ISSR-PCR Fingerprinting of *Kappaphycus* and *Eucheuma* (Rhodophyta, Gigartinales) Seaweed Varieties From Sabah, Malaysia

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ABSTRACT *Kappaphycus* and *Eucheuma* seaweeds are commonly cultivated for carrageenan production in Sabah, Malaysia. Identification of the different varieties of *Kappaphycus* and *Eucheuma* seaweeds is important because certain seaweed varieties of the same species exhibit desired characteristics such as higher carrageenan content, faster growth rates and resistance to disease. The present study set forth to characterize *Kappaphycus* and *Eucheuma* seaweed varieties based on the genetic fingerprinting using inter-simple sequence repeat–polymerase chain reaction (ISSR-PCR) method. A total of eight ISSR primers were used to produce distinct and reproducible patterns of polymorphic bands. A total of 1494 bands of 180 to 4,600 bp were successfully amplified, of which 94.3% were polymorphic. The dendogram results showed a clear differentiation among the cultivar varieties of *Kappaphycus* seaweed only; however *Eucheuma* seaweed varieties have the same band profiles. Interestingly, 22 identical variety-specific ISSR bands were obtained from 30 seaweed samples and these can be applied as molecular tools for the identification of seaweed varieties. The data obtained from ISSR analysis can be used in breeding technology and various other applications for development of seaweed industries.

KEYWORDS: *Eucheuma*; genetic fingerprinting; inter-simple sequence repeat–polymerase chain reaction (ISSR-PCR); *Kappaphycus;* seaweeds.

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TRANSACTIONS ON SCIENCE AND TECHNOLOGY

INTRODUCTION

Seaweeds are one of the economically important crops for the carrageenan industry in Malaysia, where the commonly cultivated seaweeds are belonging to genera *Kappaphycus* Doty and *Eucheuma* J. Agardh. Increasing demands have led to the rapid increase in the farming of these carrageenophytes, where more than 15 varieties have been reported as cultivars in the Malaysian waters (Tan *et al.*, 2013). The production of these economically important seaweeds has increased significantly in recent decades, but farmers had encountered challenges with identification of seaweed varieties. The rudimentary method of identification currently practiced by farmers involves phenotypic observations based on coloration, branch structure, size and other physical morphologies, which are greatly influenced by environmental factors. This has led to confusion in the identification and classification of the varieties of *Kappaphycus* and *Eucheuma* seaweeds in Malaysia. In addition, their biodiversity is still poorly known because the assessment of intraspecific genetic biodiversity of seaweeds is still in its infancy.

Previous studies reported that several molecular approaches have been applied in characterization and differentiation of seaweed species by using DNA molecular markers such as RAPD (Patwary *et al.*, 1993), M13-fingerprinting (Coyer *et al.*, 1994), microsatellites (Wattier *et al.*, 1998), AFLP (Donaldson *et al.*, 1998), and SSCP (Zuccarello *et al.*, 1999). More recently, sequence-based comparative analysis of polymerase chain reaction targeting the ribosomal ribonucleic acid (28S rRNA, 23S rRNA, ITS1, 5.8S rRNA and ITS2), mitochondrial cytochrome C oxidase (*cox1, cox2*

and *cox2-3* spacer), and plastid *rbcL* gene have been utilized to evaluate the genetic relationship between the *Kappaphycus* seaweeds (Zuccarello *et al.*, 2006; Conklin *et al.*, 2009; Zhao & He, 2011; Tan *et al.*, 2012; de-Barros Barretto *et al.*, 2012; Tan *et al.*, 2013). Eventually these approaches could only differentiate up to species level, and cannot differentiate the variation level of *Kappaphycus* and *Eucheuma* seaweed species.

In this study, inter-simple sequence repeat (ISSR) method was used to reveal the variation among different varieties of *Kappaphycus* and *Eucheuma* seaweeds. This technique is a reliable, simple, cost effectiveness, and quick method which involves the use of highly polymorphic ISSR markers to study the genomic fingerprinting, genetic diversity, phylogeny, gene tagging, genome mapping, and evolutionary biology (Sarwat, 2012). These data will demonstrate the ISSR technique is a valuable molecular method for the authentication of *Kappaphycus* and *Eucheuma* seaweed varieties at genomic DNA level.

METHODOLOGY

Seaweed samples

Table 1. Details of collected seaweed samples in this study

Collection ID	Local name	Species name	Nature
EDDM_1	*Denticulatum*	*E. denticulatum*	Cultivated
EDDM_2	*Denticulatum*	*E. denticulatum*	Cultivated
EDAD_3	*Alien denticulatum*	*E. denticulatum*	Wild
EDAD_4	*Alien denticulatum*	*E. denticulatum*	Wild
KAAB_5	*Alien brown*	*K. alvarezii*	Wild
KAAB_6	*Alien brown*	*K. alvarezii*	Wild
KACG_7	*Cacing*	*K. alvarezii*	Cultivated
KACG_8	*Cacing*	*K. alvarezii*	Cultivated
KAAG_9	*Alien green*	*K. alvarezii*	Wild
KAAG_10	*Alien green*	*K. alvarezii*	Wild
KAGT_11	*Giant*	*K. alvarezii*	Cultivated
KAGT_12	*Giant*	*K. alvarezii*	Cultivated
KATN_13	*Tangan*	*K. alvarezii*	Cultivated
KATN_14	*Tangan*	*K. alvarezii*	Cultivated
KABA_15	*Buaya*	*K. alvarezii*	Cultivated
KABA_16	*Buaya*	*K. alvarezii*	Cultivated
KABN_17	*Brown*	*K. alvarezii*	Cultivated
KABN_18	*Brown*	*K. alvarezii*	Cultivated
KSGTF_19	*Giant flower*	*K. striatus*	Cultivated
KSGTF_20	*Giant flower*	*K. striatus*	Cultivated
KSYF_21	*Yellow flower*	*K. striatus*	Cultivated
KSYF_22	*Yellow flower*	*K. striatus*	Cultivated
KSGF_23	*Green flower*	*K. striatus*	Cultivated
KSGF_24	*Green flower*	*K. striatus*	Cultivated
KAABA13_25	*Alien buaya 13*	*K. alvarezii*	Wild
KAABA13_26	*Alien buaya 13*	*K. alvarezii*	Wild
KAABA14_27	*Alien buaya 14*	*K. alvarezii*	Wild
KAABA14_28	*Alien buaya 14*	*K. alvarezii*	Wild
KAS12_29	*Alien 12*	*Kappaphycus* sp.	Wild
KAS12_30	*Alien 12*	*Kappaphycus* sp.	Wild

A total of 30 farmed and wild *Kappaphycus* and *Eucheuma* seaweed samples (Table 1) were collected from various locations around the Semporna area (Sebangkat and Selakan Island) in Sabah via snorkeling and scuba diving. Cultivated samples were classified and named based on their external morphological criteria differentiating local varieties; while the wild samples were individually described.

DNA isolation and PCR amplification

Total DNA was extracted using the protocol of Yang *et al*. (2013) and used immediately for polymerase chain reaction (PCR) amplification using eight ISSR primers (Table 2). PCR amplification were carried out in a volume of 20 µl consisted of the genomic DNA (50-60 ng/µl), 1 x PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs of each nucleotide, 10 pmol primer, and 2.5 U *Taq* DNA polymerase. The PCR was performed with initial denaturation at 95°C for 3 min followed by 30 cycles of 20 s denaturing at 95°C, 40 s annealing at 40 - 50°C according to primer used and 1 min extension at 72°C, and lastly final extension at 72°C for 10 min on a thermal cycler (O'Doherty & Sherwood, 2007). Banding patterns were analyzed with the QIAxcel Advanced System using QIAxcel DNA High Resolution Kit.

Table 2. ISSR primers tested in this study

ISSR Primer	Sequence (5′ – 3′)	Tm (˚C)	Reference
BRIAAC6	GGG AAC AAC AAC AAC AAC AAC	53.0	This study
BRICTT4	GGG CTT CTT CTT CTT	44.5	
BRIAGG4	CCC AGG AGG AGG AGG	53.7	
BRIAAC4	GGG AAC AAC AAC AAC	44.4	
ISSR 1	CAC ACA CAC ACA GG	46.2	O'Doherty and Sherwood (2007)
ISSR 2	CTC TCT CTC TCT CTC TAC	46.7	
ISSR 5	CTC TCT CTC TCT CTC TGC	50.5	
ISSR 10	GAG GAG GAG CC	41.0	
ISSR 12	CAC CAC CAC GC	44.7	

Data analysis

Scoring was performed for each primer based on absence (1) and presence (0) of bands. For comparison, the Nei's genetic distance matrix (Nei, 1972) was generated among the 30 varieties and cluster analysis was performed to develop a dendrogram by unweighted pair group method with arithmetic mean (UPGMA).

RESULT AND DISCUSSION

Table 3. ISSR fingerprints data for the molecular characterization of *Kappaphycus* and *Eucheuma* seaweed varieties.

Primer	No. of alleles	Product size (bp)	Total number of scored bands	Total number of polymorphic bands	Polymorphism	H	PIC
BRICCT4	1 - 5	600 - 2100	80	74	93%	0.39	0.31
BRIAGG4	5 - 12	300 - 1900	272	268	98%	0.48	0.36
BRIAAC4	1 - 8	230 - 2900	138	122	88%	0.29	0.25
ISSR 1	3 - 9	300 - 3000	166	164	99%	0.36	0.30
ISSR 2	3 - 8	180 - 1800	148	138	93%	0.35	0.29
ISSR 5	5 - 13	370 - 4600	238	224	94%	0.35	0.29
ISSR 10	5 - 11	280 - 3100	212	206	97%	0.43	0.34
ISSR 12	6 - 12	430 - 4300	240	224	93%	0.38	0.31

H= Heterozygosity, PIC = Polymorphic information content

Genetic diversity and genetic relationship between 30 seaweed samples of the 3 species (*K. alvarezii*, *K. striatus* and *E. denticulatum*) were analyzed. According to the ISSR-PCR results using the 8 ISSR primers (not shown here), clear and reproducible DNA bands ranging in size from approximately 180 to 4,600 bp were successfully observed. Total number of bands and polymorphism rates that each tested ISSR primer produced is shown in Table 3. Table 3 showed that

there were a total of 192 ISSR loci with 1,494 DNA fragments, which were used for UPGMA cluster analysis. The vast majority (94.3%) of fragments produced from the 30 seaweed varieties were polymorphic. Primer ISSR1 exhibited the highest degree of polymorphism (99%) as compared to primer BRIAAC4 (88%). PIC values of the ISSR markers used ranged from 0.25 to 0.36 and heterozygosity ranged from 0.29 to 0.48. ISSR markers usually show high polymorphism although the level of polymorphism has been shown varies with the detection method (Palai & Rout, 2011). The ISSR technology is sensitive to low levels of genetic variation, providing a very useful molecular tool for studying population genetics (Zietkiewicz *et al.*, 1994; Raina *et al.*, 2001).

UPGMA cluster analysis using the standardized genetic distances were used to visualize the degree of similarity between varieties of *Kappaphycus* and *Eucheuma* seaweeds. All the binary data from eight primers were combined together to construct a coalescent tree and observed the overall relationship between the 30 varieties of *Kappaphycus* and *Eucheuma* seaweeds (Figure 1), where the tree was divided into 3 clades, which are *E. denticulatum* (100% BS value), *K. striatus* (59% BS value), and *K. alvarezii* (77% BS value).

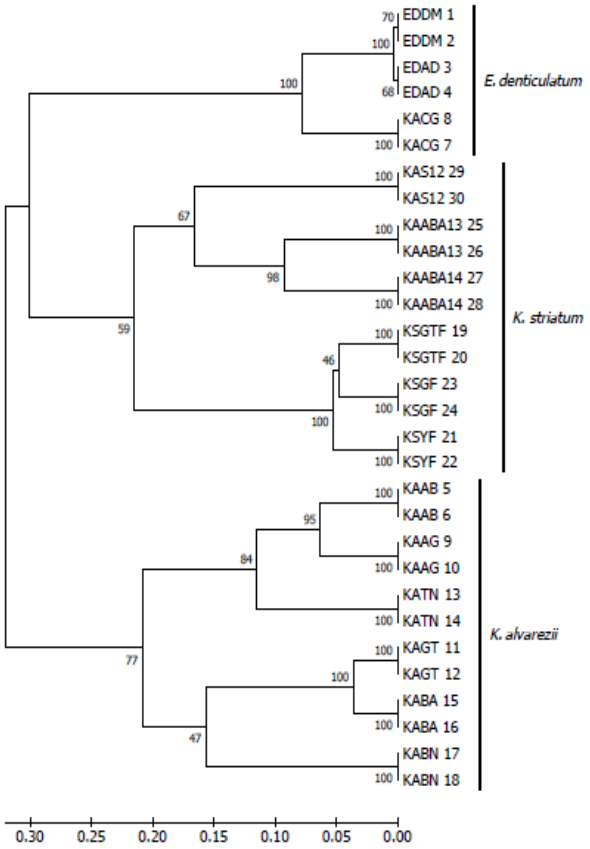

Figure 1. Coalescent tree showed the relationship between varieties of *Kappaphycus* and *Eucheuma* seaweed (combination of all primers). The numbers given at nodes are the percentage of frequencies with which a given branch appeared in 1000 bootstrap replications.

Sample KAS12_29 and KAS12_30 which were initially identified as *Kappaphycus* spp. were grouped together with *K. striatus*. The sequence of both samples also demonstrated 85% similarity with the sequence of *K. striatus* deposited in the NCBI GenBank databases (Acc. no.: JN645178).

Although the *E. denticulatum* was clustered together in the same group with *K. striatus* or *K. alvarezii*, it is interesting to note that there were genetic variations in *Kappaphycus* species detected in this study. In contrast, all the ISSR primers were unable to differentiate the varieties among the *Eucheuma* seaweed, producing the same fingerprinting profiles. All eight ISSR primers revealed the amplified DNA fragments that were unique to ten varieties from *Kappaphycus* and *Eucheuma* which

are Brown, Cacing, Green flower, Yellow flower, Alien brown, Tangan, Alien green, Alien buaya 14, Alien buaya 13 and Alien 12 (Table 4). These ISSR primers amplifying specific DNA fragments represent molecular tools for the authentication of seaweed varieties, especially markers ISSR5 and ISSR12 which produced about five unique ISSR genotyping profiles. These data demonstrated the ISSR technique is a valuable molecular method for the authentication of *Kappaphycus* and *Eucheuma* seaweed varieties at genomic DNA level.

Table 4. List of ISSR specific locus for identification of *Kappaphycus* and *Eucheuma* seaweed varieties

Primer	Size (bp)	Species	Variety	Collection ID
BRICCT4	1239	*K. alvarezii*	*Brown*	KABN
	2014	*E. denticulatum*	*Cacing*	KACG
BRIAGG4	410	*K. striatus*	*Green flower*	KSGF
	495	*K. striatus*	*Green flower*	KSGF
BRIAAC4	238	*K. alvarezii*	*Brown*	KABN
	300	*K. alvarezii*	*Brown*	KABN
	818	*K. alvarezii*	*Alien brown*	KAAB
ISSR 1	500	*K. alvarezii*	*Tangan*	KATN
ISSR 2	185	*K. alvarezii*	*Alien green*	KAAG
	1704	*K. alvarezii*	*Brown*	KABN
ISSR 5	433	*K. striatus*	*Alien buaya 14*	KAABA14
	464	*Kappaphycus sp.*	*Alien 12*	KAS12
	520	*Kappaphycus sp.*	*Alien 12*	KAS12
	560	*K. striatus*	*Yellow flower*	KSYF
	1338	*K. striatus*	*Alien buaya 13*	KAABA13
ISSR 10	357	*Kappaphycus sp.*	*Alien 12*	KAS12
	483	*Kappaphycus sp.*	*Alien 12*	KAS12
ISSR 12	646	*Kappaphycus sp.*	*Alien 12*	KAS12
	676	*K. alvarezii*	*Alien green*	KAAG
	1935	*K. striatus*	*Alien buaya 14*	KAABA14
	2260	*Kappaphycus sp.*	*Alien 12*	KAS12
	2781	*K. alvarezii*	*Tangan*	KATN

Our study has demonstrated that the ISSR technique using primers developed for this study can be a valuable molecular method for the discrimination and identification of seaweeds. ISSR markers which may be linked to a trait or a group of traits with agronomic importance can be used as specific markers and applied in marker assisted selection (MAS).

CONCLUSION

Although the *Kappaphycus* and *Eucheuma* seaweeds are similar at the phenotypic level, the varieties of *K. alvarezii* and *K. striatus* still showed a genetic variation except for *E. denticulatum*. The ISSR profiles obtained from this study provided a molecular diagnostic tool for the authentication of valuable and unique ISSR locus, which specifically identified the varietal differences of *Kappaphycus* and *Eucheuma* seaweeds. This will lead to the development of variety-specific ISSR markers and will facilitate the understanding of inter- and intra-species gene flow, genetic diversity and evolutionary relationships among *Kappaphycus* and *Eucheuma* seaweeds.

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