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Morphological and molecular analysis of two populations of *Chara zeylanica* Willd.

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Abstract: Characeae, are a prominent group of green macroalgae, which are being extensively studied due to their closeness to land plants, and their ecological and economic importance, such as aquatic feed and in limnology. In the present work, morphological studies were carried out in the two populations of *Chara zeylanica* Willd., collected from Mahabalipuram and Kovalam, on the South east coast of Chennai, Tamil Nadu. Variation in the vegetative characters were observed between the two different populations, therefore both the samples were brought under culture in the lab condition. Since the variations between the two populations were consistent even under culture condition, molecular work such as RAPD and rbcL sequencing were carried out.

Key words: Characeae, *Chara zeylanica*, rbcL sequencing, RAPD

INTRODUCTION:

Charales are a group of green macroalgae, characterised in the field by their unique complex morphological features. The vegetative body is divided into nodes and internodes, the internodes being made of a single coenocytic cell, 1-2 cm long, in mature specimens (Wood & Imahori, 1965). The Charales are usually found in the alkaline waters and are encrusted with lime of calcium. They are in general found in association with other aquatic plants or microalgae, such as *Ceratophyllum* species, *Hydrilla* species, blue green algae *etc*. The Charales are of specific interest in research due to their economic and ecological importance and most of all for their close relatedness to the ancestors of land plants (Karol *et al.* 2001; Chalotra *et al.*2013).

The members of the genus *Chara* are generally highly variable, and the variations in most of their qualitative morphological characters overlap, which makes any type of taxonomical differentiation extremely difficult. This high range of differentiation probably depends on the ecological (environmental) conditions of their growth (such as contents of elements in water and light availability) and their inherent genetic differences (Urbaniak, 2010).

The genus *Chara*, consists of corticated and ecorticated specimens, and is represented by a number of variants in India. The species *Chara zeylanica* Willdenow in south India has four varieties or subspecies (Sundaralingam, 1959). These have been classified on the basis of morphological characters only. However, the morphological characters are open to environmental impacts, such as temperature, mineral content of water, light etc. Hence, further molecular investigations along with the study of oospore ornamentation are necessary to precisely classify the Characeae members.

In the present study, two populations of *Chara zeylanica*, namely, Sample1 and Sample 2, were collected from Mahabalipuram and Kovalam respectively, which is in the South east coast of Chennai, Tamil Nadu from 2014 – 2015 at an interval of every three months . Studies on the morphological characters were done in both, field material and sample brought under culture of the two populations. The morphological characters were compared with Sundaralingam's subspecies of *Chara zeylanica* for further identification below species level. Since Sample 1 and Sample 2 showed the same morphological characters with their corresponding field materials even under culture, the field material of the two populations (Sample 1 and 2) were subjected to molecular analysis.

MATERIALS AND METHODS:

Fresh samples collected from both the locations were cleaned to remove the epiphytes. The sterile terminal apical region of the plant with two internodes were brought under culture in sterile soil water media in the lab at 22^0C with 12 hours of light and dark period under florescent lamp. Field samples were preserved as dried herbarium material and deposited in the Department of Plant biology and Plant biotechnology, S.D.N.B.Vaishnav College for Women, Chennai, and also fixed in 4% formalin. Morphological characters of the field and unicharalean culture samples, maintained in biphasic media (soil water media), were studied under Binocular Olympus CH20i microscope and photographed with Sony Cybershot DSC-T77 10.1 MP Digital camera.

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DNA extraction was carried out using CTAB method (Doyle, 1991). Spectrophotometry and 0.8% agarose gel were used for determination of quality and purity of DNA before amplification. For determination of DNA concentration, nanodrop UV-Vis spectrophotometer was used. Absorbance of DNA and protein were measured in OD 260 and 280 respectively. If the calculation of OD ratio 260/280 was between 1.7-2, DNA is considered as good quality and suitable for PCR procedure.

The oligonucleotide primers and rbcL primers with their GC content used for the recent work are provided in Table 1. PCR reactions were carried out in a total volume of 50 µl, containing 2 µl of template DNA (50 ng), 8 µl of oligonucleotide primer (2 µM), 5 µl of PCR buffer (10x), 5 µl of dNTP mix (2 mM), 0.5 µl of *Taq* DNA polymerase (5 unit). The final volume was made up by adding 29.5 µl of double distilled water.

Table 1: Primers with their GC content used in this study

PRIMER	NUCLEOTIDE SEQUENCE	GC CONTENT (%)
PGF01	5' – GAAACAGCGG – 3'	60
PGF02	5' – GGAGCCCAC – 3'	77.7
PGF04	5' – GGCATCGGCC– 3'	80
OPI 07	5' – CAGCGACAAG– 3'	60
PGA14	5' – CGCTCCATCC – 3'	70
rbcLa-F	5' – TGTCACCACAAACAGAGACTAAAGC – 3'	42.3
rbcLa-R	5' – GTAAAATCAAGTCCACCRCG – 3'	45

Amplifications were carried out in Applied Biosystems Veriti Thermal Cycler. The thermal cycler was programmed as initial denaturation at 94^0C for 5 min followed by 34 cycles of 94°C for 40 sec for denaturation, 36°C for 30 sec for annealing 72°C for 90 sec for extension and a final extension of 72°C for 10 min. Bands were resolved in a 1% agarose gel in 1X TBE buffer and stained with Ethidium bromide.

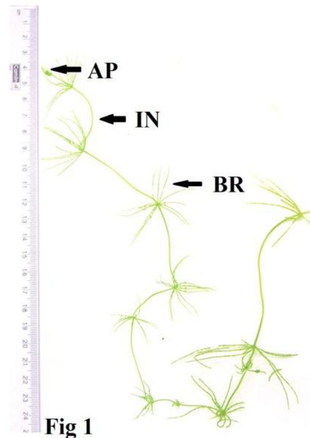
RESULTS:

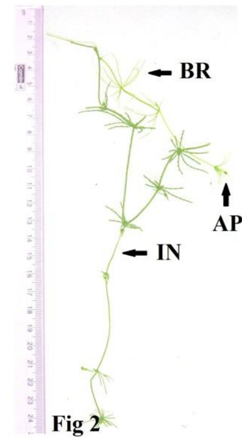
Consistent morphological variations observed between the two populations in both the field and culture samples are given in Table 2 (Plate 1).

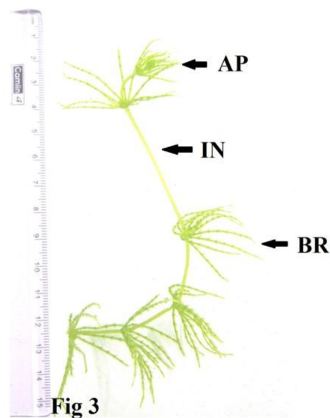
Sample 1: In these specimens the stipulodes were well developed with the upper row of stipulodes, measuring 656.6X80 µm (±8), compared to the lower row of stipulodes, which measured 383.3X73 µm (±1) (Fig 6). The lowest ecorticate region of the primary lateral measured 266X233 µm (±2). The spine cells were 113X43 µm (±0.5) long and acute. Terminal cells of the primary lateral were solitary and mucro (Fig 8). The bracts measured 393X70 µm (±3) long.

Sample 2: In these specimens both the row of stipulodes were well developed and the upper and lower row of stipulodes measured as 303.3X46 µm (±1), 170X43.3 µm (±2) (Fig 7) respectively. The lowest ecorticate internode of the primary lateral measured 283X150 µm (±3). The spine cells were 53X36 µm (±0.5) long and acute. Three terminal cells were found in the apex of the primary laterals (Fig 9). The bracts measured 100X90 µm (±3) long. The vegetative and reproductive characters of both the samples is given in Table 2.

The oospore characters were distinctly different in the two samples. In sample 1, the oospore was elliptical, clawed, flange present, length 635 µm (±2) and width 295 µm (±3). While in sample 2, the oospore was oblong, not clawed, absence of flange, length 607 µm (±3) and width 379 µm (±1).







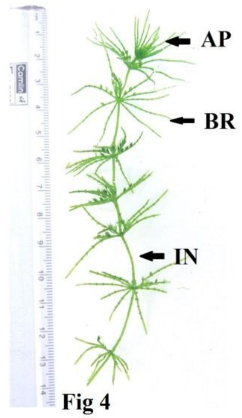
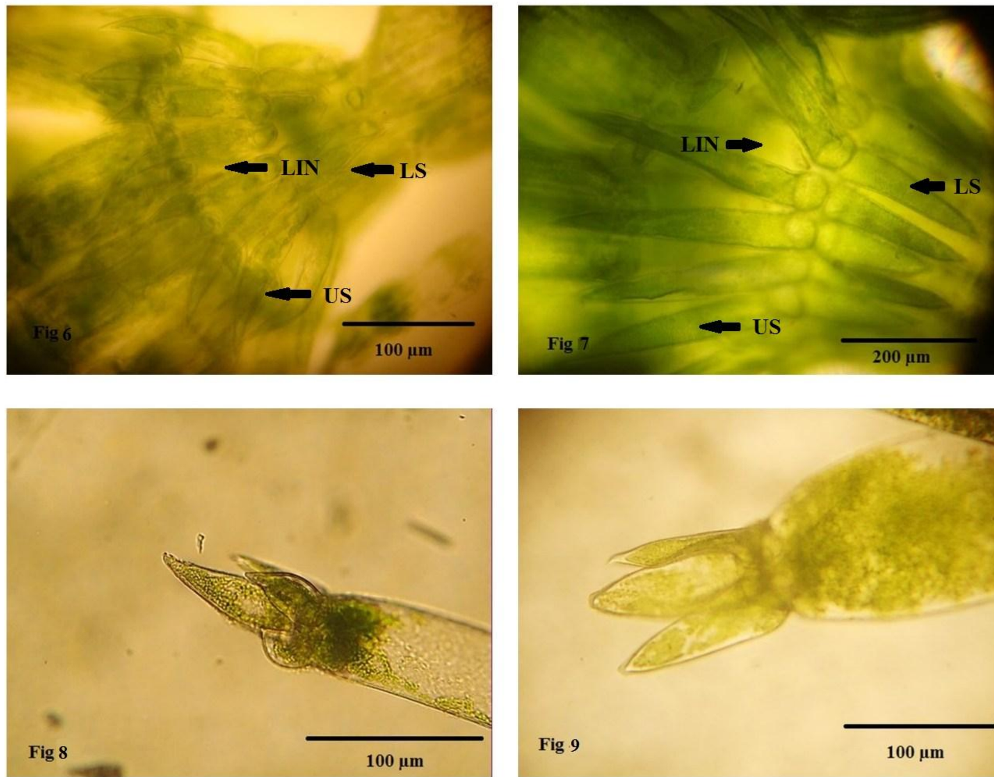
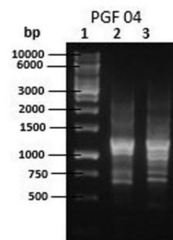
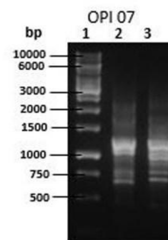


Fig 5

Plate 1: **Fig 1: Habit of field material of Sample 1; Fig 2: Habit of field material of Sample 2; Fig 3: Habit of culture Sample 1; Fig 4: Habit of culture Sample 2; Fig 5: Sample 1 in culture (AP – Apical region, BR – Branchlet, IN – Internode)**







Lane 1-Marker
Lane 2--Sample 1
Lane 3-Sample 2

Fig 10

Plate2: Fig 6: Stipulodes of Sample 1; Fig 7: Stipulodes of Sample 2; Fig 8: Terminal cell of Sample 1; Fig 9: Terminal cells of Sample 2; Fig 10: Gel photograph of RAPD primers. (US - Upper row of stipulodes, LS – Lower row of stipulodes, LIN – Lowermost internode of the branchlet)

Table 2: Comparison of morphological characters of sample 1 and sample 2

MORPHOLOGICAL CHARACTERS	SAMPLE 1		SAMPLE 2	
	FIELD	CULTURE	FIELD	CULTURE
Axis thickness	484 µm (±1)	450 µm (±1)	424 µm (±1)	410 µm (±2)
Number of stipulodes	19	21	17	16
Size of upper row of stipulodes	656.6x80 µm (±8)	475x75 µm (±1)	303.3x46 µm (±1)	230x60 µm (±1)
Size of lower row of stipulodes	383.3x73 µm (±1)	295x71 µm (±2)	170x43.3 µm (±2)	176x40 µm (±1)
Number of branchlets	11	10	9	8
No: of segments	8	8	5	7
Size of the lowest internode	266x233 µm (±2)	308x300 µm (±2)	283x150 µm (±3)	262x139 µm (±1)
Shape of spine cell	Acute	Acute	Acute	Acute
Size of spine cell	113x43 µm (±0.5)	115x39 µm (±1)	53x36 µm (±0.5)	61x37 µm (±0.5)
Number of bract cells	7	7	6	6
Position of gametangia	2nd &3rd node	2nd &3rd node	2nd,3rd &4th node	2nd,3rd &4th node
Size of the globule	370x376 µm (±2)	270x288 µm (±1)	220x210 µm (±1)	310x300 µm (±2)
Size of the nucule	635x295 µm (±2)	740x448 µm (±2)	607x379 µm (±2	621x415 µm (±2)
Size of the coronal cell	103x66 µm (±1)	83x73 µm (±1)	86x53 µm (±0.5)	73x50 µm (±1)
Number of spirals	12	12	11 (± 1)	11

RAPD PCR ANALYSIS:

RAPD PCR amplification of DNA of two *Chara zeylanica* samples was carried out with five oligonucleotide random primers of which only PGF04 and OPI07 showed amplification. 1 kb DNA ladder was used for the estimation of molecular size. Table 3 and 4 shows various amplified products using random oligonucleotide primers in the two samples. The size of the loci that has been amplified using these two primers is between 700-3100bp.

Table 3: Loci produced for Sample 1 and Sample 2 using primer PGF04

Molecular size of the band (bp)	810	1000	1300	1500	1800	2100	2400	2800
Sample 1	0	1	0	1	1	0	1	0
Sample 2	1	1	1	1	1	1	0	1

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Table 4: Loci produced for Sample 1 and Sample 2 using primer OPI07

Molecular size of the band (bp)	700	1000	1100	1200	1500	1600	1800	2400	2600	3100
Sample 1	0	1	1	0	0	1	1	1	0	1
Sample 2	1	0	1	1	1	0	1	0	1	1

DISCUSSION:

Chara zeylanica collected from Mahabalipuram agrees closely with Sundaralingam's description of the forma *hiledebrandtiana* (A. Br) comb. nov. in number of terminal cells and the size and the number of stipulodes specifically, and also other characters such as thickness of the main axis, number of branchlets, number of segments, number of bract cells, the shape of the spine cells, diameter of the antheridium, dimension of the oospore. But differs from Sundaralingam's description of var. *hiledebrandtiana* where the size of the spine cell 434-616µm long and in Sample 1 it is 113 µm (±0.5) long.

f. *hiledebrandtiana* (A. Br) comb. nov.

= *C. gymnopus* var. *hiledebrandtiana* A.Br., Braun and Nordstedt, 1882, p 196.

Chara zeylanica collected from Kovalam closely agrees with Sundaralingam's forma *humboldtii* (Kütz.) in terms of the number of terminal cells, size and number of the stipulodes, thickness of the main axis, number of branchlets, number of segments, number of bract cells, the shape and size of the spine cells, diameter of the antheridium, dimension of the oospore.

f. *humboldtii* (Kütz.) Groves, H. & J., 1912, p. 42

= *Chara poylphylla* var. *humboldtii* Kützing, Species Algarum, 522, 1849.

= *Chara gymnopus* var. *humboldtii* Braun, in Braun and Nordstedt, 1882, p. 196

However the oospore variations in shape, size, clawed base, presence/absence of flange are promising morphological characters in distinguishing these two samples. According to Casanova (1997) the variation in the oospore characters is not ecological and it is only genetic variation. Hence RAPD work and rbcL sequencing were carried out in these two samples.

Though RAPD analyses is not sufficient to distinguish at variety or forma level, it showed mild band variations between the two samples. Hence by the semi log plotting of the DNA bands, the molecular weight of the various bands were determined. Primer PGF04 amplified 1000bp, 1500bp and 1800bp and Primer OPI07 produced 1100bp, 1800bp and 3100bp as common loci in both the populations. However PGF04 amplified 2400bp only in sample 1 and 810bp, 1300bp, 2100bp and 2800bp in sample 2. Primer OPI07 amplified 1000bp, 1600bp and 2400bp only in sample 1 and 700bp, 1200bp, 1500bp, 2600bp in sample2.

The GC content of the rbcL gene of sample 1 was calculated as 37.5% and that of sample 2 is 38.2%. Since the GC content of rbcL variation is very minimum between the two varieties of *Chara zeylanica*, further molecular studies on the ITS and 18S rDNA would reveal the taxonomic delineation of these two samples. Similarly the oospore characters of these two samples should be analysed by SEM work to study the variation in ornamentation.

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