Emir. J. Food Agric. 2014. 26 (3): 247-258
doi: 10.9755/ejfa.v26i3.16498
http://www.ejfa.info/

PLANT SCIENCE

Genetic relationship between roselle (*Hibiscus sabdariffa* L.) and kenaf (*Hibiscus cannabinus* L.) accessions through optimization of PCR based RAPD method

Omalsaad[1], A. K. M. Aminul Islam[2*], Murshida Akhter Jahan[3], Zahira Yaakob[4] and Mohamad Osman[5]

[1]School of Environmental and Natural Resource Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi 43600, Malaysia
[2]Department of Genetics and Plant Breeding, Faculty of Agriculture, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur 1706, Bangladesh
[3]B-1/8, BSMRAU Campus, Gazipur 1706, Bangladesh
[4]Department of Chemical and Process Engineering, Faculty of Engineering & Built Environment, Universiti Kebangsaan Malaysia (UKM), Bangi, Selangor 43600, Malaysia
[5]Faculty of Plantation and Agrotechnology, Universiti Technology Mara (UiTM), Shah Alam, Malaysia

Abstract

The PCR based RAPD analysis was used to study genetic relationship among roselle and kenaf accessions. Extraction of genomic DNA samples of nine roselle (*Hibiscus sabdariffa* L.) and seven kenaf (*Hibiscus cannabinus* L.) accessions were successfully performed by Cetyl Trimethyl Ammonium Bromide (CTAB) method. RAPD analysis using two chloroplast primers and one mitochondrial primer produced about 62 bands of different sizes. Most of the fragments were polymorphic where 2-3 fragments were monomorphic in each primer. Sixteen accessions were classified into two main groups A and B through dendogram generated using RAPD polymorphism. Five kenaf accessions were included in group A, whereas nine roselle accessions and two kenaf accessions were included in group B. A group was formed with a range of similarity coefficients of 0.5333 to 0.8780 having all roselle accessions. Bengkalis was different from *Hibiscus* sp but it was close to roselle accessions. Noonsoon was very close to roselle accessions having a range of jaccard similarity coefficients of 0.5000 to 0.6326. It was evident from jaccad similarity coefficient of 0.8780 that the roselle varieties UKMR 1 and UKMR 2 were genetically closer to each other. On the other hand, V36 and Thainung showed the highest similarity coefficient (0.7878) among kenaf accessions in group A. This study has successfully differentiated between two species by using chloroplast and mitochondrial markers which was due to high level of polymorphism. However, the chloroplast primers were more effective than M13 to study the genetic variation between roselle and kenaf.

Key words: DNA extraction, RAPD-PCR optimization, genetic similarity, phylogenetics

Introduction

Hibiscus L. is the type genus of the tribe Hibisceae of the family Malvaceae (Barssum-Waalkes, 1966). The genus contains about 300 species that grow in tropical and subtropical regions throughout the world (Anderson and Pharis, 2003). It includes both annual and perennial herbaceous plants. Some of the species are economically important as a source of food, beverage, fiber, medicines and other species as ornamentals (Wilson and Menzel, 1964; Mohamad et al., 2005; Bolade et al., 2009).

One species of *Hibiscus*, known as roselle (*Hibiscus sabdariffa*) which is originated in Africa (David and Adam, 1988) and widely cultivated in West Africa as vegetable plant. Roselle also known as sorrel, mesta, karkade and popular plant in Middle Eastern countries (Morton, 1987; Abu-Tarboush et al., 1997). It is also found in almost all tropical countries including Malaysia, South East Asia, Indonesia, and Thailand (Chewonarin et al., 1999; Rao, 1996). It has been introduced to Malaysia in early 1990s (Mohamad et al., 2002) and have been brought to Malaysia from India (Tsai

Received 11 May 2013; Revised 10 August 2013; Accepted 09 September 2013; Published Online 11 October 2013

*Corresponding Author

A. K. M. Aminul Islam
Department of Genetics and Plant Breeding, Faculty of Agriculture, Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur 1706, Bangladesh

Email: pulak292003@gmail.com

et al., 2002). Roselle is currently an important cash crop grown in the East Coast of Malaysia especially in Terengganu and Kelatan. The first commercial cultivation has been promoted by DOA, Terengganu in 1993. There are two varieties currently available for growers to cultivate, “Terengganu” and “Arab” (Mohamad et al., 2002). In April 2009, UKM released three new varieties named UKMR-1, UKMR-2 and UKMR-3, respectively. Roselle is locally known as "asam kumbang", "asam susur" and, "asam paya". It is grown mainly for its red acid succulent calyces that can be made into a drink or to make jams or jellies. The red coloring makes it a popular ingredient of commercial herbal teas. In some places its leaves are also used as a vegetable and its stem has a fiber that is sometimes used for domestic purposes. The seeds contain oil, which is good as a lubricant fuel, and used for making soap.

Another one, kenaf (*Hibiscus cannabinus* L.) is extensively used in fiber making. Kenaf is an annual herbaceous crop of the Malvaceae family, which is known for both its economic and horticultural importance. It is a fiber plant native to east central Africa where it has been grown for several thousand years for food and fibre (LeMahieu et al., 2003). Kenaf is a short day, annual herbaceous plant cultivated for the soft bast fibre in its stem (Dempsey, 1975). According to Dempsey (1975), there are more than 129 common names for kenaf worldwide. For instance, mesta (India, Bengal), stokroos (South Africa), Java jute (Indonesia), ambari (Taiwan) (Lin et al., 2004; Li and Mai, 2006). Kenaf grows in tropical and temperate climates and thrives with abundant solar radiation and high rainfall. Under good conditions kenaf can grow at a height of 5-6m in 6-8 months (Wood, 2003). Kenaf is composed of various useful components and within each of these plant components there are usable portions e.g. fibres and fibre strands, proteins, oils, and allelopathic chemicals. The combined attributes of these components provide ample potential product diversity to continue use and development of this crop (Webber and Bledsoe, 2002). Much research has been done in kenaf, and a large number of varieties have been developed to meet the demands of high-fiber-yielding and disease-resistant kenaf in the recent decades (Dempsey, 1975; Bitzer et al., 2000). Kenaf is commercially cultivated in more than 20 countries, particularly in India, China, Thailand, and Vietnam as an important crop (FAO, 2003).

Identification and understanding of genotypic relationships between roselle and kenaf germplasm is important, which significantly promote their effective utilization and conservation. Traditionally, identification was based only on morphological and agronomical features. Since it is difficult to identify cultivars based entirely on these features, it is important to find an effective method to accurately identify roselle and kenaf varieties to meet research needs. Several kinds of methods which can be used to measure levels and patterns of genetic variation, which range from morphological characterization to various DNA-based markers such as restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and simple sequence repeats (SSR) (Crawford, 2000; Newton et al., 2002; Martinez et al., 2003; Fontaine et al., 2004; Murtaza, 2006, Ferdousi Begum et al. 2013). Different types of molecular markers are used to detect differences between individuals (Bernardo, 2008). The following markers are mostly used in genetic diversity studies listed in chronological order: RFLP (restriction fragment length polymorphism), SSR (simple sequence repeats or just microsatellites), RAPD (randomly amplified polymorphic DNA) (Williams et al., 1990) or AP-PCR (arbitrarily primed PCR) ISSR (inter-simple sequence repeats) AFLP (amplified fragment length polymorphism), SNPs (single nucleotide polymorphisms) and, more recently, DarT (diversity array technology) and other high throughput platforms (Karp et al., 1997, Chen and Sullivan, 2003; Kilian et al., 2005). DNA fingerprinting techniques such as random amplified polymorphic DNA (RAPD) (Williams et al., 1990) permits to identify taxa and to determine the phylogenetic relationships for systematic analysis. The narrow genetic base of cultivars may lack sufficient allelic diversity. But investigations of genetic relatedness are possible based on mtDNA or cpDNA markers (Skuza et al., 2013). Chloroplast and mitochondrial markers showed high level of polymorphism than DNA markers. That is why the chloroplast primers were more effective than M13 to study the genetic variation between roselle and kenaf.

This study was carried out to determine the genetic relationship between roselle and kenaf through optimization of PCR based RAPD method. This study will contribute basic knowledge in the aspect of their phylogenetic relationships and intraspecific diversity.

Table 1. Roselle and kenaf accessions used in this study with their traits.

Accession	Stem	Leaf	Flower	Fruit
Roselle:				
Accession 3 (Malaysia)	Reddish	3-5 lobed	Red	Red
Accession 6 (Malaysia)	Reddish	3-5 lobed	Red	Red
Accession 8 (Malaysia)	Reddish	3-5 lobed	Yellow	Red
Accession 12 (Malaysia)	Red	3-5 lobed	Yellow	Red
Accession 21 (Exotic)	Dark red	3-5 lobed	Red	Dark red
Nigeria	Red	3-5 lobed	Red	Dark red
UKMR 1 (Malaysia)	Smooth reddish	3-5 lobed	Red	Red
UKMR 2 (Malaysia)	Smooth reddish	3-5 lobed	Light red	Dark red
Bengkalis (Indonesia)	Smooth green	3-5 lobed	Yellow	Green
Kenaf:				
Cuba	Rough green	3-7 lobed	Cream	Green
V36 (Exotic)	Rough green	3-7 lobed	Yellow	Green
Noon soon (Exotic)	Smooth green	3-7 lobed	Yellow	Green
Thai nung (Thailand)	Rough green	3-7 lobed	Cream	green
Kho ken (Thailand)	Rough red	3-7 lobed	Cream	Red
G4 (Exotic)	Smooth green	3-7lobed	Yellow-red	Green
G393 (Exotic)	Rough green	3-7 lobed	Yellow-red	Green

Materials and Methods

Plant material

Sixteen accessions of Hibiscus sp among which nine accessions of roselle (*Hibiscus sabdariffa*) and seven accessions of its relative kenaf (*Hibiscus canabinus*) were included in the present investigation (Table 1) were collected from five countries (Malaysia, Thailand, Nigeria, Cuba, Indonesia). Seeds of all accessions were germinated in different tray in the glasshouse, Faculty of Science and Technology (FST), UKM, Bangi. After a week, the geminated seeds are transferred into a small poly bag and left to grow for four weeks. Seedlings of roselle and kenaf accessions were planted in a pot inside the Greenhouse (temperature 20°C and relative humidity 60%).

DNA extraction

DNA extracted from young leaves of roselle and kenaf by using Cetyl Trimethyl Ammonium Bromide (CTAB) method (Murray and Thompson, 1980) which is a modified version of Doyle and Doyle's (1990) method. Approximately 2-3 g leaves were crushed into fine powder using a mortar and pestle in liquid nitrogen. Leaf powder was then placed in eppendorf tubes containing 1 ml buffer solution of CTAB and 2 µl β-mercaptoethanol and then incubated at 65ºC for 4 hours. Samples were kept at room temperature for five minutes after incubation. The mixture was then centrifuged at 13000 rpm for five min. The supernatant was transferred to a fresh tube after centrifugation. The mixture was treated with 200 µl chloroform: isoamyl alcohol (CIA) (24:1) and shaken gently by inverting the tubes 8-10 times then centrifuged at 13000 rpm for 15 min. Centrifugation steps were repeated to ensure no contamination of DNA genome with protein once the supernatant was transferred into new tubes and 500 µl of 24 chloroform : 1 isoamyl alcohol was added. DNA sample was treated with RNase to remove all RNA. A total of 0.6 volume of isopropanol kept in the refrigerator was added and then turned the tube gently until mixed well. Tubes were then stored overnight at -20ºC. The precipitate tube was centrifuged at a speed of 13000 rpm for 15 minutes and supernatant removed. Pellets formed at the bottom of the tube wre dried at room temperature for 10 minutes. Then a total of 1 mL CTAB wash buffer solution was added into the tube and incubated at room temperature for 30 minutes and centrifuged. Supernatant was discarded and resulting DNA pellet left to dry at room temperature after washing with 70% ethanol. Finally, 20-25 µl of TE buffer solution was added into the eppendorf tube containing the dried DNA pellet above. Then the samples were stored at -20 º C in the refrigerator.

Confirmation and quantification DNA quality

Quantification and analysis of quality are necessary to ascertain the approximate quantity of DNA obtained after isolation and the suitability of DNA sample for further analysis. The quality of DNA samples were determined by PCR analysis using primer specific gene nad5-F and nad5-R. Nad5 gene is multi-copy genes that are conserved in the mitochondrial genome and occurs naturally

in the genome of higher plants (Mannerlof and Tenning, 1997). Electrophoresis was carried out in 1% agarose gel using molecular marker size 800 bp. and when there is, indicating that the DNA quality of samples is good. On the other hand, if there is no DNA bands produced, suggesting that the low quality of DNA samples and DNA extraction process must be repeated. Table 2 shows the reaction time and number of PCR cycle used in PCR-NAD.

Nad5-F: 5' – TAG CCC GAC CGT AGT GAT GTT AA – 3'
Nad5-R: 5' – ATC ACC GAA CCT GCA CTG AGG AA – 3'

RAPD Polymerase Chain Reaction

Reaction mixture and conditions

PCR-RAPD reaction was performed in 25 µl reaction mixture consisting of 10x buffer (Promega), 25 mM $MgCl_2$ (Promega), 10 mM dNTPs (Promega), 20 µM primers, template DNA and 500 U Taq DNA polymerase (Promega) using Eppendorf Master Cycle. The thermal cycle profiles for PCR-RAPD reaction are presented in Table 2.

Primers

Three primers (Chl-1, Chl-4, and M13) were used for the PCR amplification. The sequences of these primers are given in Table 3. The primers were selected on the basis of their high polymorphisms when tested with poplar clones except for the M 13 universal primer (Castiglione et al., 1993). The 20 nucleotide primers are from a collection of primers copied on the complete sequence of rice chloroplast DNA obtained from Masahiro Sugiura, Chikusa, Nagoya 464, Japan (Castiglione et al., 1993).

Optimization of PCR parameter

Experiments were carried out with varying concentrations of DNA template and annealing temperature to optimize the PCR amplification condition. Optimization of PCR reactions needed to produce a protocol prior to RAPD amplification to generate a clear and consistent. The parameters for PCR were as PCR buffer, $MgCl_2$, dNTPs, OPERON primers (OPERON Technologies Inc., USA), the enzyme Taq polymerase, template DNA and sterile distilled water (dH_2O), annealing temperature and number of PCR cycles. Parameters involved in optimization are the quantity of template DNA. Four different concentrations of DNA template were used (0.3µl, 0.5µl, 1 µl, 2 µl) and the annealing temperature was varied from (36ºC, 37ºC , 38ºC, 39ºC, 40ºC, 41ºC and 42ºC).

Gel electrophoresis

Electrophoresis on 1.5% agarose gels in a 1X TBE buffer for 1 hour 30 minutes with a voltage of 80V to allow strips of DNA generated by PCR amplification can be separated. The gels were stained with ethidium bromide (EtBr) for 20 seconds and washed in distilled water for 15 minutes before photograph taken over UV light.

Table 2. Master PCR amplification cycles.

Step	Temperature (°C)	Duration (minutes)	Number of cycles
Initial Denaturation	95	3 min	1
Denaturation	94	1min	1
Annealing	38	1 min	40
Extension	72	2 min	1
Final Extension	72	5 min	1
hold	4		

Table 3. Sequence of primers used in this study and their expected amplified fragment size.

Primer	Sequences
M 13	5'-TTATGAAACGACGGCCAGT-3'
Chl-1	5' – GAGGCCTACGCCCCATAGAA – 3'
Chl-4	5' – TTCCCGTGTCTTCCGGCTTAC – 3'

DNA band scoring and determination of relatedness

Strips of DNA that differ between roselle and kenaf were scored to analysis data. The RAPD fragments were scored as present (1) or absent (0) of homologous DNA bands considering each amplified DNA fragment as an unit character. The molecular size of the amplification products was measured by comparing with marker DNA ladder. The presence (1) and absence (0) of the bands was scored in a binary data matrix. Polymorphic bands were scored and used for further analysis. The genetic distance were calculated based on the similarity coefficient and the dendrogram was created with the UPGMA (unweighted pair-group method using arithmetical averages) method of the NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System for personal computers) software, Version 2.1 to clarify the genetic relationships (Rohlf, 2000). Jaccard coefficients were used to calculate the value of genetic identity and the formula is shown below:

$$J = a / (a + b + c)$$

Where, J = Jaccard coefficients

a = total number of bands in two for the two accessions x and y

b = Total number of bands in accession x,

c = total number of bands in accession y.

Results and Discussion

DNA extraction and quantification

The CTAB method has been used successfully to isolate DNA from leaf tissues of all the accessions of roselle and kenaf. DNA extracts often contain RNA, proteins, polysaccharides, tannins and pigments, which may interfere with the extracted DNA. Most proteins were removed by precipitation using chloroform and isoamyl alcohol. RNAs was removed using RNase enzymeA. DNA samples of all accessions of roselle and kenaf were electrophoresed in 1% agarose gel with DNA marker λ HindIII. The concentration of genomic DNA was determined by comparing the brightness of the DNA band with the bands of λ HindIII DNA marker. The concentration of genomic DNA extracted from sixteen roselle and kenaf accessions ranged from 2.4 ng to 18 ng per µl of the diluted DNA (Table 4). The results of the extraction of DNA for the sixteen accessions, control (without DNA) and λ HindIII DNA marker after electrophoresis was shown in Figure 1.

Table 4. Concentration of genomic DNA extracted from different accessions of roselle and kenaf.

Roselle	DNA concentration (ng/ µl)	Kenaf	DNA concentration (ng/ µl)
UKMR 1	2.4	Kho ken	9.6
UKMR 2	18	Thai Nung	9.6
Accession 3	2.4	Noon Soon	2.4
Accession 6	18	V36	9.6
Accession 8	9.6	G393	9.6
Accession 12	9.6	Cuba	18
Accession 21	2.4	G4	18
Bengkalis	8.4		
Nigeria	8.4		

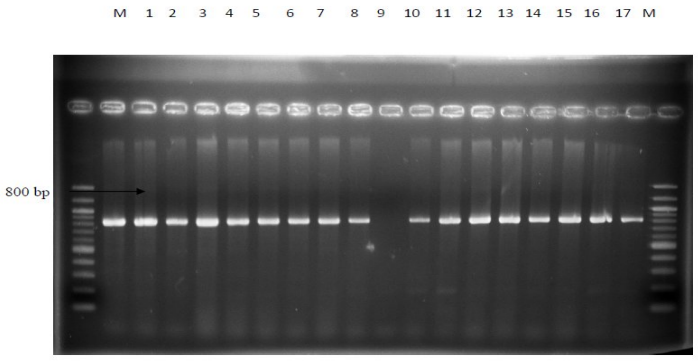

Figure 1. PCR products using nad5 primer for nine roselle and seven kenaf accessions. Lanes 1–17 = accessions except 9 (control: without DNA) as in Table 1; M = M: DNA size marker 100bp.

Determination the quality of genomic DNA

The extracted genomic DNA must be of high quality and pure so that DNA samples can be amplified using polymerase chain reaction (PCR) with efficiency. Before the genomic DNA used for PCR-RAPD reaction, DNA amplification using the primer nad5-F and nad5-R was done to ensure the reliability and quality of DNA. The expected amplified fragment size was 800 bp for good quality DNA after PCR amplification. NAD was able to produce 800 bp fragment size for DNA samples for all accessions studied (Figure 1). Nad5 gene is multi-copy genes that are conserved in the mitochondrial genome and occurs naturally in the genome of higher plants (Mannerlof and Tenning, 1997). Nad5 gene was used as internal control to ensure that genomic DNA was extracted with high quality and suitable for use in a subsequent PCR reaction.

PCR optimization

According to Williams et al. (1993) optimization is very important step to obtain a high intensity pattern of stripping the maximum number of bands of DNA. The concentrations of $MgCl_2$, primer, template DNA, dNTPs and Taq DNA polymerase were optimized using template DNA (0.2 µl, 0.3 µl, 0.5 µl, 1.0 µl and 2 µl) at the beginning of the PCR reaction to obtain clear banding pattern during amplification. However, it appears some of the PCR products did not produce any DNA band. After optimization found that 0.5 µl and 1µl of the diluted template DNA were used in the PCR-RAPD reaction to provide clarity and sharpness of the bands. Optimization of the annealing temperature was also done using temperatures of 34ºC, 36ºC, 37ºC, 38ºC, 39ºC, 40ºC, 41ºC. It was found that the optimum annealing temperature was 38ºC for most of the amplified DNA band compare to others. The genetic relationship in roselle (*Hibiscus sabdariffa* L.) varieties using M13 primer found that the optimum annealing temperature is 38ºC. Hence the concentration of $MgCl_2$, dNTPs mixture concentration, the concentration of primers, and Taq polymerase enzyme concentration were also varied in order to obtain the appropriate results. The initial concentration of $MgCl_2$ was used 2 mM. Various concentrations of $MgCl_2$ (2 mM, 2.5 mM and 3 mM) were studied and found that 3 mM was the optimal. The concentration of dNTP mixture was raised to 1 mM in the amplification reaction. The original mix of dNTP concentrations was 0.25 mM. It was found out that the concentrations of 0.5 and 1 mM dNTP mixture respectively produced bright band of DNA in amplification reaction.

RAPD profiles

Genetic relationships and genetic diversity in a number of plant taxa have widely been studied using RAPD markers because of its simplicity, speed and relatively low cost compared to other DNA-based markers (Esselman et al., 2000). But dominant inheritance and repeatability of the bands have limits the use of RAPD technique in the assessment of genetic diversity and genetic relationships (Fischer, 2000). Three primers were used for identification based on the DNA polymorphism of the accessions. All primers were amplified multiple fragments in each accession. Each sample of roselle and kenaf produced a specific band after running the PCR reaction and electrophoresis in 1% agarose gel irrespective of primers used (Figure 2A-C). This was because each of the amplified DNA fragment has a number of different repeating unit of minisatellite and size of the different lines represent different loci. The marker that used to determine the size of DNA fragment was 100 bp.

Band scoring

Three primers (Chlo 1, Chlo 4 and M13) were produced clear and reproducible bands across all the accessions of reselle and kenaf (Figure 2A-C). These primers generated 62 bands with different size range. Some polymorphisms were easy to score whereas other bands appeared to produce ambiguous fragments. Chlo 1 primer produced 25 fragments which range from 200 to 2381 bp with two monomorphic fragments (Table 5). The primer Chlo 4 produced 20 fragments which range from 200 to 2021 bp and among them two fragments were monomorphic. Whereas M13 primer produced 17 fragments that range from 300 to 1976 bp and three fragments were monomorphic fragments.

Table 5. The number of fragments scored with three primers.

Primer	The range size of bands	Total number of bands	Polymorphic bands	Polymorphic (%)
Chlo 1	200- 2381 bp	25	23	90
Chlo 4	200- 2381 bp	20	18	92
M13	300-1976 bp	17	14	82
Total		62	55	

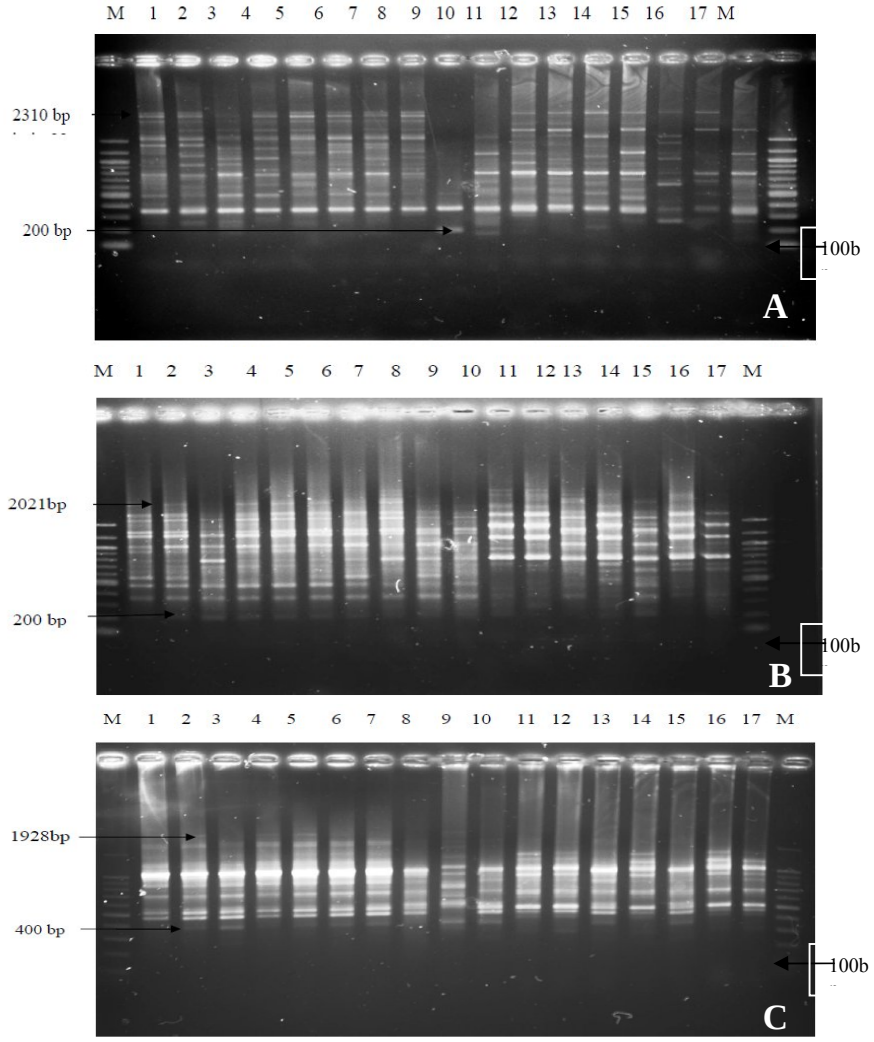

Figure 2. The RAPD profile using primers (A) chlo 1, (B) chlo 4, and (C) M 13 for nine roselle and seven kenaf accessions, Lanes 1–17 = landraces except 9 (control: without DNA) as in Table 1; M = M: DNA size marker 100bp

Jaccard similarity coefficient analysis

The genetic distance was calculated based on Jaccard's similarity coefficient using SIMQUAL program in NTSYS-pc software to clarify the genetic relationships (Table 6). An UPGMA dendrogram was constructed using NYSYSpc 2.10 software. A clear divergence between the two related species was found from the cluster analysis. Jaccard similarity coefficient between roselle and kenaf ranged from 0.2800 to 0.8780 which revealed a wide range of genetic identity. Jaccard mean genetic similarity values between accessions of roselle was 0.7052 and the accession of kenaf was 0.5489. While similarity coefficients between both roselle and kenaf accession was 0.5370. Based on this value it was noticed that the relationship between roselle accessions was closely related than between the accessions of kenaf. The mean identity of the polymorphism of the entire sample was 0.5370, indicating that high levels of polymorphism (Besse et al., 1993) for the accession of roselle and kenaf.

Table 6. The similarity coefficient by Jaccard in NTSYSpc 2.10e software between nine roselle and seven kenaf accessions.

	Acc3	Acc6	Acc8	Acc12	Acc21	UKMR-1	Nigeria	UKMR-2	Bengkalis	Cuba	V36	Noonsoon	Thainung	Khoken	G4	G393
Acc3	1.0000															
Acc6	0.8409	1.0000														
Acc8	0.6170	0.6808	1.0000													
Acc12	0.7200	0.7800	0.7500	1.0000												
Acc21	0.7500	0.8125	0.7083	0.8775	1.0000											
UKMR-1	0.7954	0.7446	0.6382	0.8125	0.8478	1.0000										
Nigeria	0.6888	0.6458	0.6818	0.6800	0.7083	0.7111	1.0000									
UKMR-2	0.7674	0.7173	0.6444	0.7500	0.8222	0.8780	0.7209	1.0000								
Bengkalis	0.5348	0.5333	0.6000	0.5744	0.6000	0.5952	0.5609	0.6000	1.0000							
Cuba	0.2978	0.2800	0.3111	0.3018	0.3673	0.3191	0.3720	0.3409	0.4000	1.0000						
V36	0.3469	0.3800	0.3913	0.4230	0.4400	0.3958	0.4883	0.4222	0.3846	0.6896	1.0000					
Noonsoon	0.5652	0.5625	0.5555	0.6326	0.5918	0.6222	0.6279	0.6279	0.5000	0.3095	0.4634	1.0000				
Thainung	0.4200	0.4230	0.4680	0.4905	0.5098	0.4693	0.5333	0.5000	0.5128	0.6363	0.7878	0.5116	1.0000			
Khoken	0.4583	0.5531	0.5111	0.5294	0.4901	0.4489	0.5454	0.4468	0.4871	0.2926	0.4141	0.6410	0.4651	1.0000		
G4	0.3913	0.3673	0.3478	0.3584	0.4000	0.3829	0.4418	0.4090	0.4444	0.7407	0.7333	0.3488	0.7272	0.3658	1.0000	
G393	0.3404	0.3469	0.3555	0.3148	0.3529	0.3333	0.4186	0.3555	0.4166	0.7692	0.6451	0.2954	0.6000	0.3414	0.7500	1.0000

High similarity coefficient of 0.8780 was measured between the accession UKMR-1 and UKMR-2. This relationship supported further by the results of cluster analysis, which showed accession UMMR-1 and UKMR-2 are members of the second group (B). These two roselle accessions have high similarity with their parent accession 21 as well (Figure 3). However, accession 21 had the highest similarity with accession 12 (0.8775). Furthermore, in the first group V36 accession and Thainung had the highest similarity coefficient between kenaf accessions (0.7878). Due to high rates of gene flow, exchange of germplasm and the pressure of natural selection the value of genetic similarity can increase (Rajasekar et al., 2005). According to analysis of the Jaccard similarity coefficient, accession 6 and Cuba had low similarity coefficient (0.2800). This indicated that both the accessions were genetically distant.

Genetic relationships between accessions of roselle and kenaf

The dendrogram constructed by the UPGMA clustering method revealed the genetic relationship among 16 accessions of roselle and kenaf (Figure 3). The cluster analysis demonstrated a considerable divergence among the included accessions, particularly between kenaf and roselle. RAPD analysis provided a relatively clear pattern of genetic relationships among the kenaf and roselle accessions. All the accession clustered into two major groups. The roselle accessions with two of kenaf accessions were clustered in one group and remaining kenaf accession was in the other group. The first group (A) consists of five accessions of kenaf (G393, Cuba, V36, Thainung and G4). Whereas, the second group (B) was consisted with eight accessions of roselle (Accession 3, Accession 6 ,Accession 12, Accession 21,UKMR 1,UKMR 2, Nigeria, Accession 8 and other species of hibiscus named Bengkalis). Two accessions of kenaf (Noonsoon and Khoken) were in the same group with roselle which indicated their genetic linkage to the related species.

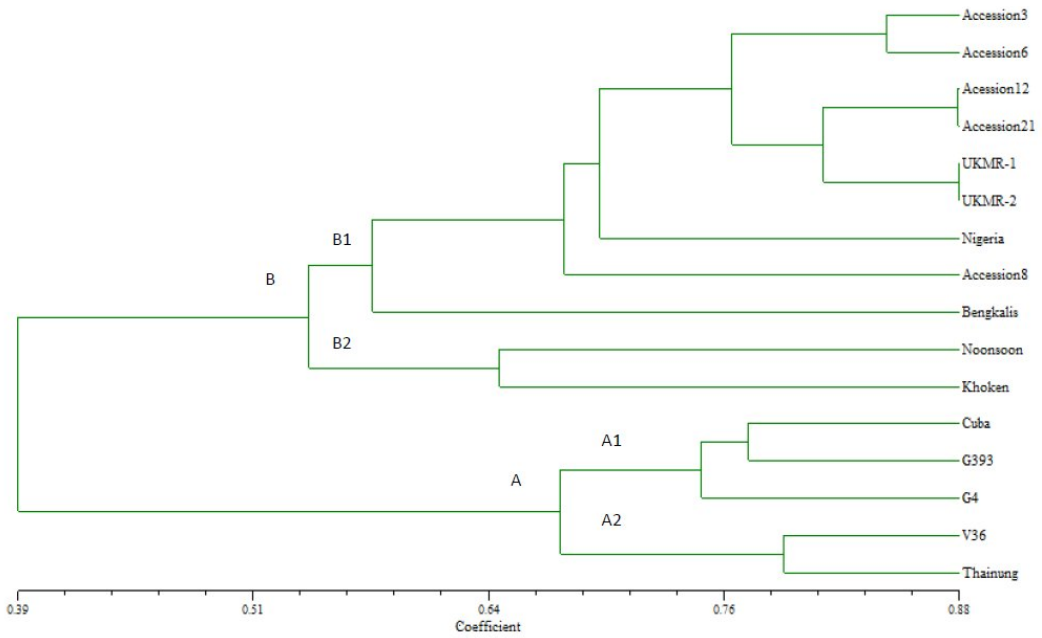

Figure 3. Dendrogram based on UPGMA analysis of genetic similarity of roselle and kenaf accessions, showing relationships among two species.

The first group (A) was divided into two subgroups namely (A1) and (A2). The subgroup A1 consisted with the accessions Cuba, G393 and G4, while the other subgroup (A2) was consisted with two accessions (V36 and Thainung). The second group (B) also consisted with two subgroups. The first subgroup B1 comprised with nine roselle accessions (accession 3, accession 6, accession 12, accession 21, UKMR 1 and UKMR 2, Nigeria, accession 8 and Bengkalis). While the second subgroup comprised of two kenaf accessions namely Noonsoon and Khoken.

Based on the results of the study, nine accessions of roselle and seven accessions of kenaf provided a range of genetic identity between 0.2800 to 0.8780. A wide range of genetic identity of these accessions showed that roselle had relatively low genetic variation. RAPD analysis showed UKMR 1 and UKMR 2 genetically was very close as well as accession 12 and accession 21 in the group B, whereas accessions 6 and Cuba showed the most distinct relationship. UKMR 1 was the closest accession with UKMR 2 having similarity coefficient of 0.8780 and UKMR 1 was close to Accession 21 with similarity coefficient 0.8478. This was because of UKMR 1 and UKMR 2 were a mutant resulted from accession 21. Very low genetic diversity (0.91-0.98 similarities) was also observed among ninety-four roselle accessions by Hanboonsong et al (2000). However, the range of similarities between the same species was much smaller than the range of different species (Figure 3). The range of genetic variation between accessions of roselle viz. UKMR 1, UKMR 2, Accession 12, Accession 21, Accession 3, Accession 6, Accession 8, Nigeria and Bengkalis were small (0.6170 to 0.8780). Accessions of kenaf had similarity coefficient range from 0.2926 to 0.7878 among the accessions G393, Cuba, V36, Thainung, G4, Noonsoon and Khoken. Results showed that the variation between kenaf accessions was higher compare to the variation between roselle accessions. It was due to two accessions of kenaf were more closed to roselle accessions. This was also supported by the dendogram which showed Noonsoon and Khoken in the same group (B) with roselle accessions.

Based on the dendogram generated clustering two kenaf accessions (Noonsoon and Khoken) have been grouped into the second group (B) together with the accessions of roselle. According to the jaccard similarity coefficient, the kenaf accession Noonsoon has low similarity value (0.2954 to 0.5116) compared to the similarity value with roselle which ranged from 0.5000 to 0.6326. Cheng et al. (2000) has concluded that the accessions of same species clustered in the same group in the dendogram. Referring to the dendogram, the kenaf accessions was generated into group A, which showed that they were closely related. However accession V36 had the closest relation with Thainung having similarity coefficient 0.7878. On

the other hand, roselle accessions were formed group B.

On the other hand, Bengkalis accession in group B showed high similarity coefficient with roselle accessions. Even though it was from different species and closed to roselle species according to jaccard similarity coefficient. The highest similarity between this accession with other roselle accession was 0.6000. Although Noonsoon and Khoken accessions are from kenaf species but jaccard similarity coefficient showed these two accessions were more related to roselle than kenaf. The similarity between the Noonsoon with other kenaf accession was also lower compared to roselle accessions. The similarity coefficient between Noonsoon and G393 was the lowest (0.2954). Khoken accession had the lowest similarity with Cuba accession with 0.2926. The same thing was noted with Khoken which is much closed with Noonsoon.

The sources of kenaf accessions in that study were narrow, and the number of DNA polymorphic fragments detected were relatively low. Cheng et al. (2002) in previous study reported that RAPD analysis was able to identify kenaf varieties and determine their genetic relationships. The present study showed that most of the roselle and kenaf accessions can be separated into two different groups. Two accessions from kenaf were found to be in the same group with roselle. This condition may occur due to amplification by RAPD primers did not occur in the same area with the other accessions of kenaf.

Conclusion

The present study on genetic relationships between roselle and kenaf accessions using chloroplast and mitochondrial primer based on RAPD markers is very useful to be able to know the intraspecific diversity. The differences between some accessions in the same species were not sufficient. Knowledge from this study is very important for taxonomic classification of roselle and kenaf to the conservation and management program and the development of varieties of these two species can be implemented more effectively.

References

Abu-Tarboush, H. M., S. A. B. Ahmed and H. A. Al Kahtani. 1997. Some nutritional properties of karkade (*Hibiscus sabdariffa*) seed products. Cereal Chem. 74:352-355.

Anderson N. and J. Pharis. 2003. Kenaf fiber-A new basket liner. Minnesota Commercial Flower Growers Bull. 52(3):7-9.

Bernardo, R. 2008. Molecular markers and selection for complex traits in plants: learning from the last 20 years. Crop Sci. 48:1649-1664.

Barssum Waalkes, J. Van. 1966. Malesian Malvacea revised. Blumea 14:1-251.

Bitzer M. J., C. G. Cook and B. S. Baldwin. 2000. The development of kenaf varieties in the United States. In: Proceeding of the International Kenaf Symposium. Hiroshima, Japan, pp. 91-94.

Besse, P., M. Seguin, P. Lebrun and C. Lanaud. 1993. Ribosomal DNA variations in wild and cultivated rubber tree (*Hevea brasiliensis*). Genome 36:1049-1057.

Bolade, M. K., I. B. Oluwalana and O. Ojo. 2009. Commercial Practice of Roselle (*Hibiscus sabdariffa* L.) Beverage Production: Optimization of Hot Water Extraction and Sweetness Level. World J. Agric. Sci. 5:126-131.

Castiglione S., G. Wang, G. Damiani, C. Bandi, S. Bisoffi and F. Sala. 1993. RAPD fingerprints for identification and for taxonomic studies of elite poplar (*Populus* spp.) clones. Theor. Appl. Genet. 87(1-2):54-59.

Chen, X. and P. F. Sullivan. 2003. Single nucleotide polymorphism genotyping: biochemistry, protocol, cost and throughput. Pharmacogen. J. 3:77-96.

Cheng, Z., B. S. Baldwin, Y. Ohtani and K. Sameshima. 2000. Identification of Method and Genetic Relationship among Kenaf (*Hibiscus cannabinus* L.) Varieties based on RAPD (Random Amplified Polymorphic DNA) Analysis. Proceedings of the Final workshop on "Application of Biotechnology in the Improvement of Jute, Kenaf and Allied Fibres- Phase II," (IJO/ AGR/10) Beijing, China 10-12 August 2000. pp. 61-72.

Cheng, Z., K. Sameshima and J. K. Chen. 2002. Genetic variability dissemination from and relationship of kenaf germplasm based on RAPD analysis (in Chinese). Plant Fibers Prods. 1:1-12.

Chewonarin, T., T. Kinouchi, K. Kataoka, H. Arimachi, T. Kuwahara, U. Initkekumnuen and Y. Ohnishi. 1999. Effects of Roselle (*Hibiscus sabdariffa* Linn), a Thai medicinal plant, on the mutagenicity of various known

mutagens in *Salmonella typhimurium* and on formation of Aberrant Crypt Foci induced by the colon carcinogens Azoxymethane and 2-Amino- 1Methyl-6-phenylimidazo (4,5-*b*) pyridine in F344 rats. Food Chem. Toxicol. 37:591-601.

Crawford, D. J. 2000. Plant macromolecular systematics in the past 50 years: one view. Taxon 49:479-490.

David, G. and P. Adam. 1988. Crops of the drier regions of the Tropics. ELBS Edt. Longman Publishing Co., Wisconsin, Madison, USA.

Dempsey, J. M. 1975. Fiber crops. The University Presses of Florida, Gainesville, USA.

Doyle, J. J. and J. L. Doyle. 1990. Isolation of plant DNA from fresh tissue. Focus 12:13-15

Esselman, E. J., D. J. Crawford, S. Brauner, T. F. Stuessy, G. J. Anderson and O. M. Silva. 2000. RAPD marker diversity within and divergence among species of *Dendroseris* (*Asteraceae*: *Lactuceae*). Am. J. Bot. 87:591-596.

FAO. 2003. The production and consumption of kenaf in China. ESC-Fibres Consultation no. 03/6.

Ferdousi Begum, A. K. M. Aminul Islam, M. Golam Rasul, M. A. Khaleque Mian and M. Mofazzal Hossain. 2013. Morphological diversity of eggplant (*Solanum melongena*) in Bangladesh. Emir. J. Food Agric. 25 (1):45-51

Fontaine, C., P. N. Lovett, H. Sanou, J. Maley and J. M. Bouvet. 2004. Genetic diversity of the shea tree (*Vitellaria paradoxa* C.F. Gaertn), detected by RAPD and chloroplast microsatellite markers. Heredity 93:639-648.

Hanboonsong, Y., T. Vinijsanun and W. Ponragdee. 2000. Molecular Characterization and Genetic Relationships of Roselle Germplasm in Thailand. Proceedings of the Final Workshop on "Application of Biotechnology in the Improvement of Jute, Kenaf and Allied Fibres-Phase II," (IJO/ AGR/10). Beijing, China 10-12 August 2000. pp. 95-106.

Kilian, A., E. Huttner, P. Wenzl, D. Jaccoud. 2005. The Fast and the Cheap: SNP and DArT-based Whole Genome Profiling for Crop Improvement. In: Proceedings of the International Congress in the Wake of the Double Helix: From the Green Revolution to the Gene Revolution (Tuberosa R, Phillips RL and Gale M, eds.). May 27-31, Bologna, pp. 443-461.

Karp, A., S. Kresovich, K. V. Bhat, W. G. Ayada, T. Hodgkin. 1997. Molecular tools in plant genetic resources conservation: a guide to the technologies. IPGRI technical bulletin no 2. International Plant Genetic Resources Institute, Rome, Italy.

LeMahieu, P. J., E. S. Oplinger and D. H. Putnam. 2003. Kenaf. In: Alternative Field crops Manual. http://www.corn.agronomy. wisc.edu/ FISC/Alternatives/Kenaf.htm (visited on 4 April 2011)

Li, Y. and Y. W. Mai. 2006. Interfacial characteristics of sisal fibre and polymeric matrix. J. Adhesion 82:527.

Lin, P., L. Lin, J. Wu and N. Lin. 2004. Breeding of FuHong4, a kenaf variety with high yielding and resistance. Plant Fiber Prod. 26:1-4.

Mannerlof, M. and P. Tenning. 1997. Screening of transgenic plants by multiplex PCR. Plant Mol. Biol. Rep. 15:38-45.

Martinez, L., P. Cavagnaro, R. Masuelli and J. Rodriguez. 2003. Evaluation of diversity among Argentina grapevine (*Vitis vinifera* L.) varieties using morphological data and AFLP markers. Elect. J. Biotechnol. 6:241.

Mohamad, O., S. Herman, B. M. Nazir, A. Aminah, S. Mamot, S. Bakhendri and R. M. Abdul. 2005. Mutation breeding of roselle in Malaysia. Paperpresented at FNCA 2005 Workshop on Mutation Breeding, 5-9 December, Kuala Lumpur, pp. 1-7.

Mohamad, O., S. Herman, B. M. Nazir, S. Shamsudin and M. Takim. 2003. A dosimetry study using gamma irradiation on two accessions, PHR and PHI, in mutation breeding of roselle. (*Hibisc sabdariffa* L.). Paper presented at 7th MSAB Symposium on Applied Biology, 3-4 June, Sri Kembangan pp.1-10.

Mohamad, O., B. M. Nazir, M. A. Rahman and S. Herman. 2002. Roselle: A new crop in Malaysia. Bulletin Genetics Society of Malaysia 7:12-13.

Morton, J. 1987. Roselle. In Morton, J. F. (Ed). Fruits of Warm Climates, Creative Resource Systems Inc., USA.

Murray, T. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acid Res. 8:4321-4326.

Murtaza, N. 2006. Cotton genetic diversity study by AFLP markers. Elect. J. Biotechnol. 9:456-460.

Newton, A. C., T. R. Allnut, W. S. Dworak, R. F. Del Castillo and R. A. Ennos. 2002. Patterns of genetic variation in *Pinus chiapensis*, a threatened Mexican pine, detected by RAPD and mitochondrial DNA RFLP markers. Heredity 89:191-198.

Rao, P. U. 1996. Nutrient composition and biological evaluation of mesta (*Hibiscus sabdariffa*) seeds. Plant Foods Human Nutr. 49:27-34.

Rajasekar, S., S. H. Fei and E. C. Nick. 2005. Analysis of genetic diversity in rough bluegrass determined by RAPD markers. Crop Science 46:162-167.

Rohlf, F. J. 2000. NTSYS-pc Numerical Taxonomy and Multivariate Analysis System version 2.1. Owner Manual.

Skuza L., E. Filip and I. Szućko. 2013. Use of Organelle Markers to Study Genetic Diversity in Soybean. In: James E. Board (Ed.). A Comprehensive Survey of International Soybean Research - Genetics, Physiology, Agronomy and Nitrogen Relationships. ISBN 978-953-51-0876-4.

Tsai, P. J., J. Mc Intosh, P. Pearce, B. Camden and B. R. Jordan. 2002. Anthocyanin andantioxidant capacity in Roselle (*Hibiscus Sabdariffa* L.) extract. Food Res. Internat. 35:351-356.

Webber, C. L. and V. K. Bledsoe, 2002. Kenaf yield components and plant composition. In: J. Janick and A. Whipkey (Eds). pp. 348-357. Trends in New Crops and New Uses. ASHS Press. Alexandria, VA.

Williams, J. G. K., A. R. Kubelik, K. J. Livak, J. A. Rafalski and S. V. Tingey. 1993. DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acid Res. 18:6531-6535.

Wilson, F. D. and M. Y. Menzel. 1964. Kenaf (*Hibiscus cannabinus*), Roselle (*Hibiscus sabdariffa*). Econ. Bot. 18:80-91.

Wood, I. 2003. Kenaf: the forgotten fiber crop. The Australian New Crops Newsletter 10. http://www.newcrops.uq.edu.au/newslett/ncn10212.htm