UGC MAJOR RESEARCH PROJECT

FINAL REPORT

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2013-2017

SUMMARY OF THE WORK DONE

Delineation of *Gracilaria* species has always been a problem due to its morphological plasticity. The present work is a wholesome approach in the delineation of the *Gracilaria* species of Tamil Nadu, with its morphological, anatomical and molecular studies. Thus five new varieties of *G.corticata* and four new varieties of *G.foliifera* are reported for the first time.

The RAPD, ISSR, DNA Barcoding results obtained in the present study supplement our understanding of the genetic diversity of *G.corticata* and *G.foliifera* and other *Gracilaria* species of Tamil Nadu. This work also highlights several interesting sampling locations for further investigation and will contribute significantly to ongoing studies. Some of the interesting features that are necessary to be exploited is to determine whether the various varieties identified in these two species correlate with the environmental changes. Further work on DNA barcoding work can also clarify if they are different haplophytes from different localities. This may provide insights into origin and evolutionary relationships of *G.corticata* and *G.foliifera*.

Identification of cyclooxygenase gene in some species of *Gracilaria* is a new approach. The identification of this gene that is responsible for the formation of prostroglandin which is highly useful in the field of medicine due to its anti-inflamatory effect and increase in platelet will be a good contribution to the society.

Bionanotechnology is an interdisciplinary field that combines the biological process and the technology to build nano sized materials for various biological

applications. In the present study the biosynthesis and characterization of gold nanoparticles from *G.corticata* is reported which has biomedical applications in different areas such as drug delivery, tissue engineering, biosensor, etc. (Paper attached)

Polyunsaturated Fatty acids profile with GC-MS is analysed for *Gracilaria* species. The presence of eicosapentaenoic acid is a PUFA which has unique biological activities in the synthesis of prostaglandins and other eicosaenoids, they are lipid bioactive from *Gracilaria* species.

15. WHETHER ANY PH.D. ENROLLED/PRODUCED OUT OF THE PROJECT: YES

Name of the candidate: S.Sugandhi M.Sc., M.Phil
Registered for Ph.D dt.6.3.2013
Topic: Studies on Rhodophyceae

University of Madras (No.Ph.D/Regn./4/FT/01/2013/2022

Status: Completing in 2018

16. NO. OF PUBLICATIONS OUT OF THE PROJECT:

3 publications
1 paper presentation

S.No	TOPIC	JOURNAL/CONFERENCE
1.	Biosynthesis and characterization of gold nannoparticles from *Gracilaria corticata*	Nannoscience and nanotechnology- An Indian Journal. 8(12):475-481, 2014
2.	Morphological and anatomical studies on some members of Rhodophyceae	Seaweed Res. Utiln. 37(1): 1-6,2015
3.	Molecular studies on four morphological variants of *Gracilaria foliifera* Boergeson	International Journal of Applied and Pure Science and Agriculture. 3(11): 1-8, 2017.

4.	**Morphological and RAPD studies on some species of *Gracilaria***	**Paper presentation-** **National symposium on "Algae for Human Welfare"-** **19-21August 2015** **Kakinada, Andhra Pradesh**

(DETAILS ATTACHED)

(PRINCIPAL INVESTIGATOR)

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(Seal)

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Biosynthesis and characterization of gold nanoparticles from *Gracilaria corticata*

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ABSTRACT

In the present study, gold nanoparticles were synthesized from the aqueous solution of red seaweed *Gracilaria corticata* using gold precursor. The formation of gold nanoparticles was primarily confirmed by the colour change from pale yellow to red. The biologically synthesized gold nanoparticles were characterized by UV-Vis spectrophotometer, FTIR, X-ray diffraction, FE-SEM, EDX, and HR-TEM. HR-TEM and FE-SEM analyses revealed the size and shape of the nanoparticles. FTIR showed that nanoparticles were capped with alga compounds. X-ray diffraction pattern and UV-Vis spectrum showed the peaks corresponding to gold nanoparticles. Thus, physiochemical characteristic results suggest that gold nanoparticles will have biomedical applications in different area such as drug delivery, tissue engineering, biosensor, etc.

KEYWORDS

Gracilaria corticata;
Gold nanoparticle;
UV (Visible);
FTIR;
Transmission electron microscopy;
Scanning electron microscopy;
X-ray diffraction.

INTRODUCTION

Bionanotechnology is an interdisciplinary field that combines the biological processes and the technology to build nano sized materials or particles for various biological applications. The use of biological process in nanotechnology can have an impact in the production, function and properties of the synthesized nanomaterials or nanoparticles. Nanobiotechnology is one of the most promising areas in modern nanoscience and technology. This emerging area of research interlaces various disciplines of science such as physics, chemistry, biology and material science[13]. Gold is one of the precious, inert, and less toxic metals, and it is utilized for curing various diseases. Gold nanoparticles play a vital role in nanobiotechnology as biomedicine because of convenient surface bioconjugation with biomolecular probes and remarkable plasmon resonant optical properties[4,23,7]. Gold nanoparticles have an important function in the delivery of nucleic acids, proteins, gene therapy, in vivo delivery, targeting, etc[21].

Generally, metal nanoparticles are synthesized and stabilized through chemical and mechanical methods[22], electrochemical techniques[14], photochemical reactions in reverse micelles and nowadays via green chemistry methods[20]. A wide variety of physical and chemical processes have been developed for the synthesis of metal nanoparticles[8], but these methods are expensive and require the use of toxic and aggressive chemicals as reducing and/or capping agents[9]. Therefore, green

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chemistry should be integrated into nanotechnologies especially when nanoparticles are to be used in medical applications, which include imaging, drug delivery, disinfection, and tissue repair[1].

The green biosynthesis of nanoparticles can be achieved via the selection of an environmentally acceptable solvent with eco-friendly reducing and stabilizing agents[6]. Therefore, biological approaches to nanoparticle synthesis have been suggested as valuable alternatives to physical and chemical methods[11]. The literature survey found that the marine red algae are rich sources of phenolic compounds especially bromophenols. Phenolic substances were reported to possess a wide range of biological effects, including antioxidant, antimicrobial, anti-inflammatory and vasodilator actions. Furthermore, tannis and flavonoids are defined as naturally occurring seaweed polyphenolic compounds which have been found only in marine algae[10].

In the present study, the red seaweed *Gracilaria corticata* was used to synthesis gold nanoparticles by the reduction of aqueous $HAuCl_4^-$ into nanoparticles. The synthesized gold nanoparticles were characterized by UV-Vis spectrophotometer, FTIR, X-ray diffraction, FE-SEM, EDX, and HR-TEM.

MATERIALS AND METHODS

In the present study *Gracilaria corticata* were collected from Kovalam, 50 km from Chennai, Tamilnadu, India. The algae were cleaned thoroughly in double distilled water and shade dried for 3 to 5 days, ground with mortar and pestle and seived to a mesh size of <0.5mm. The dried biomass powder was used for the synthesis of gold nanoparticles. About 30 mg of seaweed powder was added with 10 ml of 10^{-3} M aqueous $HAuCl4^-$ solution in a 20 ml test tube and incubated at room temperature. The colour change from yellow to deep red colour indicates the formation of gold nanoparticles. The optical property of synthesized gold nanoparticles was characterized using a ultraviolet–visible (UV–vis) spectrophotometer; morphological characters was studied by performing TEM and SEM; crystalline nature was analyzed using X-ray diffraction (XRD) patterns; and functional molecules involved in the reduction process were comprehensively studied using Fourier transform-infrared (FTIR) spectroscopy.

RESULTS AND DISCUSSION

The colour change from yellow to deep red was the visual inspection of reduction of gold ions to gold nanoparticles. The gold ions exhibits yellow colour in double distilled water (Figure1a), when it is exposed to seaweed powder it turns to dark pink (Figure1b) after 2 hours of incubation. Mukherjee *et al*., (2002) reported that the appearance of the purple colour clearly indicates the formation of gold nanoparticles in the reaction mixture during the studies carried out on *Fusarium oxysporum*. Synthesized gold nanoparticles were extracted by centrifugation with 5,000 rpm for 15 min at 4ºC.

Figure 2 shows the UV-Vis spectra recorded at 546 nm corresponding to the formation of gold nanoparticles. The reaction was optimized since the size and shape of nanoparticles depend on bio-extract concentration[3]. Rajathi *et al*. (2012) reported the formation of gold nanoparticles using *Stoechospermum marginatum* (kützing) confirmed by the presence of an absorption peak at 550 nm. In this present study, the colour change and absorption peak at 530 nm was an evidence for the bioreduction of gold ions extracellularly. The Plasmon resonance for gold nanoparticles

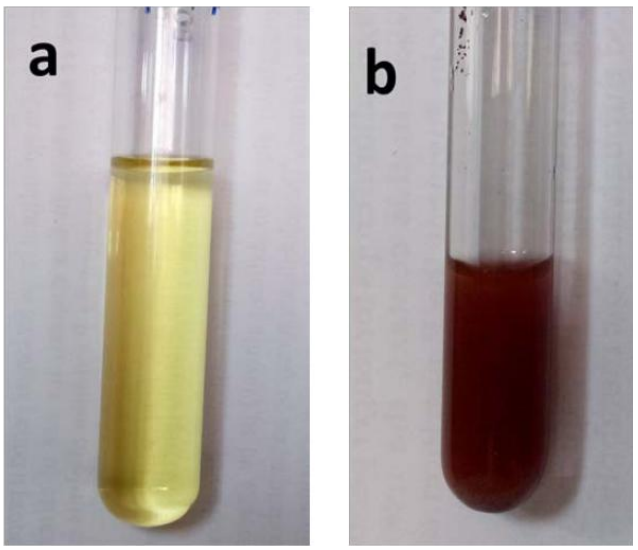

Figure 1 : (a) Aqueous solution of 1×10^{-3} M chloroauric acid in double distilled water (b) Deep red colour due to the addition of seaweed powder to the choroaurate solution after 2 hours incubation

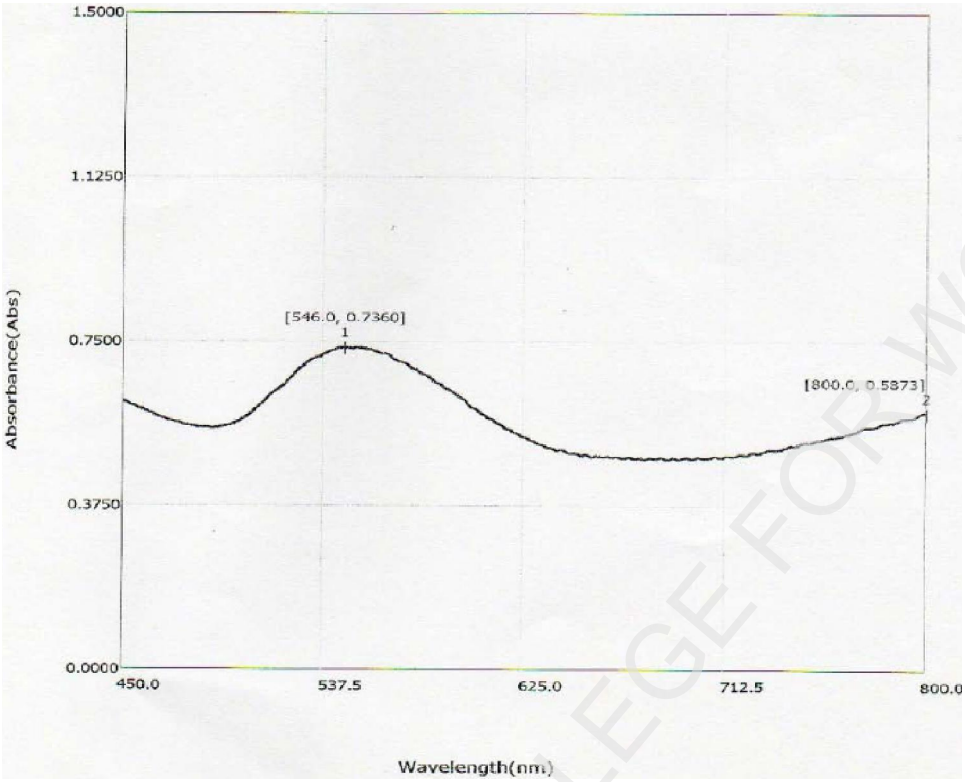

Figure 2 : UV-Visible range spectrum of gold nanoparticles showing the surface Plasmon band

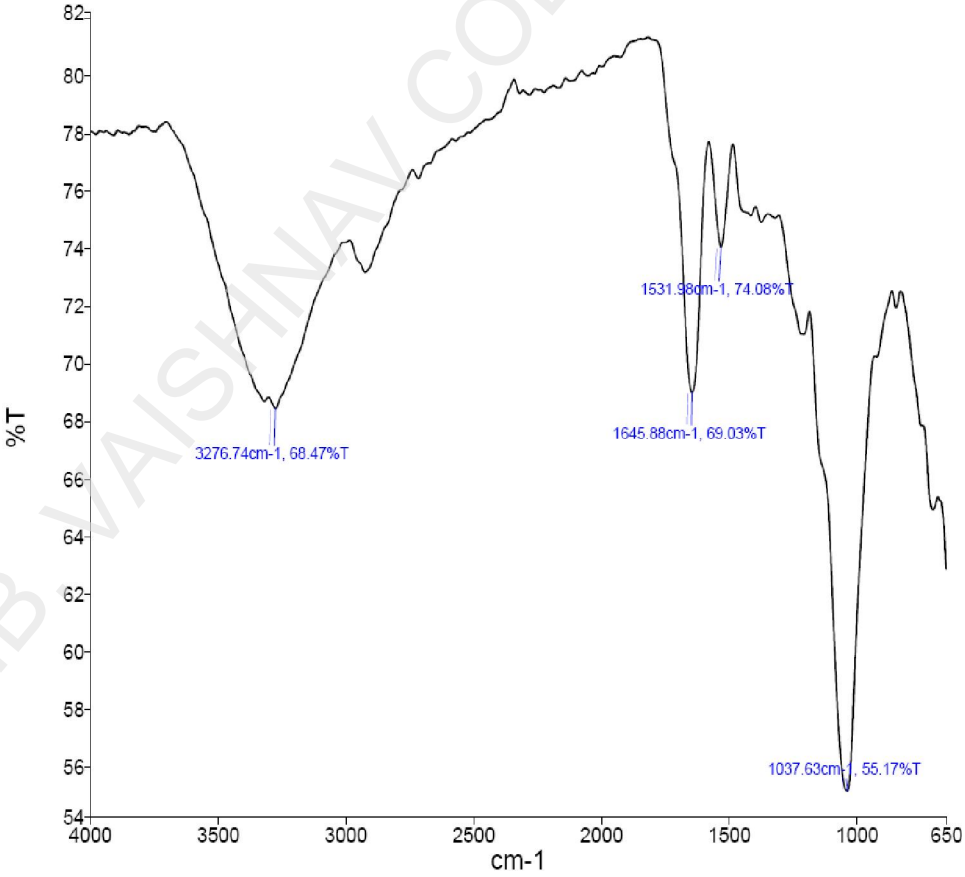

Figure 3 : FT-IR spectrum of gold nanoparticles synthesized by *G. corticata*

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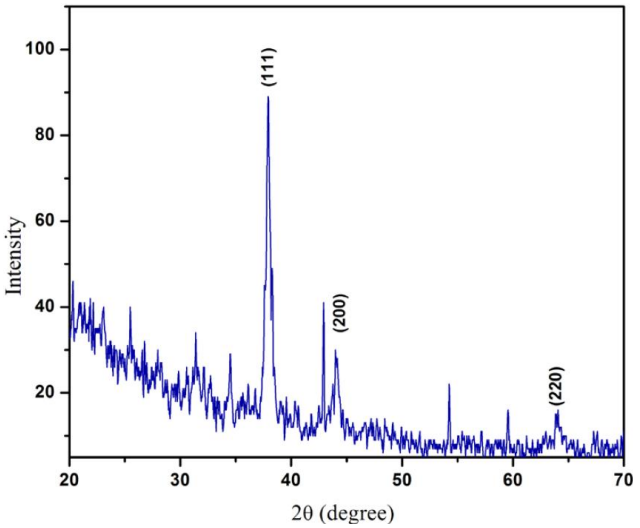

Figure 4 : X-ray diffraction pattern of the gold nanoparticles obtained from *G. corticata*

in aqueous solution appeared at approximately 540 nm and changed according to the size of the nanoparticles[16].

FTIR spectrum analysis of gold nanoparticles showed intense absorption bands at 3276.74, 1645.88, 1531.98 and 1037.63 cm^{-1} (Figure 3). The intense broad absorbance peak at 3276.74 cm^{-1} (O-H stretch) is the characteristic of the hydroxyl functional group in alcohols and phenolic compounds. The band at 1645.88 cm^{-1} (C=C stretch) can be assigned to the functional group alkenes. The intense medium absorbance at 1531.98 cm^{-1} (N-O asymmetric stretch) is the characteristic of nitro compounds. The intense absorbance at 1037.63 cm^{-1} (C-N stretch) may be assigned as aliphatic amines group. A previous report reveals that the hydroxyl group (O–H) has a strong ability to interact

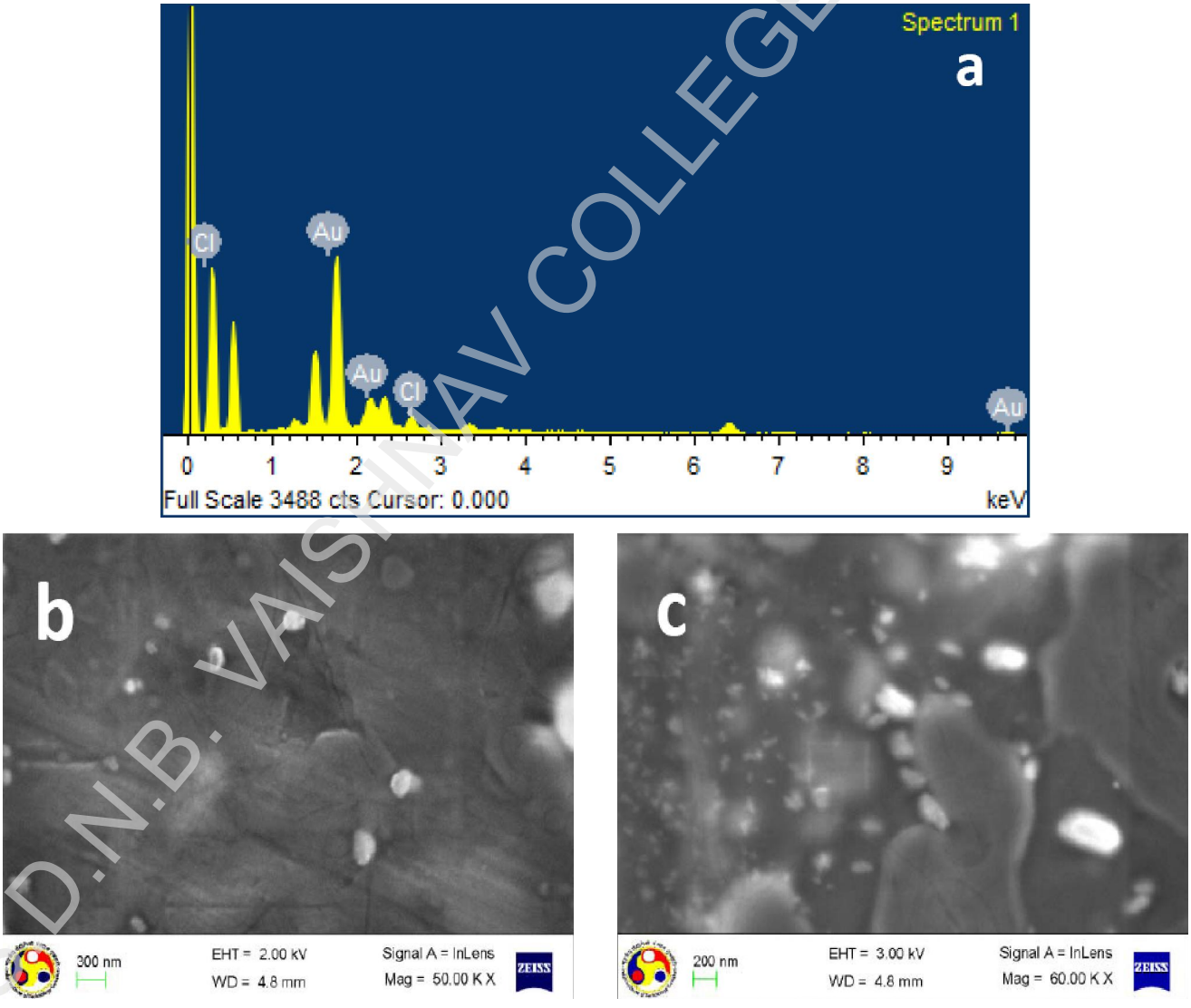

Figure 5 : (a) EDX analysis of gold nanoparticles synthesized by *G. corticata* and (b&c) FE-SEM images of gold nanoparticles

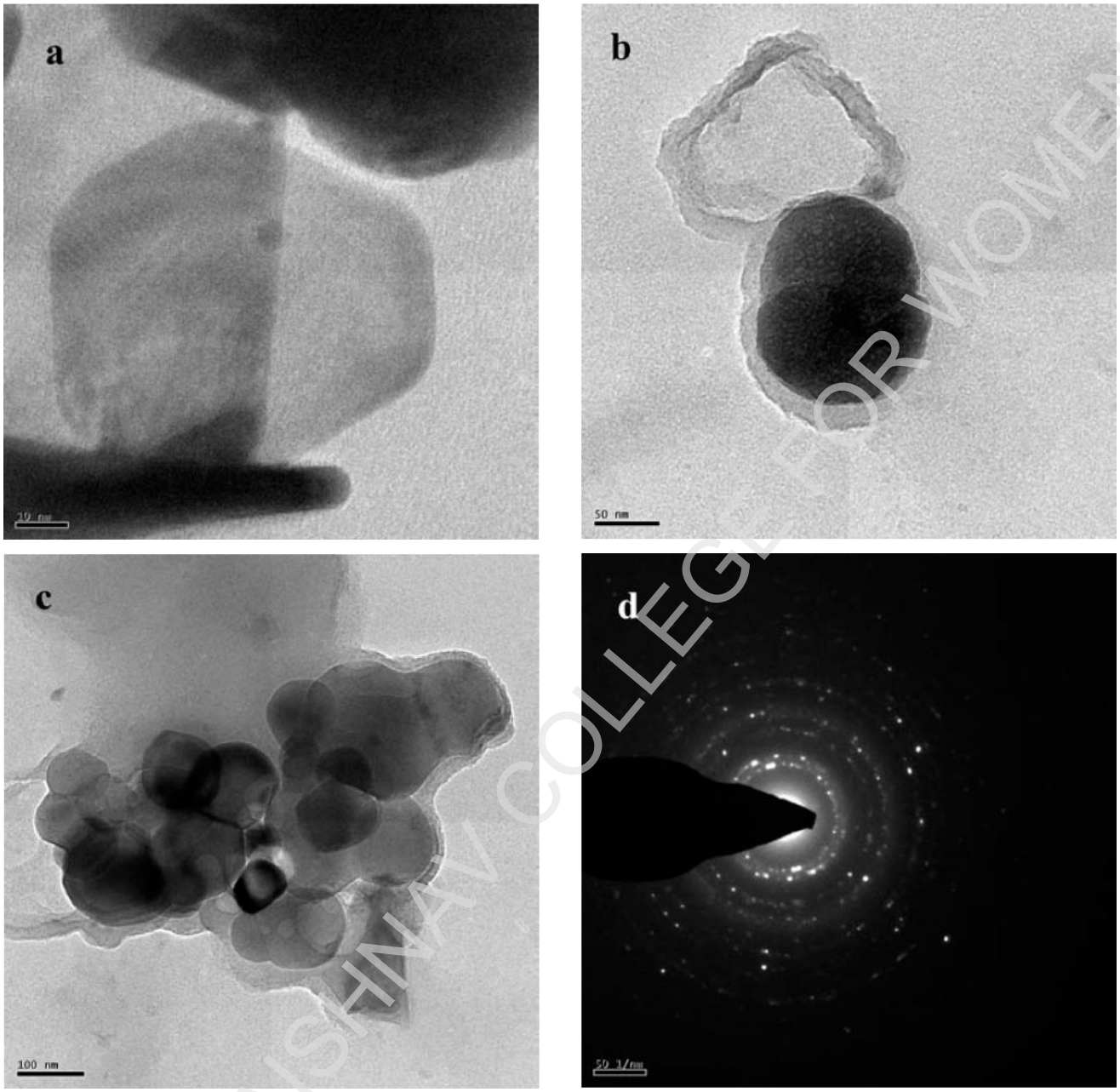

Figure 6 : HR-TEM images of gold nanoparticles formed by reduction of gold ions using *G. corticata* (a) 10 nm scale (b) 50 nm scale (c) 100 nm scale and (d) selected area diffraction pattern

with nanoparticles[19,2,5].

The crystalline nature of synthesized gold nanoparticles of *G. corticata* was analysed using XRD patterns as observed by Singh *et al*., (2013) in *Padina gymnospora*. Figure 4 shows the XRD pattern of gold nanoparticles synthesized using red seaweed. The XRD patterns revealed that gold nanoparticles corresponded to the crystalline gold fcc phase. The diffraction peaks obtained at 2θ= 37.92° (1 1 1), 44.01° (2 0 0), and 64.13° (2 2 0) are identical with those reported for the standard gold metal (Au^0) (Joint Committee on Powder Diffraction Standards-JCPDS, USA). The presence of these three intense peaks corresponding to the nanoparticles was in agreement with the Bragg's reflections of gold identified with the diffraction pattern[17]. The other unidentified peaks reveals the association of algal biomass with the synthesized gold nanoparticles.

FE-SEM micrographs show gold nanoparticles at 200 to 300 nm range (Figure 5b and 5c). A few larger

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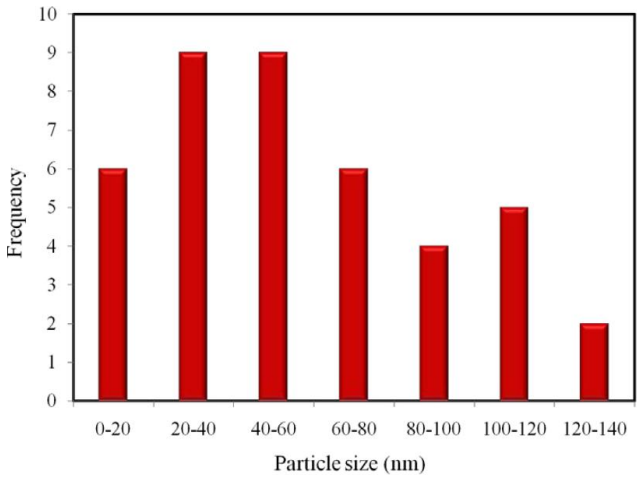

Figure 7 : Particle size distribution obtained from HR-TEM micrograph

particles are formed by the aggregation of smaller particles. In EDX, strong signals were observed for the gold atoms and weak signals for carbon, oxygen and chloride. This weaker signals indicate the presence of biomolecule of *G. corticata* (Figure 5a)

The TEM study gives clear shape and size of the nanoparticles. The diameter of the nanoparticles ranges from 5 to 135 nm. The nanoparticles synthesized were hexagonal and spherical shaped (Figure 6a-d). The particle size distribution histogram constructed from the TEM micrograph is shown in Figure 7. The size distribution varies from 10 to 140 nm with an average particle size of 75 ± 41.83 nm. The reason behind the aggregation of the gold nanoparticles may be the change of the pH during the synthesis of the nanoparticles.

CONCLUSION

In the present study, we have used economically important red seaweed *G. corticata* which is a rich marine source available in abundance. The biomolecules present in the algae must be responsible for the reduction of gold ions to gold nanoparticles. This biological method of synthesis of gold nanoparticles is less time consuming, ecofriendly, non-toxic and single-step process. The nanoparticles thus formed are spherical and hexagonal shaped with smooth edges. Hence, this has high potential in biomedical applications. This method is inexpensive and highly recommended for large-scale production of gold nanoparticles.

ACKNOWLEGEMENT

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Seaweed Res. Utiln., 37(1) : 1 - 6, 2015

Morphological and anatomical studies on some members of Rhodophyceae

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ABSTRACT

The morphological and anatomical studies on the red seaweeds *Gelidiopsis*, *Rhodymenia* and two species of *Gracilaria viz.*, *G. corticata* and *G. prolifera* were studied. These algae were collected from Kovalam, Tuticorin, Muttam and Kanyakumari .It was interesting to note the distinct morphological and anatomical variations in the species of *G. corticata* collected from different localities. The variations among the five different populations of *G. corticata* collected from different places lead to the presumption that these could be different varieties of *G. corticata*. However, these morphological and anatomical studies along with the molecular analysis confirmed that the variation is at the molecular level.

Introduction

In India, seaweeds are mainly used for the commercial production of agar, alginates and also liquid seaweed fertilizer. Thus, the seaweed industries offer employment to hundreds of people living in the coastal areas. About 8.0 million tons per year of wet seaweeds value at US $ 6.0 billion are utilized for human consumption, phycocolloids, feed supplements, agrochemicals, nutraceuticals and pharmaceuticals (McHugh, 2003). There are about 25 agar industries and 10 algin industries situated at different places in the maritime states of Tamilnadu, Kerala, Karnataka, Andhra Pradesh and Gujarat. Red algae such as *Gelidiella acerosa*, *Gracilaria edulis*, *G. crassa*, *G. foliifera* and *G. verrucosa* are used for agar manufacture.

Over one hundred species of commercially important agarophyte of *Gracilaria* (Gracilariaceae, Rhodophyta) are widely distributed throughout the tropical and temperate waters of the world. Proper classification is important for both cultivation and industrial exploitation of *Gracilaria*. *Gracilaria* species are identified traditionally by their morphological features, such as branching pattern, extent of thallus construction and reproductive structures (Lim *et al*., 2001). However, species delineation of *Gracilaria* is problematic due to intergradations in morphological and reproductive characteristics (Bird 1995).

Taxonomic investigations of members of the family Gracilariaceae of the seas around India are very limited and many of the *Gracilaria* species hither-to reported from this area are inadequately described (Umamaheswara Rao, 1972). The genus *Gracilaria* is well represented in Indian seas. Boergesen (1933, 1934,1937a,b and 1938) reported the following eleven species and one variety of *Gracilaria* from different localities. *G. compressa*

(Agardh) J.Agardh, *G. confervoides* (Linnaeus) Greville, *G. corticata* J.Agardh, *G. corticata* var. *ramalinoides* J.Agardh, *G. crassa* (Harvey) J.Agardh, *G. debilis* (Forsskal) Boergesen, *G. disticha* J.Agardh, *G. fergusonii* J.Agardh, *G. foliifera* (Forsskal) Boergesen, *G. lichenoides* (Linnaeus) Harvey, *G. pygmaea* Boergesen and *Corallopsis cacalia* J.Agardh (*G. cacalia* (J.Agardh) Dawson). The nomenclature of some of these species has been changed in recent years (Papenfuss, 1950; Silva, 1952; Newton, 1953 and Dawson, 1954) and Boergesen (1942) also revised the names of some Indian plants, while working on the algae of Mauritius.

In view of the economic importance of *Gracilaria*, *Gelidiopsis* and *Rhodymenia,* detailed morphological and anatomical studies have been carried out in the present study. Morphological characters such as height of the thallus, attachment, branching type, length and width of main axis, branches & branchlets and anatomical characters such as number of cortical layers, dimensions of cortical cells, subcortical cells, medullary cells and size of the cystocarp were analysed. Based on the morphological and anatomical features, *Gelidiopsis* and *Rhodymenia* are identified as *Gelidiopsis variabilis* and *Rhodymenia dissecta* by referring the manual published by Desikachary *et al.* (1998). However, *Gracilaria corticata* showed much variation in both morphologically and anatomically in five different populations collected from intertidal zone, sublittoral zone, infralittoral fringe zone in the intertidal region exposed to heavy surf action and in rock pools of intertidal zone. The distinct variations lead to the presumption that these could be varieties of *G. corticata*. However, these morphological and anatomical studies along with the molecular analysis confirmed that the variations between the different populations of *G. corticata* is at the molecular level.

Materials and methods

In the present investigation, some Rhodophyceae members were collected from different localities along the east coast of Tamil Nadu viz.Kovalam, Muttam, Tuticorin and Kanyakumari. Collections were made from September 2012 to May 2014. Every collection was assigned a collection number and herbarium was prepared and the materials were also preserved in 4% formalin. The morphological features of the collected materials were observed under stereo-dissection binocular microscope and identified.

Morphometric examination

Morphological features of five specimens of *Gracilaria corticata* were drawn by using Camera lucida (Figs. 1 - 5). Hand sections were taken and stained with Toludene blue O. Cell dimensions were recorded with stage micrometer. The morphometric characters used in the analysis were size of the cortical cells, number of cortical layers, diameter of medullary layers, number of medullary layers, height of the plant, length of the stipe, length of the primary branches and secondary branches. Field materials and cross section of the thallus were photographed using Sony Cyber-shot (16.1 Megapixels)-DSC

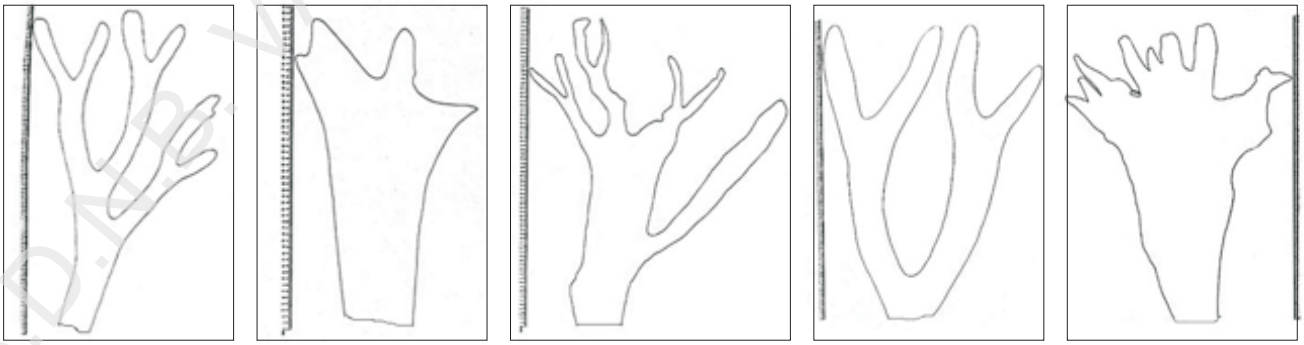

Fig. 1. Gel Gc5 Fig. 2. Gc2 Fig. 3. Gc3 Fig. 4. Gc4 Fig. 2. Gc5

Figs. 1-5. *Branching pattern of five individuals of* Gracilaria corticata

w690. Numbers were assigned to different populations of *G. corticata* as Gc1, Gc2, Gc3, Gc4 and Gc5.

Results and Discussion

Morphological and Anatomical characteristics

Different populations of *G. corticata* showed distinct morphological and anatomical characteristics. External and internal characters of *Gelidiopsis*, *Rhodymenia* along with the five populations of *G. corticata* are given in Figs. 6-12 .

Gelidiopsis variabilis (Grev.) Schmitz

Schmitz, F. Marine Florideen von Deutsch-Ost-Afrika, Bot. Jahrb. fur. Systems, 21, p. 148, 1895. De Toni, J.B. Sylloge Algarum, 4, p. 410, 1900.

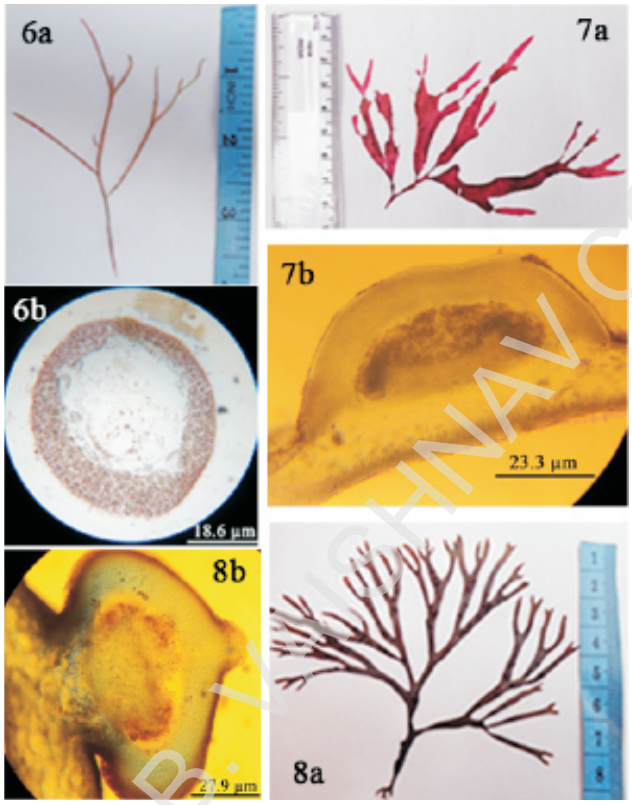

Plate I :

Fig. 6a. Gelidiopsis variabilis *thallus; Fig. 6b. Cross section of thallus of* Gelidiopsis variabilis*; Fig. 7a.* Rhodymenia dissecta *thallus; Fig. 7b. Cross section of cystocarp of* Rhodymenia dissecta*; Fig. 8a.* Gracilaria corticata *1 thallus; Fig. 8b. Cross section of cystocarp of* Gracilaria corticata *1*

= *Gelidium variabile* (Grev.) J. Ag. Harvey, W. Ceylon Algae No. 33, Agardh, J.G. Species, Genera et Ordines algarum, 2, p. 468, 1851. Agardh, J.G. Epicrisis, p. 547, 1876. Kuetzing, F.T. Tabulae Phycologicae, 19, t. 23, fig.c-d, 1869.

Thallus 8.5 - 10 cm in height, erect, cylindrical, filiform, primary axis erect, sparsely branched below, dichotomous; stipe length 2.5 - 3 cm and width 1 mm, attached by circular discs, axes less branched, branching alternate, with long branches above and shorter ones below, branches constricted below, primary branch length 2 - 3 cm and width 0.5 - 1 mm, secondary branch length 2 - 3 cm and width 0.5 - 1 mm, secunds rare 0.7 – 1.5 cm. Thallus with 9 - 12 layers of cortical cells, cells ovoid, 10 – 12.5 µm, decreasing in size outwards.

Rhodymenia dissecta Boergesen

Boergesen, F. Contributions to a South Indian marine algal flora III, p.226, fig. 7, 1938. Gopalakrishnan, P. *Rhodymenia dissecta* Boergesen from Gujarat, Phykos, 10 : 154-155, 1971.

Thallus 17 - 20 cm in height, pink to red, thin, stipe length 1 - 1.5 cm and width 3 - 5 mm, attached by oval discs, axes more branched, branching opposite with long branches above and shorter ones below, branches constricted below, primary branch leafy, length 3.5 - 6 cm and width 0.5 - 1.5 cm, secondary branch length 3 - 5 cm and width 0.5 - 0.8 cm, dichotomously branched and proliferous. Thallus with 3 layers of cortical cells, cells circular, 3.5 – 7.5 µm, progressively smaller towards periphery, 2 rows of circular medullary cells, 85 - 100 µm, cystocarp scattered all over the surface, hemispherical, 0.5 – 1 mm in diameter, 400 - 450 µm in length and 700 - 750 µm in diameter. Gonimoblast filaments in a compact globular mass.

Gracilaria corticata (Gc1): (from Littoral zone)

Agardh, J.G. Species, Genera et Ordines algarum, 2, p. 602, 1852.. Agardh, J.G. Epicrisis, p.423, 1876. Boergesen, F. Some Indian Rhodophyceae especially from the shores of the Presidency of Bombay, III. Kew. Bull. 1933, no. 3, p. 124, 1933. Boergesen, F. Contributions to a South Indian marine algal flora III.

J. Indian bot. soc., 17, p. 225, 1938. Durairatnam, M. A contribution to the study of the marine algae of Ceylon. Bull. Fish. Res. Stn., Ceylon, 10, p. 10, 1961. Durairatnam, M. Some marine algae from Ceylon – 1. Bull. Fish. Res. Stn. Ceylon. 15, p. 14, fig. 1, 1962. Umamaheswara Rao, M. On the Gracilariaceae of the seas around India J. mar. boil. Assn., India, 14(2), p. 677, fig. 1 k-m; 2 a-b; pl. II E, 1972.

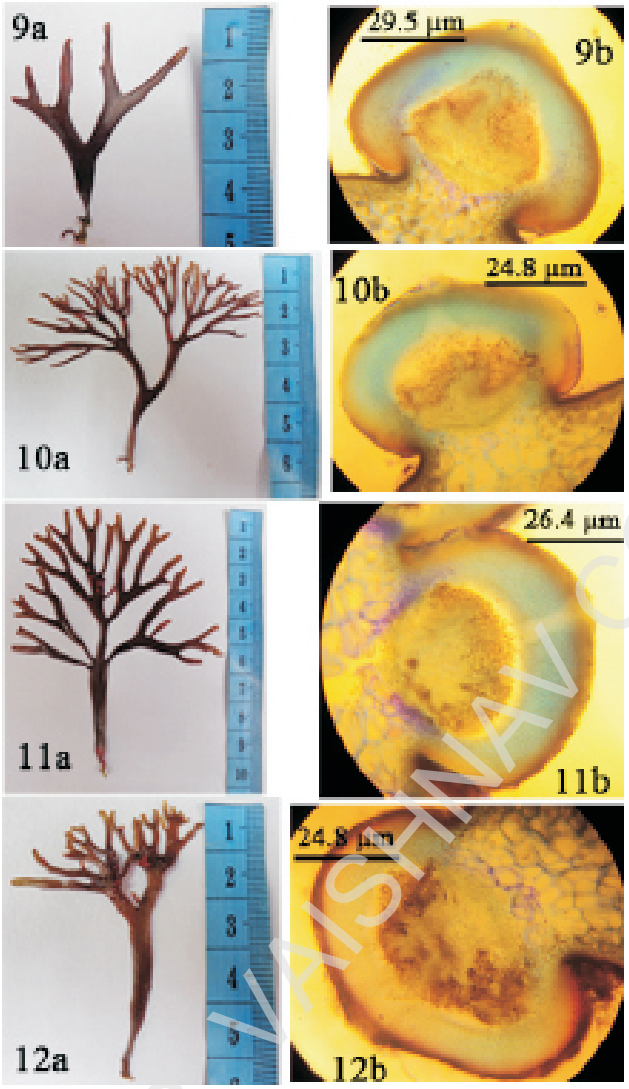

Plate II

Fig. 9a. Gracilaria corticata *2 thallus; Fig. 9b. Cross section of cystocarp of* Gracilaria corticata *2; Fig. 10a.* Gracilaria corticata *3 thallus; Fig. 10b. Cross section of cystocarp of* Gracilaria corticata *3; Fig. 11a.* Gracilaria corticata *4 thallus; Fig. 11b. Cross section of cystocarp of* Gracilaria corticata *4; Fig. 12a.* Gracilaria corticata *5 thallus; Fig.12b. Cross section of cystocarp of* Gracilaria corticata *5*

= *Rhodymenia corticata* J. Ag. Agardh, J.G. Nya alger fran Mexico, oefvers Kongl. Vet. 15 : 1-50, 1841.

Thallus 8 - 10 cm in height, cartilaginous, rigid, attached by irregular discs, dichotomously branched, axes more branched, branching alternate, with long branches below and shorter ones above, stipe 2 - 3 cm long and 2 - 3 mm wide, primary branch length 6 - 7 cm and width 1.5 - 2 mm, secondary branch length 4 - 5 cm and width 1.5 - 2 mm, acute. Thallus with 3 - 4 layers of cortical cells, cells circular, 3 - 7.5 µm diameter, 5 rows of angular medullary cells, 85 - 100 µm diameter with thick wall, cystocarp hemispherical, elevated, 1 - 1.5 mm wide with constriction below, gonimoblast filament compact, 500 - 560 µm in height and 700 – 730 µm in width.

Gracilaria corticata (Gc2): (from Supra littoral zone)

Thallus 4 - 5 cm in height, cartilaginous, rigid, attached by irregular discs, dichotomously branched, axes less branched, branching opposite, stipe length 1 - 2 cm and width 4 mm, primary branch length 2 - 3 cm and width 3 - 5 mm, secondary branch length 1 - 1.7 cm and width 2 mm, acute. Thallus with 3 layers of cortical cells, cells circular, 3 - 7.5 µm, 5 rows of angular medullary cells, 68 - 115 µm with thick wall, cystocarp 410 - 450 µm high and 750 - 780 µm width, hemispherical, elevated, 1 - 1.5 mm wide with constriction below, gonimoblast filament compact.

Gracilaria corticata (Gc3): (from Littoral zone)

Thallus 5 - 6 cm in height, cartilaginous, less rigid, attached by irregular discs, axes more branched, branching opposite, tri - tetrachotomously branched, profusely branched above, stipe length 2 - 2.5 cm and width 2 mm, primary branch length 3 - 4 cm long and width 2 mm, secondary branch 2.5 - 2.7 cm long and width 1 mm, tertiary branches 1 - 1.5 cm long, forked, narrow and acute. Thallus with 3 layers of cortical cells, cells circular, 1.2 - 2.5 µm, 2 rows of angular medullary cells, 9 - 102 µm, cystocarp many in number, sometimes in pairs, 200 - 220 µm in height and

420-450 µm wide, hemispherical, elevated, 1.5 - 2 mm wide with constriction below, gonimoblast filament compact.

Gracilaria corticata (Gc4): (from Sub littoral zone)

Thallus 12 - 14 cm in height, cartilaginous, less rigid, attached by irregular discs, axes more branched, dichotomously and trichotomously branched, stipe length 3.5 - 5 cm and width 3 - 4 mm, primary branch 6 - 7 cm long and 2 - 3 mm wide, secondary branch 4.5 - 5.5 cm long and 2 mm wide, tertiary branches 2.5 - 3 cm long. Thallus with 2 layers of cortical cells, cells circular, 3 - 5 µm, 4 rows of angular medullary cells, each measure 100 - 115 µm in diameter, cystocarp 180 - 200 µm in height and 450 - 485 µm in width, hemispherical, elevated, 1 - 1.5 mm wide with constriction below, slightly flattened above, gonimoblast filament compact.

Gracilaria corticata (Gc5): (from Intertidal zone in rock pool)

Thallus 5 - 5.5 cm in height, attached by irregular discs, main axes dichotomously branched, further branches dichotomous and trichotomous, stipe length 3 - 3.5 cm and width 3 - 4 mm, primary branch length 2 - 2.5 cm in height and width 1 - 1.5 mm, secondary branch 1 - 1.5 cm long and width 1 mm, tertiary branches 3 - 5 or forked, 0.2 - 0.5 cm long, acute. Thallus with 2 layers of cortical cells, cells circular, 3 - 5 µm, 4 rows of angular medullary cells, measuring 80 - 110 µm in diameter, cystocarp of 400 - 480 µm in height and 600 - 690 µm in width. Cystocarp bilobed at the top, apiculate, protruding, not constricted at the base.

The present study on morphological and anatomical characters of some Rhodophyceae members has lead to the critical analysis of *Gracilaria corticata* which showed much variations among them. The five populations of *G. corticata* collected from littoral, sublittoral, intertidal and the intertidal rock pools shared common characters such as cartilagenous thallus, dichotomous branching, with primary and secondary branches. The common anatomical features of the thallus were the presence of the cuticle, cortical layers, medullary cells and the cystocarp. However, there was wide variation in the colour, height of the plant, branching pattern, number of cortical layers, dimensions of cortical cells and the medullary cells. Morphologically the five populations of *G. corticata* appeared to be different, making the species delineation of *Gracilaria* difficult. Hence, they are presumed to be different varieties of *G. corticata*. The present study revealed that Gc5 could be *G. corticata* var *cylindrica* based on the characters described by Umamaheswara Rao (1972). However, this study should be confirmed further by the molecular studies of these five populations.

Acknowledgement

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MOLECULAR STUDIES ON FOUR MORPHOLOGICAL VARIANTS OF *GRACILARIA FOLIIFERA* BOERGESON

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Abstract

The red algae Gracilaria foliifera was collected from different localities in Tuticorin, Tamilnadu, India. Based on morphological and anatomical observations, G. foliifera was differentiated into four variants. The four variants showed distinct morphological and anatomical variations. Hence, molecular studies were carried out to investigate the molecular characteristics using RAPD technique. Eight primers were used of which five primers namely PGF04, PGA01, PG04, PG10 and PG07 gave good amplification. The data were analysed using NtSys software. The dendrogram obtained have differentiated the four variants based on their molecular data. However further molecular studies with species specific primers could support the results obtained from this current study.

Keywords: Gracilaria folifera, RAPD, Delineation, Tuticorin

I. INTRODUCTION

Among the seaweeds, the red algae (Rhodophyta) are important source of agar and carrageenan and also has various industrial applications. They are used in food and pharmaceutical industries. The taxonomy of this commercially important *Gracilaria* is still problematic as there is a limitation of distinct morphological characteristics (Bird, 1995). Due to the economic importance of *Gracilaria*, several studies on their taxonomy have resulted in taxonomic revisions and nomenclature changes (Silva et al., 1996). The high degree of morphological variation in seaweeds is due to their plasticity which causes difficulties and confusions in the taxonomy of *Gracilaria* species (Bird & McLachlan, 1982) and species identification based on morphological characters alone is unreliable (Bellorin et al., 2008). Hence, it is essential to include molecular techniques for taxonomic studies.

There are approximately over 150 species distributed widely from subboreal to tropical marine waters (Byrne *et al*., 2002). *Gracilaria* is cultivated in several countries and regions including the Philippines, Chile, China, the Republic of Korea, Indonesia,Vietnam and Argentina at a commercial scale (McHugh 2003). Over one hundred species of the commercially important agarophyte of *Gracilaria* (Gracilariaceae, Rhodophyta) are widely distributed throughout the tropical and temperate waters of the world. Proper classification is important for both cultivation and industrial exploitation of *Gracilaria*. *Gracilaria* species are identified traditionally by their morphological features, such as branching pattern, extent of thallus constriction, and reproductive structures (Lim *et al*., 2001). However, species delineation of *Gracilaria* is problematic due to intergradations in morphological and reproductive characteristics (Bird 1995).

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In the present study *Gracilaria foliifera* (Gf) was collected from Tuticorin. The absence of definite morphological characters in the delineation of the species of *Gracilaria*, has made it more difficult through phenotypic convergence and plasticity. Uncertainties in the classification can be overcome by using molecular techniques because they measure genetic rather than phenotypic changes (Donoghue & Sanderson, 1992). Since the random amplified polymorphic DNA (RAPD) assay was successfully used in the classification of seaweeds at the genus and species level, RAPD markers were used to study the genetic relationships among the four morphologically different *G. foliifera* collected from different sites of Tuticorin. Based on the morphological, anatomical and RAPD results, a comparative study was carried out with four different specimens of *G. foliifera*.

II. MATERIALS AND METHODS

In the present investigation *G. foliifera* was collected from Tuticorin, Tamil Nadu. Every collection was assigned a collection number and herbarium sheets were prepared and the materials were also preserved in 4% formalin. The morphological features of the collected materials were observed under stereo-dissection binocular microscope and identified with Rhodophyta Vol.II (Desikachary *et al*., 1998). Field materials and cross sections were photographed using Sony Cyber-shot DSC w690. Numbers were assigned to different variants of *G. foliifera* as Gf1, Gf2, Gf3 and Gf4.

2.1 Morphological and Anatomical studies

Morphological characters namely colour and texture of the thallus, thallus length, stipe length, stipe width, number of primary and secondary branches, length and width of primary and secondary branches were recorded. Handsections were made and stained with Toludene blue O. Cell dimensions were recorded with stage micrometer. Anatomical characters namely number of cortical layers, number of medullary layers, dimensions of cortical, medullary cells and cystocarp were analysed.

2.2 DNA isolation

The algal samples were washed in filtered seawater to remove all epiphytes and debris. The washed samples were air-dried in the air-conditioned culture room. The isolation buffer contains 0.1mM Tris Cl, 0.05M EDTA, 0.5mM NaCl, 10% SDS (Sodium Dodecyl Sulphate), 100µg ml^{-1} proteinase K. The buffer was incubated in a 65°C water bath for about 30 min. The air-dried samples (200mg) were ground using mortar and pestle along with few pinch of acid washed sand until powder form for DNA extraction. About 1ml of isolation buffer was added to the samples during grinding process to enhance the DNA isolation. The homogenized mixture was incubated in 65°C waterbath for 30 min. The mixture was centrifuged at 13000 rpm for 15 min to remove the sand particles. The supernatant was collected in a new eppendorf tube. An equal volume of chloroform : isoamyl alcohol (24:1) was added and centrifuged at 13000 rpm for 15 min. The upper aqueous layer was transferred to a new eppendorf tube and 700µl ice cold isopropanol was added. The mixture was incubated at room temperature for 2 hr or overnight and centrifuged at 13000 rpm for 30 min at 4°C. The supernatant was discarded and the pellet was airdried. The dried pellet was suspended in 250µl TE buffer. About one tenth volume of ammonium acetate and 2.5 volume of 95% ethanol was added. The mixture was incubated at -20°C for 2 hr and centrifuged at 7000 rpm for 10 min. The supernatant was discarded and the pellet was washed with 1ml of 70% ethanol. The pellet was then airdried and suspended in 200µl TE buffer.

2.3 Random amplified polymorphic DNA (RAPD)

Polymerase chain reaction (PCR) amplification was performed in a final volume of 25µl containing 0.5µl of *Taq* polymerase, 2.5µl of 10X *Taq* DNA polymerase buffer, 2.5µl of 10mM dNTP mix, 1µl of 10µM primer, 50 ng of genomic DNA. Eight primers used were PG02, PG03, PG04, PGF04, PGA01, PG04, PG07 and PG10. Amplification was performed in a Eppendorf Mastercycler®

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programmed at 94°C for 5 minutes denaturation and followed by 34 cycles of 45 seconds denaturation at 94°C, 1 min at 36°C, 72°C for 1 min and a final extension at 72°C for 10 minutes. The amplified products were separated by electrophoresis through 2% agarose gels in 1X TBE at 50 V for 1 hr. The gel was stained with ethidium bromide and the amplified product was visualized under a UV transilluminator. Agarose gels were photographed.

RAPD data analysis was carried out using the NtSys software with results obtained from the primers PG04, PGF04, PGA01, PG04, PG10. The calculation of the matrix of similarities was based on the Dice coefficients (S_D) and the clustering was carried out using the unweighted pair group method using arithmetic averages (UPGMA).

III. RESULTS

3.1 Morphological and anatomical characteristics

Gracilaria foliifera (Gf) collected from different localities presented distinct morphological variations in thallus height, length and breadth of the stipe, primary branches, secondary branches and tertiary branches. Gf1, Gf2 and Gc4 ranged between 11 to 16cm in height of the thallus and Gf3 ranged between 7 to 8cm in height. Gf1, Gf3 and Gf4 were dichotomously branched with branching alternate branching and profusely branched while Gf2 dichotomously branched with opposite branching. There was also anatomical variations among the four specimens in the number of cortical cells, number of medullary layers and the size of the medullary cells. Variations were also observed in the length and breadth of the cystocarp among the four specimens (Fig.1-4).

***Gracilaria foliifera* Boergeson**

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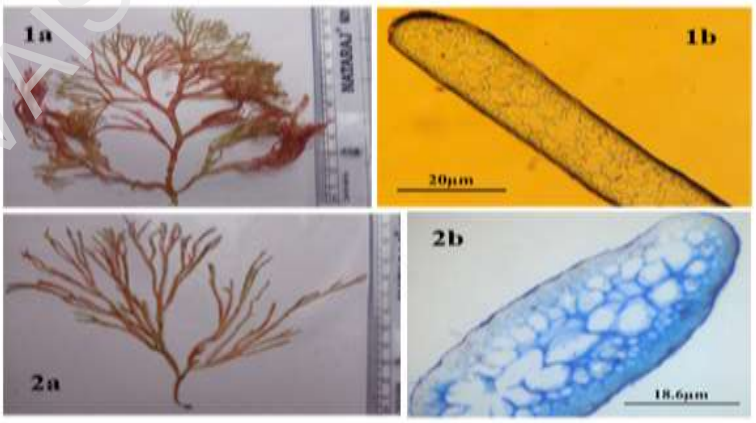

Figure1: 1a. *Gracilaria foliifera* 1 thallus; 1b. Cross section of Gf1 thallus; 2a. *Gracilaria foliifera* 2 thallus; 2b. Cross section of Gf2 thallus

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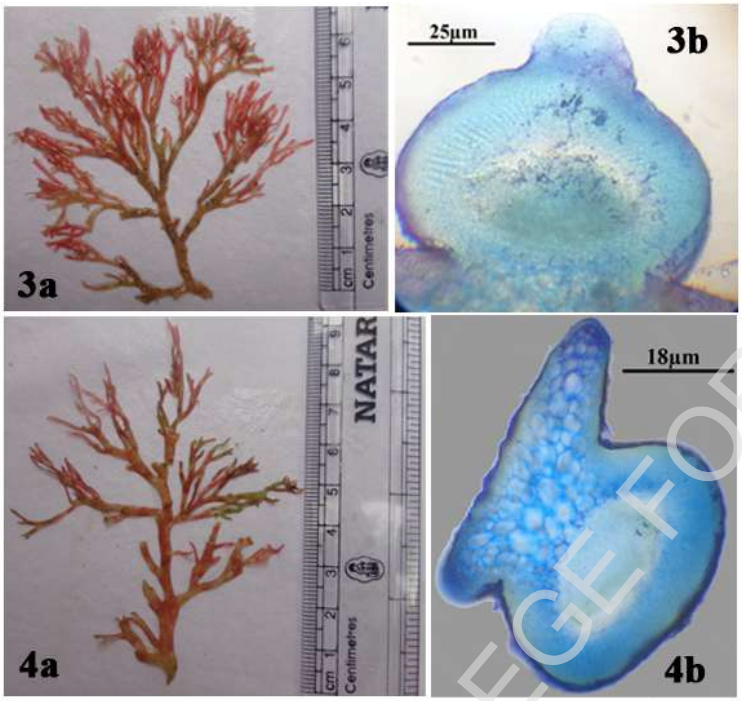

Figure 2: 3a. *Gracilaria foliifera* 3 thallus; 3b. Cross section of cystocarp of Gf3; 4a. *Gracilaria foliifera* 4 thallus; 4b. Cross section of cystocarp of Gf4

***Gracilaria foliifera* (Gf1):**

Thallus 12-13cm in height, thin, attached by irregular discs, dichotomously branched, axes more branched with slender tertiary branches and profusely branched above, branching alternate, with short branches below and longer ones above, stipe 1-1.5cm long and 3mm wide, primary branch length 1.5-2cm and width 4mm, secondary branch length 0.8-1cm and width 2mm, acute. Thallus with 2-3 layers of cortical cells, cells circular, 5-10µm diameter, 3-4 rows of angular medullary cells with wavy margin, 80-132.5µm diameter with thick wall.

***Gracilaria foliifera* (Gf2):**

Thallus 15-15.5cm in height, attached by irregular discs, dichotomously branched, axes more branched with slender tertiary branches and profusely branched above, branching alternate, with short branches below and longer ones above, stipe 1-1.5cm long and 2mm wide, primary branch length 5.8-6.1cm and width 4mm, secondary branch length 1-1.5cm and width 2mm, with sharp end. Thallus with 2 layers of cortical cells, cells circular to ovoid, 7.5-10µm diameter, 3-4 rows of angular medullary cells, 100-110µm diameter with thick wall.

***Gracilaria foliifera* (Gf3):**

Thallus 7.5-8cm in height, attached by irregular discs, dichotomously branched, axes more branched, branching alternate, with short branches below and longer ones above, stipe 1cm long and 2mm wide, primary branch length 1.3-1.8cm and width 2mm, secondary branch length 1.6-1.9cm and width 2mm, with sharp end. Thallus with 2 layers of cortical cells, cells circular, 7.5-10µm diameter, 3-4 rows of angular medullary cells, 40-87.5µm diameter with thick wall, cystocarp elevated, 756.84µm in height and 656.88µm width.

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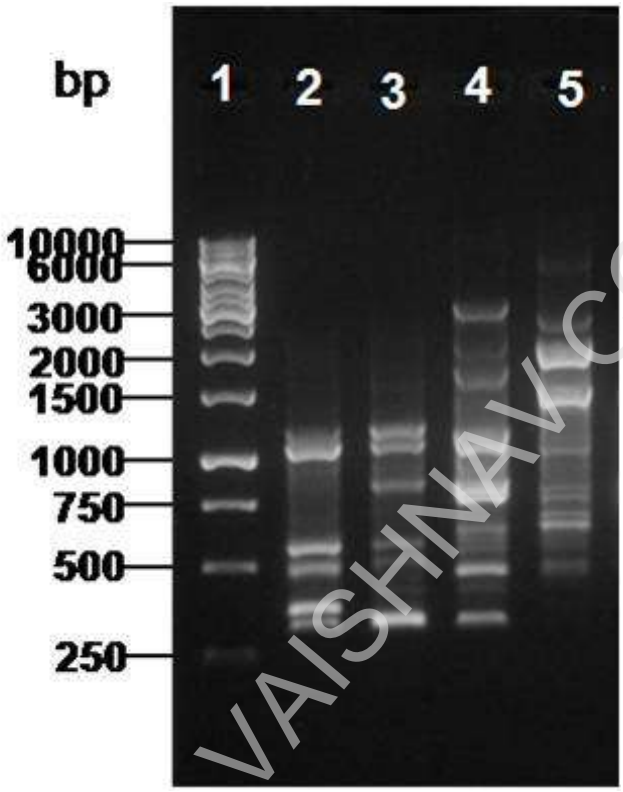
***Gracilaria foliifera* (Gf4):**

Thallus 11-12.5cm in height, attached by irregular discs, dichotomously branched, axes more branched, branching alternate, stipe 1.8-2cm long and 3mm wide, primary branch length 1.8-1.9cm and width 3mm, secondary branch length 2.8-2.9cm and width 1mm, with sharp end. Thallus with 2 layers of cortical cells, cells circular, 5-10µm diameter, 3 rows of angular medullary cells, 80-145µm diameter with thick wall, cystocarp 885.36µm in height and 999.6µm width.

3.2 RAPD analysis:

A total of 15 amplicons were generated by the primer PGF04. The amplicons ranged between 0.3 – 6 kb in size (Fig. 5). All bands were found to be polymorphic. The bands 1 - 1.2 kb and 0.4 – 0.6 kb were amplified by the primer PGF04 in all the four specimens of *Gracilaria foliifera*. The band 6 kb was observed only in Gf4. 3 kb band was amplified in Gf3, 2.5 kb and 1.8 kb in Gf4, 1.7 kb in Gf3, 1.4kb in Gf4. The band 1.2 kb was amplified in Gf1 and Gf2. 0.9 kb band was amplified in Gf3. 0.8 kb was amplified in Gf2, Gf3 and Gf4. 0.75 kb was amplified in Gf3 and Gf4, 0.7 kb in Gf4, 0.65 kb in Gf3 and Gf4, 0.35 kb in Gf1. The band 0.3 kb was amplified in Gf1, Gf2 and Gf3.



Lane 1-Marker, 2-Gf1, 3-Gf2, 4-Gf3, 5-Gf4

Figure 3 RAPD profile of DNA amplified by PGF04 of four specimens of *Gracilaria foliifera*

IV. DISCUSSION

The four individuals of *Gracilaria foliifera* collected from different locations of Tuticorin shared common characters such as cartilaginous thallus, dichotomous branching, with primary and secondary branches. However there was wide variation in the height of the plant, breadth of the plant and branching pattern. Morphologically the four specimens of *Gracilaria foliifera* appeared to be different, making the species delineation of *Gracilaria* difficult.

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The genetic diversity among the four specimens was evaluated by five primers which yielded species specific DNA profiles and proved to be informative. A total of 15 amplicons were generated by one primer (PGF04). The amplicons ranged between 0.3 and 3 kb in size. All bands were found to be polymorphic. The polymorphic information content was found to be 0.29 and marker index was found to be 1.09 (Table 1).

Table 1: Degree of polymorphism and polymorphic information content for RAPD primers in Gf1, Gf2, Gf3 and Gf4

Primer code	Total no. of bands	Total no. of polymorphic bands	POL (%)	PIC	MI
PGF04	15	15	100	0.29	1.09

Based on the RAPD analysis, the similarity coefficient among all the four specimens of Gf was calculated based on DICE genetic distance. Resulting clusters were expressed as UPGMA (Unweighted Pair-Group Mean Arithmetic Method) dendrograms constructed using SHAN neighbour-joining tree for RAPD, molecular marker used. The coefficients on the x-axis represent the similarity indices (DICE) of the different specimens chosen for the study. The genetic similarity value derived from the RAPD data given in Table 2. Based on UPGMA algorithm from RAPD, the genotype were grouped into two major clusters (Fig. 6). Cluster I consisted of three variants; Cluster II consisted of only one specimen (Gf4 as out group). Cluster I consisted of two sub groups. Subgroup 1 consisted of Gf1 and Gf2 in which the genetic similarity between Gf1 and Gf2 is 69%. Sub group II consisted of Gf3 and the genetic similarity between Gf3 and Gf1 is 72% and genetic similarity between Gf3 and Gf2 is 73%.

Table 2: Similarity matrics obtained by DICE similarity coefficient of Gf1, Gf2, Gf3 and Gf4

Rows\Cols	Gf1	Gf2	Gf3	Gf4
Gf1	0.000			
Gf2	0.648	0.000		
Gf3	0.721	0.735	0.000	
Gf4	0.762	0.849	0.735	0.000

The separation approach as revealed by the Mantel test comparing the results of RAPD indicated a significant correlation within the four specimens of *Gracilaria foliifera*. The cophenetic correlation coefficient between dendrogram and the original similarity matrix was also significant for RAPD (r = 0.79) supporting a good degree of confidence in the association obtained for the four specimens of Gf. Principal Coordinate Analysis (PCA) showed the multidimensional relationships that describe portions of the genetic variance in a data set for four specimens of Gf (Fig. 7). Screening genetic diversity at the interspecific level, the average values of na, ne, and h were 2.00, 1.54 and 0.33 respectively. The mean Shannon's indexes (I) for all the four specimens was 0.51 (Table 3).

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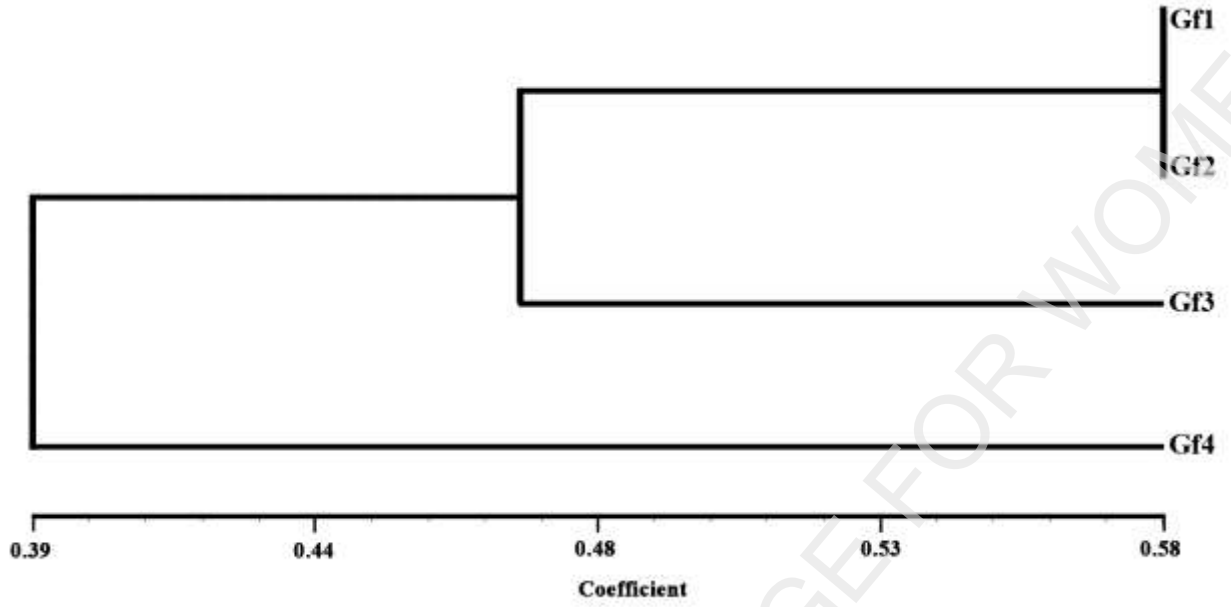

Figure 4: Dendrogram representing the genetic variability among Gf1, Gf2, Gf3 and Gf4, as revealed by UPGMA cluster analysis. The genetic distances were from Dice similarity coefficient.

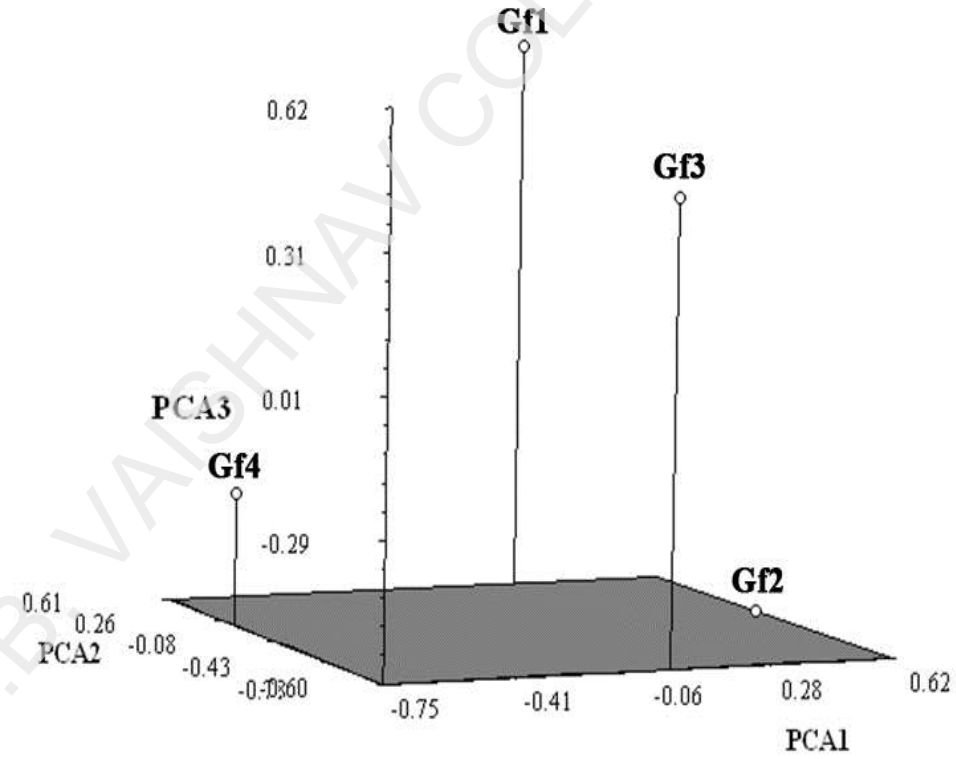

Figure 5: Principle co-ordinate map for the first, second and third principle coordinate estimated for RAPD marker for Gf1, Gf2,Gf3 and Gf4

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Table 3: Genetic diversity parameters Gf1, Gf2, Gf3 and Gf4.

Parameter	Value
The number of observed alleles, na	2.0000 ± 0.000
The mean number of effective alleles, ne	1.5434 ± 0.2714
The mean Nei's gene diversity index, h	0.3332 ± 0.1101
Shannon index, I	0.5092 ± 0.1247

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