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Genetic analysis of selected *Sargassum fusiforme* (Harvey) Setchell (Sargassaceae, Phaeophyta) strains with RAPD and ISSR markers*

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Abstract Since the 1980s, *Sargassum fusiforme* has been cultivated in Zhejiang, South China, and nowadays it becomes one of the important commercial seaweeds in China. With traditions of eating habits in the East Asian countries, this brown alga is used as food, because it contained functional oligo/polysaccharides and chemical components, and was regarded playing roles in antioxidant activities and regulating immunology. Through over 15 years' selection, breeding and cultivation, we obtained three strains with good traits and testified their characters during the production, which included the cultivars with high yield and other two good characters, either all the selected strains were applied in the *Sargassum* production. To avoid confusion during the selection and nursery, it was preferred to establish one fingerprint for distinguishing the *Sargassum* cultivars from different strains. Random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) methods were adopted to analyze the genetic diversities of the selected *S. fusiforme* strains. With that, one fingerprint with RAPD markers was constructed, and one sequence characterized amplified region (SCAR) marker to *S. fusiforme* was obtained. It is indicated that the applied fingerprint could be valid in *S. fusiforme* genetic and germplasm justification, and will be positive to molecular marker assistance in its selection and cultivation.

Keyword: *Sargassum fusiforme*; random amplified polymorphic DNA (RAPD); inter-simple sequence repeat (ISSR); sequence characterized amplified region (SCAR); genetic analysis

1 INTRODUCTION

Sargassum fusiforme is discontinuously distributed in the lower intertidal zones along the coast of China. Historically, it was regarded as a healthy food in Japan and China (Zhang et al., 2002). Due to the existence of many functional oligo/polysaccharides, and was regarded as healthful seaweed food, and functioned in antioxidant, regulation immunology etc. (Karawita et al., 2004; Ji et al., 2004; Choi et al., 2009; 2010; Zhu et al., 2010). Nevertheless, the present *S. fusiforme* wild biomass cannot meet the fast need for human consumption and economic requirements, it is urgent to conduct its selection and cultivation with the good varieties.

Since the 1980s, the application and cultivation of *S. fusiforme* became one of commercial seaweeds industry in Zhejiang and Fujian in south China, and the present output reached up to 8 700 tons/a in Dongtou, Zhejiang, its exhibit northward expansion tendency in China. Nevertheless, a number of wild-type of *S. fusiforme* were used for propagations in

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Table 1 Backgrounds of the *Sargassum fusiforme* strains applied in the study

Serial No.	Name	Characteristics
1	*S. fusiforme* strain 1	Brittle branch, pneumathodes first emerged
2	*S. fusiforme* strain 2	Long lateral branch
3	*S. fusiforme* stain 3 (Lufeng No.1)	Long frond, with the largest yield; the main cultivated strain applied in the *Hizikia* production
4	*S. fusiforme* strain 4 (Lufeng No.1)	Long frond, with the largest yield; the main cultivated strain applied in the *Hizikia* production
5	*S. fusiforme* wild population	The spores mature period appeared earliest; collected in Luxi Island, Zhejiang Province
6	*Sargassum horneri*	

Stain 1, 2, 3, 5 were collected in 2007; stain 4 was collected in 2008, *S. hornei* was used as the out-group.

different areas in China, and this caused serious influence to the *Sargassum* wild resources (Zhang et al., 2002; Haraguchi and Sekida, 2008), and even led to high heterogeneities to *S. fusiforme* populations. Previously, Wang (2003) investigated the natural distributions of *S. fusiforme* in Zhejiang and indicated that its natural resources tend to be declined. We suspected that this was mainly due to the collection of natural *S. fusiforme* for artificial breeding, except for climate warming and coastal environmental side effects.

During the past 15 years' for the *S. fusiforme* selection in Dongtou, Zhejiang and northern areas in Shandong, it proved that the selective characteristics were stable and the production was high (Li et al., 2010). Nevertheless, it will be confused if judged merely by the morphological features during the selection, it, therefore, needs to conduct the genetic and fingerprint analysis to these cultivars.

Previously, Zhao et al. (2007, 2008) studied the population genetic of *S. thunbergii* and *S. muticum* with RAPD and the inter-simple sequence repeat (ISSR) markers, while to *S. fusiforme*, Park et al. (1998) applied RAPD markers to identify wild isolates from the six sites in Korea. Shan et al. (2009) used AFLP markers to assess the genetic diversities of one *S. fusiforme* strain. Yu et al. (2012) used ISSR and sequence-related amplified polymorphism (SRAP) markers to study the genetic structure of nine wild *S. fusiformis* populations in China. Hu et al. (2013) applied mtDNA Cox1 sequences to illustrate the genetic structure and geographic distribution patterns to *S. fusiforme*. Xu et al. (2014) and Nan et al. (2015) used ISSR markers to investigate genetic diversity, genetic variation to *S. fusiforme* between the wild and breeding strains.

Based on the selection and cultivations, we adopted the populations of *S. fusiforme* that cultivated in Dongtou and Zhejiang to analyze their genetic structures with RAPD and ISSR markers. We aim to construct the fingerprint and obtain the screened sequence characterized amplified region (SCAR) markers, in a molecular assistant to *S. fusiforme* selection and breeding, and for the extraction of brown seaweeds bioactive substance in the future.

2 MATERIAL AND METHOD

2.1 Selection and breeding of *S. fusiforme*

From 1993, the selection of the *S. fusiforme* was initiated during the cultivation. Each year, the selected parental cultivars were applied for the next year's nursery and cultivation, and the objective characters of selected *S. fusiforme* with high yield was obtained until 2000. After that, the selection and breeding were continuously conducted from 2001, and the successive 9 generations of selected *S. fusiforme* cultivar were proved to be stable in the practice.

2.2 Strains morphological background

The three selected cultivars of *S. fusiforme* were collected from the cultivated populations in Dongtou, Zhejiang. The phenotype of strain 3 and 4 namely "Lufeng No.1" was illustrated in our previous study (Li et al., 2010), and was now applied in *S. fusiforme* production in Zhejiang and Fujian of China, all the *Sargassum* morphological features were illustrated in Table 1.

2.3 RAPD and ISSR amplification

To each *Sargassum* population, usually, 24 individuals were adopted for DNA extraction and further analysis. Plant Genomic DNA Extraction Kit (Tiangen Biotech, Beijing) was applied for DNA extraction, the extracted algal DNA were then electrophoresis on 0.8% agarose gels. Totally, 300 RAPD primers were synthesized from Sangon (Shanghai), after selection and tests, 12 RAPD primers were selected (Table 2).

Table 2 Primers applied in RAPD and ISSR analyses

Primer	Sequence (5′→3′)	Loci
RAPD-S17	AGGGAACGAG	12
RAPD-S62	GTGAGGCGTC	9
RAPD-S102	TCGGACGTGA	8
RAPD-S201	GGGCCACTCA	17
RAPD-S1025	GTCGTAGCGG	9
RAPD-S1027	ACGAGCATGG	9
RAPD-S1028	AAGCCCCCCA	14
RAPD-S1213	GGGTCGGCTT	12
RAPD-S2025	GGGCCGAACA	11
RAPD-S2031	TGCGGGTTCC	10
RAPD-S2039	TTGCGGACAG	11
RAPD-S2118	AGCCAAGGAC	13
ISSR-807	$(AG)_8T$	12
ISSR-810	$(GA)_8T$	10
ISSR-811	$(GA)_8C$	5
ISSR-823	$(TC)_8C$	8
ISSR-828	$(TG)_8A$	9
ISSR-836	$(AG)_8YA$	12
ISSR-840	$(GA)_8YT$	7
ISSR-851	$(GT)_8YG$	15
ISSR-855	$(AC)_8YT$	8
ISSR-890	$VHV(GT)_7$	6

RAPD reactions were carried out in a 20-μL reaction volume, which contained 1 μL of template DNA solution, 0.25 μmol/L primers, 200 μmol/L each of dATP, dGTP, dCTP, dTTP (Promega, Shanghai), 2.0 mmol/L Mg^{2+}, 1× polymerase buffer (Promega, Shanghai) and 1.0 unit of *Taq* polymerase (Promega, Shanghai). PCR amplification reaction was performed by Master Thermal Cycler (Effendorf, German) in the procedure of initial denaturation for 5 min at 94°; 45 cycles of 1 min at 94°C, 1 min at 37°C and 2 min at 72°C; and the final extension of 10 min at 72°C.

To ISSR amplification systems, 90 primers were synthesized by Sangon (Shanghai), finally, 10 primers were adopted (Table 2). The PCR reactions were performed in a 20-μL reaction volume containing 1 μL template DNA solution, 5 pmol primers, 0.1 mmol each of dATP, dGTP, dCTP, and dTTP (Promega, Madison, WI, USA), 1.5 mmol Mg^{2+}, 1× polymerase buffer (Promega, USA), and 1.0 U *Taq* DNA polymerase (Promega, USA). PCR amplification reaction was conducted using a Master Thermal Cycler (Effendorf, Germany). The cycling parameters were 94°C for 5 min and 40 cycles of denaturation at 94°C for 30 s, annealing at the 52°C for 45 s, and extension at 72°C for 2 min.

Fig.1 Collected *S. fusiforme* "Lufeng No. 1" (upper) and wild-type (lower)

All the RAPD and ISSR reaction products were analyzed on 1.5% agarose gels in the 1× TAE buffer then stained with ethidium bromide, and photographed under UV light for the record by a digital imager (Bio-rad, USA).

2.4 Statistical analysis

Those clear and stable repeated amplified bands were scored as present (1), or absent (0) according to the observation. Software POPGENE version 1.31 (Yeh et al., 1997) was used for evaluating genetic variability from the percentage of polymorphic loci (*P*) at the 99% criterion, the average expected heterozygosis (*H*) assuming a Hardy-Weinberg equilibrium, and Shannon's information index of genetic diversity (*I*). Software TFPGA 1.3 (Miller, 1997) was utilized to calculate the Nei's unbiased genetic distance (*D*) (Nei, 1978) and to construct a dendrogram by means of UPGMA (unweighted pair group method with arithmetic averages) grouping method. The *S. horneri* was used as the out-group. By bootstrapping 1 000 permutations were performed to assess the robustness of the groupings.

2.5 Sequence characterized amplified region (SCAR) generation

To the amplification of S1027 band, the exhibition of loci (700 bp) which specifically to strain 3 (Lufeng No.1) was selected and sequenced (Fig.1), and the objective loci were recovered with DNA Recovery Kit (Fastagen, Shanghai). The selected DNA fragments were ligated and transformed with the

Fig.2 Observation of *S. fusiforme* "Lufeng No. 1" in cultivation

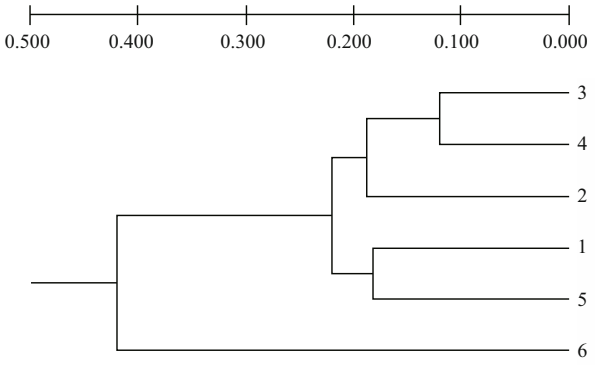

Fig.3 UPGMA dendrograms constructed with Nei's (1978) unbiased genetic distance of *S. fusiforme* populations with RAPD analysis

Population 1: *S. fusiforme* strain 1; population 2: *S. fusiforme* strain 2; population 3: *S. fusiforme* stain 3 (Lufeng No.1); population 4: *S. fusiforme* strain 4 (Lufeng No.1); population 5: *S. fusiforme* wild population; population 6: *Sargassum horneri*.

pGEM-T Easy Vector System I (Promega, USA). After the transformation, white colonies were selected and verified through PCR tests.

SCAR-PCR amplifications were performed in the Master Thermal Cycler (Effendorf, Germany) with a 20-μL reaction volume. Each PCR reaction contained 1 μL template DNA solution, 1× polymerase buffer (Promega, USA), 2.0 mmol Mg^{2+}, 0.1 mmol each of dATP, dGTP, dCTP, and dTTP (Promega, USA), 10 pmol each specific SCAR primer and 1.0 U *Taq* DNA polymerase (Promega, USA). The thermal profile for PCR was an initial denaturation at 94°C for 5 min and 40 cycles of denaturation at 94°C for 30 s, annealing at the 52°C for 45 s, and extension at 72°C for 1 min. The PCR products were distanced on 1.5% agarose gels in the 1× TAE buffer, stained with ethidium bromide, and were photographed under UV light and recorded with a digital imager (Bio-rad, USA).

Table 3 The Nei's (1978) genetic distances (*D*) calculations to the populations of *S. fusiforme* with RAPD analysis

Population	1	2	3	4	5	6
1	*****					
2	0.177 8	*****				
3	0.173 6	0.162 7	*****			
4	0.178 5	0.196 1	0.111 6	*****		
5	0.173 4	0.225 1	0.256 3	0.251 7	*****	
6	0.393 1	0.450 3	0.459 0	0.396 6	0.368 6	*****

Population 1: *S. fusiforme* strain 1; population 2: *S. fusiforme a* strain 2; population 3: *S. fusiforme* stain 3 (Lufeng No.1); population 4: *S. fusiforme* strain 4 (Lufeng No.1); population 5: *S. fusiforme* wild population; population 6: *Sargassum horneri*.

3 RESULT

3.1 Field tests and morphological comparisons of the selected *S. fusiforme*

The objective characters of *S. fusiforme* "Lufeng No.1" was started from 2001, and was successive artificially inbred for over 9 generations till 2009, which was cultivated for 2 532 m^2 and finally more than 400 tons were yielded, with an average of 6 300 kg/hm^2. Compared with the wild-type of *S. fusiforme*, the selected *Sargassum* strains, it is obvious in air vesicles, main branch, lateral branch, and growth rate (Figs.1, 2).

3.2 Genetic analysis

Generally, 135 loci ranging from 300 to 2 000 bp were scored, which yielded 11.3 loci per primer averagely. The Nei's (1978) genetic distances (*D*) between pairs of *S. fusiforme* populations with RAPD analyze were from 0.111 6 to 0.459 0 (Table 3). The lowest value was between the population's strains 3 and 4, and the highest between the populations 3 and the out-group. The UPGMA dendrograms (Fig.3) based on pairwise *D* with RAPD data indicated that populations 3 and 4 clustered together first, and then with populations 2; meanwhile population 1 and 5 clustered together, finally with the out-group.

The yielded 92 loci ranging from 300 to 2 000 bp were scored, with 9.2 loci per primer averagely. The Nei's (1978) genetic distances (*D*) between pairs of *S. fusiforme* populations with ISSR analysis were from 0.086 3 to 0.650 3 (Table 4). The lowest value was between populations 3 and 4, and the highest between populations 3 and the out-group. The UPGMA dendrograms (Fig.4) based on pairwise *D* with RAPD

data indicated that populations 3 and 4 clustered together first, and the populations 1 and 2 clustered together then with populations 5, finally with the outgroup.

3.3 DNA fingerprints construction

Base on the RAPD analysis, four loci yielded from the selected two primers were chosen for the DNA fingerprints construction (Table 5), and each of the three *S. fusiforme* strains exhibited the unique loci pattern and could be distinguished from each other.

3.4 SCAR marker verification

After the sequences analysis, the selected SCAR primers for strain "Lufeng No.1" verification were ACGAGCATGGTGATGCGTTGAAG (forward) and ACGAGCATGGGCATATTGAACGT (reverse), it was proved that the SCAR markers for *S. fusiforme* strain "Lufeng No.1" was valid (Fig.5).

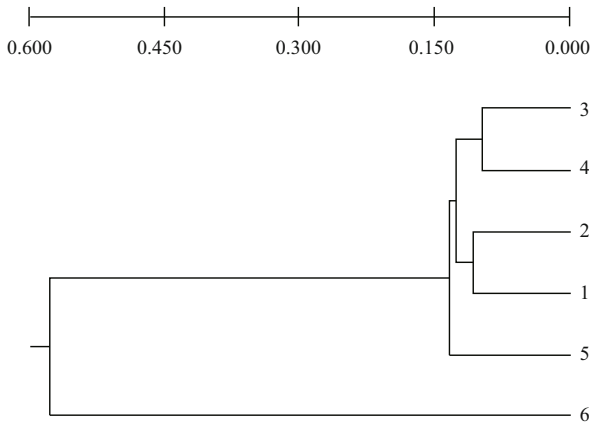

Fig.4 UPGMA dendrograms constructed with Nei's (1978) unbiased genetic distance of *S. fusiforme* populations with ISSR analysis

Population 1: *S. fusiforme* strain 1; population 2: *S. fusiforme* strain 2; population 3: *S. fusiforme* stain 3 (Lufeng No.1); population 4: *S. fusiforme* strain 4 (Lufeng No.1); population 5: *S. fusiforme* wild population; population 6: *Sargassum horneri*.

Table 4 The Nei's (1978) genetic distances (*D*) calculations to the populations of *S. fusiforme* with ISSR analysis

Population	1	2	3	4	5	6
1	*****					
2	0.098 3	*****				
3	0.121 1	0.098 5	*****			
4	0.101 5	0.141 1	0.086 3	*****		
5	0.115 4	0.145 4	0.119 1	0.117 8	*****	
6	0.560 6	0.558 8	0.650 3	0.555 9	0.535 0	*****

Population 1: *S. fusiforme* strain 1; population 2: *S. fusiforme* strain 2; population 3: *S. fusiforme* stain 3 (Lufeng No.1); population 4: *S. fusiforme* strain 4 (Lufeng No.1); population 5: *S. fusiforme* wild population; population 6: *Sargassum horneri*.

Table 5 DNA fingerprints established for *S. fusiforme* selected strains

	$S1027_{1150}$	$S1027_{800}$	$S1213_{1100}$	$S1213_{700}$
Population 1	-	-	-	
Population 2	-	-		
Population 3	-			-
Population 4	-			-
Population 5			-	-

Population 1: *S. fusiforme* strain 1; population 2: *S. fusiforme a* strain 2; population 3: *S. fusiforme* stain 3 (Lufeng No.1); population 4: *S. fusiforme* strain 4 (Lufeng No.1); population 5: *S. fusiforme* wild population. - means having the amplified DNA sequence.

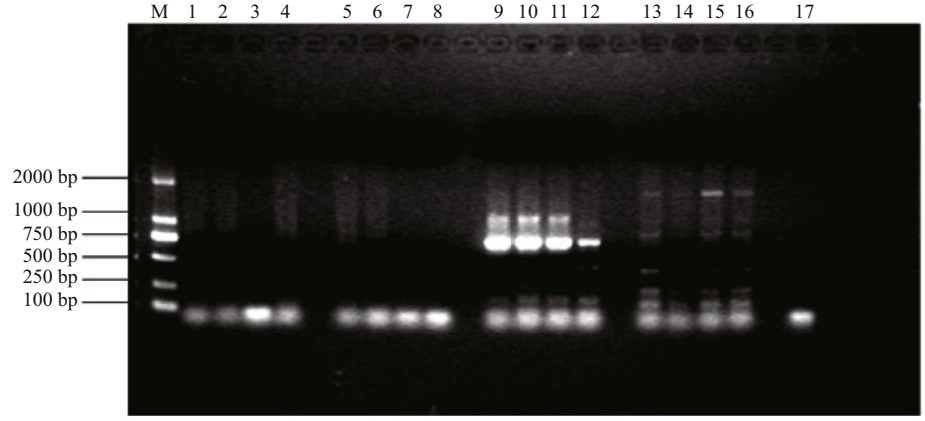

Fig.5 SCAR marker verification for the *S. fusiforme* strains

M: DL2000, 1, 2, 3, 4: population 1; 5, 6, 7, 8: population 2; 9, 10: population 3; 11,12: population 4; 13, 14, 15, 16: population 5; 17: population 6. Population 1: *S. fusiforme* strain 1; population 2: *S. fusiforme* strain 2; population 3: *S. fusiforme* stain 3 (Lufeng No.1); population 4: *S. fusiforme* strain 4 (Lufeng No.1); population 5: *S. fusiforme* wild population; population 6: *Sargassum horneri*.

4 DISCUSSION

To the application of RAPD and ISSR markers systems, it was used mainly for the genetic relations analysis of the five *S. fusiforme* populations (Figs.3, 4); and it is shown that there were slight differences in the dendrograms with the two marker systems. Usually, ISSRs markers were more discriminative in cultivar identification than RAPDs system, due to its smaller genetic distances difference. Even with minor genetic diverge, all the strains belong to *S. fusiforme* clad judged, within the same clade, strains 3 and 4 which collected continuously for the followed two years could be clustered into one group (Figs.3, 4).

Compared with the wild population, the genetic variation of stain 3 ("Lufeng No.1") could be easily distinguished with RAPD and ISSR fingerprint analysis (Figs.3, 4). The genetic distances among the wild population and strain 3 and strain 4 ("Lufeng No.1") are 0.187 7 and 0.184 8 respectively, this indicated genetic divergences already occurred during the selection during in the past 15 years (Tables 3, 4).

The phenotypes of selected *S. fusiforme* strains were stable, however, highly polymorphic loci (83.8%) existed in the selected *S. fusiforme* populations if detected with AFLP markers (Shan et al. 2009), here in our study, the RAPD and ISSR analysis results either showed that it exhibited highly heterogeneities. We suspected that frequent transplantation and breeding could enhance the gene flows exchange between the alien *S. fusiforme* and local populations, and this enriched the gene pool to local *S. fusiforme* population; another reason is that *Sargassum* phyla have high genetic diversities (Zhao et al., 2007, 2008). Finally, we believed that some loci will be lost if the selection sustained for more time.

It is reported that the SCAR markers are more reproducible and reliable than the RAPD systems (Paran and Michelmore, 1993). Considering the stabilities and reproductions, we generated the SCAR marker for verifying *S. fusiforme* strain 3 and 4 ("Lufeng No.1"), and proved that it is stable and reproducible in distinguishing the selected *S. fusiforme* cultivars, further work is needed to conduct verification of the *S. fusiforme* cultivars to those from the successive selection, this will be positive to *S. fusiforme* natural and wild populations.

5 CONCLUSION

RAPD and ISSR marker were proved to be valid for analyzing the five strains of *S. fusiforme*. The constructed fingerprints and SCAR marker to *S. fusiforme* strain (Lufeng No.1) were applicable to the *Sargassum* selection, it could be useful in molecular markers assistance in *S. fusiforme* selection and cultivars identification.

6 DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

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