

Application of SSR and AFLP to the analysis of genetic diversity in *Gracilariopsis lemaneiformis* (Rhodophyta)

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Abstract The agarophyte *Gracilariopsis lemaneiformis* is both important for biological research and of significant economic value. However, the genetic diversity of wild populations of the alga has not been studied. We used amplified fragment length polymorphism (AFLP) PCR and simple sequence repeat (SSR) analysis to investigate diversity in four field populations, three from the coast of Qingdao and one from Weihai, China. Forty *G. lemaneiformis* isolates collected from the four different geographical groups were analyzed using 16 pairs of SSR primers for PCR amplification. However, no polymorphisms were detected, indicative of a degree of genetic homogeneity. A total of 347 reproducible bands were then amplified using eight AFLP primer pairs, and genetic indices of diversity within and between populations were calculated. This analysis revealed only low levels of genetic diversity both within and between the four geographical groups of *G. lemaneiformis*. The Weihai population showed a higher within-population genetic diversity than any of the Qingdao populations. The data suggest that there is only limited gene flow between populations. An UPGMA dendrogram revealed two main clusters, and one of these included all the Qingdao isolates. The order of clustering was in accordance with their geographical distribution. These results suggest that the wild *G. lemaneiformis* populations are closely related and that there is little

genetic diversity within wild germplasm in the regions sampled.

Keywords AFLP · Genetic diversity · *Gracilariopsis lemaneiformis* · SSR

Introduction

The red alga *Gracilariopsis lemaneiformis* (Bory) Dawson, Acleto, and Foldvik has long been adopted as a model organism due to several important traits that are generally absent from other macroalgae. The species is culturable under laboratory conditions throughout its complete life cycle and produces large numbers of tetraspores (haploid) and carpospores (diploid) that permit genetic investigations including tetrad analysis, multi-gene profiling, and the induction of mutations (Zhang and van der Meer 1988). Since the 1990s, genetic analysis has led to significant advances in our understanding of the life cycle, growth requirements, and basic molecular biology of the organism (Li 1999; Sui and Zhang 2002; Zhang and van der Meer 1988). *Gracilariopsis lemaneiformis* is well known as the major agarophyte in China because of its high yields and commercially valuable extracts (Lapointe and Ryther 1978; Troell et al. 1999; Wang 2002). Agar produced from *G. lemaneiformis* accounts for more than 90% of the total annual production in China (Fei et al 1999). In addition, *G. lemaneiformis* can remove nutrients including nitrogen and phosphate from the culture medium, and therefore has considerable potential in ecological restoration applications (Yang and Fei 2003). However, despite the importance of the alga in biological research and in commercial applications, the genetic backgrounds of field populations have not been studied in detail.

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Amplified fragment length polymorphism (AFLP) PCR and simple sequence repeat (SSR) analysis have been widely used for genetic linkage mapping, and provide markers for genetic breeding, species identification, gene localization, and the identification of quantitative trait loci (Bruin et al 2003; Chen et al 2007), although AFLP and SSR have both advantages and disadvantages compared to other parallel techniques (Xu et al. 2007). In the present study, we used AFLP and SSR to analyze the genetic diversity of four field populations of *G. lemaneiformis* in China. Three population samples were collected from sites several miles apart on the coast of Qingdao, whereas the fourth group of samples was from Weihai, several hundred kilometers away. The aim of the study was to investigate the genetic background and diversity of wild populations in China in order to facilitate further biological studies on this species.

Materials and methods

Forty *Gracilariopsis lemaneiformis* isolates were collected from four different geographical sites: Zhanshan Bay (QDZS), Huiquan Bay (QDHQ), and Taiwan Road (QDTW) of the Qingdao region, and the Shidao area of the Weihai region (WHSD), China. Ten isolates were collected from the intertidal zones at each site, and isolates were randomly selected within a moderate distance. The three Qingdao sampling sites were located several kilometers apart. The sampling site in Weihai was several hundred kilometers away. After collection, isolates were washed in tap water to remove excess salts and sand, and then dried to remove surface water prior to nucleic acid extraction.

Nucleic acid extraction Genomic DNA was extracted using DNA MagExtractor TIANamp Genomic Kit (TianGen, Beijing, China) according to the instructions of the manufacturer. Extracted genomic DNA was stored at -20°C .

SSR analysis Genomic DNA libraries enriched for $(\text{GA})_n$ repeats were constructed by a modification of a published method for AFLP PCR amplification of sequences containing repeats (Zane et al. 2002). In brief, genomic DNA was sheared by ultrasound treatment, fragments were converted to blunt ends by a standard technique, ligated with synthetic adaptors, and hybridized to a $(\text{GA})_n$ probe bound to nylon membrane in order to enrich for fragments containing microsatellite sequences. Bound fragments were eluted, PCR amplified, and ligated into a cloning vector. Clones containing microsatellite sequences were PCR-amplified and their DNA sequences were determined (Pang et al. 2009). Based on these *G. lemaneiformis* sequences, 16 PCR primer pairs were randomly selected that gave reproducible amplification products (Pang et al. 2009). The sequences of the primers used in SSR analysis are shown in Table 1. PCR was performed using 20 ng of template DNA, 50 ng primers, 1.0 μl dNTP (2.0 mM), 1.2 μL MgCl_2 (25 mM), and 1 U Taq DNA polymerase in a final volume of 20 μL . PCR amplification products were separated on 6% denaturing polyacrylamide gels, and electrophoresis was performed at constant power of 60 W for 1.5 h. Following electrophoresis, DNA bands were visualized using the Silver Sequence DNA Sequencing System (Saini et al. 2004).

AFLP analysis AFLP reactions were according to the protocol of Vos et al. (1995) with the modification that 500 ng of DNA were digested at 37°C for 6 h with 5 U each of EcoRI and MseI. The resulting fragments were ligated to EcoRI and MseI adaptors; pre-amplification was performed using primers based on the EcoRI and MseI adaptors plus one selective nucleotide (EcoRI-A, MseI-C) under the conditions described by Vos et al. (1995) except that 1 U of Taq polymerase (MBI) was used. Selective amplification was then conducted using eight primer combinations, each with three nucleotides for MseI- and two nucleotides for EcoRI-specific primers. PCR products was denatured at 95°C for 3 min then separated on 5% denaturing polyacryl-

Table 1 Sequences and characteristics of the SSR primers used in this work

Name	Primer Sequence	Core sequence	Ta	Product length
08j2-14	f:aagactcggataaacggaagc r:cgtacatatcgtggcacc	$(\text{GA})_9$	57°C	177 bp
08j2-34	f:cctgagccgctgctaaatc r:tgtgacaacacccactctg	$(\text{TC})_{13}$	56°C	362 bp
08j2-45	f:gggaggacgaagcgataag r:accgcaagagccaagtgtaa	$(\text{AG})_{14}$	56°C	392 bp
08j2-76	f:gctgtgtgtcgtggaatg r:gtggaatcgtagcgggtgg	$(\text{CT})_{20}$	58°C	339 bp
08j2-24	f:gtcaaacctcacaacgcacca r:catttctccctccaatcct	$\text{A}_9\text{GA}_9+(\text{GA})_8\text{TAT}(\text{GA})_4$	55°C	212 bp

Table 2 Polymorphism rates (P) observed using eight AFLP primer pairs in *Gracilariopsis lemaneiformis*

Primers	Total bands	Polymorphic loci	Polymorphism rate
M-CAA/E-AAA	59	37	62.7%
M-CAT/E-AAA	36	15	41.7%
M-CAA/E-AAA	47	17	36.2%
M-CAA/E-AAT	59	28	47.5%
M-CAT/E-AAT	51	31	60.8%
M-CAA/E-AAG	27	11	40.1%
M-CAG/E-AAG	36	22	61.1%
M-CAT/E-AAG	32	16	50.0%
Total	347	177	51.0%

amide gels. Electrophoresis was performed at a constant power of 60 W for 2.5 h including a 0.5 h pre-run. Following electrophoresis, DNA bands were visualized using the Silver Sequence DNA Sequencing System (Saini et al. 2004).

Data analysis Ten *G. lemaneiformis* isolates from each of the four different geographical groups (QDZS, QDHQ, QDTW, and WHSD) were analyzed for genetic diversity by SSR and AFLP. Individual AFLP bands were scored for presence (1) or absence (0) and entered into a binary matrix representing the AFLP profile of each sample. Analysis was restricted to bands that were generated reproducibly. Statistical analysis employed PopGen32 and MEGA3.1 software.

Results

SSR analysis

Ten *G. lemaneiformis* isolates from each of the four different geographical populations were analyzed for genetic polymorphisms using 16 different PCR primer pairs. All of the 16 SSR primer pair PCR products were monomorphic, and consistent SSR products of the same sizes were generated in all displays. This analysis indicates a degree of genetic homogeneity between the different *G. lemaneiformis* samples.

To refine our analysis, isolates were subdivided according to agar content. Ten isolates with a high agar (~20%),

ten with moderate content (15–20%), and ten with low content (~15%) were also analyzed using four of the 16 primer pairs. No genetic variation was detected by this method.

We also investigated whether the primer pairs selected were able to amplify bands from other closely related *Gracilaria* species. Twelve of the 16 primers consistently generated PCR products from 981 *G. lemaneiformis*, a strain obtained from wild-type *G. lemaneiformis* by selective breeding (Zhang and Fei 2008), while only one primer pair of the 16 (6.25%) consistently generated PCR products from the more distantly related *Gracilaria verrucosa* and *Gracilaria textorii*.

AFLP analysis

Eight of the 16 primer pairs that gave consistent AFLP results were selected for the analysis of the 40 *G. lemaneiformis* isolates from four different geographical locations. This generated a total of 347 reproducible bands. The polymorphism rates (P) identified using eight primer pairs are shown in Tables 2 and 3. Polymorphism rates identified using the different primer pairs ranged from 36.2% to 62.7%, and ranged from 9.8% to 20.4% within different groups, a much lower extent of polymorphism than has been reported for other algae including *Undaria pinnatifida* (~80%; Zhang et al. 2009a, b) and *Laminaria japonica* (80.95%; our unpublished data).

The diversity indices of the four geographical groups are shown in Table 4. The observed mean number of alleles (Na) ranged from 1.1066 to 1.3026 while the effective number of alleles (Ne) ranged from 1.0477 to 1.1130. The

Table 3 Polymorphism rates (P) revealed by AFLP analysis of four geographical groups of *Gracilariopsis lemaneiformis*

Different groups	Total bands	Polymorphic loci	Polymorphism rate
QDZS	347	66	19%
QDHQ	347	45	13.0%
QDTW	347	34	9.8%
WHSD	347	71	20.4%

Table 4 Diversity indices revealed by AFLP analysis of four geographical groups of *Gracilariopsis lemaneiformis*

Different groups	Na	Ne	H	I
QDZS	1.1931	1.1007	0.0601	0.0918
QDHQ	1.1210	1.0669	0.0400	0.0604
QDTW	1.1066	1.0477	0.0292	0.0457
WHSD	1.3026	1.1130	0.0715	0.1151
Total	1.6081	1.3779	0.2092	0.3085

Na observed number of alleles, Ne effective number of alleles, H Nei's gene diversity, I Shannon diversity index

Nei measure of genetic diversity (*H*) ranged from 0.0292 to 0.0715 whereas Shannon diversity indices (*I*) ranged from 0.0457 to 0.1151. Furthermore, the indices of diversity were significantly higher within the Weihai isolates than within the Qingdao isolates, and were also increased when all individual isolates were considered. Overall, these data suggest that the levels of genetic diversity within the four geographical groups of *G. lemaneiformis* are very low. The Weihai population showed the highest within-population genetic diversity, and inter-group genetic diversity was significantly higher than intra-group diversity.

The genetic diversity between the four groups was analyzed mathematically (Table 5). The values of the mean genetic diversity index (*Ht*) and within-population genetic diversity index (*Hs*) of the three Qingdao groups ranged from 0.0804 to 0.1078 and from 0.0346 to 0.05, respectively, while those between Weihai and Qingdao groups were 0.2106 and 0.0909, respectively. The *Ht* and *Hs* values between all of the groups were 0.1887 and 0.0502, respectively. The gene flow as reflected by the *Nm* value ranged from 0.1812, when all of the groups were considered, to 0.6498 between two of Qingdao groups. Values were all less than 1.000, indicating little gene flow between the four groups. The coefficient of gene differentiation (*Gst*) ranged from 0.5368 to 0.8246, indicating that the majority of genetic diversity was

Table 5 Inter-group gene diversity revealed by AFLP analysis of four geographical groups of *Gracilariopsis lemaneiformis*

Different groups	Ht	Hs	Nm	Gst
QDTW-QDZS	0.0958	0.0447	0.5337	0.5368
QDHQ-QDTW	0.0988	0.0346	0.6498	0.5694
QDHQ-QDZS	0.0804	0.0500	0.3775	0.8246
QDZS-QDHQ-QDTW	0.1078	0.0431	0.3328	0.6003
WHSD-QD	0.2106	0.0909	0.3797	0.5864
Mean	0.1887	0.0502	0.1812	0.7340

Ht average gene diversity index, Hs inner gene diversity index, Nm gene flow, Gst coefficient of gene differentiation

Table 6 Inter-group genetic distances and similarities revealed by AFLP analysis of four geographical groups of *Gracilariopsis lemaneiformis*

Groups	QDZS	QDHQ	QDTWL	WHSD
QDZS		0.9388	0.8953	0.7395
QDHQ	0.0632		0.8687	0.6797
QDTWL	0.1106	0.1408		0.7318
WHSD	0.3154	0.3861	0.3132	

(Values above the diagonal line were the genetic similarity coefficient, values below the diagonal line were genetic distance)

represented by inter-group differences (Sokal and Michener 1958).

Genetic distances and similarities between populations are shown in Table 6. The genetic similarity coefficient ranged from 0.6797 to 0.9388. The coefficient between the Qingdao populations was higher than between each Qingdao group isolate and the Weihai isolates. The genetic distance ranged from 0.0632 to 0.3861, however, the distance between the Weihai isolates and any of the Qingdao isolates was larger than the distance between different Qingdao group isolates.

A distance tree was then constructed using clustering with the unweighted pair group method with arithmetic mean (UPGMA; Fig. 1). Two principal clusters were generated, and one included all of the Qingdao isolates. One isolate, No 31 collected from Weihai, did not cluster



Fig. 1 UPGMA dendrogram of the *G. lemaneiformis* groups used in this study

strongly with any other isolates. QDZS clustered most strongly with QDTW, then with QDHQ, and lastly with WHSD. The order of clustering was in accordance with their geographical distributions.

Discussion

Sixteen SSR primers were developed from *G. lemaneiformis* by a random selection method (Pang et al. 2010). However, this failed to reveal any genetic polymorphism between the 40 isolates, including between isolates of different agar contents. A similar low rate of SSR polymorphism was also reported in a recent study on *G. lemaneiformis* (Zhang et al. 2009b) and in another red alga, *Porphyra* (our unpublished data), although this technique has demonstrated high rates of genetic polymorphism in terrestrial plants and marine animals (Guo et al. 2009; Waldbieser et al. 2001; Zhang et al. 2008).

The core sequences of the SSR sites employed in this study were short, and were repeated from seven to 38 times. It has been reported that the rate of SSR polymorphism is proportional to the repeat interval of the core sequence (Rico et al. 1997), and this may explain our SSR findings in *G. lemaneiformis*. However, it is possible that simple repeat sequences in this alga are stable, with little copy number variation, and this could potentially reflect differences in the DNA replication machinery of these algae compared to other species.

In addition, only one of 16 SSR primers (6.25%) tested consistently generated PCR products in other *Gracilaria* species, showing a similar level of transferability that genome-derived (normally randomly picked) SSR primers instead of conserved region-derived ones may produce (Yang et al. 2008).

The AFLP results suggest that there are only low levels of genetic diversity in wild *G. lemaneiformis* populations from northern yellow sea of China. Genetic diversity indices indicated that the different isolates are closely related at the genetic level. This low level of diversity differs from findings in other cultivated macroalgae including *U. pinnatifida* (Zhang et al. 2009a) and *L. japonica* (Shi et al. 2007), where a far higher level of genetic diversity was reported.

G. lemaneiformis is distributed across North America, South Africa, Asia, and the Hawaiian Islands (Abbott 1985a, b, 1994; Ganesan 1990; Norris 1985; Xia and Zhang 1999). In China, the species has been reported to be distributed in the North of the country (Zhang et al. 2004; Qingdao, Weihai, and Dalian, personal observations). It is possible that more genetic variation might have been revealed by analysis of isolates from Dalian, a coastal city about 160 km in Liaoning province of Northern Huanghai.

However, we have no data on this possibility due to sampling failure from Dalian.

Each local geographical population of *G. lemaneiformis* is normally restricted to a limited site with areas of intertidal rock and sand substrata ranging from several tens to several hundreds of square meters. In view of the large number of progeny of each carposporophyte or tetrasporophyte, ten samples were collected from different sites within the area in order to maximize genetic diversity. Nevertheless, only low levels of genetic polymorphism were detected within each *G. lemaneiformis* population (Table 4). It is notable that these species are known to have been present at the sampling sites for many years (Xia and Zhang 1999), and it is unlikely that these populations reflect recent colonization by a genetically restricted substrain; a recent population bottleneck is unlikely to explain the low levels of genetic diversity. It is possible that isolates from each sampling site derive from a single ancestral strain; however, we cannot exclude that possibility of genetic homogenization following establishment of the population. The low levels of genetic diversity between the different geographical groups indicates a paucity of wild germplasm resources in this species, and this is likely to impact negatively on selective breeding programs based on wild isolates of *G. lemaneiformis*.

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