

MOLECULAR CHARACTERIZATION OF SOME ALGAE BY PROTEIN BANDING PATTERN AND ISSR MARKERS COLLECTED FROM THE GULF OF AQABA, SAUDI ARABIA

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Abstract

Algae have high ecological and commercial importance; the estimated turnover of the seaweed industry is USD 10 billion. In order to understand the distribution of seaweed, their identification is necessary, which is generally based on morphological characteristics, often resulting in the wrong identification at the species level. DNA fingerprinting could be used for species-level identification. The technique can resolve many intrinsic problems of morphological taxonomy as only a small amount of tissue is required for species identification. Along with conservation biology, DNA fingerprinting could also be used in the identification of invasive and endangered species. In the case of algae, DNA fingerprinting was beneficial for the recognition of high-yielding agar strains as well as for the identification of cryptic species. In this study, several identification-based problems of algae have been discussed by using different intraspecific markers, such as protein banding pattern and ISSR, for eight algae (*Padina pavonica*, *Turbinaria gracilis*, *Carpomitra costata*, *Pterocladia capillacea*, *Cladostephus spongiosus*, *Ulva lactuca*, *Sporochnus comosus*, and *Sargassum muticum*), were collected from the Gulf of Aqaba, specifically from the beaches of Haql (28°45'N, 34°45'E). According to the available data, the ISSR marker is more effective than protein banding pattern for the measurement of algal genetic diversity.

Keywords: *Padina pavonica*, *Turbinaria gracilis*, *Carpomitra costata*, *Pterocladia capillacea*, *Cladostephus spongiosus*, *Ulva lactuca*, *Sporochnus comosus*, and *Sargassum muticum*, ISSR marker, SDS-PAGE.

Introduction

Algae represent a group of phytoplankton with a great ecological and economic significance. Therefore, to understand the distribution of seaweed, species identification is necessary, which is generally based on morphological characteristics, such as size, form, and color of a morphotype. This method is outdated and is slightly complex because of its three major limitations (Hebert *et al.*, 2003; Pires & Marinoni, 2010). Firstly, the key for the morphological characterization is not complete or available for all taxa (May, 1988). Some taxa such as flowering plants and vertebrates are well studied than others such as algae and nematodes. Secondly, the morphological characteristics and their complexity depend on the group under consideration; therefore, sometimes the identification keys need well-trained taxonomists, who are rare to find, and are not permanently available for consistent documentation; this is referred to as taxonomic impediment. Thirdly, the organism to be identified may be tiny or at a developmental stage, where even trained taxonomists face difficulty in characterization and identification. In addition, the morphological approaches are time-consuming and are unable to classify up to the species level (Rindi *et al.*, 2008; Packer *et al.*, 2009). Molecular biology has revealed that many biological species showed genetic divergence without accompanying morphological disparities and therefore such species could not be identified by traditional methods. The recognition of these cryptic species remains a challenge in current taxonomy (Heinrichs *et al.*, 2011). Species

identification using DNA fingerprinting has been effectively documented in animals, plants, and fungi (Mihdhir *et al.*, 2016; Khalik & Osman, 2017) and algae (Munshi & Osman, 2010). Here, we discuss the molecular characterization of some algae by protein banding pattern and ISSR markers collected from the Gulf of Aqaba, Saudi Arabia (Woelkerling, 1990; Alothyqic *et al.*, 2016). The algae are commonly known as red algae and have several types of shape, from unicellular-filamentous (simple) to blade-like-pseudoparenchymatous (most complex) (Cole & Sheath, 1995). According to previous studies, there are approximately 7282 species of red algae, the total number of estimated species that are described is 7000, and total number of undescribed species is 7000 (Guiry, 2012; Latif *et al.*, 2018 a, b). This information highlights the importance of identification and classification of Rhodophyta, which is environmentally economically valuable. Several genera of red algae are found in freshwaters, but most red algae are marine and they are generally reported on tropical and temperate marine shores. At these shores, they play the role of "keystone species" by building and maintaining coral reefs (Freshwater *et al.*, 1995). This gives banded coral, giant clams, clownfish, shrimps, and other animals a reliable habitat to dwell and maintain an ecological balance. Furthermore, these algae form flat sheets that fuse and stabilize reef crest and protect reefs from wave damage. The molecular assessment of red algae fossils indicates that these coralline red algae have been playing this important role from hundreds of millions of years (Freshwater *et al.*, 1995; Graham *et al.*, 2009). Rebours *et al.*, (2014) reported the

estimated value of seaweed industry, which is approximately USD 10 billion. The most commonly used species are the members of Rhodophyta (*Eucheuma/Kappaphycus*, *Porphyra*, and *Gracilaria*). Among these species, *Kappaphycus* alone accounts for USD 1.3 billion, while the nori market generates US\$1.5 billion. *Porphyra yezoensis/Pyropia yezoensis*, *Gracilaria*, and some other species are grown in mariculture for use as human food. In fact, most algae contain amino acids, proteins, carbohydrates, vitamin B1, B2, B12, and C, and carotenoids that are essential for the normal functioning of the human body. Algae also contain minerals such as potassium, magnesium, iron, selenium, and large amounts of iodine. In addition, the fat content in algae is very low (around 0.2%–2%), which makes algae a good source of balanced nutrition (Luo *et al.*, 2006). *Kappaphycus* along with other genera are cultivated for the extraction of gel-forming agar, agarose, and carrageenan. These gelling polysaccharides are commonly used in the preparation of laboratory cell culture media Ramadan *et al.*, 2019), genomics/proteomics-based research (Siow *et al.*, 2012), and food processing (Van de Velde *et al.*, 2002). Red algae are also considered as a source of compounds that can be used as antimicrobials or herbicides as well as a source for human medicine (Bixler, 1996; Villanueva *et al.*, 2008; Graham *et al.*, 2009; Yow *et al.*, 2013). The algal industry has been increased multifold with value of over USD 6 billion (Anon., 2014; Loureiro *et al.*, 2015). Furthermore, algae cultivation has much greater potential ranging from integrated fish farming to biofuel production. Therefore, the identification of correct species for different uses is required, and DNA fingerprinting seems to be a promising solution.

Materials and Methods

Samples: Eight samples of algae were collected from the Gulf of Aqaba, Saudi Arabia, specifically from the beaches of Haql (28°45'N, 34°45'E). Specimens were washed and dried at room temperature (Table 1).

SDS-PAGE: Fresh algae were analyzed using SDS-PAGE for their protein content. The eight algal samples were homogenized in liquid nitrogen in a sample buffer (0.2 M Tris-HCl, pH 8, and 2% sodium dodecyl sulfate, 10% sucrose, and 1% beta-mercaptoethanol). Total protein profiles were separated as described by Laemmli (1970). The results were analyzed statistically as described by Steel & Torrie (1980).

DNA extraction and purification: DNA purification was performed using a DNeasy Kit (Qiagen) according to the manufacturer's instructions. The eight algal samples were ground using liquid nitrogen, and then liquid nitrogen was allowed to evaporate. To the sample, 400 µL of Buffer AP1 and 4 µL of RNase (10 mg/mL) were added. The mixture was then incubated for 1 h and mixed 2–3 times by inverting tubes to lyse the cells. Then, 130 µL of Buffer AP2 was added to the solution, mixed, and kept on ice for 5 min. In order to remove detergent, polysaccharides, and proteins, the solution was loaded onto a QIAshredder spin column (Qiagen) placed in a

2-mL collection tube and spun for 2 min at the highest speed. The flow-through was collected in a new tube and 450 µL of lysate was recovered. After this, 1.5 mL of AP3/E Buffer was added to the lysed tissues. The precipitate was spun for 1 min at 8000 rpm to get rid of flowthrough and the last stage was repeated for the remaining sample. The flowthrough and collection tube were removed. A DNeasy column was placed in a new 2-mL collection tube, and 500 µL of AW Buffer was added to the DNeasy column and centrifuged at 9000 ×g for 1 min. The flowthrough solution was removed and the collection Eppendorf tube was reused and 500 µL of AW Buffer was added to the DNeasy column and spun for 5 min at the maximum speed to dry the membrane. The column was transferred to a new Eppendorf tube and 100 µL prewarmed (65°C) AE Buffer was poured straight on the DNeasy membrane. The solution was kept at 25°C for 5 min and then spun for 1 min at 8000 rpm for elution. The final elution volume was made to 50 µL and the total DNA concentration was determined.

Estimation for DNA concentration: The DNA sample (2 µL) was resolved on 1% agarose gel along with 10 µL of a DNA size marker (lambda DNA Hind III digest Phi X174/HaeIII digest). The DNA concentration was determined by comparing the fluorescence intensity with the DNA marker.

ISSR-PCR reactions: A group of primers (Table 2) was used for determining polymorphism. PCR reactions were carried out in a total volume of 25 µL that included 1.5 mM MgCl₂, 1X PCR buffer, 0.2 mM dNTPs, 25 pmol of each primer, 1 unit of Taq polymerase, and 0.1 µg of template DNA.

Table 1. Name of the eight samples.

Sample number	Sample name
Sample 1	<i>Padina pavonica</i>
Sample 2	<i>Turbinaria gracilis</i>
Sample 3	<i>Carpomitra costata</i>
Sample 4	<i>Pterocliadiella capillacea</i>
Sample 5	<i>Cladostephus spongiosus</i>
Sample 6	<i>Ulva lactuca</i>
Sample 7	<i>Sporochneus comosus</i>
Sample 8	<i>Sargassum muticum</i>

Table 2. The sequence of ISSR primers.

Primer	Sequence
ISSR- 1	5'-AGAGAGAGAGAGAGAGC-3'
ISSR- 2	5'-AGAGAGAGAGAGAGAGG-3'
ISSR- 4	5'-ACACACACACACACACG-3'
ISSR- 5	5'-GTGTGTGTGTGTGTGTGYG-3'
ISSR- 10	5'-GACAGACAGACAGACAAT-3'
ISSR- 11	5'-ACACACACACACACACYA-3'
ISSR- 12	5'-ACACACACACACACACC-3'
ISSR- 13	5'-AGAGAGAGAGAGAGAGT-3'
ISSR- 14	5'-CTCCTCCTCCTCCTCTT-3'
ISSR- 15	5'-CTCTCTCTCTCTCTCTG-3'

A: Adenine, T: Thymine, G: Guanine and C: Cytosine

PCR: PCR was performed in a Perkin-Elmer system. At first, denaturation at 95°C for 5 min was carried out. Then 35 cycles were completed, where a cycle included denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1.5 min, and a final extension at 72°C for 7 min. The PCR-amplified fragments were resolved by electrophoresis on 1.5% agarose gel at 95 V in 1X TAE buffer with ethidium bromide (0.5 µg/mL). A 100-bp DNA ladder marker was used as standard. PCR amplification bands were visualized under UV light using a Gel Documentation System BioRad 2000.

Data analysis: The DNA bands were analyzed for individual typical bands. The PCR reactions for primers showing the polymorphism were repeated to prove the reproducibility. The presence or absence of a fragment was counted as 1 or 0, correspondingly. Assessment for genetic similarity (GS) was expressed as Nei & Li (1979) coefficient. UPGMA was done through the matrix of GS evaluations to calculate the informativeness for individual markers.

Results and Discussion

Statistical analysis: The protein profiles and ISSR markers were investigated to determine the genetic affinity among the eight algal species. Distinct amplified DNA fragments were counted as “1” for presence and “0” for absence in the contrasting lanes for different species. The fragments appearing in parallel were considered as same. The similarity between two genotypes was assessed by the dice coefficient (Sneath & Sokal, 1973) and a similarity matrix was constructed for the cluster analysis.

The phylogenetic investigation is complementary in the progressive taxonomies. At the primary stage, each accession was characterized as a distinct group and the relations among these accessions were defined through the distance (dice coefficient). However, when some related accessions were obtained, the distance between two clusters was considered as the average distance for all couples of accessions in the two dissimilar clusters. This technique is called UPGMA (Sneath & Sokal, 1973). In the current study, the initial full molecular phylogenetic examination of the eight algae based on protein banding pattern and ISSR is provided. Several phylogenetic-based DNA markers, such as SSU and LSU (18S rRNA and 5.8S+28S rRNA), internal transcribed spacers (ITS) regions of the nuclear-encoded ribosomal operon and the plastid-encoded ribosomal operon (16S rRNA and 23S rRNA), and several chloroplast (*rbcL*, *atpB*), mitochondrial (*coxI*) and nuclear protein-coding genes (e.g., *actin*), have been used for the revisions of many taxa (Coleman & van Oppen, 2008). The second assembly of the rRNA ITS2 area revealed important patterns in the evolution of Acroporidae corals. However, the nuclear small subunit ribosomal ribonucleic acids (SSU rRNA) are possibly one of the most commonly used molecular markers (Ouvrard *et al.*, 2000). There are many explanations for the use of these markers such as the presence of (1) a huge record, (2) common primers, (3) suitable number of nucleotide sequences for BLAST

search, and (4) no report of cross-gene transmission in eukaryotes. Protein banding patterns are also used to determine genetic polymorphism. The goal of our study was to yield molecular markers for the documentation of eight algae from Saudi Arabia. The SDS-PAGE examination of the whole protein banding profiles showed no remarkable variations in the total protein banding patterns (Fig. 1).

ISSR 10 primers were used and revealed that the molecular weight of the bands ranged between 250 and 10,000 bp. The average number of produced bands was five. Altogether, the genotypes showed marked distinctions and the results were reproducible and could be used for DNA fingerprinting. The dissimilar primers showed a diverse level of polymorphism between the eight algae (Figs. 2–11). UPGMA revealed two main clusters. Cluster 1 was composed of cultivar sample 4 (Alshehri *et al.*, 2019), which was not seen in the other seven algae (Figs. 12, 13), with Nei and Li's coefficient. Cluster 2 was composed of seven algae showing similarity according to Nei and Li's coefficient (Figs. 12, 13).

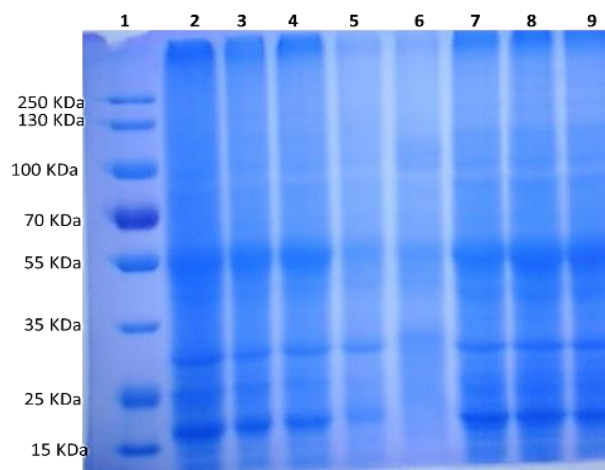


Fig. 1. SDS-page (12%) of the total protein extracts from the eight samples (Algae). 1: pre-stained protein marker and 2–9: eight algal samples.

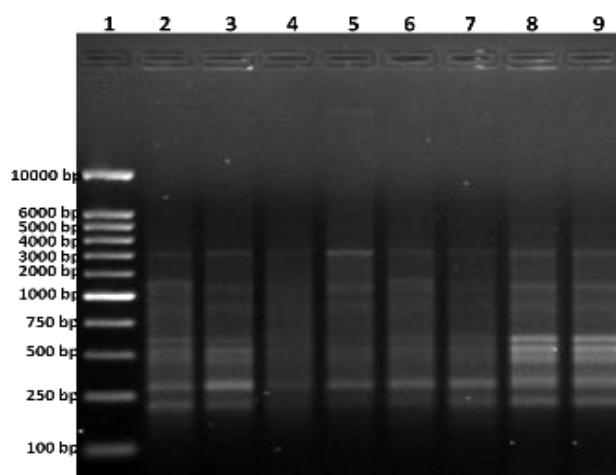


Fig. 2. Agarose gel (1.5%) showing the PCR amplification of the products using DNA extracted from the eight algal samples and primer ISSR-1. 1: 1 Kb DNA marker and 2–9: eight algal samples.

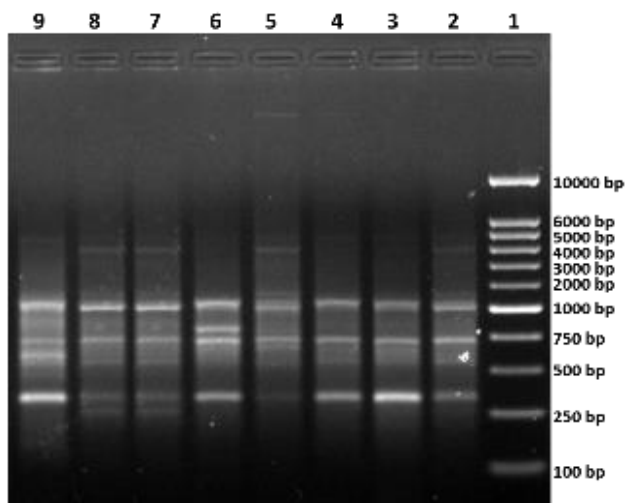


Fig. 3. Agarose gel (1.5%) showing PCR amplification products using DNA extracted from the eight algal samples and primer ISSR-2. 1: 1 Kb DNA marker and 2-9: eight algal samples.

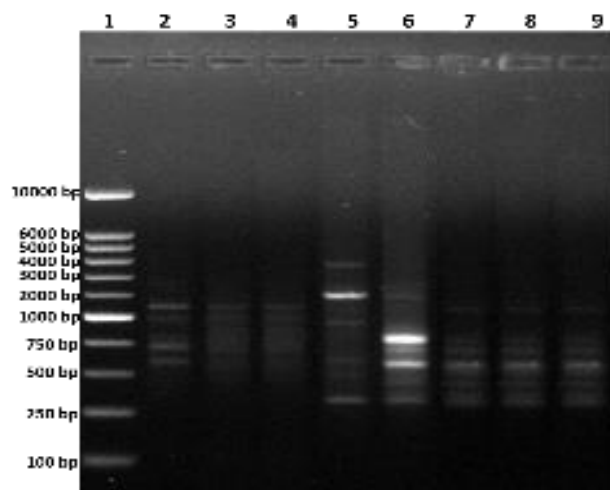


Fig. 6. Agarose gel (1.5%) showing PCR amplification products using DNA extracted from the eight algal samples and primer ISSR-10. 1: 1 Kb DNA marker and 2-9: Eight Algae samples.

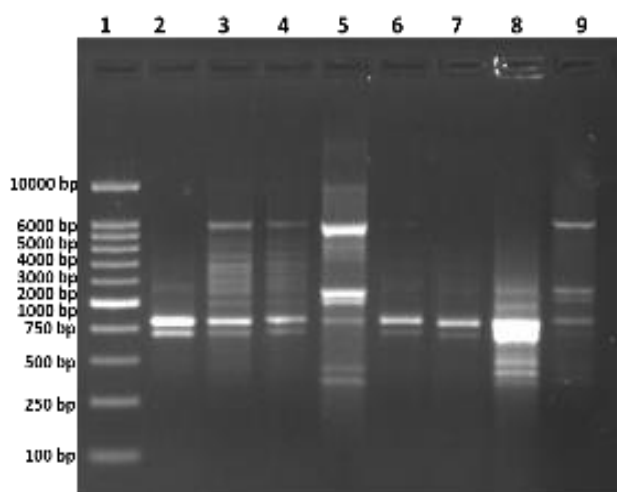


Fig. 4. Agarose gel 1.5% showing PCR amplification products using DNA extracted from the eight samples (Algae) and primer ISSR- 4. 1: 1 Kb DNA marker and 2-9: eight Algae samples.

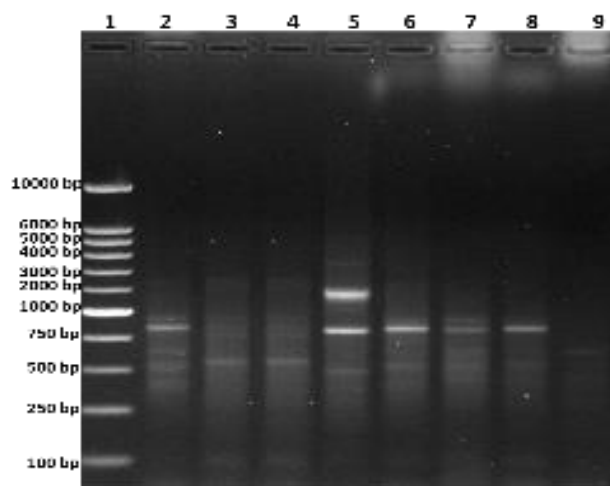


Fig. 7. Agarose gel (1.5%) showing PCR amplification products using DNA extracted from the eight samples and primer ISSR- 11. 1: 1 Kb DNA marker and 2-9: Eight Algae

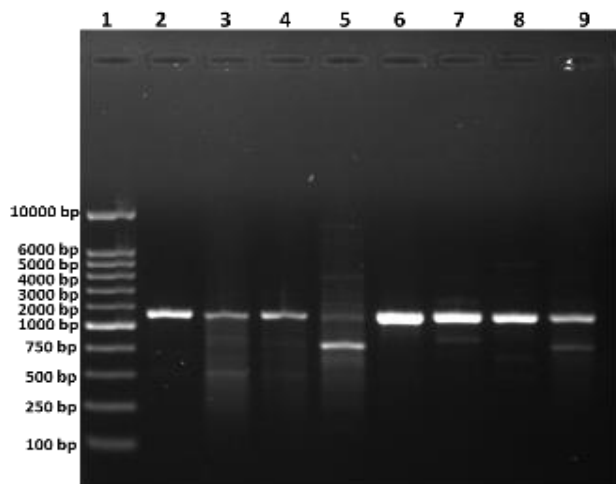


Fig. 5. Agarose gel 1.5% showing PCR amplification products using DNA extracted from the eight samples (Algae) and primer ISSR- 5. 1: 1 Kb DNA marker and 2-9: eight Algae samples.

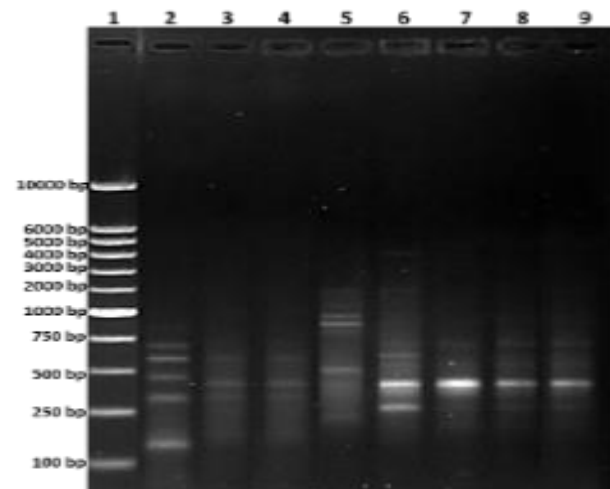


Fig. 8. Agarose gel 1.5% showing PCR amplification products using DNA extracted from the eight samples (Algae) and primer ISSR- 12. 1: 1 Kb DNA marker and 2-9: eight Algae samples.

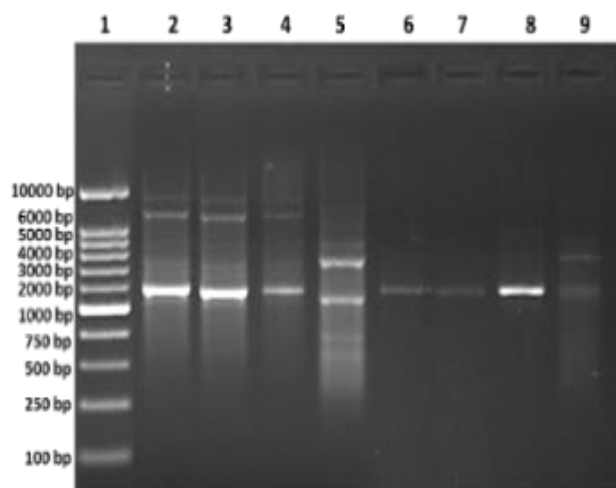


Fig. 9. Agarose gel 1.5% showing PCR amplification products using DNA extracted from the eight samples (Algae) and primer ISSR- 13. 1: 1 Kb DNA marker and 2-9: eight Algae samples.

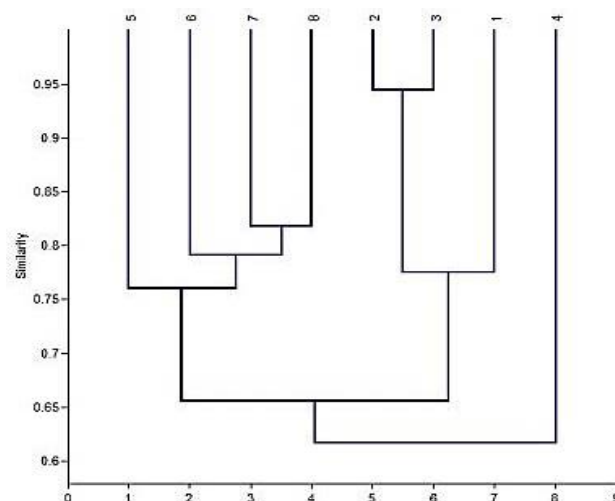


Fig. 12. Dendrogram of phylogenetic relationships among the eight algae based on Nei and Li's similarity coefficient obtained from ISSR primers.

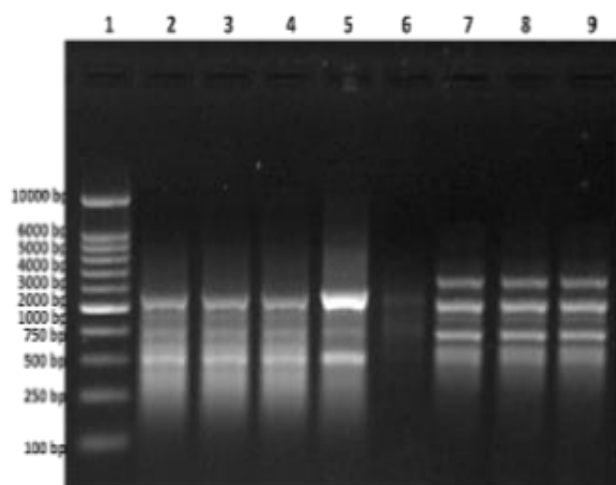


Fig. 10. Agarose gel 1.5% showing PCR amplification products using DNA extracted from the eight samples (Algae) and primer ISSR- 14. 1: 1 Kb DNA marker and 2-9: eight Algae samples.

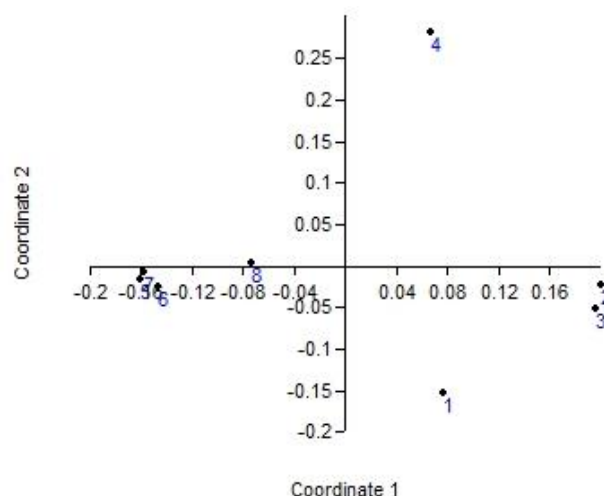


Fig. 13. PCO scatter diagram showing relationships among the eight algae based on ISSR primers.

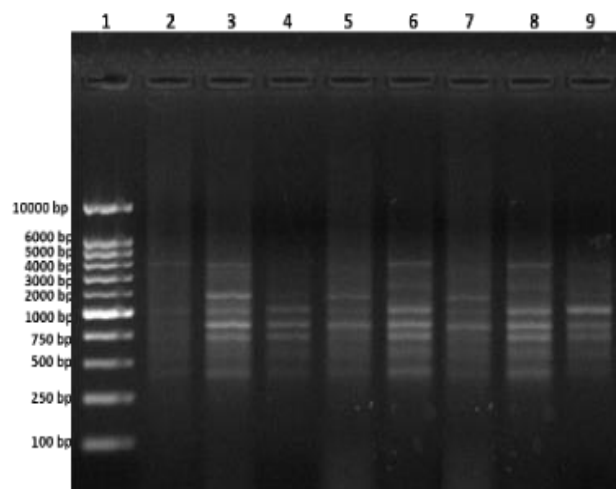


Fig. 11. Agarose gel 1.5% showing PCR amplification products using DNA extracted from the eight samples (Algae) and primer ISSR- 15. 1: 1 Kb DNA marker and 2-9: eight Algae samples.

As shown by Nei and Li's coefficient, samples 6 and 5 were the most closely related, 7 and 8 were marginally related, and 2 and 3 were weakly related, while sample 1 was related to samples 2 and 3 (Figs. 12 and 13). The usual likeness among the seven algae was >55%. All eight algae showed slight genetic relevance. These data show that ISSR and DNA fingerprinting can be used for the documentation of algae. These data will help in the categorization of the germplasm in a gene bank. The ISSR markers and DNA fingerprinting are highly useful in the identification of the algal species (Farooq *et al.*, 2008; Yang & Quiros, 1993; Khalik *et al.*, 2012).

We believe that the use of ISSR markers seems to be highly promising for the classification and genetic conservation of algal germplasm. It is essential to consider a high number of primers while evaluating the phylogenetic relationship among a large population. Besides ISSR markers, we also employed SDS-PAGE. The narrow genetic diversity observed among the eight algal species could be attributed to the loss of diversity among the plantation areas and clonal propagation of

ecotypes. In conclusion, our study showed that protein banding pattern and ISSR markers independently have their advantages in algal cultivar fingerprinting, and a mixture of different markers can be used to obtain robust results (Al-Khalifah & Askari, 2003; Osman *et al.*, 2013; Osman *et al.*, 2015).

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