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Evaluation of the genetic diversity of germplasms of Kaempferia galanga collected in Kon Ka Kinh National Park, Gia Lai Province, Vietnam

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SUMMARY

Gia Lai is a province in the Central Highlands of Vietnam, home to Kon Ka Kinh National Park, which boasts rich biodiversity and is one of 27 parks in the Southeast Asian region recognized as ASEAN heritage parks. Among the medicinal species in the park is Kaempferia galanga, a native medicinal herb with various benefits that requires conservation and development. To ensure the conservation and development of this herb, we conducted a study to investigate and collect samples, assess the genetic diversity of Kaempferia galanga species collected from 5 buffer zones of Kon Ka Kinh National Park and 5 provinces across the country (1. Me Linh, 2: Kon Ka Kinh 1, 3: Kon Ka Kinh 2, 4: Kon Ka Kinh 3, 5: Kon Ka Kinh 4, 6: Kon Ka Kinh 5, 7: Hiep Hoa, 8: Hue, 9: Dong Hy, 10: RCMP). The analysis evaluated the genetic diversity of Kaempferia galanga L species naturally distributed in Kon Ka Kinh National Park, Gia Lai Province, and some other provinces in the country. Through

the study using 13 ISSR primers on a population of 10 samples of Kaempferia galanga, the genetic relationship of these samples was determined with genetic similarity coefficients ranging from 0.59 to 0.97. The genetic diversity of the 10 contiguous samples fluctuated relatively high. These samples can be used for genetic resource conservation and new breeding selection. Additionally, 5 ISSR primers including UBC 807, UBC 834, UBC 840, UBC 844, and UBC 880 were identified for polymorphism in the 10 contiguous samples studied. Among them, primers UBC 807 and UBC 880 had the highest percentage of polymorphic loci and an average PIC index of 0.27 - 0.29. Moreover, primer UBC 807 was also found to have the highest Rp index at 3.4, followed by primers UBC 834, UBC 840, and UBC 844 with Rp indices ranging from 2 to 2.8.

Keywords: Kon Ka Kinh National Park, Database, Genetic diversity, *Kaempferia galanga* L.

1. INTRODUCTION

Kon Ka Kinh National Park is situated in the northeast of Gia Lai Province, 50 km northeast of Pleiku City. It spans the administrative boundaries of six communes across three districts, namely Kbang, Mang Yang, and Dak Doa. The total natural area of Kon Ka Kinh National Park covers 41,913.78 hectares, with natural forests occupying 38,991.58 hectares, accounting for 93%, while planted forests cover 163.47 hectares. The remaining area consists of bare land and agricultural land.

Kon Ka Kinh National Park boasts a diverse flora and fauna system, with 1,754 plant species belonging to 753 genera and 181 families, representing approximately 14% of the country's flora. Additionally, the park is home to over 300 species of medicinal plants, more than 100 species of mushrooms, and over 850 species of animals. Particularly noteworthy are the numerous valuable medicinal plants with high economic significance, such as ginseng, Gynostemma pentaphyllum, lime pot, royal jelly, lingzhi mushroom, golden orchid, and seven leaves one flower. Herbaceous plants constitute the majority of medicinal plants at 25%, followed by vines and epiphytes at 9%, shrubs at 8%, and other life forms accounting for about 3%. Woody plants represent 46% of the total.

The conservation value of plants in Kon Ka Kinh National Park is determined by the presence of rare plant species listed in the Vietnam Red Book, the IUCN Red List, and Government Decree 32/2006 ND-CP (Kon Ka Kinh National Park, 2016; Do Tat Loi, 1999; Do Huy Bich, 2004).

Among the medicinal species found in Kon Ka Kinh National Park, Kaempferia galanga L. stands out as an indigenous medicinal plant with a long history of use for various purposes such as pain relief, anti-inflammatory, and antipyretic effects, as well as in perfumes, cosmetics, and cuisine. However, overexploitation and trade by local communities have resulted in a significant decline in the wild population of this important species. Furthermore, there has been a lack of research and assessment on the current status of land plants in the area, hindering efforts to find sustainable solutions for exploitation and development.

It is imperative to conduct accurate identification and specific differentiation of species for research on cross-breeding, breeding, and the development of technical procedures for cultivation to ensure the correct species identification and clear origin. Utilizing molecular marker methods to identify the scientific names of land plants collected in Kon Ka Kinh National Park is crucial. This identification process will serve as a fundamental basis for research focused on the conservation and sustainable development of this medicinal species at the local level. (Kon Ka Kinh National Park (2016), According to The Resolution No. 9 - NQ/TU dated July 3, 2019).

Genetic diversity analysis can be conducted using various markers, including agronomic, biochemical, physiological, morphological, and molecular markers (Moulin, Rodrigues, Gonçalves, Sudré, & Pereira, 2012). However, morphological and biochemical characteristics have limitations in this evaluation as they can be significantly influenced by environmental factors. In contrast, molecular markers offer independence from environmental effects, stability, and a high level of polymorphism (Prashanth, Yugander, & Bhavani, 2015). Therefore, the selection of effective molecular markers is crucial for studying genetic diversity among plant accessions.

ISSR presents several advantages as a tool for assessing plant genetic diversity compared to other molecular marker techniques such as Random Amplified Polymorphic DNA (RAPD). ISSR offers enhanced reliability and reproducibility of DNA fragments, along with the ability to reveal a greater number of polymorphic bands. Moreover, ISSR primers are longer than RAPD primers, anneal at higher temperatures, and do not require prior sequence information (Prashanth, Yugander, & Bhavani, 2015; Zheng et al., 2015).

Furthermore, ISSR has been successfully utilized in studying the interspecific and intraspecific genetic diversity of the Zingiberaceae family, including species such as Curcuma alismatifolia, Kaempferia galanga, Zingiber officinale, Alpinia galanga, Curcuma sp., Elettaria cardamomum, Zingiber sp., and Etlingera elatior (Taheri, Abdullah, Abdullah, & Ahmad, 2012; Devi et al., 2015).

Given the importance of genetic diversity information for K. galanga, particularly for further conservation efforts and medicinal plant databases, this research aimed to study the genetic diversity of K. galanga. The study focused on selected ethnic groups in Kon Ka Kinh National Park of Gia Lai Province, employing ISSR markers.

2. MATERIALS AND METHODS

2.1. Plant Materials

Samples for this project were collected from the buffer zone of Kon Ka Kinh National Park, Vietnam, with sampling conducted in November and December 2023. This initiative is part of a research branch focused on investigating, statistically analyzing, and conserving medicinal plant genetic resources in Kon Ka Kinh National Park, Gia Lai Province, Vietnam. The collection of K. galanga genetic resources aims to facilitate genetic diversity assessment.

Leaf samples were collected directly, comprising 5 samples of K. galanga within the buffer zone of Kon Ka Kinh National Park and 5 samples from various provinces across the country (Table 1). Leaves were harvested directly from K. galanga plants, preserved using silica gel, and then wrapped in paper bags for storage (as used for DNA samples).

Table 1. Sample of K. galanga from 12 locations in Vietnam

No	Local names	Species	Region/province
1	Me Linh	K. galanga	Me Linh district, Hanoi city
2	Kon Ka Kinh 1	K. galanga	Core area 1 of Kon Ka Kinh National Park
3	Kon Ka Kinh 2	K. galanga	Core area 2 of Kon Ka Kinh National Park
4	Kon Ka Kinh 3	K. galanga	Core area 3 of Kon Ka Kinh National Park

5	Kon Ka Kinh 4	K. galanga	Core area 4 of Kon Ka Kinh National Park
6	Kon Ka Kinh 5	K. galanga	Core area 5 of Kon Ka Kinh National Park
7	Hiep Hoa	K. galanga	Bac Giang
8	Hue	K. galanga	Thua Thien Hue
9	Dong Hy	K. galanga	Thai Nguyen
10	Ha Noi Research Centre for Cultivation and Processing of Medicinal Plants (RCMP)	K. galanga	Thanh Tri district, Hanoi city

- ISSR primers used in the study

Table 2. ISSR primers used to evaluate genetic diversity of K.galanga samples

No	**Primer No**	**Primer sequence**
1	UBC807	(AG)8T
2	UBC 814	(CT)8A
3	UBC 834	(AG)8YT
4	UBC840	(GA)8CTT
5	UBC 844	(CT)8RC
6	UBC 856	(AC)8YA
7	UBC857	(AC)8YG
8	UBC 864	(ATG)6
9	UBC880	(GGAGA)3
10	UBC887	DVD(TC)7
11	UBC888	BDB(CA)7
12	UBC889	DBD (AC)7
13	UBC902	CTC (GT)8
B=C/G/T; D=A/G/T; R=A/G; Y=C/T); V=A/C/G		

2.2 Research method

2.2.1. Methods for extracting and investigating DNA concentration and quality

DNA extraction: DNA of samples studied was extracted with CTAB method. This method maximizes the amount of DNA extracted and minimizes the DNA breakage in the extraction process. After being extracted, these DNA were electrophoresed on a 1% agarose gel to check integrity and purity.

Polymerase Chain Reaction: Their volume was 20 µl including 10 mM Tris HCl (pH 8,4); 2 mM $MgCl_2$, 2mM dNTPs; 1,5 µM primer, 1U/µl Taq polyme0072ase and 2 µl ADN samples 10ηg/µl. The PCR process include 5 steps: (1) 94°C for 4 minutes; (2) 94°C for 1 minute; (3) from 43°C to 58°C for 45 seconds, it depend on the annealing temperature of each primer; (4) 72°C for 8 minutes and the incomes of this process were refrigerated at 4°C.

Electrophoresis of PCR products: PCR products were electrophoresed on a 1% agarose gel machine in 1X TAE environment with a voltage of 100V for 1 hour 30 minutes. These products were dyed by Ethidium Bromide 0,5ug/ml for 30 minutes. Pictures were analized by gel DOC machine.

2.2.2. Data analysis.

Based on the images of PCR products electrophoresis result, the appearance or not of DNA the presence or absence of a certain state on the gel will be recorded sequentially as 1 and 0. The data of Kaempferia galanca breeds with ISSR primers would be contained in Excel software. NTSYSpc.v2.10e solfware (Numberical Taxonomy System Personal Computer) were used to establish the genetic coefficients table and UPGMA branch diagram about the genetic relationship among Kaempferia galanca breeds based on the data of ISSR primers .

Excel software was used to calculate PIC and Rp index.

Pic index (polymorphism information content) was calculated according to the formula of Roldan-Ruiz et al. (2000):

$PIC_i = 2f_i (1 - f_i)$

I was the locus calculated, fi is the frequency of the allele appearing, (1- fi) is the frequency of the allele disappearing. The final PIC index result would be the average PIC of the total locus calculated according to the formula above.

Rp index (Resolving power) was the difference index of each primer pair according to Prevost and Wilkinson (1999). This index is calculated according to formular

$Rp = \sum Ib$

In particular, Ib (Band informativeness) has the formula: $Ib = 1 – [2 \times |0.5-p|]$ (p is the frequency of band appearance)

3. RESULTS AND DISCUSSION

3.1. Results of testing the purity of extracted DNA

The leaf samples for each accession from the field collections were weighed to 0.1 g and stored in a deep freezer (at -80°C) until use. Total genomic DNA was extracted from these samples using DNA kit isolation (Sigma GenEluteTM Plant Genomic DNA Miniprep Kit, Catalog Number G2N70) following the kit manual. The quality and quantity of extracted genomic DNA were determined using a spectrophotometry method with a UV-Vis spectrophotometer (UV-Vis spectrophotometer from Shimadzu, Japan) at absorbance readings of 260/280 nm. Additionally, the extracted DNA was checked using electrophoresis on 1% agarose gels at 100 V for 30 minutes (Bio-Rad, USA).

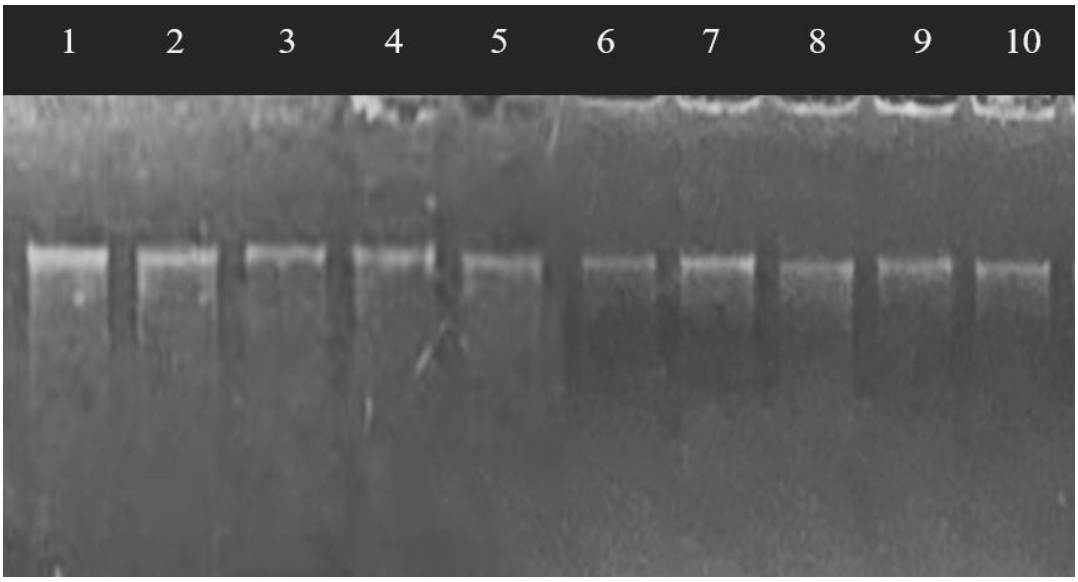

Fig 1. Electrophoresis image of DNA sample

The results of DNA sample electrophoresis showed compact band images, proving that the extracted DNA is intact and suitable for use in PCR reactions with ISSR primers.

3.2. The results of electrophoresis of the Kaempferia galanga DNA samples with ISSR primers.

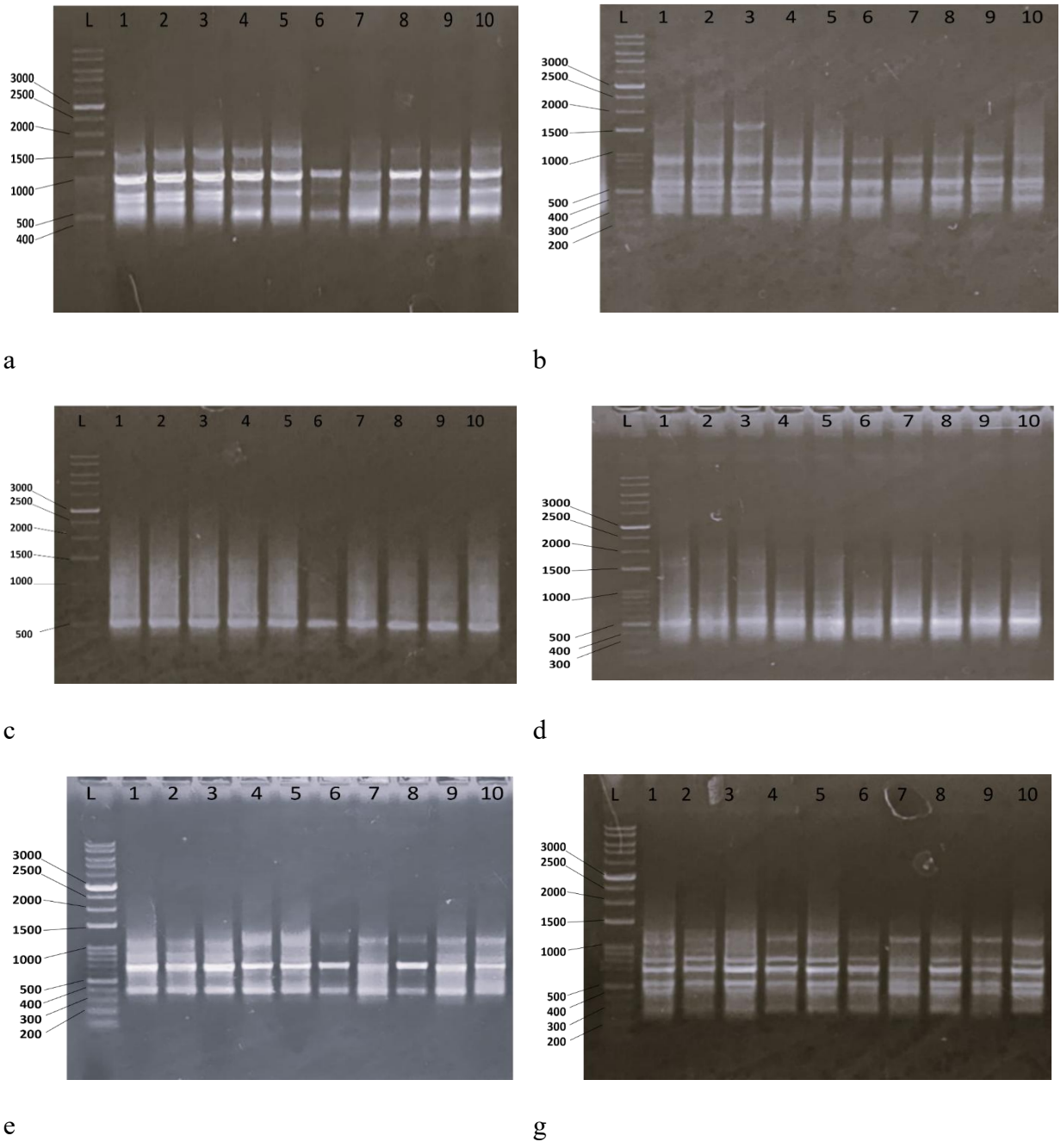

Fig 2. *DNA fragments amplified from K. galanga using ISSR primers (a). UBC840, (b). UBC844, (c). UBC856, (d). UBC880, (e). UBC807, (g). UBC834. (L) Ladder 1kb Opti-DNA ladder (ABM, UK) 1. Me Linh, 2: Kon Ka Kinh 1, 3: Kon Ka Kinh 2, 4: Kon Ka Kinh 3, 5: Kon Ka Kinh 4, 6: Kon Ka Kinh 5, 7: Hiep Hoa, 8: Hue, 9: Dong Hy, 10: RCMP.*

Table 3. Results of polymorphism analysis of ISSR markers across 10 samples of K. galanga.

No	Primer No	Primer sequence	Total locus	The number of polymorphic locus	The percentage of polymorphic locus (%)	PIC	Rp
1	UBC807	(AG)8T	6	4	66.67	0.29	3.4
2	UBC 834	(AG)8YT	7	3	42.86	0.166	2.8
3	UBC840	(GA)8CTT	5	2	40	0.164	2.6
4	UBC 844	(CT)8RC	6	3	50	0.18	2
5	UBC 856	(AC)8YA	1	0	0	0	0
6	UBC 864	(ATG)6	3	0	0	0	0
7	UBC880	(GGAGA)3	3	2	66.67	0.27	1.2

Among the 13 ISSR primers used to assess genetic diversity across 10 samples of Kaempferia galanga, 7 primers produced clear banding patterns, with 5 of them resulting in polymorphic outcomes. The percentage of polymorphic loci ranged from 40 to 66.67% (Table 3). ISSR primers UBC 807 and UBC 880 showed the highest percentage of polymorphism. Therefore, these two primers demonstrate effectiveness in evaluating genetic diversity within the studied population of Kaempferia galanga.

Rp index (Difference index between primer pairs): A higher Rp index indicates greater effectiveness of the primer in genotypic grouping. The highest Rp index is 3.4 for primer UBC807, while the lowest is 1.2 for primer UBC880. Primers UBC834, UBC840, and UBC844 have Rp indices of 2.8, 2.6, and 2 respectively. Thus, in this study, primer UBC807 is the most effective in assessing the polymorphism of Kaempferia galanga samples.

Polymorphic Information Content (PIC): PIC index is used to evaluate the polymorphic nature of primers within the studied population. A PIC index > 0.5 indicates high polymorphism, 0.25 < PIC < 0.5 indicates moderate polymorphism, and PIC < 0.25 indicates low polymorphism (Botstein et al., 1980). From the results in Table 1, PIC indices range from 0.18 to 0.29. The PIC indices of primers UBC807 and UBC880 are 0.29 and 0.27 respectively, falling into the moderate PIC index group. The remaining primers have low PIC indices. Therefore, primers UBC807 and UBC880 yield moderate polymorphism results within the studied population of Kaempferia galanga.

Table 4. Genetic similarity coefficients of 10 Kaempferia galanga genotypes with ISSR primers based on the similarity matrix. Data processed using NTSYSpc.v2.10e software.

	1	2	3	4	5	6	7	8	9	10
1	1.00									
2	0.93	1.00								
3	0.93	0.93	1.00							
4	0.62	0.69	0.62	1.00						
5	0.66	0.66	0.66	0.97	1.00					
6	0.66	0.59	0.59	0.83	0.86	1.00				
7	0.79	0.72	0.72	0.69	0.72	0.86	1.00			
8	0.76	0.69	0.69	0.79	0.83	0.90	0.90	1.00		
9	0.86	0.79	0.79	0.76	0.79	0.79	0.93	0.90	1.00	
10	0.86	0.79	0.79	0.76	0.79	0.79	0.86	0.90	0.93	1.00

Note: *1: 1. Me Linh, 2: Kon Ka Kinh 1, 3: Kon Ka Kinh 2, 4: Kon Ka Kinh 3, 5: Kon Ka Kinh 4, 6: Kon Ka Kinh 5, 7: Hiep Hoa, 8: Hue, 9: Dong Hy, 10: RCMP.*

The genetic relationships among the 10 samples of Kaempferia galanga are depicted through genetic similarity coefficients, ranging from 0.59 to 0.97 (Table 4). The genetic similarity coefficients of the 10 Kaempferia galanga samples exhibit relatively high variability, which may be attributed to their collection from various geographical regions.

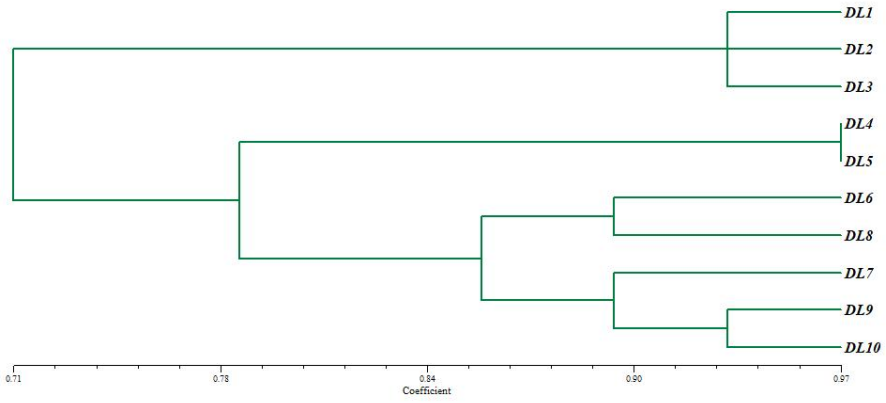

Fig 3. UPGMA dendrogram illustrating the genetic relationships among 10 genotypes of Kaempferia galanga based on ISSR primer data. Generated using NTSYSpc.v2.10e software.

Note: *1: 1. Me Linh, 2: Kon Ka Kinh 1, 3: Kon Ka Kinh 2, 4: Kon Ka Kinh 3, 5: Kon Ka Kinh 4, 6: Kon Ka Kinh 5, 7: Hiep Hoa, 8: Hue, 9: Dong Hy, 10: RCMP.*

Based on the dendrogram, the 10 samples of Kaempferia galanga can be divided into two main groups with an average similarity index of 0.78% (Figure 3). Group 1 comprises samples from Me Linh, Kon Ka Kinh 1, and Kon Ka Kinh 2. The samples Kon Ka Kinh 1 and Kon Ka Kinh 2 exhibit the closest similarity coefficient of 0.93, similar to that of Hanoi with Kon Ka Kinh 1 and Kon Ka Kinh 2. This indicates a very close genetic relationship among these three samples of Kaempferia galanga in group 1. Group 2 consists of the remaining 7 samples of Kaempferia galanga: Kon Ka Kinh 3, Kon Ka Kinh 4, Kon Ka Kinh 5, Hiep Hoa, Hue, Dong Hy, and RCMP. Samples Kon Ka Kinh 3 and Kon Ka Kinh 4 show the closest genetic relationship with a similarity coefficient of 0.97, while Kon Ka Kinh 3 and Hiep Hoa exhibit a distant genetic relationship with a similarity coefficient of 0.67 (Table 4). The remaining samples in group 2, including Kon Ka Kinh 4, Kon Ka Kinh 5, Hue, Dong Hy, and RCMP, show relatively close genetic relationships, with similarity coefficients ranging from 0.76 to 0.93 (Table 4, Figure 3).

4. CONCLUSION

The study conducted analysis and DNA extraction of Kaempferia galanga samples collected at Kon Ka Kinh National Park as a basis for further research.

ISSR molecular markers were used for grouping and assessing genetic diversity of Kaempferia galanga accessions collected from 10 locations. The genetic Similarity Index of K. galanga ranged from 59 – 97%, indicating a high level of genetic diversity among accessions. In general, high genetic diversity in K. galanga was due to the various environmental conditions found in its wide distribution area in Park Kon Ka Kinh Gia Lai provin and Some Provinces In Vietnam. Analysis of more accessions from different locations and using another molecular marker would provide complete information of genetic diversity of K. galanga accessions in Viet Nam.

Through research, 5 ISSR primers were identified, including UBC 807, UBC834, UBC840, UBC844 and UBC880, for polymorphism in 10 studied land samples. Among them, primers UBC807 and UBC880 are the two primers that give the highest percentage of polymorphic loci and an average PIC index. Besides, primer UBC807 was also determined to have the highest Rp index, followed by primers UBC834, UBC840 and UBC844.

5. ACKNOWLEDGEMENT

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