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Identification and genetic relationships of kenaf (*Hibiscus cannabinus* L.) germplasm revealed by AFLP analysis

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Abstract

Kenaf (*Hibiscus cannabinus* L.) is one of the world's most economically important fiber crops. In order to identify different varieties, and investigate its diversity and genetic relationships, twenty-three kenaf accessions and two accessions of its relative, roselle (*H. sabdariffa* var. *altissima*), were analyzed by morphological characterization and AFLP fingerprinting. It is very difficult to identify kenaf accessions based merely on morphological characters, due to their limited variation. For the AFLP study, a total of 505 polymorphic markers (out of 560) were produced by six selected AFLP primer combinations. The AFLP fingerprinting was effective in identifying all kenaf accessions included in the study. Kenaf and roselle are independent species with close relationships, and great genetic diversity was also detected among the kenaf accessions with different origins, based on the analysis of the AFLP markers. The AFLP analysis strongly supports the opinion that kenaf originated in Africa. It also demonstrated that the dissemination of kenaf was from Africa through Asia to Central and North America.

Introduction

Kenaf (*Hibiscus cannabinus* L.) is one of the most economically important crops for soft fiber production, particularly in the Asian-Pacific region. The traditional uses of kenaf have mainly focused on it as a source of fiber for making ropes, sacks, canvas, and carpets (Dempsey 1975; Li 1980). Recently, more applications of kenaf have been developed, for example, pulping and papermaking, oil absorption and bioremediation, board and filtration media making, and animal feed (American Kenaf Society (AKS) 2000; Japan Kenaf Association (JKA) 2000; Sellers and Reichert 1999).

Kenaf is a member of the *Hibiscus* in the Malvaceae family. A list of over 120 common names has been compiled for the kenaf plant, including mesta, teal, ambari hemp, and rama (Sellers and Reichert 1999), which reflects the diversification and common use of this fibrous species. Another botanically different but closely related species, usually referred as roselle (*H. sabdariffa* L. var. *altissima* westet), is also sometimes called kenaf. Most authors agree with the opinion that the origin of kenaf is in Africa, where diversified forms of the kenaf species and its relative species in the genus *Hibiscus*, including roselle, are found growing widely in many countries of the eastern Africa (Wilson and Menzel 1964; Dempsey 1975; Li 1990). All proposals of kenaf's origin are merely based on field surveys and investigations, but to date no data from genetic studies have been collected to support these opinions.

In Asia, kenaf was first cultivated and commercially utilized in India around 1900, whereas roselle was introduced to Java around 1938 (Dempsey 1975). However, little is known about how kenaf was established in India, only some of the earliest literature state that kenaf was introduced from Africa (Rox-

burgh 1795; Royle 1855; Hooker 1875; Howard and Howard 1910). Also, there is very little information on kenaf breeding prior to the 1900′s, although varietal selection, studies of flower structures, and pollination mechanisms were reported (Howard and Howard 1911). Kenaf is now commercially cultivated in more than 20 countries, particularly in China, India, and Thailand (Food and Agriculture Organization (FAO), 1998; Liu 2000), but knowledge on dissemination of kenaf worldwide is still limited.

A large numbers of kenaf varieties have been developed to meet the demands for high-fiber-yielding and disease-resistant kenaf in the recent decades (Dempsey 1975; Institute of Bast Fiber Crops, Chinese Academy of Agricultural Sciences 1985; Bitzer et al. 2000). These varieties have played an important role in the further improvement of kenaf as genetic resources. However, identification of kenaf varieties based only on morphological and agronomical characters is always problematic (Deng et al. 1991, 1994; Siepe et al. 1997). In addition, our understanding of the relationships of kenaf germplasm is still very limited. All these together have significantly hindered the effective utilization and conservation of these valuable genetic resources. Our previous study indicated that RAPD analysis was able to identify kenaf varieties and determine their genetic relationships to a certain extent, but the sources of kenaf accessions used in that study were narrow, and the number of DNA polymorphic fragments detected were relatively low (Cheng et al. 2002). For its efficient utilization, more indicative molecular data need to be accumulated to determine diversity and genetic relationships of kenaf germplasm worldwide.

The AFLP DNA fingerprinting technique, based on the selective PCR amplification of restriction fragments from a total restriction digest of genomic DNA (Vos et al. 1995), has advantages in its reproducibility, high levels of DNA polymorphism detection, genome-wide distribution of markers, and no requirement of prior sequence information of the genome being studied (Prabhu and Gresshoff 1994; Lu et al. 1996). As a consequence, the AFLP technique has been widely used to study genetic relationships of many different plants species such as soybean (Maughan et al. 1996), sunflower (Hongtrakul et al. 1997), tea (Paul et al. 1997), maize (Ajmone et al. 1998), strawberry (Degani et al. 2001), apple (Goulao et al. 2001), daylily (Tomkins et al. 2001), and wheat (Aytiil and Akkaya 2001). The objectives of this study were to (i) identify kenaf varieties with wide sources from different geographical regions by AFLP fingerprinting, in comparison with their morphological characters; (ii) estimate their variations and genetic relationships; and (iii) reveal origin and dissemination of kenaf, through which kenaf formed its current distribution pattern worldwide.

Materials and methods

Plant materials and DNA extraction

A total of 23 accessions of kenaf germplasm representing a variation of cultivated and wild types from 14 countries were included in this study. The 21 accessions of the cultivated kenaf were selected from a large number of kenaf varieties collected over the world based on morphological and RAPD screening, and can represent genetic diversity of the current kenaf varieties cultivated worldwide. For comparison, two accessions of the closely related species, roselle, were also included for analysis (Table 1). All the accessions were planted for morphological characterization on May 25, 2001 at the Handan campus of Fudan University in Shanghai, China. The main morphological characters, such as 1000-seed weight, stem color, leaf shape, and maturity were measured (Table 2). The total genomic DNA was isolated from 3-day-old fresh shoots, following the modified protocol by Hayagawa (1997) from the CTAB (cetyltrimethylammonium bromide) method (Murray and Thompson 1980).

AFLP analysis

AFLP technique was carried out following the description of Gibco BRL (Life Technologies, USA) AFLP Analysis System I for plants with large genomes. Genomic DNA (0.4 μg/per sample) was digested using both *Eco*RI and *Mse*I enzymes for 4 h at 37 °C, and *Eco*RI and *Mse*I adapters were ligated to the digested DNA fragments for 12 h at 37 °C. About 5 μL ligation mixture was used as the template DNA for pre-amplification reaction with primers of *Eco*RI+C and *Mse*I+A. Twenty cycles were run at 90 °C for 30 s, 56 °C for 60 s and 72 °C for 60 s in a Eppendorf Mastercycler Gradient 5331 (Germany). The pre-amplification products were diluted 1:10 and used as a template for selective amplification with primers adding three selective nucleotides (*Eco*RI+3 and *Mse*I+3). The selective amplification was per-

Table 1. Kenaf germplasm with source and country of origin.

Species	Name of accession	Code	Line	Source	Country of origin
Kenaf (*H. cannabinus*)	Kenya	Keny	PI 292207	MSU[1], USA	Kenya
	Sudan 1	Sud1	PI 273463	MSU[1], USA	Khartoum, Sudan
	Sudan Pre (Precoce)	SudP	PI 267666	MSU[1], USA	Sudan
	Sudan Tra (Tradiff)	SudT	PI 267667	NARC[2], Japan	Sudan
	Rama	Rama	PI 268079	MSU[1], USA	Kaduna, Nigeria
	Indian Selection	InSe	–	MSU[1], USA	India
	Khon Kaen 60	KK60	PI 538258	Thailand	Khon Kaen, Thailand
	Krasnador	Kras	PI 318726	MSU[1], USA	Russian Federation
	Florida A65–656	Flor	PI 318723	NARC[2], Japan	Iran
	Tainung 1	Tan1	PI 365441	MSU[1], USA	Taiwan
	Tainung 2	Tan2	PI 532872	MSU[1], USA	Taiwan
	Aokawa 3	Aok3	–	China	Vietnam
	Sekkou Chuziku	SeCh	–	China	Vietnam
	IX 51	IX51	PI 189210	MSU[1], USA	Java, Indonesia
	EI Salvador	EISa	–	NARC[2], Japan	Java, Indonesia
	Cuba 108	C108	–	MSU[1], USA	Cuba
	Cubano	Cubn	PI 208832	MSU[1], USA	Cuba
	Guatemala 4	Gut4	PI 270104	MSU[1], USA	Guatemala
	Guatemala 7	Gut7	PI 270105	MSU[1], USA	Guatemala
	Master Fiber	MasF	PI 329185	MSU[1], USA	San Salvador, EI Salvador
	Everglades 41	EV41	PI 532873	MSU[1], USA	USA
	Everglades 71	EV71	PI 532874	MSU[1], USA	USA
	SF 459	S459	PI 586657	MSU[1], USA	Texas, USA
Roselle	Noon Soon 2	Noo2	–	Thailand	Thailand
(*H. sabdariffa* var. *altissima*)	Keawyai	Keay	–	Thailand	Thailand

[1]MSU: Mississippi State University, USA.
[2]NARC: National Agricultural Research Center, Japan.

formed for one cycle at 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 60 s; then for 12 cycles with a 0.7 °C annealing temperature decrease per cycle, and finally for 23 cycles at 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 60 s. Ex Taq™ DNA polymerase (TaKaRa, Japan) was used for all PCR reactions.

The AFLP amplified products were electrophoresed on 26-lane 6% polyacrylamide denaturing gels in 1X TBE using Shelton electrophoresis apparatus (Shelton Scientific Mfg., Shelton, USA). Gels were pre-run at 30 w for 30 min, then 4 μL denatured and selectively amplified products were loaded and the gel was run at 30 W until the forward running dye (bromophenol blue) reached the two thirds of the gel. PBR322 DNA/Hae III Markers ranging in size from 21 to 587 bp was used to determine fragment sizes. After electrophoresis, the AFLP amplified products were detected using silver sequence™ DNA staining reagents (Promega, USA). The dried gel was scanned (ScanMaker 6400XL, Microtek) using the Adobe Paintshop6.0 (Adobe Systems, Mountain View, USA).

Data analysis

AFLP bands were manually scored as 1 (for presence) and 0 (for absence) from the images of the gels. Differences in intensity of the bands among different samples were not considered during the scoring. Both monomorphic and polymorphic bands were included in the data set to provide unbiased estimation of genetic variation. Pairwise similarities were computed using the index of Jaccard (a/n-d), (*a*, shared fragments between *i* and *j* accessions, *d*, polymorphic fragments absent in both the *i* and *j* accessions, and *n*, total number of polymorghic fragments). Similarity coefficient, cluster analysis (UPGMA), and dendrogram construction were performed with the NTSYS-pc analytical software (Rohlf 1998).

Table 2. Morphological characters of 25 kenaf accessions (Only one measurement for each variable was made per accession)

Code	Seed weight (g/1000 seed)	Seed shape	Seed hilum	Stem color	Leaf shape	Flower color	Maturity[1]
C108	25.0	Irregularly subreniform	Yellow-brown, inconspicuous	Red	Palmate	Cream	Late
Cubn	33.6	Idem	Idem	Red	Palmate	Cream	Late
Gut4	26.8	Idem	Idem	Red	Entire	Cream	Late
Gut7	35.2	Idem	Idem	Red	Entire	Cream	Late
MasF	34.7	Idem	Idem	Red	Palmate	Cream	Late
EISa	29.9	Idem	Idem	Red	Entire	Cream	Late
EV41	26.0	Idem	Idem	Red	Entire	Cream	Late
EV71	30.2	Idem	Idem	Red	Entire	Cream	Late
S459	31.5	Idem	Idem	Red	Palmate	Cream	Late
InSe	23.8	Idem	Idem	Red	Palmate	Cream	Late
Tan1	32.6	Idem	Idem	Red	Palmate	Cream	Late
Tan2	31.1	Idem	Idem	Red	Palmate	Cream	Late
Aok3	26.2	Idem	Idem	Green	Palmate	Cream	Late
SeCh	27.9	Idem	Idem	Green	Palmate	Cream	Medium
IX51	29.8	Idem	Idem	Red	Palmate	Cream	Late
KK60	32.7	Idem	Idem	Red	Palmate	Cream	Late
Kras	26.6	Idem	Idem	Red	Palmate	Cream	Early
Flor	19.5	Idem	Idem	Red	Palmate	Cream	Early
Sud1	12.5	Idem	Idem	Bronze	Entire	Cream	Late
SudP	32.3	Idem	Idem	Red	Palmate	Cream	Late
SudT	36.2	Idem	Idem	Red	Palmate	Cream	Late
Keny	9.7	Idem	Idem	Green	Deep palmate	Purple	Extreme-late
Rama	33.1	Idem	Idem	Green	Entire	Cream	Late
Noo2	23.3	Subreniform	Brownish-red, conspicuous	Green	Deep palmate	Yellow	Extreme-late
Keay	26.3	Idem	Idem	Green with red spot on node	Deep palmate	Yellow	Extreme-late

[1]According to the days to 50% flowering from seed germination, Early: < 130 days; Medium: 131–165days; Late: 166–200 days; and Extreme-late: > 201.

Results

Variation in morphological and agronomical characters

Variation of morphological characters in the 23 kenaf and 2 roselle accessions are summarized in (Table 2). Considerable morphological differences in characters, such as seed character, leaf shape, and flower color were observed between kenaf and roselle. The maturity of roselle was much later than that of kenaf accessions (normally roselle do not flower in nature at latitudes higher than 30 °N). However, morphological variation among the kenaf accessions was small. Most of the kenaf accessions had red or green stems, yellow flowers, and big-sized seeds, entire- or palmate-leaves, expect for ‘‘Keny’’ and ‘‘Sud1’’ that had small-sized seed, bronze stems, and purple flowers. Four maturity types were observed among the kenaf accessions, and most of the kenaf accessions were late maturing types, especially the accession ‘‘Keny’’ that was an extreme-late-maturing type similar to the roselle accessions.

Variation in ALFP patterns

In the preliminary analysis, 64 AFLP primer combinations were assayed using two kenaf accessions (’’EV71’’ and ‘‘Gut4’’) to select appropriate primer combinations. The number of generated bands from different primer combinations was greatly variable, ranging form 0 (e.g. E-ACA/M-CTT) to 91 (E-AGC/M-CAA). Forty-nine primer combinations produced bands from the two kenaf accessions, but only 11 primer combinations produced five or more distinct bands (Table 3). The primers, M-CAA, M-CTG, E-AAC, and E-AGC, produced more polymorphic bands, and eventually six primer combinations were selected for use in this study (Table 4).

A total number of 505 polymorphic bands (out of

Table 3. AFLP primer combination selection using two kenaf accessions ''EV71'' and ''Gut4''

	M-CAA	M-CAC	M-CAG	M-CAT	M-CTA	M-CTC	M-CTG	M-CTT
E-AAC	A	B	NR	A	B	NR	A	NR
E-AAG	C	C	C	C	C	C	B	C
E-ACA	NR	NR	C	B	B	B	B	NR
E-ACC	NR	NR	C	C	C	B	A	C
E-ACG	A	A	NR	B	B	B	B	B
E-ACT	B	B	B	NR	B	NR	NR	NR
E-AGC	A	NR	A	B	A	A	B	B
E-AGG	B	B	B	B	NR	B	A	B

NR = No reaction
A = 5 or more polymorphic fragments when comparing both accessions
B = 4 or less polymorphic fragments between both accessions
C = No polymorphic fragments in the two accessions

560 bands) were produced from all kenaf and roselle accessions using the six AFLP primer combinations (Table 4). The rate of polymorphism varied from 87% by the primer combination E-AGC/M-CAA to 96% by E-ACC/M-CTG, with an average of 90% (Table 4). All the accessions can be easily distinguished based on differences in AFLP banding patterns. A total number of 396 polymorphic bands were generated from the 23 kenaf accessions, and 5 polymorphic bands from the 2 roselle accessions, respectively, by the six primer combinations. The average number of polymorphic fragments was 33.1 per primer combination for each of the 23 kenaf accessions.

A dendrogram based on the similarity coefficient of the 505 AFLP markers from the 25 accessions was constructed (Figure 1) The cluster analysis demonstrated a considerable divergence among the included samples, particularly between kenaf and roselle accessions. In the dendrogram, three major groups can be identified at different similarity levels. The two roselle accessions formed an independent group that was significantly separated from all other accessions at the similarity level of 0.31. The two roselle accessions were almost identical, with the similarity coefficient of 0.98. The wild and semi-wild kenaf, ''Keny'' and ''Sud1'' clustered quite distinctly between the roselle accessions and all the cultivated kenaf accessions, although the wild and semi-wild types were separate from each other significantly, with the semi-wild type more closely linked to the cultivated accessions with the similarity coefficient of 0.66. All the cultivated kenaf accessions were clustered together in a large group with obvious subdivision into two subgroups. All African and Asian kenaf accessions were scattered in one subgroup, referred as ''African and Asian subgroup'' here, except for the two Taiwanese accessions ''Tan1'' and ''Tan2.'' In addition, three accessions, ''MasF'' ''S459'' and ''Cubn'' from Central and North America are also included in this subgroup. Similarity coefficients varied between 0.72 and 0.85 among accessions in this African and Asian subgroup. Most kenaf accessions currently grown in North and Central America were included in another subgroup. This subgroup was referred to as the ''newly derived subgroup'' here, because many varieties in this subgroup were derived mainly from one Asian strain. Similarity coefficient ranged from 0.67 to 0.85 among accessions in this subgroup.

Table 4. Polymorphism generated by six selected oligonucleotide primer combinations from the kenaf germplasm.

Primer combination	Total number of fragments			Polymorphic fragments			Polymorphic (%)		
	All 25 accessions	23 Kenaf accessions	2 Roselle accessions	All 25 accessions	23 Kenaf accessions	2 Roselle accessions	All 25 accessions	23 Kenaf accessions	2 Roselle accessions
E-AAC/M-CTG	104	82	57	93	64	1	89	78	2
E-ACC/M-CTG	77	56	38	74	49	4	96	88	12
E-ACG/M-CAA	76	55	42	69	43	0	91	78	0
E-ACG/M-CAC	80	72	39	72	60	0	90	83	0
E-AGC/M-CAA	124	121	54	108	102	0	87	84	0
E-AGC/M-CTA	99	90	57	89	78	0	90	87	0
Total	560	476	287	505	396	5	Mean 90	83	2

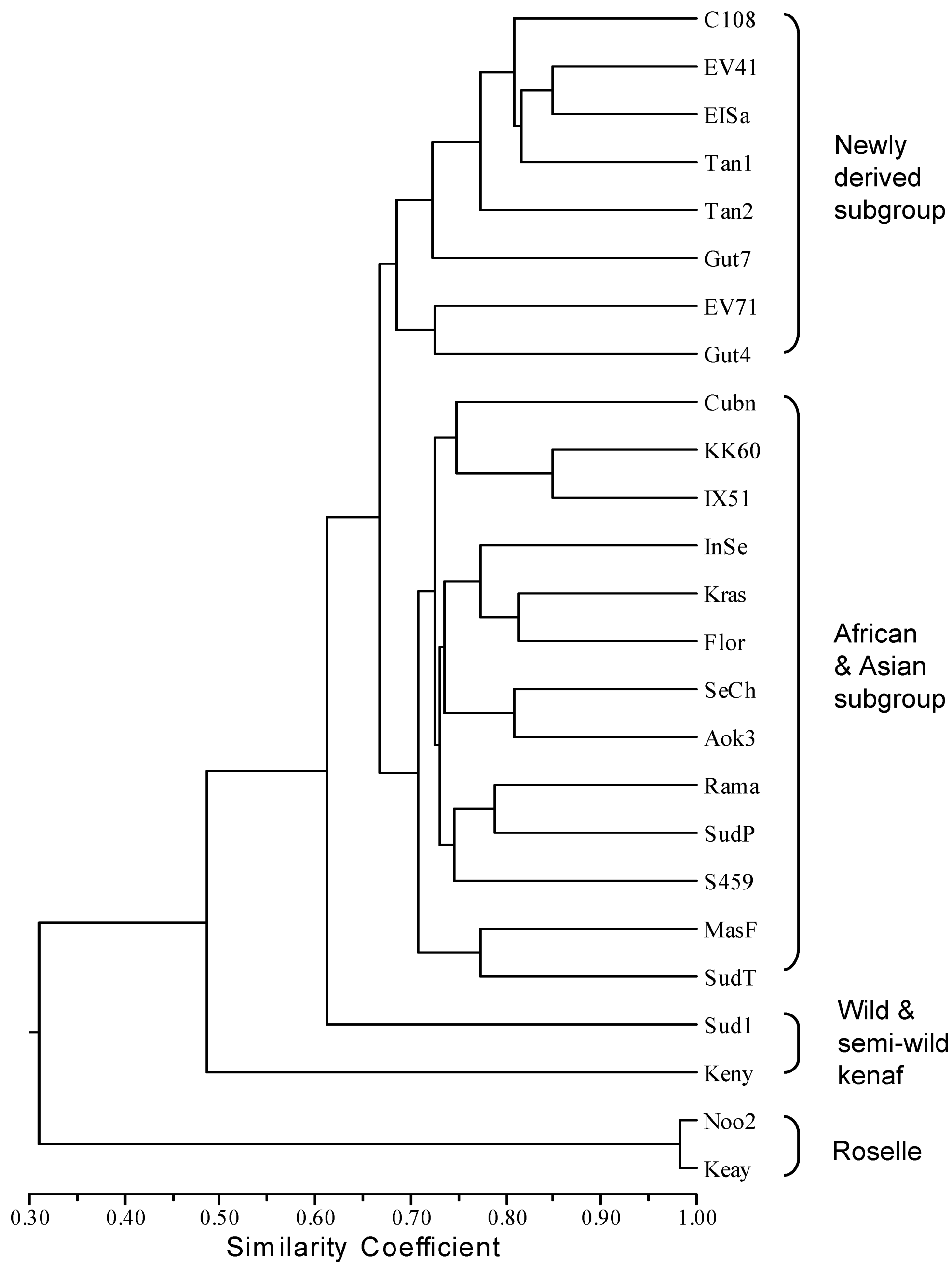

Figure 1. UPGMA dendrogram of genetic relationships of kenaf germplasm, based on the Jaccard similarity coefficient from the 560 AFLP fragments.

Discussion

Identification of kenaf germplasm

As in many previous reports (Deng et al. 1991, 1994; Siepe et al. 1997; Cheng et al. 2002), morphological characters in this study provided very limited information for varietal identification of kenaf germplasm. It is only possible to distinguish the two roselle samples from kenaf accessions by their leaf shape and flower color, and it is impossible to identify all kenaf accessions merely by the selected morphological

characters that are commonly used for kenaf varietal identification. On the contrary, the AFLP analysis in this study provided a powerful and reliable molecular tool for the identification of kenaf samples, although generally low genetic diversity was found in kenaf varieties. This paper is the first attempt at using AFLPs for identifying kenaf germplasm and its wild relatives from wide sources, and the use of only six primer combinations met the objectives, even for the two roselle accessions for which nearly identical morphological variations were observed. In fact, only one or two informative AFLP primer combinations will generate sufficient markers able to identify all the accessions used in this study. We also found through the comparison that AFLP technique is a more powerful tool than RAPD analysis in distinguishing kenaf varieties (Cheng et al. 2002). The AFLP analysis has been widely used in many other crop species for studying their variation (Aytiil and Akkaya 2001; Tomkins et al. 2001), reflecting the effective and accurate performance of this method for germplasm identification. Therefore, we recommend applying AFLP fingerprinting technique for kenaf germplasm identification, particularly when confused kenaf materials are involved in utilization.

Diversity and genetic relationships of kenaf germplasm

Morphological characterization and AFLP fingerprinting showed dramatic differences between kenaf and roselle accessions, and the AFLP analysis clearly separated the roselle accessions into one independent group. Genetic variation between kenaf and roselle based on the AFLP markers supported their independent taxonomic status, although both species are sometimes referred to as kenaf. Genetic diversity within the roselle accessions was very low, probably because of the limited number of samples that was included in this study. However, based on the RAPD analysis for ninety-four roselle accessions, very low genetic diversity (0.91–0.98 similarities) was detected (Hanboonsong et al. 2000).

The variation among the kenaf accessions was detected based on the AFLP fingerprinting with similarity coefficients ranging between 0.47–0.85, despite the low morphological variation that is unusable for identification. However, genetic variation among the cultivated kenaf varieties was relatively low (0.65–0.85 similarities), indicating low genetic diversity of kenaf germplasm in general, given that the sample set used in this study was selected from a large number of kenaf varieties worldwide. In addition, the AFLP analysis also provided a relatively clear pattern of genetic relationships among the kenaf accessions studied. All kenaf accessions were clustered into two major groups, i.e. the wild and semi-wild group and the cultivated group (Figure 1) The wild ''Keny'' and semi-wild ''Sud1'' were clustered between the roselle group and cultivated kenaf group, with ''Keny'' closer to roselle, and ''Sud1'' closer to the cultivated kenaf, indicating their genetic linkage to the relative species.

The cultivated kenaf accessions were clustered into two subgroups, the ''African and Asian subgroup'' and ''newly derived subgroup'' although with low differentiation. Most of the kenaf accessions from African and Asian countries were included in the ''African and Asian subgroup'' with the inclusion of three Central and North American accessions, ''MasF'' ''S459'' and ''Cubn.'' This might indicate the close relationship between the African and Asian kenaf accessions and strong linkage of the American accessions to the African and Asian kenaf germplasm. For example, the ''S459'' showed a close relationship with the two African accessions ''SudP'' and ''Rama,'' and the Cuban ''Cubn'' showed its close genetic link with the Indonesian accession ''IX51''

The ''newly derived subgroup'' included the kenaf accessions that were developed from a few Javanese ancestors in Central and North America. During and after the World War II, intensive breeding programs for high-yielding and disease-resistant kenaf varieties were conducted initially in Cuba, Guatemala, and then in Florida of USA (Wilson and Menzel 1964; Dempsey 1975). Five kenaf accessions with Javanese pedigree released by these programs were included in this subgroup. (Dempsey 1975) proposed that the Javanese varieties possessed components of Indian ancestors. This study demonstrates the strong linkage between the Asian and American kenaf accessions.

Origin and dissemination of kenaf

Most authors agree that kenaf originated in Africa (Wilson and Menzel 1964; Dempsey 1975; Li 1990), and that roselle more likely originated in the western African countries of Angola and Congo, like many other relative species in the genus *Hibiscus* (Wilson and Menzel 1964; Dempsey 1975). Our study based

on the AFLP analysis supported the observation that cultivated kenaf remained a close affinity with their African relative species, such as roselle and wild/semi-wild kenaf accessions (Figure 1) The existence of semi-wild kenaf in Africa might be an indication of the origin of the cultivated kenaf from this continent.

Asia is supposedly the earliest continent to introduce kenaf and becomes the most important kenaf producing area. According to the literature, kenaf was introduced from Africa directly to India for commercial cultivation at the beginning of the 20th century (Dempsey 1975). The cultural interaction between the ancient Egypt and Indus may have played an important role for kenaf's dissemination from Africa to India, from where kenaf cultivation was expanded to other Asian countries. Genetic relationships between the African and Asian kenaf accessions revealed by the AFLP analysis in the ''African and Asian'' subgroup strongly indicate the close linkage of kenaf germplasm from the two areas, supporting the assumption that the cultivated kenaf was introduced to Asia and diversified there in different Asian countries before it spread out to other places. This postulation gains supports from the fact that most of the kenaf germplasm collected from Central and North America were bred from some Javanese strains, such as ''EISa'' introduced to Central and North America in the 1940s (Dempsey 1975). Therefore, most American kenaf accessions were clustered in the ''newly derived subgroup'' although close genetic relationships are still detectable between the Asian and American accessions.

Genetic diversity of the studied kenaf accessions among the geographically separated continents appropriately reflects the history of origin, dissemination, and utilization of kenaf germplasm as genetic resources. This knowledge will be useful for the efficient varietal improvement, and effective conservation of kenaf germplasm by selecting accessions with different genetic backgrounds.

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