BIODIVERSITAS
Volume 23, Number 8, August 2022
Pages: 4124-4130

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d230833

Genetic diversity of eight maize (*Zea mays*) cultivars from East Nusa Tenggara (Indonesia) based on inter simple sequence repeat markers

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Manuscript received: 29 June 2022. Revision accepted: 26 July 2022.

Abstract. *Yani A, Chasani AR, Daryono BS. 2022. Genetic diversity of eight maize (Zea mays) cultivars from East Nusa Tenggara based on inter simple sequence repeat markers. Biodiversitas 23: 4124-4130.* East Nusa Tenggara (NTT) is one of Indonesia's island that has many local maize cultivars. However, existence of this local corn is threatened by the presence of high-yielding maize seeds. Therefore, characterization of local maize cultivars using molecular marker is needed to conserve the germplasm. This study aims to determine the genetic diversity of local maize cultivars (*Zea mays* L.) from East Nusa Tenggara based on ISSR markers. This research used eight local maize cultivars (Watar Ohaq, Watar Puluq, Wata Bura, Wata Kuma, Wata Bunga, Pena Muti, Boti Pulut, and Latun Wara). Young leaves of maize were used for DNA isolation. Total genomic DNA was isolated using Genomic DNA Mini Kit for Plant (Geneaid). DNA was then amplified using 5 ISSR molecular markers (ISSR-807, ISSR-808, UBC-809, UBC-811, UBC-834). DNA band profiles were scored based on the presence or absence of bands. Genetic variability was analyzed based on the UPGMA method using the MVSP ver application. 3.1. Local maize from NTT has high genetic diversity (similarity index between 0.29-0.65). Local maize cultivars were separated into two main clusters at a similarity index of 0.43 and the grouping pattern was not influenced by the geographic location of local maize origin.

Keywords: DNA polymorphism, genetic variability, Indonesia, ISSR, maize

INTRODUCTION

Maize (*Zea mays* L.) is recognized as one of the most important staple food crops in the world for meeting primary carbohydrate needs, especially in East Nusa Tenggara, Indonesia. Maize production must be increased to meet the community's food needs, which necessitates research into technology improvement and the selection of superior varieties. East Nusa Tenggara's maize production in 2020 is low, at only 685.083 tons, when compared to other maize producing centers in Indonesia, such as East Java and Central Java, which have total production of roughly 6.1 million tons and 3.2 million tons, respectively (BPS NTT 2020). The loyalty of farmers to continue to plant local maize is most likely causes of the low maize productivity in East Nusa Tenggara. However, although local maize yield is inferior when compared to hybrid maize, local maize has flexibility to adapt in unique agroecology (Yulita et al. 2015). Therefore, characterization of local maize in term to produce high-yielding seed from local maize is totally needed.

Conserving maize genetic diversity is critical for understanding and mitigating the risk of species extinction (Plenk et al. 2019), since low genetic diversity and inbreeding have a negative impact on population fitness (Jiménez et al. 2014). Numerous studies show that limited genetic diversity is the primary driver of species extinction, resulting in low survival rates and decreased population growth (Jiménez et al. 2014; Plenk et al. 2019). One of the efforts of the Indonesian Government to increase maize production in numerous parts of East Nusa Tenggara is to distribute superior types of maize seeds to the community. This poses a long-term threat to the future of local maize cultivars, whereas the presence of cultivars is critical since they might serve as a source of germplasm for efforts to develop maize cultivars in order to obtain varieties with expected superior qualities (Prasanna 2012). Therefore, maize breeding program based on local maize is required to create new high-yielding varieties.

In the development of superior cultivars, genetic information and genetic relationship analysis play a vital role, particularly in selecting effective and efficient parent plants. Parent plants with distant relationship will produce offspring plant with considerable segregation, making it easier to pick types with the required features (Amzeri et al. 2011). Morphological and molecular characteristics can be used to determine genetic information and kinship (Govindaraj et al. 2015; Arif et al. 2020; Uslan and Pharmawati 2020). However, physical features have a shortcoming in identifying genetic variability since they are frequently impacted by the environment (Govindaraj et al. 2015). As a result, the use of molecular markers can support the weakness of recognizing genetic variation based on morphological characteristics.

The use of molecular markers is valid and can be observed in all plant tissues regardless of growth, differentiation, development, or cell status. Moreover, they are unaffected by environmental, pleiotropic, or epistatic

influences (Agarwal et al. 2008). As a result, the usage of DNA markers, as opposed to the phenotypic aspect, becomes increasingly significant. The detection methods of genetic diversity using molecular markers are commonly categorized into three groups: DNA sequence-based (Nikmah et al. 2016), hybridization-based, and PCR-based (Wahyudi et al. 2020b). Several DNA molecular methods are frequently used to understand genetic characteristics (Govindaraj et al. 2015), such as Restriction Fragment Length Polymorphism (RFLP) (Hapsari et al. 2015), Random Amplified Polymorphic DNA (RAPD) (Uslan and Pharmawati 2020), Variable Number Tandem Repeats (VNTR) (Hermenegildo et al. 2021), and Sequence-Related Amplified Polymorphism (Wahyudi et al. 2020b). One of the approaches, Inter Simple Sequence Repeat (ISSR), is thought to be more robust than others and generates more polymorphism bands than RAPD (Das et al. 2018; Poerba et al. 2018). Furthermore, ISSR markers are extremely polymorphic, making them valuable for investigations of genetic diversity, phylogeny, genetic coding, genomic mapping, and evolutionary biology (Reddy et al. 2002).

Several analyses of maize genetic variability have been conducted using molecular markers to assess aspects of plant evolution or to obtain genotype characterization (Idris and Abuali 2011). The use of hybrid vigor or heterosis in maize culture is a remarkable example of applied genetics success since it provides for a better knowledge of genetic phenomena and plant breeding in general (Baldauf et al. 2016). In East Nusa Tenggara, research on the genetic variability of local maize cultivars is still limited to Timor region (Yulita and Naiola 2013; Yulita et al. 2015; Bani et al. 2018). To bridge a knowledge gap, this study investigated the genetic diversity of different local maize cultivars from several East Nusa Tenggara islands. The aims of this study are to determine the genetic diversity and relationship of local maize cultivars from several East Nusa Tenggara islands based on ISSR markers. The result of this study is expected to be used as basic information for local maize conservation and breeding program especially in producing high-yielding varieties based on local maize of NTT.

MATERIALS AND METHODS

Study area

Eight seeds of local maize cultivars were obtained and collected from local farmers of Lembata, Adonara, Timor, Alor, and Flores in East Nusa Tenggara Province, Indonesia (Table 1). The seeds were cultivated in January 2022 at the Center for Agrotechnology Innovation at Universitas Gadjah Mada, based in Kalitirto, Berbah, Sleman, Yogyakarta, Indonesia, while the molecular character analysis was carried out at the Genetics and Breeding Laboratory, Faculty of Biology, Universitas Gadjah Mada, Indonesia. The leaves of the maize were processed for further molecular analysis.

Genetic diversity analysis

DNA isolation

The DNA isolation procedure followed the standard Genomic DNA Mini Kit for Plant (Geneaid) protocol. Leaves of 2 weeks old maize were cut as much as 0.1 g then added with liquid nitrogen and finely ground to a powder state. The samples were transferred into a 1.5 mL microtube and added with GP1/GPX1 buffer and 5 µl of RNAse A, then mixed using a vortex. The mixture samples were incubated at 60°C for 10 minutes by inverting the tube every 5 minutes. Then 100 µL of buffer GP2 was added to samples, vortexed, and incubated on ice bath for 3 minutes. The sample was transferred to a filter column which had been placed in a 2 mL collection tube and centrifuged at 1000 x g. The supernatant was transferred to a new 1.5 mL microtube and added with 1.5 volumes of GP3 buffer, to which isopropanol was previously added, and vortexed for 5 seconds. As much as 700 µL of the mixture samples were transferred into the GD column, which has been placed in a 2 mL collection tube, then centrifuged at 15000 x g for 2 minutes. The liquid was discarded, and the GD column was placed back into the collection tube. The remaining mixture sample was added to the GD column and centrifuged at 15000 x g for 2 minutes.

As much as 400 µL of W1 buffer was added to the GD column and centrifuged at 15000 x g for 30 seconds. The liquid was discarded, and the GD column was placed back in the collection tube. A total of 600 µL of wash buffer, to which absolute ethanol was added priorly, was put into the GD column and then centrifuged at 15000 x g for 30 seconds. The liquid was discarded, and the GD column was put back in the collection tube and centrifuged at 15000 x g for 3 minutes to dry the matrix column. The dry GD column of samples were transferred to a new 1.5 mL microtube and added with 100 µL of preheated Elution Buffer/TE to the center of the matrix column. The mixture was rested for 3-5 minutes to ensure the Elution buffer is fully absorbed. The mixture samples were centrifugated at a speed of 15000 x g for 30 seconds to elute the purified DNA. The isolated DNA was stored at -20°C as stock for further analysis.

Inter Simple Sequence Repeat (ISSR)

DNA was tested quantitatively using a nanodrop spectrophotometer at a wavelength of 260nm/280nm (Spectronic 20D). DNA was then amplified using 5 ISSR molecular markers (i.e., ISSR-807, ISSR-808, UBC-809, UBC-811, UBC-834) with annealing temperatures ranging from 52.0-58.4°C (Table 2). PCR reactions were carried out with a total volume of 25 µL consisting of 8.5 µl Nuclease-free Water, 8.5 µL Dream TaqTM Green PCR Master Mix 2x, 2 µL primer (10 pmol/µL), and 2 µL DNA template (25 ng/µL). DNA was amplified using PCR (BioRad) with one initial denaturation cycle at 94°C for 2 minutes, 40 denaturation cycles at 94°C for 1 minute, 40 annealing cycles at a temperature according to the specific primer (Table 2) for 1 minute, and 40 extension cycles at 72°C for 1 minute. The amplification was terminated with one final extension cycle at 72°C for 5 minutes.

Table 1. Cultivars, source's locations, and islands of 8 local maize cultivars from East Nusa Tenggara, Indonesia

Cultivar	Source's location	Island
Watar Ohaq	Leuburi Village, Buyasuri Sub-district, Lembata District	Lembata
Watar Puluq	Leuburi Village, Buyasuri Sub-district, Lembata District	Lembata
Wata Bura	Nelelamawangi Village, Ile Boleng Sub-district, Flores Timur District	Adonara
Wata Kuma	Nelelamawangi Village, Ile Boleng Sub-district, Flores Timur District	Adonara
Wata Bunga	Nelelamawangi Village, Ile Boleng Sub-district, Flores Timur District	Adonara
Pena Muti	Bileon Village, Fautmolo Sub-district, Timor Tengah Selatan District	Timor
Boti Pulut	Kabola Village, Kabola Sub-district, Alor District	Alor
Latun Wara	Nanga Mbaling Village, Sambi Rampas Sub-district, Manggarai Timur District	Flores

Table 2. List ISSR primers used in this study

Primer	Sequence (5'–3')	Annealing (°C)
ISSR-807	(AG)8T	55.8
ISSR-808	(AG)8C	55.8
UBC-809	(AG)8G	58.4
UBC-811	(GA)8C	55.1
UBC-834	(AG)8YT	52.0

Electrophoresis gel

For the preparation of the agarose gel, agarose powder was weighed using an analytical balance and poured into an Erlenmeyer flask and dissolved with 1x TBE buffer. The Erlenmeyer flask was covered with plastic wrap and pierced with a needle to allow steam to escape. The flask was then heated in a microwave until the solution was homogenous, as indicated by the absence of cloudiness (clear). The isolated DNA was electrophoresed using 2% agarose gel which had been mixed with Florosafe DNA Staining (1st Base) in 1x TBE buffer (pH 8.3). A total of 6 µL of each sample was placed in a gel well. Electrophoresis was carried out at 100 volts for 40 minutes. When the sample reaches the fourth line from the end pole (anode), the electrophoresis process can be terminated. The gel was then observed under Geldoc UV-Transilluminator for visualization of DNA bands and documented for tape measurement requirements.

Data analysis

GelAnalyzer ver.19.1 was used to assess DNA bands based on band migration distances on gels for ISSR molecular markers analysis, and the gels were scored for the presence or absence of bands, resulting in a binary matrix. Jaccard's similarity coefficient was calculated to evaluate genetic distances and tree construction among accessions (Wahyudi et al. 2020b). Later, the Unweighted Pair-Group Method Using Arithmetic Average (UPGMA) with MVSP ver.3.1 was used to represent genetic distances and build dendrogram among samples.

RESULTS AND DISCUSSION

DNA concentration and purity of 8 local maize cultivars from East Nusa Tenggara

According to DNA quantification results from 8 local maize cultivars, the concentration of isolated DNA ranges from 32.7-127.4 ng/L, and DNA purity is based on the A260/A280 absorbance ratio ranges from 1.779-2.109. (Table 3). The absorbance ratio of A260/A280 in four samples of maize cultivars from Watar Ohaq, Wata Bura, Wata Kuma, and Latun Wara was generally between 1.8-2.0, indicating high DNA purity. Other samples with absorbance ratios outside the 1.8-2.0 range were found in Wata Bunga and Pena Muti (<1.8), Watar Puluq and Boti Pulut (>2.0).

The DNA purity was determined by the ratio of 260/280 nm wavelength, where good purity (less or no contaminant) is when the value is in the range of 1.80-2.00 (Widyastuti 2017). According to Lucena-Aguilar et al. (2016), the purity value of less than 1.60 indicated the presence of a contaminant such as protein and phenol. When visualized by Geldoc UV-Transilluminator, a contaminated material can induce PCR process inhibition and a poor form of the DNA band (Widyastuti 2017). If the purity value is greater than 2.00, the sample was contaminated by RNA because the range for RNA purity is 2.00-2.20 (Lucena-Aguilar et al. 2016).

Based on these results using ISSR as molecular markers on 8 local maize cultivars from East Nusa Tenggara, revealed that some of the cultivars show a variety of DNA concentration and DNA purity (Table 3). DNA profiling based on polymerase chain reaction (PCR) does not require high concentration and purity. According to Weising et al. (2005), the isolated DNA can be used in the PCR amplification stage.

Table 3. DNA quantification results based ISSR molecular markers on 8 local maize cultivars from East Nusa Tenggara, Indonesia

Cultivar	DNA concentration (ng/µL)	Purity (A260/A280)
Watar Ohaq	32.7	1.901
Watar Puluq	127.4	2.109
Wata Bura	37.2	1.940
Wata Kuma	42.2	1.946
Wata Bunga	52.2	1.787
Pena Muti	58.2	1.779
Boti Pulut	33.1	2.056
Latun Wara	46.2	1.993

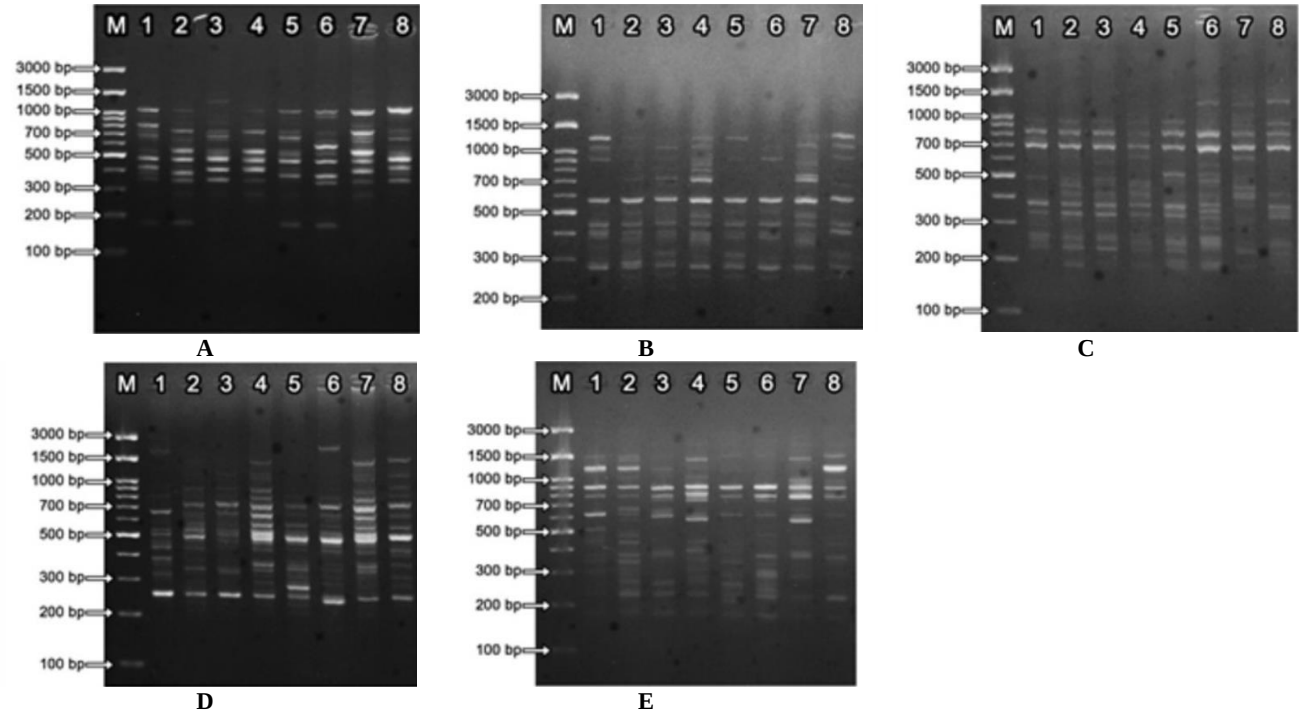

Figure 1. ISSR profile of each primer. A. ISSR-807, B. ISSR-808, C. UBC-809, D. UBC-811, and E. UBC-834) on 8 local maize cultivars from East Nusa Tenggara. Note: 1. Watar Ohaq, 2. Watar Puluq, 3. Wata Bura, 4. Wata Kuma, 5. Wata Bunga, 6. Pena Muti, 7. Boti Pulut, 8. Latun Wara. M: DNA ladder100 bp (Geneaid

ISSR profile of 8 local maize cultivars from East Nusa Tenggara

DNA amplification from the extraction of leaf samples derived from 8 local maize cultivars from Lembata, Adonara, Timor, Alor, and Flores in East Nusa Tenggara using 5 ISSR molecular markers (ISSR-807, ISSR-808, UBC-809, UBC-811, UBC-834) resulted in a total of 276 bands with a fragment range between 227 to 1675 bp. More than 75% of polymorphic band resulted (Figure 1 and Table 4) where ISSR-807 produced the most polymorphic band (Figure 1).

Further examination showed that ISSR-807 produced DNA band ranging from 227-1328 bp, 57 for number of bands, and 13 for number of amplified loci; ISSR-808 generated DNA band ranging from 325-1287 bp, 52 for number of bands, and 12 for number of amplified loci; UBC-809 resulted in 265-1675 bp, 59 for number of bands, and 17 for number of amplified loci; UBC-811 produced 281-2263 bp, 57 for number of bands, and 15 for number of amplified loci; and UBC-834 generated DNA band ranging from 230-1663 bp, 51 for number of bands, and 17 for number of amplified loci. Based on the polymorphism, it showed a variety on number of polymorphic loci for each ISSR molecular markers, where the highest was produced by UBC-809 and UBC-834 (15 loci), followed by UBC-811 (13 loci), ISSR-807 (12 loci), and ISSR-808 (9 loci). The total number of amplified loci was 74 while the average was 14.8. In total, there were 64 loci for number of polymorphic loci where the average was 12.8%. The polymorphism percentage showed a variety for each ISSR molecular markers, where the highest was produced by ISSR-807 (92.31%), followed by UBC-809 and UBC-834 (88.24%), UBC-811 (86.67), and ISSR-808 (75.00%), where the average was 86.09%. The summary of these value on polymorphism is shown in Table 4.

PCR parameters, buffer solution, and polymerase enzyme type can all affect the success of the amplification process (Lorenz 2012; Abdel-Latif and Osman 2017). Moreover, Uslan and Pharmawati (2020) noted that DNA extraction method, DNA concentration and purity, primers, and inhibitors such as proteins, phenols, RNA, and other compounds in cells can influence the success of DNA amplification. According to Wahyudi et al. (2020b), the absence of DNA bands may potentially be attributable to primers that do not match the template DNA, resulting in insufficient amplification. A difference in a single base pair is sufficient to cause a mismatch in the mold, resulting in ineffective amplification. In addition, the polysaccharide and other organic chemical content of flour isolates can inhibit the activity of DNA polymerase by mimicking the structure of nucleic acids (Schrader et al. 2012). Primers' performance, especially their specificity and sensitivity, also affects amplification results. Primer specificity refers to the ability of the correct primer to amplify only the target DNA, while primer sensitivity refers to the minimum primer limit in amplifying DNA (Uslan and Pharmawati 2020). Primer sensitivity is necessary to determine local maize cultivars' genetic diversity, which has been shown by the ISSR molecular markers.

When primer nucleotides are paired with plant genome nucleotides, DNA bands form (Lorenz 2012). A large number of polymorphic can be explained by the use of

DNA templates or methods. According to Wahyudi et al. (2020a), the ability of the primer to detect the complementary DNA sequence in the DNA template has a significant impact on the power and authenticity of produced DNA bands. Furthermore, the primer's compatibility with the plant DNA sequence of the local maize cultivars is required for DNA amplification. The greater the number of sticking sites from the primer used, the greater the number of DNA bands produced (Yulita and Naiola 2013). According to Reddy et al. (2002), in general, primers with AG, GA, CT, TC, AC, and CA show more polymorphism than primers with other di-, tri-, or tetra-nucleotides. The size difference between the DNA bands is related to the length of plant DNA sites, which can be extended by primers. The longer and more noticeable the new DNA will be, the greater the distance between the primer site and the remaining nucleotides in the DNA template (Wahyudi et al. 2020b). DNA polymorphisms are differences in DNA sequences that occur in individuals, groups, or populations. Polymorphism occurs when two or more alleles at the same locus exist in the same population at a substantial enough frequency (Sukhumsirichart 2018).

ISSR molecular markers have a large polymorphism proportion and have been frequently used in maize plant to detect genetic variability, which is consistent with our findings. Muhammad et al. (2017) reported a polymorphism proportion of the 20 ISSRs molecular markers of 92.15 percent in order to determine maize variety in Pakistan. Valiyeva et al. (2019) studied the variety of dark seeded maize in Azerbaijan using an ISSR molecular marker with a polymorphism proportion of 94.6 percent. According to Amoon and Abdul-Hamed (2020), the predominant polymorphism proportion of ISSR was 88.3 percent that showed the effectiveness of ISSR for detecting genetic variability of maize. The same result was also shown in this study where more than 75% of polymorphic band were produced by ISSR markers.

Genetic variability and clustering of 8 local maize cultivars from East Nusa Tenggara

The summary of similarity matrix is shown in Table 5. Despite their geographical separation, Wata Kuma and Boti Pulut have the largest genetic distance (0.651), according to the similarity matrix. Furthermore, the genetic distance between Pena Muti and Boti Pulut is the smallest (0.291).

Further examination of the clustering manifested in the dendrogram based on the ISSR marker showed two main clusters at a similarity index of 0.43, namely group A and group B, each of which is further divided into two subgroups. Group A is divided into two subgroups with a similarity index of 0.52. Subgroup A1 consists of the cultivar Pena Muti (Timor Island) and subgroup A2 consists of cultivars Wata Bura, Wata Bunga, and Watar Puluq (Adonara and Lembata Islands) with similarities ranging from 0.56-0.61, despite the difference in geographical location. Cluster B is divided into two subgroups with a similarity index of 0.45 into subgroup B1 consisting of Latun Wara, Boti Pulut, and Wata Kuma (Alor, Flores, and Adonara Islands) with similarity ranging from 0.58-0.65, despite the difference in geographical location. Subgroup B2 consists only of Watar Ohaq (Lembata Island). The dendrogram of local maize cultivars from East Nusa Tenggara is shown in Figure 2.

Table 4. Results on range of molecular weight, number of bands, number of amplified loci, number of polymorphic loci, and polymorphism percentage of each ISSR molecular marker on local maize cultivars from East Nusa Tenggara

Primer	Sequence (5'-3')	Annealing (°C)	Molecular weight (bp)	Number of bands	Number of amplified loci	Number of polymorphic loci	Polymorphism percentage
ISSR-807	(AG)8T	55.8	227-1328	57	13	12	92.31
ISSR-808	(AG)8C	55.8	325-1287	52	12	9	75.00
UBC-809	(AG)8G	58.4	265-1675	59	17	15	88.24
UBC-811	(GA)8C	55.1	281-2263	57	15	13	86.67
UBC-834	(AG)8YT	52.0	230-1663	51	17	15	88.24
Total				276	74	64	
Average				55.2	14.8	12.8	86.09

Table 5. Similarity matrix based on UPGMA method clustering using MVSP software of 8 local maize cultivars from East Nusa Tenggara based on Jaccard's coefficient similarity

	Watar Ohaq	Watar Puluq	Wata Bura	Wata Kuma	Wata Bunga	Pena Muti	Boti Pulut	Latun Wara
Watar Ohaq	1.000							
Watar Puluq	0.396	1.000						
Wata Bura	0.455	0.548	1.000					
Wata Kuma	0.429	0.479	0.545	1.000				
Wata Bunga	0.444	0.610	0.575	0.408	1.000			
Pena Muti	0.429	0.543	0.478	0.396	0.533	1.000		
Boti Pulut	0.396	0.388	0.477	**0.651**	0.347	**0.291**	1.000	
Latun Wara	0.511	0.440	0.468	0.630	0.400	0.415	0.532	1.000

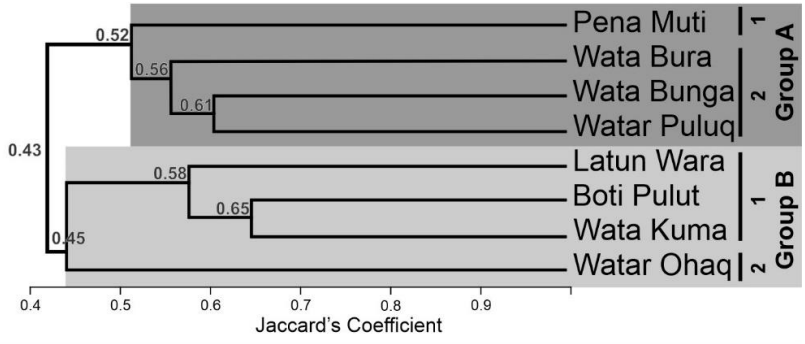

Figure 2. Dendrogram of 8 local maize cultivars from East Nusa Tenggara based on Jaccard's coefficient similarity with UPGMA method in MVSP software. Note: Arabic number represent sub-cluster

The greater the similarity index between the cultivars compared, the closer the relationship. A genetic similarity matrix in Table 5 demonstrates that local maize cultivars from East Nusa Tenggara have similarity values ranging from 29.1-65.1 (in percentage), indicating that the cultivars have high genetic diversity. The limited similarity of populations indicates the genetic diversity that can result from isolation, which facilitates the evolution of a specific feature. A diverse environment or geographical condition will also result in additional adaptation patterns and genetic traits (Poerba et al. 2018).

According to previous studies, our results differ slightly from those of Yulita and Naiola (2013), who studied several maize varieties from East Nusa Tenggara and found similarity values ranging from 30-80%. Yulita and Naiola (2013) stated that this difference may be due to close relationship used for their studies (local maize from the same island, namely Timor Island), whereas this study used local maize from different islands, namely Timor Island (Pena Muti), Lembata Island (Watar Ohaq and Watar Puluq), Adonara Island (Wata Bura, Wata Kuma, and Wata Bunga), Flores Island (Latun Wara), and Alor Island (Boti Pulut). Individuals of diverse origins will, in general, have varied features due to the presence of natural selection. The presence of barriers and diversity in habitat that divides the islands can cause genetic difference. According to Jannah et al. (2022), isolated populations with distinct habitats or geographical conditions can stimulate the development of certain characteristics, resulting in different adaptation patterns and distinct genetic characteristics. Furthermore, Raji and Siril (2021) noted that geographical dispersion, isolation, and segregation within the species all influence genetic diversity at the species level.

It can be concluded that ISSR molecular markers (ISSR-807, ISSR-808, UBC-809, UBC-811, and UBC-834) were found to be capable of detecting genetic diversity in 8 local maize cultivars from East Nusa Tenggara, where populations were separated into multiple clusters. A conservation action plan is needed to secure the future survival of local maize cultivars in order to boost maize production utilizing local maize cultivars in East Nusa Tenggara to meet the community's food needs. Other DNA molecular markers and genomic analysis will be significant in the future breeding of local maize cultivars in Indonesia.

ACKNOWLEDGEMENTS

The authors thank Agrotechnology Innovation Center of Universitas Gadjah Mada and Genetics and Breeding Laboratory, Faculty of Biology, Universitas Gadjah Mada, Indonesia for facilitating this research. Thanks also to Budi and Kristanto who have helped in maintaining the plants. This study was financially supported by Lembaga Pengelola Dana Pendidikan (LPDP).

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