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Cluster analysis of *Dioscorea alata* accessions of Purwodadi Botanic Gardens (Indonesia) collection based on morphological characteristics and SSR markers

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Abstract. *Mas'udah S, Fauziah, Hapsari L. 2019. Cluster analysis of* Dioscorea alata *accessions of Purwodadi Botanic Gardens (Indonesia) collection based on morphological characteristics and SSR markers. Cell Biol Dev 3: 6-12.* The morphological characteristics of D. alata accessions show a high diversity, especially the tubers with varying form, size, weight, color, and flesh. Classification and naming of *D. alata* accessions generally use different local names in each region, causing problems. This study aimed to classify 20 accession numbers of *D. alata* collection of Purwodadi Botanic Garden collected from East Java based on tuber morphological characteristics and simple sequence repeat (SSR) molecular markers. Based on the morphological characteristic, the accessions showed high variation. However, from the seventeen characters observed, two characters were similar, and 15 others were diverse. The main morphological characteristics that contribute to the cluster are tuber skin color, tuber shape, and the color of the inner skin of the tuber. Clustering results based on DNA amplification showed different groups compared to the morphological characters cluster, although some accessions with close morphological characters were in the same molecular Group. The accession origin could not be used as the group marker. Marker E11 and A7 have the highest polymorphisms in this experiment. As conservation strategies, accessions with high-level similarity could be represented by one accession for maintenance efficiency. Conserving species until varieties level with different characters could enrich germplasm for breeding development.

Keywords: Classification, diversity, local varieties, microsatellite, phenotype, yam

INTRODUCTION

Dioscorea spp. is a tuber plant with a climbing stem; it belongs to Dioscoreaceae. The Dioscorea family comprises more than 600 species spread in the Tropical and Sub-Tropical regions. Still, less than 200 species have been identified (Ayensu and Coursey 1972), and only about 50 - 60 species have been cultivated as food and medicine (Coursey 1976; Onwueme 1996). Indonesia has many types of Dioscorea; there is *D. warbugiana*, *D. alata*, *D. keduensis*, *D. nummularia*, *D. esculenta*, *D. pentaphylla*, *D. sansibarensis*, *D. hispida*, *D. bulbifera*, etc. (Onwueme 1996; Herison et al. 2010; Trustinah 2013; Fauziah and Mas'udah 2015; Solikin 2017).

Dioscorea alata was one of the most important species, much cultivated and utilized by citizens, especially in Southeast Asia, including Indonesia (Onwueme and Ganga 1996). *D. alata* is a substitute for rice/sago as a staple food in the dry season (Sulistyono and Marpaung 2004). *D. alata* is locally known as uwi (Javanese), huwi (Sundanese), lame (Celebes), obi (Madura), and lutu (Maluku Island), which in other country known as greater yam, water yam, or ten-months yam (Onwueme dan Ganga 1996), Tuber of *D. alata* has a high content of starch and protein, vitamin C and antioxidant but low on sugar. The tuber was also a source of minerals, so *D. alata* was prospective to be functional food and supported the diver antioxidant. The tuber was the source of minerals considered functional food and supported food diversification (Hapsari 2014). Some studies of tuber processing were found, and its tuber could be used for bakery, cookies, flakes, muffins, noddle, vermicelli (Hapsari 2014); flour (Afidin et al. 2014); chips (Putri et al. 2017); *fries* (Munawaroh et al. 2018), etc.

Dioscorea alata in Indonesia showed high morphological diversity, especially in tuber shape, size, skin, and flesh color. Accessions clustering and naming of *D. alata* used different local names depending on the origin of the plant/tuber, which confused identification (Onwueme and Ganga 1996; Herison et al. 2010; Purnomo et al. 2017; Kinasih et al. 2017). Characterization and clustering of *D. alata* in Indonesia are needed as base information to make valid accession names and arrange development programs. Morphological characters and genetic markers could hold characterization and clustering. Many studies used morphological characterization to observe the diversity of plant germplasm used as base information. Furthermore, some molecular marker was developed to confirm the diversity of plant identity by morphological characters (Azrai 2005; Zulfahmi 2013)

Purwodadi Botanic Garden, Indonesian Institute of Science is an ex-situ plant conservation institute that collects *D. alata* from some region in Indonesia especially Java (Lestarini et al. 2012) some Dioscorea collected from Malang (Fauziah 2013), Nganjuk (Trimanto and Hapsari 2015) and Pasuruan (Fauziah and Mas'udah 2015). Some of

local variety was collected are Uwi Kelopo, Uwi Putih, Uwi Jaran, Uwi Sego, Uwi Perti, Uwi Bangkulit, Uwi Ireng, Uwi Alas, Uwi Klelet, Uwi Randu, Uwi Senggrani, Uwi Bangkong, Uwi Ndoro, Uwi Dusono, etc. A study of its utility and potential was needed to add *D. alata* tuber value and support the food security program. This study aimed to cluster accessions of D. alata collected in Purwodadi Botanic Garden based on morphological characteristics and molecular markers using simple sequence repeats (SSR) or microsatellites. SSR marker was a very useful genetic marker as if it is dominant, high polymorphism, and could detect variation between the population in a different region, individual variation in population and microsatellite was also cheap in an experiment (Powell et al. 1996: Siqueira et al. 2012; Tamiru et al. 2015; Vieira et al. 2016). The results of this study could be used as base information to choose a valid name for *D. alata* accessions as a conservation strategy and development program consideration.

MATERIALS AND METHODS

Area of Study

This research used 20 accessions of *Dioscorea alata* L. collection of Purwodadi Botanic Garden from Nganjuk, Malang, and Pasuruan. The research was divided into two experiments. The first experiment was morphological characters observation, and the second was a molecular analysis of the accessions. Morphological characters were observed in 2015 and 2016. Molecular analysis conducted in Biotechnology Laboratorium, Faculty of Agriculture, University of Brawijaya, Malang. Molecular analysis using fresh leaves from February-March 2015 planting and extract with Purelink® Plant Total DNA Purification Kit (Invitrogen 2012).

Methods

Morphological observation

Observation on vegetative morphological characters was held in March-April 2015 and 2016. Tuber morphological characters were observed after harvest, during which the harvesting period was from September until October 2015 and 2016. Morphological characterization used Descriptors for Yams (Dioscorea spp) by IPGRI/IITA (1997) with modification. The result of morphologic characterization showed in Table 2

Molecular analysis

DNA extraction was taken from the fresh leaf using Purelink® Plant Total DNA Purification Kit (Invitrogen 2012). Then, DNA amplification was done using nine SSR markers (Tostain 2006) from Macrogen, South Korea. Amplification processing used Techne PCR Thermocycler. The amplification cycle consisted of 25 cycles of denaturation for 30 seconds at 95 °C, and annealing depends on the primer (Table 3) for 1 minute and extension for 30 seconds at 72 °C. The final extension was performed using 72 °C for 8 minutes, and the final result of the amplification was confirmed by electrophoresis in 4% agarose gel with 1 μg/ml ethidium bromide in TBE buffer and visualized in UV transilluminator (Bio-Rad Universal Hood II Gel Doc System).

Data analysis

Morphological data were analyzed by similarity coefficient based on Euclidean coefficient, and the result was used to construct dendrograms by unweighted pair group method with arithmetic mean (UPGMA) (Sokal and Michener 1958) using PAST 3.21 program (Hammer et al. 2001). In addition, principal component analysis (PCA) of morphological characters was conducted to identify characters with the highest contribution.

Molecular data were analyzed by DNA band scoring, which showed up in agarose gel. An SSR band in agarose gel represented an allele from each accession. Each band of markers presented shows the allele of each accession, and the allele was scored as 1 when present and 0 when absent. Polymorphism percentage was counted by polymorphism allele in each primer. Cluster analysis used UPGMA methods and similarity coefficient with Euclidean by Paleontological Statistics (PAST) 3.21 (Hammer et al. 2001).

Table 1. Twenty accessions of *Dioscorea alata* observed

Accession code	Local name	Origin
DA 23	Uwi Kelopo	Pasuruan
DA 24	Uwi Putih	Pasuruan
DA 27	Uwi Ulo	Pasuruan
DA28	Uwi Perti	Pasuruan
DA29	Uwi Ulo	Pasuruan
DA30	Uwi Perti	Pasuruan
DA31	Uwi Bangkulit	Pasuruan
DA32	Uwi Kelopo	Pasuruan
DA36	Uwi Bangkulit	Pasuruan
DA43	Uwi Bangkulit	Nganjuk
DA44	Uwi Klelet	Nganjuk
DA46	Uwi Dusono	Nganjuk
DA48	Uwi Putih	Nganjuk
DA57	Uwi Ketan Putih	Malang
DA58	Uwi Biru	Malang
DA59	Uwi Lajer	Malang
DA63	Uwi Budeng	Malang
DA64	Uwi Ulo	Malang
DA66	Uwi Legi	Malang
DA67	Uwi Sego	Malang

Table 2. Scoring of morphological characters of *Dioscorea alata*

Abbreviation	Variable	Score
R	Roots on the tuber surface	3: few, 7: many
SH	Tuber shape	1: round, 2: oval, 3: oval-oblong, 4: cylindrical, 5: flattened, 6: irregular, 10: other
CR	Cracks on the tuber surface	0: absent, 1: present
NU	Number of tubers	1: one, 2: 2-5, 3: more than 5
HD	Hardness of tuber when cutting with a knife	1: easy, 2: hard
TEX	Texture of flesh	1: smooth, 2: grainy, 3: very grainy
SK	Tuber skin color	1: light brown, 2: brown, 3: yellowish, 4: greyish, 5: maroon, 6: purple
FS	Flesh color at a central transverse cross-section	1: white, 2: yellowish white, 3: yellow, 4: orange, 5: light purple, 6: purple, 7: purple with white, 8: white with purple, 9: outer purple/inner yellowish, 10: other
SKH	Skin color at the head of the tuber	1: white, 2: yellowish white, 3: yellow, 4: orange, 5: light purple, 6: purple, 7: purple with white, 8: white with purple, 9: outer purple/inner yellowish, 10: other
G	Gum is released by cutting the tuber	3: low, 5: intermediate, 7: high
DRM	Dormancy period/sprouting after dorman	1: 0-2 months, 2: 3-4 months, 3: more than 4 months
SZ	Tuber size (weight)	1: less than 1 kg, 2: 1-4 kg, 3: more than 4 kg
SKT	Tuber skin thickness	1: < 1 mm, 2: ≥ 1 mm
OXT	Flesh oxidation time after cutting	1: < 1 minute, 2: 1-2 minutes, 3: > 2 minutes
OXC	Flesh oxidation color	1: greyish, 2: purple, 3: orange, 7: other
STC	Stem color	1: green, 2: purplish green, 3: brownish green, 4: brown, 5: purple, 7: other
WGC	Wing color	1: green, 2: green with a purple edge, 3: purple, 5: other

Table 3. SSR marker used in DNA amplification

SSR code		Primer sequence	Allele size (bp)	Annealing temp. (°C)
B5[1]	F	TTCCCTGTAGGAAAAATAGTGA	233	60
	R	CGTCCCTAGAAAATTCAACCTC		
C5[1]	F	AACCAATTACCCTTTGTCATGG	441	58
	R	GCCTTGCAAGCAATTTTGA		
E11[1]	F	ATGGTGTTCTCCCATGCTTC	291	63
	R	ACCAAAAATCAGGCTTGTGC		
H12[1]	F	TTGTAATTGGGTGTTGTATTTGC	245	53
	R	CGGCCAAAACATTTTCTGAT		
H2[1]	F	AAACCAAACAGGCAAAGCAT	331	58
	R	TGCCCTGCTTGTAAGATTGA		
E10[1]	F	GAATACTGATGATGCATAAAGCAA	284	58
	R	CCATGGTGAAGAGGATGGAT		
F1[1]	F	ATGGCTCAAGAGCACACG	403	58
	R	GGGCCTCATAAACATGCAAT		
A4[1]	F	TTCGTTCTCGATAGCGGACT	348	53
	R	CCAGTTCCCAGCCTCTTGT		
A7[2]	F	GCCCCACCTTAATTTCAT	267	52
	R	GGAATGAGATGGGACGAGAA		

Noted: [1] Siqueira et al. (2011); [2] Siqueira et al. (2012)

RESULTS AND DISCUSSION

Accession clustering based on morphological characters

The result of 17 morphologic characters on 20 accessions of *D. alata* showed three main groups of *D. alata*. Group I consisted of five accessions, Uwi Bangkulit (DA31), Uwi Dusono (DA46), Uwi Klelet (DA44), Uwi Biru (DA58) and Uwi Budeng (DA63). Flesh color (FS) and skin color at the head of the tuber (SKH) showed the character of Group I. Their flesh color is purple or contains purple. Group II was divided into 2 subgroups. Subgroup II.1 consisted of Uwi Kelopo (DA32), Uwi Ulo (DA27), Uwi Perti (DA30), Uwi Bangkulit (DA36), Uwi Bangkulit (DA43) and Uwi Ulo (DA64). Subgroup II.1 has white and yellowish-white tuber flesh color. Although Uwi Bangkulit DA30 and DA43 were in this Group, these two varieties have purple inner skin color but white until yellowish white in tuber flesh color. Genetically, there are 3 colors in the

tuber flesh color of *D. alata*, white, yellow, and purple (Purnomo and Susandari 2009). Other characteristics for Subgroup II.1 were few roots in the tuber surface, and tuber size was small or under 4 kg plant^{-1}. Subgroup II.2 consisted of nine accessions, Uwi Perti (DA 28), Uwi Putih (DA48), Uwi Lajer (DA59), Uwi Legi (DA66), Uwi Sego (DA67), Uwi Ketan Putih (DA57), Uwi Putih (DA24), Uwi Ulo (DA29) and Uwi Kelopo (DA23). Subgroup II.2 has more than 1 tuber number per plant, skin thickness >1 mm, and outer skin color of tuber was brown until dark brown. The highest Euclidean Distance, with 12.5 degrees, was between Uwi Kelopo (DA23) and Uwi Bangkulit (DA31). It showed Uwi Kelopo (DA23) and Uwi Bangkulit (DA31) have different characteristic although found in the same district. The lowest Euclidean Distance, with 2.00 degrees, was between Uwi Putih (DA48) and Uwi Lajer (DA59). Uwi Putih (DA48) and Uwi Lajer (DA59) were collected from different districts but had similar characteristics.

Purnomo and Susandari (2009) showed that *D. alata* in Yogyakarta has an oval shape, cylindrical and oblong, with white, yellow, and purple flesh. Based on this experiment, the tuber shape of *D. alata* in East Java has high variation; in another way, the diversity of *D. alata* in East Java is higher. The character variations in plants may be influenced by the gene as an internal factor and supported by environmental factors as a stimulant to express the characters (Lestari and Apriyadi 2017). The local name of *D. alata* found that given by its shape. Key characteristics of *D. alata* were on the young stem, which winged and/or spines at the base and top of the stem, while in the tuber could be seen from the shape, inner skin color, root on tuber surface, skin thickness, tuber skin color, and time of tuber oxidation (IPGRI/IITA 1997). Results showed local names based on tuber shape did not represent the same characters. Clustering based on morphological characters did not represent tuber shapes as key characters of the Group. Mwirigi et al. (2009) identified four major clusters of 43 Kenyan local landraces that used morphological characters. Still, morphological and agronomic features showed inconsistent identification and could not be used as cluster key characters. Gene expression could be seen from morphologic characters, and the influencing genes could be traced through DNA bands. Molecular analysis and morphological analysis would complement each other.

Principal component analysis (PCA) showed 7 main characters with eigenvalue > 1. The main component with the highest proportion was PC4, with 78.9%. This value showed that PC4 significantly contributed to the clustering of 20 accessions tested. Characters with positive values in PC4 were found in SK or tuber skin color. Characters with the maximum contribution to genetic material diversity are characters with the highest and positive vector value (component) (Haydar et al. 2007).

Accession clustering based on molecular marker

The molecular analysis of nine markers showed five markers had polymorphism on the accession tested and the other four markers had no band after PCR. Table 5 shows the polymorphism of the SSR marker used in the experiment and polymorphic information content (PIC). PIC showed the informativeness level of the molecular marker. PIC is used as a standard to evaluate genetic markers based on DNA band after PCR amplification. PIC value is divided into three classes, PIC> 0.5 = very informative, then 0.25> PIC> 0.5 = moderate, and PIC <0.25 = low. The results of DNA amplification show that the E11 primer has the highest PIC value of 0.55, which means that the E11 primer had a fairly high informative value. In contrast, the primers C5 and A7 have a moderate informative value.

Dendrogram based on DNA band showed 2 group clusters of *D. alata* accessions, and Group I and Group II had different characters. Group II consisted of accessions with white tuber flesh and inner skin color white until yellowish.

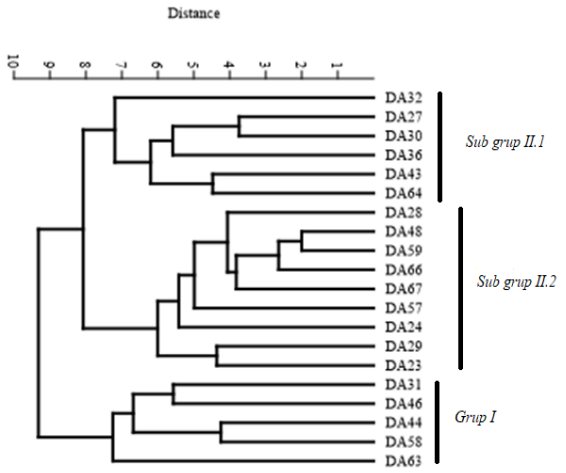

Figure 1. Clustering of *Dioscorea alata* accessions based on morphological characters

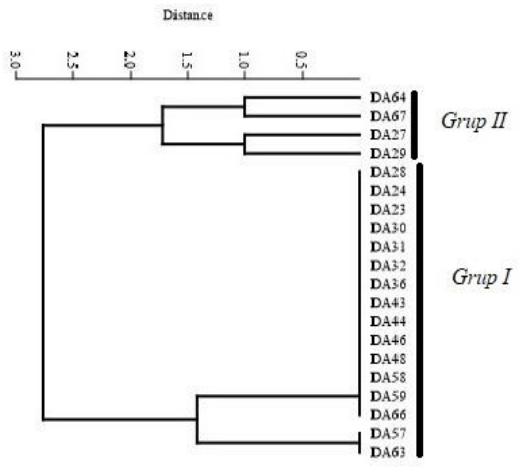

Figure 2. Clustering of *Dioscorea alata* accessions based on molecular markers

Clustering based on molecular marker analysis differed from clustering based on morphological characters. That could happen due to the gene flow between accessions as mentioned in *Manihot esculenta* (Halsey et al. 2008), *Ipomoea batatas* (Roullier et al. 2013), and *Dioscorea bulbifera* (Silva et al. 2016). The loss of genetic structure in *D. alata* is related to the behavior of farmers who often exchange planting material (in this case in the form of tubers) between farmers and even bring the planting material to other regions and causing the mixing of genotypes (Siqueira 2011; Siqueira et al. 2014). Farmers' preference to grow *D. alata* depends on the plant's usefulness to farmers. Furthermore, this affects the genetic diversity of these plants because, generally, plants that farmers and not of economic value do not sufficiently utilize will be lost from the region. The farmers selected variants/accession, maintained, and multiplied by clonal propagation. Many clonally propagated crops exhibit a mixed reproductive system in which evolutionary dynamics result from interaction. (Roullier et al., 2013) Vegetative propagation used for crops affects gene flow from experimental or commercial material. (Halsey et al. 2008). Uwi (Yam) is a dioecious plant, and crossing that occurs spontaneously contributes to some accessions, besides selection in plants that experience somatic mutations can be the main source of genetic diversity at the farm level (Obidiegwu et al. 2009).

Implications in ex-situ conservation strategies

Grouping of accessions *D. alata* based on the region cannot be determined because the character possessed by accession originating in one area also appears in accessions from other regions. For this reason, conservation strategies that can be carried out in rescuing *D. alata* diversity in Indonesia must be accomplished to varieties. (local varieties). That is supported by the results of the study of Purnomo et al. (2017), which states that the *D. alata* kinship tree based on the results of RAPD molecular analysis shows no groups formed based on regional origin.

Table 4. Principal component analysis of 17 morphological characters of *Dioscorea alata* accessions

Variable	PC 1	PC 2	PC 3	PC 4	PC 5	PC 6	PC 7	PC 8
R	-0.0383	0.1517	-0.3449	-0.1334	-0.0556	0.6431	0.4531	-0.1236
SH	-0.0030	-0.3363	0.2951	-0.2935	0.5527	-0.1527	0.5346	0.1709
CR	-0.0022	0.0164	0.0192	0.0435	0.0394	0.0330	0.0450	0.0071
NU	-0.0914	0.1193	-0.0269	0.1130	0.0615	0.3107	0.1696	0.1035
HD	-0.0189	-0.0311	-0.0711	-0.1470	0.0289	0.1184	0.2059	-0.0302
TEX	0.0027	0.0121	-0.0587	-0.0133	-0.0787	0.1017	0.0920	0.3952
SK	0.3009	-0.3775	0.1297	0.7409	0.2214	0.2540	0.0504	-0.1704
FS	0.5393	0.6756	-0.1276	0.0521	0.4474	-0.1368	0.0074	-0.0246
SKH	0.7690	-0.3418	-0.0442	-0.3642	-0.3105	0.1116	-0.0564	0.0827
G	0.0900	0.0884	-0.1428	0.3325	-0.4938	-0.4533	0.5964	0.0707
DRM	0.0083	-0.0059	0.1032	0.0958	-0.0330	0.0905	0.0042	-0.2067
SZ	-0.0031	-0.0019	0.0120	-0.0711	-0.0907	0.1219	-0.0385	-0.4375
SKT	-0.0199	0.0768	0.0251	0.0327	0.0311	-0.0210	0.1401	-0.0310
OXT	-0.0363	0.0329	-0.0904	0.1552	-0.0031	0.2520	-0.1864	0.6316
OXC	0.0655	0.3428	0.8403	-0.0349	-0.2798	0.2248	0.0950	0.0432
STC	0.0388	-0.0006	-0.0018	0.0894	0.0247	-0.0272	-0.0262	0.2559
WGC	0.0385	-0.0129	0.0564	0.1271	-0.0211	0.0413	-0.0160	0.2119
Eigenvalue	14.703	582.592	344.201	256.358	191.139	147.266	110.139	0.551
Proportion	45.273	17.939	10.599	78.937	58.855	45.346	33.914	16.962

Table 5. Polymorphism of SSR Marker used in this experiment

Primer	Number of polymorphism allele	Present on accession number-	Polymorphism (%)	PIC
B5	1	DA64	5	0.05
C5	1	DA27, DA29, DA57, DA63, DA64, DA67	30	0.3
E11	3	DA27,DA29, DA57, DA63, DA64, DA67	88	0.55
H12	1	DA29	5	0.05
A7	1	DA64, DAA67	40	0.1
H2	0	-	-	-
E10	0	-	-	-
F1	0	-	-	-
A4	0	-	-	-

The diversity of plant populations is needed to prepare future conservation and breeding strategies to improve crop varieties. Accessions with close similarity could be represented by one accession so that only one accession can be chosen for germplasm collection if the facilities and infrastructure are very limited. In contrast, remote-related accessions are accessions that are well used for breeding activities (Sukartini 2007). Based on the results of this study, Uwi Putih (DA24) from Pasuruan and Uwi Putih (DA48) from Nganjuk were accession with close similarities. Therefore, one of the accessions can be selected as an ex-situ conservation priority to avoid duplication and optimize facilities. Furthermore, based on the results of the grouping analysis with SSR markers, as many as 14 accessions from Group I had a large similarity distance, but it is known that there were differences in morphology, so they are still considered for conservation as the materials for further breeding and development.

In conclusion, morphological characterization of 20 access numbers of *D. alata* showed a very varied appearance. From 17 tuber morphological characters observed, there were two uniform and 15 diverse characters. The analysis of the main components of the morphological characters showed seven characters with a diversity contribution value of 17.9 - 78.9%. The character of the outer (tuber) skin color has the largest vector value and is thought to have a large contribution to the diversity of *D. alata*. In addition to the character of the skin color (outer) of the tuber, the shape of the tuber and the color of the inner skin of the tuber are characters with a large contribution to the diversity of *D. alata*.

The amplification of 9 molecular markers showed a level of polymorphism of 5 -88%. The highest polymorphism was found in E11 (88%) and A7 (40%) primers. Grouping accessions based on molecular markers SSRs give different results from grouping patterns based on morphological characters.

Conservation strategies that could be done to maintain the existence of *D. alata* and avoid duplication to streamline the use of facilities and infrastructures, namely choosing one of the accessions that have the same local name and the same morphological character (high level of similarity) and conserving at the cultivar level for accession with characters different but has a high level of similarity based on the grouping of DNA banding patterns.

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