

Genetic diversity of local corn (*Zea mays*) cultivars from South Amarasi, Kupang District, Indonesia by Inter Simple Sequence Repeats marker

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Abstract. *Uslan, Jannah N. 2020. Genetic diversity of local corn (Zea mays) cultivars from South Amarasi, Kupang District, Indonesia by Inter Simple Sequence Repeats marker. Biodiversitas 21: 1208-1214.* Corn (*Zea mays* L.) is one of the most important food crops in Indonesia. However, the studies described their genetic variation is relatively poor. Therefore, the aim of this study was to analyze the genetic diversity of local corn cultivars from South Amarasi, Kupang District, East Nusa Tenggara (NTT), Indonesia using ISSR markers. The sampling was conducted in 4 different sites in Sub-district of Amarasi, Kupang District. A total of 11 corn cultivars from Sub-district of South Amarasi was collected. DNA isolation was performed by using CTAB Method. Clustering analysis was conducted on MSVP 3.2 software. It was shown that all ISSR-primers used (UBC 811, UBC 814 and UBC 824) were successfully produced polymorphic bands and represents the high genetic diversity of the local corn cultivars. The genetic distance index indicated that several corn cultivars from two different populations were geographically unclustered, although there are samples from several populations that have a low genetic distance. The genetic variation index also showed high genetic diversity among the populations. Further research on the exhaustive sample collection was needed to give an insight into the genetic diversity of local corn cultivars (*Zea mays* L.) from South Amarasi, Indonesia. Please write implementation of this research

Keywords: ISSR, genetic diversity, East Nusa Tenggara, *Zea mays*

INTRODUCTION

Zea mays (corn) is one of the most important food crops in the world especially in Indonesia (Djaini et al. 2014). The demand for corn is used by producers as a substitute for rice which processed into various forms (Yusuf et al. 2013). The high demand for corn was making several areas in Indonesia as the largest producer of corn including East Java, Central Java, Lampung, South Sulawesi, North Sumatra, and East Nusa Tenggara (NTT) (BPS 2018).

Kupang District with an area of 26.138 ha, is capable of producing 64.979 tons of corn, making Kupang the highest producer of corn in East Nusa Tenggara (NTT) after Timor Tengah Selatan and Belu Sub-districts (BPS 2009). Sub-district of South Amarasi, Kupang District, East Nusa Tenggara Province is an area whose food plants are dominated by corn. Ironically, corn productivity in this region was considered low (2.48 tons/ha in 2014), lower than the national corn productivity (BPS 2015). This case is due to the local farmers that prefer to use local cultivars rather than hybrid corn (Faesal and Syuryawati 2011). Specifically, corn plants in the province of East Nusa Tenggara are dominated by local corn cultivars (37%) and the remainders are varieties of Jagung Bersari (16% of lamuru varieties) and hybrid corn (6%) (Subagio and Aqil 2013; Murningsih et al. 2015). In this case, breeding efforts are needed to increase corn production through plant breeding by knowing the information of its genetic diversity. Information on genetic variation, genetic

distance, and genetic relationship of corn from each population, can be used as references in further breeding programs in producing corn with high-yielding varieties in the South Amarasi Sub-district (Reni 2015).

Variation or genetic diversity of plants can be assessed by morphological and molecular approaches (Beyene et al. 2005; Iqbal et al. 2015; Sundari et al. 2019). However, the use of morphological characters is considered weak to infer the genetic diversity, due to the similarity of characters, subjectivity of examiners, and similar characters as a result of convergent evolution (Sundari et al. 2017; Gusmiati et al. 2018; Probojati et al. 2019). Therefore, the use of molecular markers is one of the reliable approaches that often used to determine the genetic diversity of corn. Molecular approaches that regularly used to assess genetic variation or diversity include Random Amplification of Polymorphic DNA (RAPD) (Lopes et al. 2017; Probojati et al. 2019), Restriction Fragment Length Polymorphism (RFLP) (Darrah et al. 2019), Simple Sequence Repeat (SSR) (Guevarra et al. 2017), and Inter Simple Sequence Repeat (ISSR) (Dar et al. 2018). ISSR is considered a reliable method that has a high reproducibility rate with a primers length of up to 16-25 mers (Costa et al. 2016). Previously, researchers have assessed the genetic diversity of some local corn cultivars in the Kupang District which is Amarasi, Fatule'u and Nekameses Sub-districts based on ISSR with a fairly high level of genetic diversity (approximately 0.30-0.80 similarity values) (Yulita and Naiola 2013). However, the genetic diversity research on

the local corn cultivars in other sub-Sub-districts is needed, especially in South Amarasi Sub-district. Therefore, the aim of this study was to analyze the genetic diversity of local corn cultivars from South Amarasi Sub-district, Kupang District, East Nusa Tenggara (NTT), Indonesia using ISSR markers.

MATERIALS AND METHODS

Sample collection

Young leaves of local corn (*Zea mays* L.) cultivars were collected from a total of 4 sampling sites location from South Amarasi Sub-district, Kupang District, East Nusa Tenggara (NTT), Indonesia; and analyzed at Animal Physiology Laboratory of Department of Biology, Brawijaya University, Malang and Biology Laboratory of FKIP Muhammadiyah University of Kupang. Three of local corn cultivars in every population were collected their leaves for DNA extraction. A total of 11 corn cultivars from South Amarasi Sub-district was collected including, Sahraen Village, Retraen Village, Nekmese Village, and Buraen Village. The chosen sites were marked its coordinates and altitude as shown in Table 1.

DNA isolation and DNA quantification

A total of 0.1 g of samples were ground to powder using mortar and pestle. DNA isolation was carried out using the CTAB method, developed by Doyle and Doyle (Doyle and Doyle 1990), with modification by Pharmawati (2009). The powdered sample was added by 1 mL extraction buffer [2% CTAB, 1,4 M NaCl, 0.2% β -mercaptoethanol, 50 mM Na₂EDTA (pH 8,0) and 100 mM Tris-HCL (pH 8,0)] that contained 0.2% β -mercaptoethanol. The samples were incubated at 65°C using a water bath for 60 minutes and turned frequently for every 10 minutes. A total of 700 μ l chloroform: isoamyl alcohol (24:1) was added into suspension and homogenized using Vortex. Suspension was centrifuged at 12.000 rpm at room temperature for 5 minutes. The supernatant was transferred into a new tube, with the addition of cold ethanol, adjusting to a total volume of supernatant. The suspension was mixed gently and incubated for 1 hour at -20°C temperature. The incubated suspension was centrifuged at 12.000 rpm for 3 minutes, and the pellets were washed by 400 μ l of 70% alcohol. Pellets were dried out, then added with 100 μ l of sterilized ddH₂O to dissolve the DNA pellets. The tubes were transferred to -20°C temperature storage (Uslan and Pharmawati 2015).

The quality of isolated DNA was evaluated by a qualitative test. The qualitative test was carried out by electrophoresis on 1,8% agarose gel with 1x TAE buffer [40 mM Tris-acetate (pH 7.9) and 2mM Na₂EDTA]. A total of 0.5 g agarose was added to 50 ml TAE buffer, then transferred into Erlenmeyer tubes and incubated for 2 minutes in a microwave. Agarose gels were placed into electrophoresis chambers and submerged on 1x buffer TAE. A total of 3 μ L of samples were mixed with loading dye on parafilm paper. Lambda DNA (50ng/ μ l) with a concentration of 2 μ l, 4 μ l, 6 μ l was inserted into the well to

estimate the DNA concentration. Electrophoresis was carried out at a voltage of 100 volts for 30 minutes, then stained by submerging the gels on Ethidium bromide (EtBr) for 30 minutes. The agarose gels were visualized using Gel DOC, UV-Transilluminator (Bio-Rad) (Sambrook and Russel 2001).

ISSR-PCR

ISSR-PCR analysis was performed using 3 primers (Table 2) with PCR Thermocycler Bio-Rad. The selection of primers based on previous study by Muhammad et al. (2017) and Dar et al. (2018). Total volume of PCR reaction mix was 20 μ l consisting of 12 μ l master mix (consisted of 2 μ l dNTP mix [dATP, dTTP, dGTP, and dCTP]; 2 μ l Taq buffer polymerase; 1.5 μ l MgCl₂; 0.2 μ l Taq polymerase; 1 μ l glycerol; 2.3 ddH₂O), 8 μ l of UBC primer, and 3 μ l DNA template. ISSR-PCR was performed by DNA pre-denaturation at 94°C for 5 min for 1 cycles, followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 40°C for 90 sec, extension at 72°C for 2 min. The final extension was performed at 72°C for 7 minutes for 1 cycle. ISSR fragments were separated by electrophoresis method on 1.8 agarose gels in 1xTAE buffer for 40 minutes at 100 volts for 40 minutes, then submerged in Ethidium bromide (EtBr) for 30 min. Then, the ISSR profiles on the gels were visualized by gel DOC, UV-Transilluminator (Bio-Rad) with a 100 bp of DNA ladder marker to determine the size of DNA amplification products (Sambrook and Russel 2001).

Table 1. Sampling Sites location of local corn cultivars (*Zea mays* L.) from South Amarasi Sub-district, Kupang District, Indonesia with notes on location coordinate and altitude

Sampling sites (village)	Total samples	Location coordinate	Altitude (m asl.)
Sahraen	3	123° 70.7396'E 10°22.9108'S	387.
		123° 83.847'E 10°15.5283'S	356
		123° 83.6104'E 10°15.3526'S	400
Retraen	3	123° 83.373 9'E 10°15.1769'S	180
		123° 82.3639'E 10°53.03'S	168
		123° 82.3137'E 10°85.91'S	174
Buraen	3	123° 81.9457'E 10°12.8541'S	300
		123° 82.8303'E 10°13.6701'S	274
Nekmese	3	123° 82.5679'E 10°18.251'S	476
		123° 82.5731'E 10° 19.1596'S	487
		123° 82.5683'E 10° 18 6489'S	500

Table 2. ISSR primer used in this study

Primer	Base sequence (5'→ 3')
UBC 811	GAG AGA GAG AGA GAG AC
UBC 814	CTC TCT CTC TCT CTC TA
UBC 824	TCT CTC TCT CTC TCT CG

Data and scoring analysis

The DNA band size was determined by plotting on semi-log paper. The bands that appear are assumed to be the ISSR allele. The migration distance from the well of visualized gels was measured to the middle of the 100 bp ladder band and scored using PyElph vers.4.1. The X-axis was the distance from the well to mid ladder band, while Y-axis was DNA size on the ladder. The points on semi-log paper were linked to produce the standard curve (Pharmawati et al. 2005). The DNA pattern obtained was scored as present (1) and absent (0). The scored were analyzed by clustering using the UPGMA method based on Nei and Li's similarity coefficient on MSVP 3.2 software. To estimate the primers' informativeness, the calculation of Polymorphic Information Content (PIC) was carried out (Laurentin and Karlysky 2007). PIC value for each primer was calculated by using Equation 1:

$$PIC_i + 2f_i(1 - f_i)$$

Where, PIC_i: polymorphism information content of the primer i, F_i: frequency of primer fragment that was present, (1-f_i): frequency of primer fragment that was absent. The genetic variation parameters were showed by the effective alleles (n_a), that was calculated by using Equation 2:

$$n_a: \frac{1}{\sum_i p_i^2}$$

and a total of observed alleles (n_e) was calculated by using Equation 3:

$$n_e: \frac{\sum a_i e_i}{\sum lokus}$$

and the mean of heterozygosity/ genetic variation (H_e) was calculated by using Equation 4:

$$H_e: 1 - \sum_i p_i^2$$

Where, P_i is frequency if the genetic type of the i. The criterion of genetic diversity level refers to Nei (Nei 1987) where the value of genetic diversity ranges from 0.1 to 0.4 is categorized as low, while the value of 0.5-0.7 is categorized as moderate, and value of 0.8-1.0 is categorized as high.

RESULTS AND DISCUSSION

ISSR profiles of local corn (*Zea mays* L.) cultivars

The results of DNA bands pattern from 12 accession of local corn (*Zea mays* L.) cultivars using 3 ISSR primers indicated the various amplification size range. The amplification products ranged up from 200 to 1200 bp, however, the smear DNA appeared on several samples with one sample from Berkaen villages that cannot appear (only 11 samples were presented as shown in Figure 1). The result of DNA amplification of 11 samples of local corn cultivars (*Zea mays* L.) from South Amarasi Sub-district, Kupang District showed that all primers used were

produced polymorphic DNA bands successfully (Figure 1). The absent and present of bands on each primer is a result of the successful attachment of primers to its homolog DNA template on a single locus (Tingey et al 1994). The technical difficulties and errors may contribute to the amplification of the DNA, resulting in the inability of primers to amplified the DNA fragment (samples from Bekaen villages). According to Uslan and Pharmawati (2015), the damaged DNA occurred as a result of released secondary metabolites when the cell was destructed by physical procedure, leading to degraded DNA shows by DNA bands that not merely be at the same intensity (shown as thick and thin DNA bands). Consequently, an evaluation of the technical procedures is needed to get an appropriate result.

The strength of ISSR primers that can amplify the DNA fragments was considered adequate. Therefore, these results exhibit that 3 ISSR primers used (Table 2) are suitable for similar studies on genetic variation of local corn cultivars (*Zea mays* L.). According to Weising et al. (2005) and Probojati et al (2019), the polymorphism is results of nucleotide base change that transform the primer binding site, and also an insertion or deletion within the amplified region. The polymorphism showed the number of genetic variations that exist among the samples. The high polymorphism appearance on amplification products showed high genetic diversity (Uslan and Pharmawati 2015). Accordingly, this research provides proof that indicates the high genetic diversity of local corn cultivars (*Zea mays* L.) from South Amarasi Sub-district, Kupang District.

Polymorphism analysis of local corn (*Zea mays* L.) cultivars

The total of 115 bands ranged from 200 to 1200 base pairs produced by 3 ISSR primers. The highest Numbers of Polymorphic Bands (NPB) was produced by UBC 824 (45 bands), giving the average of Polymorphic Bands (PB) to 100 % (Table 3). Polymorphic Information Content (PIC) values are 0.91 on each primer. PIC as an index that indicated the most capable primers to detect the polymorphism bands, emphasize that all of these 3 ISSR primers are able to amplify the 11 samples of local corn cultivars (*Zea mays* L.) from South Amarasi Sub-district, Kupang District. The higher the PIC values, the more likely the primer to be used for detecting genetic diversity in a population (Roldan-Ruiz et al 2000).

Table 3. Polymorphism analysis results of 3 ISSR primers amplification of local corn cultivars (*Zea mays* L.) from South Amarasi Sub-district, Kupang District.

Primer	DNA amplification size (bp)	TNB	NPB	PB (%)	PIC
UBC 811	300-1100	34	34	100	0.91
UBC 814	200-1100	36	36	100	0.91
UBC 824	300-1200	45	45	100	0.91
Total		115	115	100	

Note: TNB: total number of bands; NPB: number of polymorphic bands; PB (%): polymorphic band percentage; PIC: polymorphism information content.

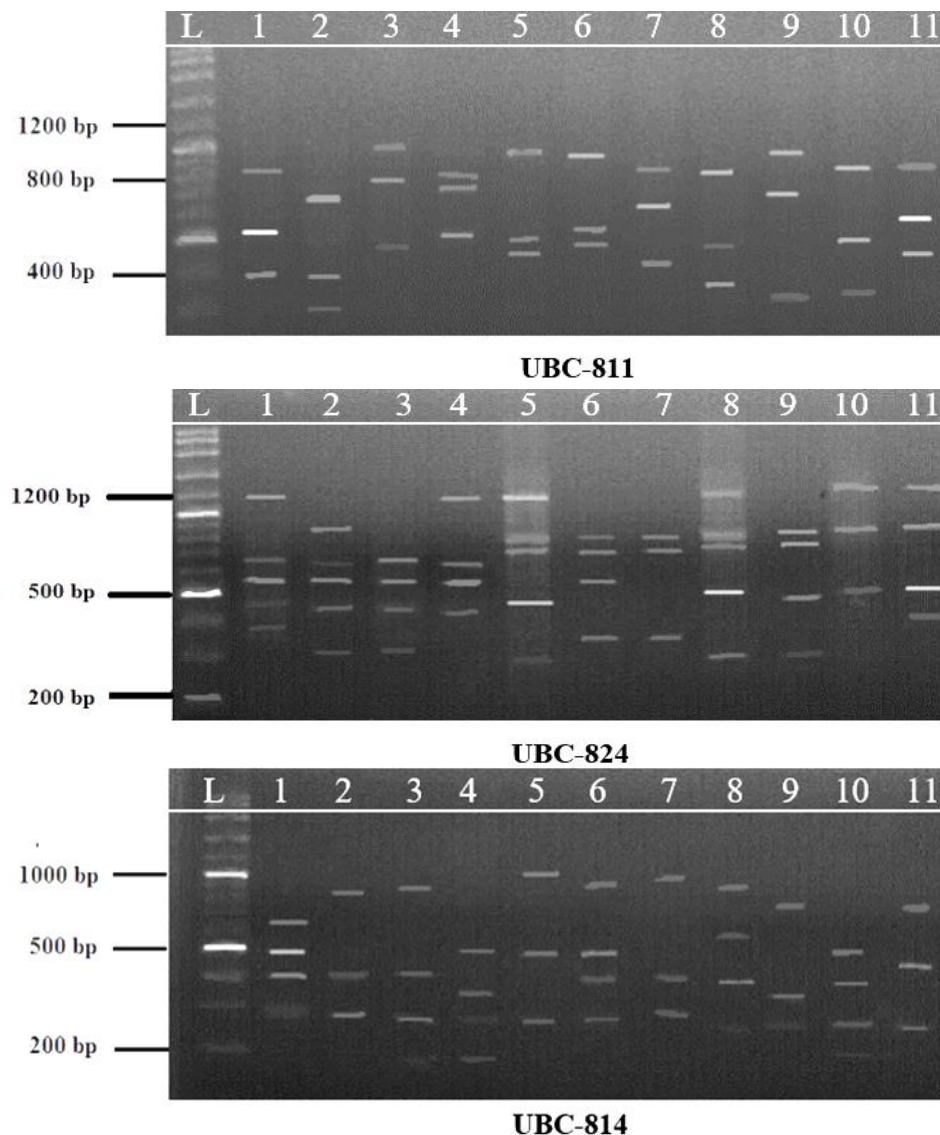


Figure 1 Electropherogram PCR-ISSR of local corn (*Zea mays* L.) cultivars from South Amarasi Sub-district, Kupang District which produced DNA polymorphic bands by several primers to with UBC-811, UBC-814, and UBC-824. (Note: Number of local corn cultivars numbers respectively, Sahraen village [1-3], Retraen village [4-6], Nekmese village [7-9] and Buraen village [10-11], DNA Ladder 100 bp indicated by a notation of L)

Genetic distance, clustering, and genetic variation of local corn cultivars

Based on scoring function to elucidate genetic distance of 11 samples of local corn cultivars (*Zea mays* L.) from South Amarasi Sub-district, Kupang District (Table 4), indicated that similarity index with the highest genetic distance showed by local corn cultivars (*Zea mays* L.) from Buraen 2 and Retraen 3 populations (similarity index 0.0004), while the lowest genetic distance indicated by local corn cultivars (*Zea mays* L.) from Sahraen 3 and Sahraen 2 populations (similarity index 0.700). The high genetic distance showed by Buraen 2 and Retraen 3

populations indicated that these 2 samples were geographically unclustered, while the low genetic distance shows that samples from Sahraen 3 and Sahraen 2 populations from the identical locations have high similarity. The unclustering samples may be caused by the acquisition of seeds from a local farmer to other farmers to a different area. Also, differences in the genetic distance may occur due to insect-assisted pollination. According to Usulan and Pharmawati (2015), the process of outcrossing that assisted by wind or insects leads to greater opportunities to distribute to wider areas.

Table 4. Similarity matrix of clustering by UPGMA methods using Nei and Li's coefficients of 11 local corn (*Zea mays* L.) cultivars from South Amarasi Sub-district, Kupang District, the lowest and highest similarity values was indicated by red colors.

		1	2	3	4	5	6	7	8	9	10	11
1	Sahraen 1 (1)	1.000										
2	Sahraen 2 (2)	0.364	1.000									
3	Sahraen 3 (3)	0.273	0.700	1.000								
4	Retraen 1 (4)	0.435	0.286	0.286	1.000							
5	Retraen 2	0.261	0.190	0.286	0.273	1.000						
6	Retraen 3	0.261	0.286	0.381	0.273	0.545	1.000					
7	Nekmese 1	0.182	0.300	0.300	0.190	0.476	0.476	1.000				
8	Nekmese 2	0.174	0.190	0.286	0.182	0.455	0.455	0.381	1.000			
9	Nekmese 3	0.095	0.005	0.105	0.200	0.500	0.400	0.316	0.300	1.000		
10	Buraen 1	0.182	0.100	0.100	0.286	0.190	0.286	0.200	0.381	0.211	1.000	
11	Buraen 2	0.091	0.200	0.100	0.286	0.095	0.004	0.100	0.095	0.105	0.400	1.000

Table 5. Result of genetic variation estimation of local corn (*Zea mays* L.) cultivars from 4 different populations of South Amarasi, Kupang District.

Population	Total samples	Na	Ne	He
Sahraen	3	2.977	10.667	0.664
Retraen	3	3.000	11.000	0.667
Buraen	2	2.980	10.000	0.664
Nekmese	3	2.000	6.667	0.500
Total	11	10.957	38.333	2.495
Mean		2.739	9.583	0.624

Note: Na: total alleles observed; Ne: total effective alleles; He: genetic variation

Dendrogram depicting the clustering of all samples (Figure 2), showed that from 11 samples of local corn (*Zea mays* L.) cultivars from South Amarasi Sub-district, Kupang District is divided to 3 main clusters and 9 sub-clusters (based on similarity coefficient 0.50/ 50 %). Cluster I consists of Buraen 2 (sub-cluster 1), Buraen 1 (sub-cluster 2). Cluster II consists of Nekmese 3 (sub-cluster 3), Nekmese 2 (sub-cluster 4), Nekmese 1 (sub-cluster 5), Retraen 3 and Retraen 2 (sub-cluster 6). Cluster III consists of Sahraen 3 and Sahraen 2 (sub-cluster 7), Retraen 1 (sub-cluster 8), Sahraen 1 (sub-cluster 9). According to Wijayanto et al. (2013), the smaller the coefficient of similarity (toward 0 values), the farther the relationship, vice versa. Some populations showed a close genetic relationship, although the remainders do not form close relationships, even though, they are originated from the identical population. This shows that some relationships between the samples do not influence by geographical factors. According to Poerba and Martanti (Poerba and Martanti 2008), this case is due to the influence of genetic recombination. According to Karuniawan et al. (2008), samples from the identical population do not invariably have a close relationship, which may be influenced by

genotypic interactions with the environment. ISSR as universal primers, randomly amplifies genomes (Kusumadewi et al. 2010) and becomes more complex in the case of outcrossing Rimbawanto et al. 2006).

The result was shown that the genetic variation index (He) from South Amarasi Sub-district, Kupang District ranging from 0.500 to 0.667, indicating the high genetic diversity. the average number of total alleles observed (Na) was 2.739 and the average number of total effective alleles (Ne) was 9.583. The average number of genetic variation (He) shows a value of 0.624. Based on the results of the analysis, the population of local corn cultivars from the Retraen population showed the highest genetic variation (He) 0.667, while the Buraen population showed the lowest genetic variations (Table 5). High variations value can be caused by the influence of environmental conditions. Soil and climate conditions in Kupang areas are considered as dry and arid, in addition to topography and environmental stress, create such a limiting factor in local corn cultivars selections for long periods. This selection process produces plants that genetically have higher environmental stress capabilities. Many factors were known to influence the degree of variations. According to Ardiyani et al. (2014), high differentiation among the population as well as low genetic diversity in a population is a result of dominating the self-pollination system compared to cross-pollination or outcrossing.

Genetic diversity that points out the amount of genetic variation is valuable information to support the conservation and breeding efforts (Ihwan et al. 2019). The process that causes whether low or high genetic variations were considered fundamental, given the genetics as the basic element in hierarchical biology (Zulfahmi 2013). As basic information, genetic variation can be used as a basis for conservation efforts, breeding programs, and sustainable use of genetic resources (Ardiansyah et al. 2018).

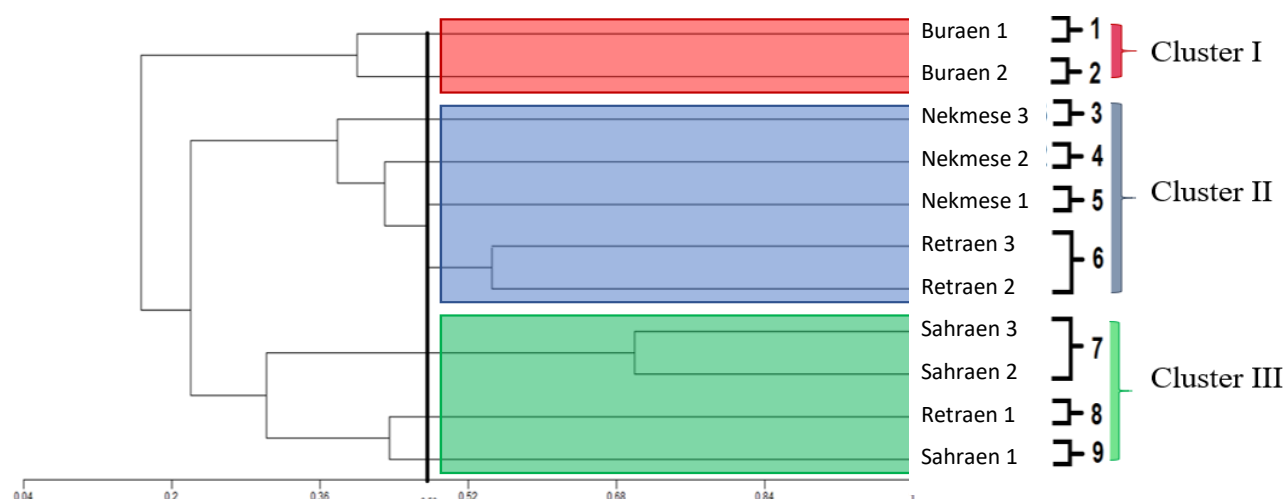


Figure 2. Dendrogram of 11 samples of local corn (*Zea mays* L.) cultivars from South Amarasi Sub-Sub-district, Kupang District based on Nei and Li's coefficient

ISSR-PCR analysis of 11 samples of local corn cultivars (*Zea mays* L.) from South Amarasi Sub-district, Kupang District, showed that all ISSR-primers used (UBC 811, UBC 814 and UBC 824) were suitable and successfully produced polymorphic DNA bands pattern, represents the high genetic diversity of the samples. The genetic distance showed by the similarity index indicated that several samples from two different populations were geographically indifferent, although there are samples from several populations that have a low genetic distance. The dendrogram divided the samples into 3 main clusters, although some populations showed a close genetic relationship and the remainders were not, indicating the absence of geographical influence. The genetic variation index also showed high genetic diversity among the populations. Further research on the exhaustive sample collection was needed to give an insight into the genetic diversity of local corn (*Zea mays* L.) cultivars from South Amarasi Sub-district, Kupang District, East Nusa Tenggara (NTT) Province, Indonesia.

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