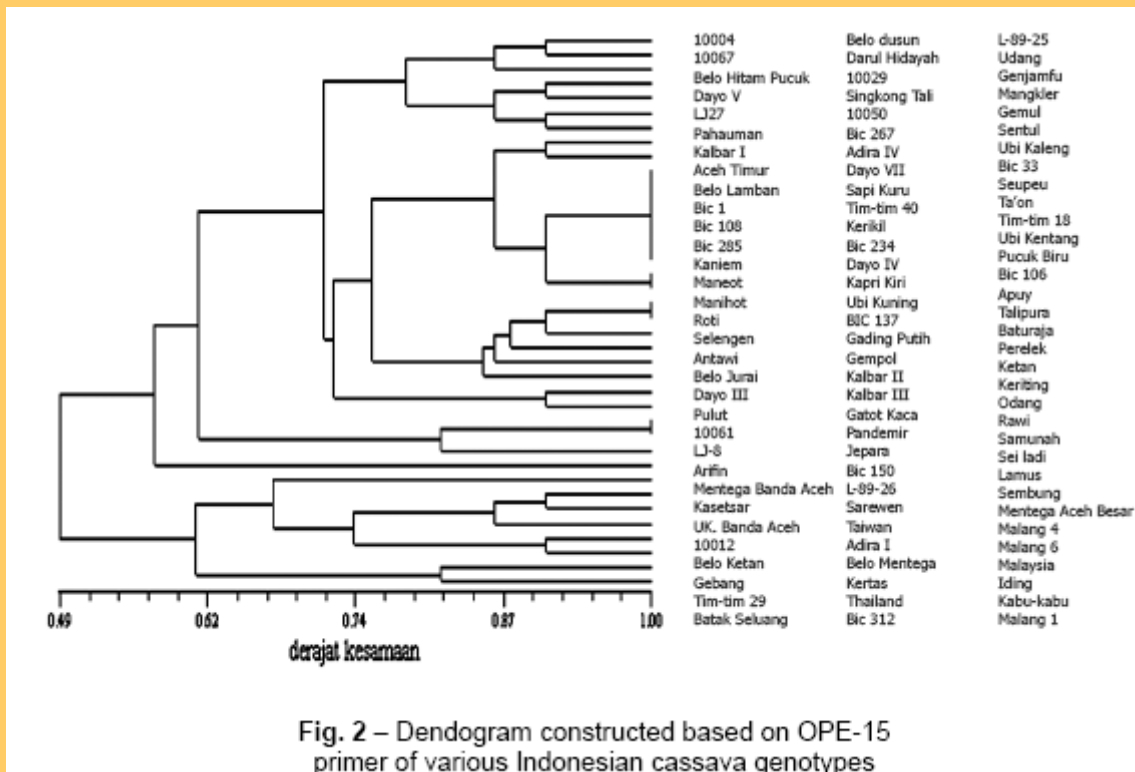
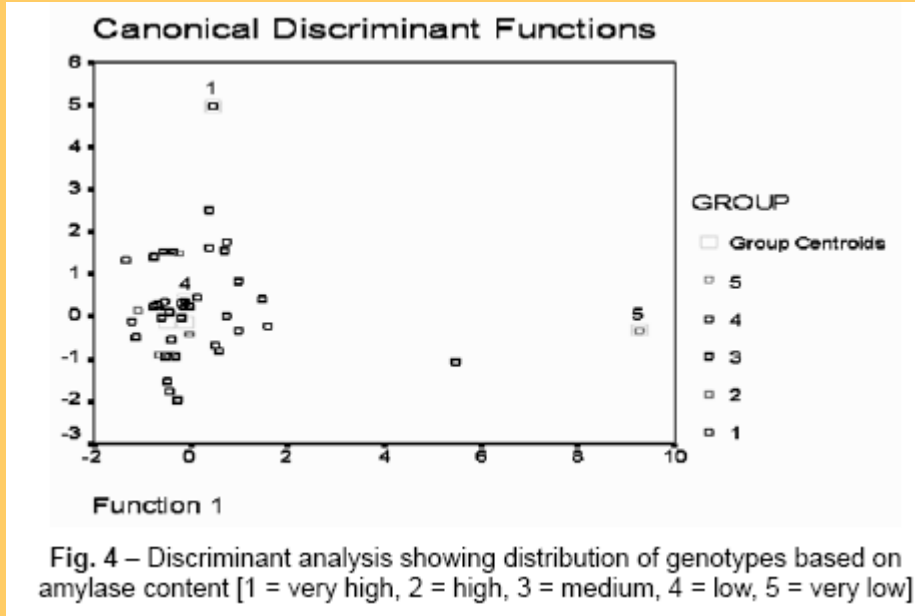
The dendrogram based on OPE-15 primer or those based on OPH-17 primer alone, or from a combination of OPE-15, OPF-4, and OPF-13 primers (Figs. 2 and 3) shows two main clusters, although the genotypes grouping in each cluster were not the same. Dendrograms based on OPE-15 primer showed that cluster 2 only consists of one genotype (Malang 1), while cluster 1 consists of two sub-clusters, which differed in the number of genotypes. Sub-cluster 2 consists of Iding and Kabu-kabu and was separated from other genotypes in sub-cluster 1, which consists of Adira 1, Adira 4, Malang 2, Malang 4, and Darul Hidayah that are cultivars released by the Indonesian Government. These genotypes were also in the same sub-cluster with Thailand and Kasetsart, which are very popular among farmers in the largest production center of Lampung. The last two genotypes are regarded as having high starch content and early maturity. Although, the dendrogram did not clearly differentiate between genotypes containing high or low amylose, Iding, which is considered as high amylose content genotype, was separated from sub-cluster 1 that included L89-26, Dayo V, 10067, Gebang and Selengan, whose amylose contents were very low to low. The genetic distance of genotypes with low amylose content was close, e.g. between 10067 and Dayo V.

The similarity among genotypes assessed by OPE-15 primer ranged between 56% and 100%, which revealed high genetic variation. The result also differentiated genotypes based on source of collection, e.g. Dayo V, Pahauman and Kalbar I from West Kalimantan Province in Kalimantan (Borneo) Island. This primer also grouped genotypes with low amylose content such as 10044, 10067, Dayo IV.

A RAPD marker appears to correlate with amylose and amylopectin content. Five candidate RAPD markers of high amylose were initially selected: OPE-15 H, OPE-15 I, OPE-15K, OPE-15 L and OPE-15O, and other three for high amylopectin: OPE-15-B, OPE-15F and OPE-15I, although further research to verify this

assertion will be needed.

Discriminant analysis on 59 genotypes revealed five groups for amylose content: very low, low, medium, high and very high, which confirmed the separation of the highest amylose content genotype (Iding, indicated by number 5 in Fig. 3) from the others. Similarly, the lowest amylose content genotypes were separated from the others as shown in Fig. 4 as number 5.



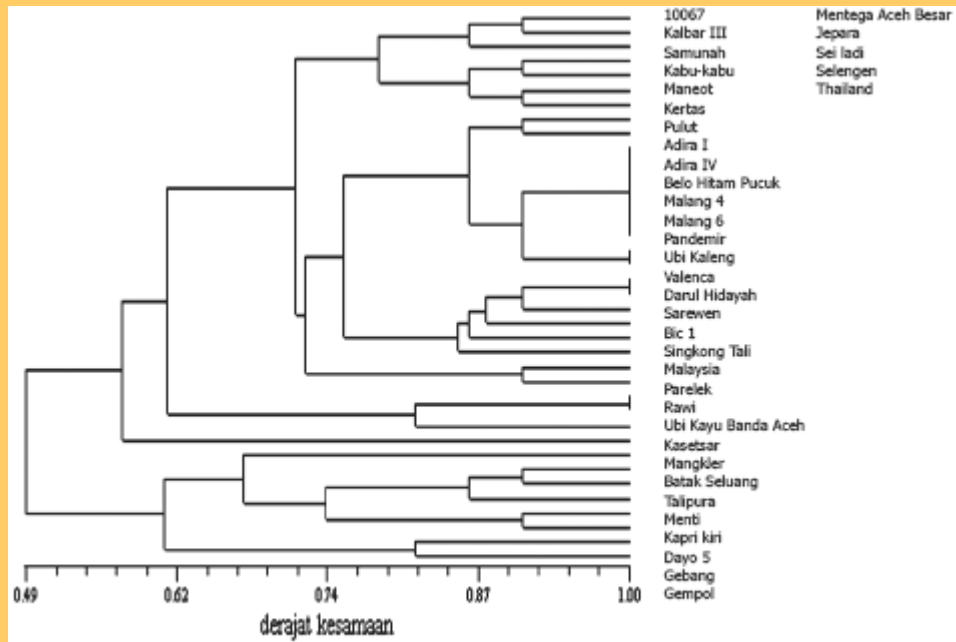


Fig. 3 – Dendrogram constructed based on OPH-17 primer of various Indonesian cassava genotypes

The dendrogram based on OPH-17 primer consisted of two clusters but the number of genotypes in cluster 2 was smaller than that of cluster 1. As per OPE-15 primer, Gebang and Selengen were also in one cluster, and that cluster 1 consisted of most Indonesian improved cultivars (Adira 1, Adira 4, Malang 2, Malang 4 and Darul Hidayah). Rawi and Menti, which are high yielding and have high starch content, were also grouped in this cluster. However, Kasetzar was not in the same cluster as Thailand (Fig. 3). The dendrogram ensuing from a combination of three primers (OPE-15, OPF-4 and OPH-17) consisted of two clusters, Adira 1 with Adira 4 in same cluster and separated from cluster 1 (Fig. 5).

**AFLP analysis** The number of primer pairs was 13 and 6 after using MseI-PstI and MseI-EcoRI enzyme restrictions, respectively. A total of 48 and 16 genotypes were assessed using PAA-MCAC (P11-700/MSe-48) and PGT-MCAC (P22-800/MSe-48) primer pairs, respectively. Of 13 primer pairs tried, only two could amplify DNA and gave a neat resolution in most genotypes analyzed (Table 2). The result also showed that MseI-PstI was the best restriction enzyme tried so far.

**Table 2. AFLP primer pairs tried and result of amplification**

| No. | Primer pairs               | Amplification |
|-----|----------------------------|---------------|
| 1.  | PAA-MCAC (P11-700/Mse-48)  | ++            |
| 2.  | PGT-MCAC (P22-800/Mse-48)  | ++            |
| 3.  | PAT-MCAC (P14-800/Mse-48)  | ++            |
| 4.  | MCT-MCAG (P10-700/Mse-49)  | + ("smear")   |
| 5.  | PAA-MCAG (P11-700/Mse-49)  | + ("smear")   |
| 6.  | EACG-MCAG (E37-700/Mse-49) | -             |
| 7.  | EACG-MCAG (E37-700/Mse-48) | -             |
| 8.  | EACG-MCAG (E37-700/Mse-49) | -             |
| 9.  | EAGA-MCAG (E38-700/Mse-49) | -             |
| 10. | EAGA-MCAG (E37-700/Mse-48) | -             |
| 11. | EAGA-MCAG (E38-800/Mse-49) | -             |
| 12. | EAC-MCAC (E32-700/Mse-48)  | -             |
| 13. | EATG-MCAC (E45-800/Mse-48) | -             |

The DNA bands from PAA-MCAC (P11-700/MSe-48) and PAT-MCAC (P14-800/Mse 48) primer pairs were used for constructing dendograms (Figs. 6 and 7). Both primer pairs resulted in two main clusters although the grouping of the two was different. P11-700/Mse-48 primer pair separated Adira 1 from Adira 4 in distinct clusters. As per with RAPD primers several genotypes that are of high amylose content, such as Taon and Kapri Kiri, were grouped in one cluster. The dendogram ensuing from P4-800/Mse-48 grouped Adira 1, Adira 4, Darul Hidayah and Iding in the same cluster. However, the two primers were similar in terms of separating cluster 1 with another cluster consisting of Taon, Genjamfu and Kapri Kiri. When the dendograms were compared with that ensuing from using RAPD primers, some likeness was noted. For example, both OPE-15 and PAT-MCAC (P14-800/Mse-48) grouped Maneot and Manihot in one cluster and the similarity index was very close; i.e., 1, which suggests that the two could be the same cultivar but with two given names. The results indicated therefore that the two DNA markers systems are complimentary and to each other.

## References

Gillies ACM, Cornelius JP, Newton AC, Navarro C, Hernandez M, Wilson J (1997) Genetic variation in Costa Rica populations of tropical timber species *Cedrela odorata* L. assessed using RAPDs. *Mol Ecol* 6:1113-1115.

Horwitz W (1990) Official methods of analysis of the association of official analytical chemist. 13th ed. Association of Official Analytical Chemist. Washington DC, USA.

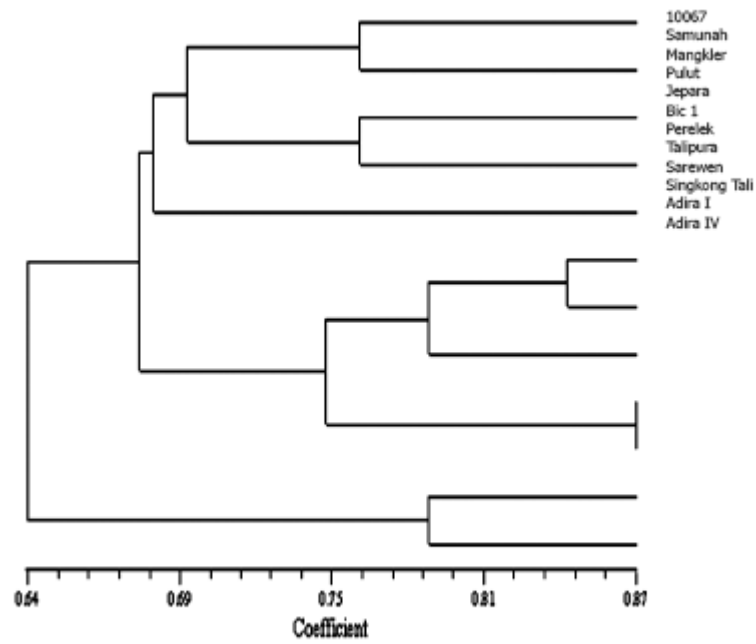


Fig. 5 – Dendrogram constructed based on a combination of three primers (OPE-15, OPF-4 and OPH-17) of various Indonesian cassava genotypes and cultivars

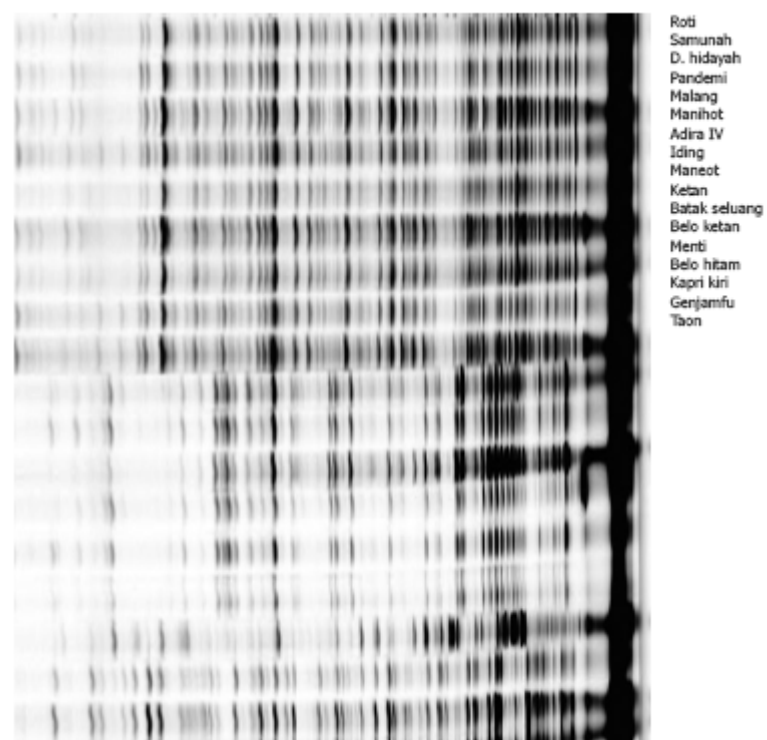


Fig. 6 – DNA bands of Indonesian cassava generated by AFLP primer (Licor/ M48-P11700- MCAC- PAA)

